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Maturing neurons are selectively sensitive to human immunodeficiency virus type 1 exposure in differentiating human neuroepithelial progenitor cell cultures

Micheline McCarthy,[1,2,3] Irving Vidaurre,[1] and Rebeca Geffin[4]

[1]Department of Veterans Affairs Medical Center, Miami, Florida, USA; Departments of [2]Neurology, [3]Microbiology and Immunology, and [4]Pediatrics, Miller School of Medicine, University of Miami, Miami, Florida, USA

Human immunodeficiency virus type 1 (HIV-1) infection of the brain is associated with neuronal injury manifested by dendritic pruning, aberrant neurofilament metabolism, and decreased synaptic density. The central nervous system (CNS) responds to neuronal injury by differentiating new neurons and astrocytes from resident populations of multipotent neuroepithelial progenitor cells (NEP) located in regions such as the subventricular zone or hippocampus. *In vitro* studies have demonstrated that the HIV-1 virion or envelope glycoprotein gp120 can injure differentiated human neurons and astrocytes, suggesting that HIV-1 proteins could similarly injure NEP or NEP-derived glial and neuronal lineage-committed precursor cells. To answer this question, human fetal brain-derived "neurospheres" containing NEP and NEP-derived precursor cells were cultured in low serum differentiation medium containing lymphotropic HIV-1(SF2), macrophage-tropic HIV-1(SF128A), or recombinant $gp120_{SF2}$ from HIV-1(SF2). These experiments indicate that exposure to HIV-1 does not affect the ability of the NEP to differentiate into cells expressing either astrocyte-specific or neuron-specific cytoskeletal antigens. However prolonged exposure to HIV-1 does selectively decrease expression of neuronal antigens (microtubule β-III-tubulin and intermediate filament neurofilament-L) but not astrocyte antigens (intermediate filament glial fibrillary acidic protein). The effects of continuous exposure to HIV-1 or gp120 may result from injury to developing neurons and/or impairment of the neuronal developmental process itself. By depressing neuronal microtubule and neurofilament protein expression, HIV-1 and gp120 exposure compromise the potential for postmitotic neuronal dendrite and axon development. *Journal of NeuroVirology* (2006) **12,** 333–348.

Keywords: cell differentiation; HIV-1; HIV envelope protein gp120; neuroepithelial cells; neurofilament proteins; oxidative stress

Introduction

Human immunodeficiency virus type 1 (HIV-1) infection is associated with progressive cognitive and motor decline, with debilitating clinical dementia (HIV-associated dementia or HAD) occurring in acquired immunodeficiency syndrome (AIDS) patients. The pathological substrate of this progression is not well understood across its full clinical spectrum. HAD correlates with pathological HIV encephalitis (HIVE) and neuronal apoptosis in both pediatric and adult AIDS brains (Adie-Baissette *et al*, 1995; Gelbard *et al*, 1995; Petito and Roberts, 1995). However,

Address correspondence to Micheline McCarthy, MD, PhD, Department of Neurology (127), VA Medical Center/University of Miami, 1201 NW 16th Street, Miami, FL 33125, USA. E-mail: mmccarth@med.miami.edu

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clinicopathologic studies suggest that HIVE is not a sufficiently sensitive or specific histopathologic marker for the less severe but measurable neurocognitive dysfunction (Masliah *et al*, 1997). Markers for dendritic and/or synaptic injury likely correlate better with clinical neurocognitive status in adult AIDS patients. Such markers include neuronal microtubule proteins, which reveal loss of dendritic complexity, or neuronal synaptic vesicle proteins, which are decreased in the thinned neocortex of patients with HIVE (Wiley *et al*, 1991; Masliah 1992a, 1992b). Decreased synapse density combined with dropout of selective pyramidal neuron subpopulations in the frontal cortex reportedly correlated highly with declining neuropsychological performance in AIDS patients (Asare *et al*, 1996; Everall *et al*, 1999). Thus changes in quantity or distribution of the neuronal structural proteins subserving synapses or dendrites may reflect the neuronal injury underlying the mild-to-moderate end of the spectrum of HIV-associated neurocognitive illness. Recent experimental studies using human neuronal cultures suggest that exposure to HIV-1 or its major envelope glycoprotein, gp120, induces dendritic injury in neurons (Iskander *et al*, 2004; Walsh *et al*, 2004). This is in addition or alternatively to neuronal toxicity mediated through chemokine coreceptor CXCR4 and/or neurotoxins produced by activated non-neuronal cells, leading to increased oxidative stress or apoptotic cell signaling (Shi *et al*, 1996; Kaul and Lipton, 1999; Ohagen *et al*, 1999; Zheng *et al*, 1999; reviewed in Kaul et al, 2001; Nath, 2002; and van de Bovenkamp *et al*, 2002).

In the human brain, multipotent neuroepithelial progenitor (NEP) cells are a potential source of cells for repair of neuronal injury. NEP can generate both neuronal and glial cell types. Most NEP cells commit to a differentiated phenotype early in central nervous system (CNS) development, but growth factor–responsive multipotent (neuronal/glial) cells have been found in adult as well as fetal mammalian forebrain (Reynolds and Weiss, 1992; Gritti *et al*, 1996; Eriksson *et al*, 1998; Roy *et al*, 2000). Fetal and adult human NEP cells have been reported to have similar molecular characteristics and growth potential (Palm *et al*, 2000). Given their capacity to induce neuronal injury, HIV-1 and/or gp120 may injure NEP cells or interfere with the differentiation of astrocytes and neurons from NEP cells. Exposure to gp120 reportedly promoted quiescence (inhibition of proliferation) in human NEP cells by signaling through CCR3 or CXCR4 chemokine receptors, though this effect was reversible (Krathwohl and Kaiser, 2004). Human fetal NEP cells have been demonstrated to support restricted HIV-1 infection, in which virus production by NEP cells can be augmented by exposure to cytokines or by differentiation towards an astrocyte phenotype (Lawrence *et al*, 2004). Thus HIV-1 could pose a threat to the NEP population in both the developing and the mature CNS.

Proliferating human NEP and lineage-restricted precursor cells can be cultured for *in vitro* viral studies as adherent monolayers (Lawrence *et al*, 2004) or spherical cell clusters in suspension (neurospheres) (McCarthy *et al*, 2000). Differentiation can be promoted by withdrawal of growth factors; then preferential differentiation towards neurons or astrocytes can be manipulated by addition of exogenous neurotrophic growth factors or serum to the culture medium. Thus NEP and NEP-derived precursor cells cultured from fetal human brain present an accessible *in vitro* system with which to study the effects of HIV-1 on neuroepithelial lineage cells in various stages of differentiation. In this investigation, primary cultures of differentiating human fetal NEP-derived cells were continuously exposed to macrophage-tropic HIV-1 strain SF128A or lymphotropic HIV-1 strain SF2 in the absence of detectable subpopulations of monocytes or microglia. Cultures were monitored for their ability to differentiate and for signs of neurotoxicity or induction of cell death pathways. Exposure to HIV-1 did not inhibit the elaboration of either neuronal or astrocyte lineage-committed cells from NEP cultures; but continued exposure appeared to selectively injure maturing neurons, depressing neuronal microtubule and neurofilament protein expression, and potentially impairing postmitotic neuronal dendrite and axon development.

Results

Cell antigen expression by differentiating NEP-derived cells exposed to HIV-1

Initial studies with mock-exposed (control) cultures confirmed that NEP differentiated into a mixed population of neurons and astrocytes in the minimum culture medium (MDM)/2.5% fetal bovine serum (fbs) differentiation medium (Figure 1). The intermediate filament protein nestin, a marker for neuroepithelial-derived progenitor cells (Reynolds and Weiss, 1992), was detected in adherent cell clusters within the first day of seeding (Figure 1A). Nestin then disappeared from cultures incubated beyond day 5. A monolayer of glial fibrillary acidic protein (GFAP)-expressing astrocytes with overlying neuronal lineage cells expressing β-III-tubulin was well developed by day 5 (Figure 1B). Hu antigens were detected in the nuclei of cells expressing β-III-tubulin or neurofilament (NF)-L by day 5, and Hu expression persisted during 3 weeks of culture (Figure 1C). Total cell numbers in mock-exposed (control) cultures started to decline by day 22 (see Figure 4B); thus a 22-day timeline was used for virus exposure studies.

Serial immunoblotting tracked the effect of HIV-1 exposure on cytoskeletal antigen expression in the differentiating NEP-derived cell cultures (Figure 2). Initially, HIV-1 exposure did not have a significant differential effect on the adherence of neurospheres, or on the expression of cytoskeletal antigens.

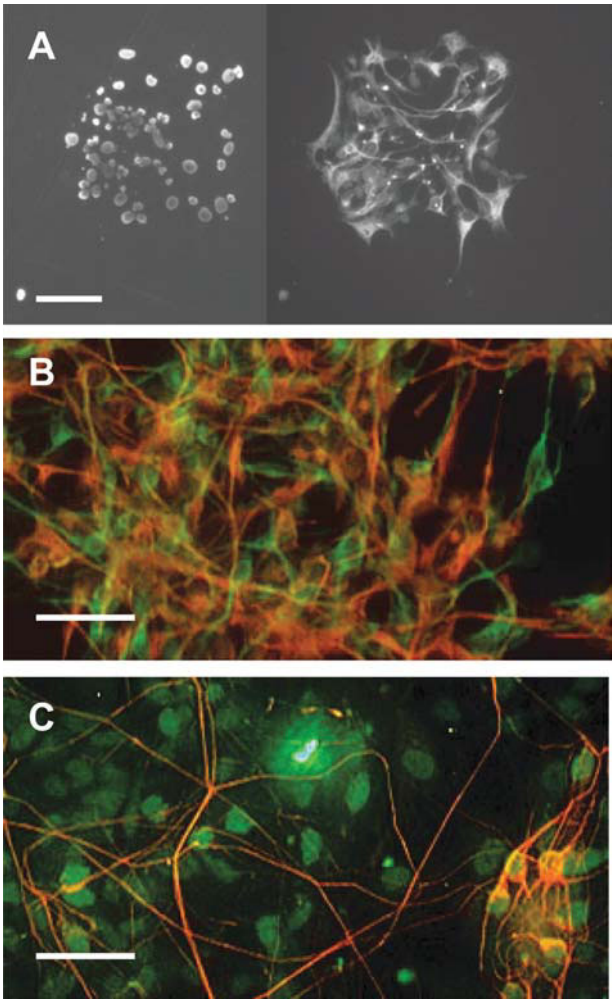


Figure 1 Indirect immunofluorescent antibody staining for cell antigen expression by human neurosphere-derived cultures incubated in MDM differentiation medium with low serum (2.5% fbs). (**A**) Cells derived from an adherent neurosphere at day 1 of culture in differentiation medium. Cells express nestin (*right panel*); left panel depicts DAPI stain for cell nuclei. Magnification bar = 100 μm. (**B**) Cell monolayer at day 5 of culture in differentiation medium containing astrocytes expressing GFAP (red, rhodamine) and neuronal lineage-committed cells expressing microtubular β-III-tubulin (*yellow-green*; fluorescein). (**C**) cell monolayer at day 22 of culture in differentiation medium. Post-mitotic neurons express Hu antigens in neuronal nuclei (*yellow-green*; fluorescein), and some of these additionally express NF-L in neuronal processes (*red*, rhodamine). Magnification bar in **B** and **C** = 50 μm.

Mock- or virus-exposed cultures harvested at day 0, that is, 3h after seeding of NEP cells, showed low-level expression of β-III-tubulin (Figure 2A), NF-L (Figure 2 B), or GFAP (Figure 2C) antigens. This was not virus specific, and resulted from stimulation of terminal differentiation of lineage-committed precursor cells present in the neurospheres at the time of adherence. Astrocyte or neuronal cytoskeletal antigens increased modestly through day 5 but were not affected by virus exposure. But by day 22, HIV-1–exposed cultures had less neuronal β-III-tubulin or NF-L compared to mock-exposed cultures (Figures 2A, B). β-III-Tubulin expression was approximately 60% lower in HIV-1(SF2)–exposed cultures and 10% lower in HIV-1(SF128A)–exposed cultures compared to mock-exposed cultures, though there was wider specimen-related variability in HIV-1(SF128A)-exposed cultures. Pairwise differences between mock-exposed and HIV-1(SF2)–exposed cultures were significant ($P \leq .05$, unpaired t test); differences between mock-exposed and HIV-1(SF128A)–exposed cultures were not significant ($P > .05$). NF-L expression at day 22 was approximately 75% lower in HIV-1(SF2)–exposed cultures and 45% lower in HIV-1(SF128A)–exposed cultures compared to mock-exposed cultures. As with β-III-tubulin expression, pairwise NF-L differences between mock-exposed and HIV-1(SF2)–exposed cultures were significant ($P \leq 0.05$, unpaired t test); differences between mock-exposed and HIV-1(SF128A)–exposed cultures were not significant. During the interval from days 5 to 22, NF-L expression in mock-exposed cultures increased by approximately 2½ fold. But exposure to HIV-1(SF2) resulted in relative loss of β-III-tubulin or NF-L between days 5 and 22, whereas exposure to HIV-1(SF128A) resulted in small increases in β-III-tubulin or NF-L that were below control levels. HIV-1 exposure had no significant effect on GFAP expression (Figure 2C) by day 22, and, overall, GFAP expression increased equally in mock- or virus-exposed cultures between days 5 and 22.

Immunofluorescent staining for neural cell antigen expression (Figure 3) showed a comparative loss of β-III-tubulin or NF-L intensity at day 21 in virus-exposed cultures. There was no virus-associated disruption in the intracellular distribution of these antigens. Reduction in β-III-tubulin or NF-L intensity was seen more readily in immunofluorescent images from HIV-1(SF2)–exposed cultures (Figure 3). GFAP was not visibly altered in either intensity or intracellular distribution in immunofluorescent images from cultures exposed to HIV-1 for 22 days. Neurite lengths were traced and measured in dual stained immunofluorescent images of cells coexpressing nuclear Hu and cytoplasmic β-III-tubulin antigens (Figure 4A). At day 22, HIV-1–exposed cultures showed no significant differences from mock-exposed cultures in mean neurite length (unpaired t test). But the mean total neurite length per cell varied significantly among the mock-exposed and viral-exposed cultures ($P = .0001$, one-way analysis of variance [ANOVA]). This value was significantly lower in HIV-1(SF2)–exposed neurons when compared to mock-exposed neurons (74 versus 97 μm; $P = .0001$ by unpaired t test), or when compared to HIV-1(SF128A)–exposed neurons (74 versus 86 μm; $P = .003$ by unpaired t test).

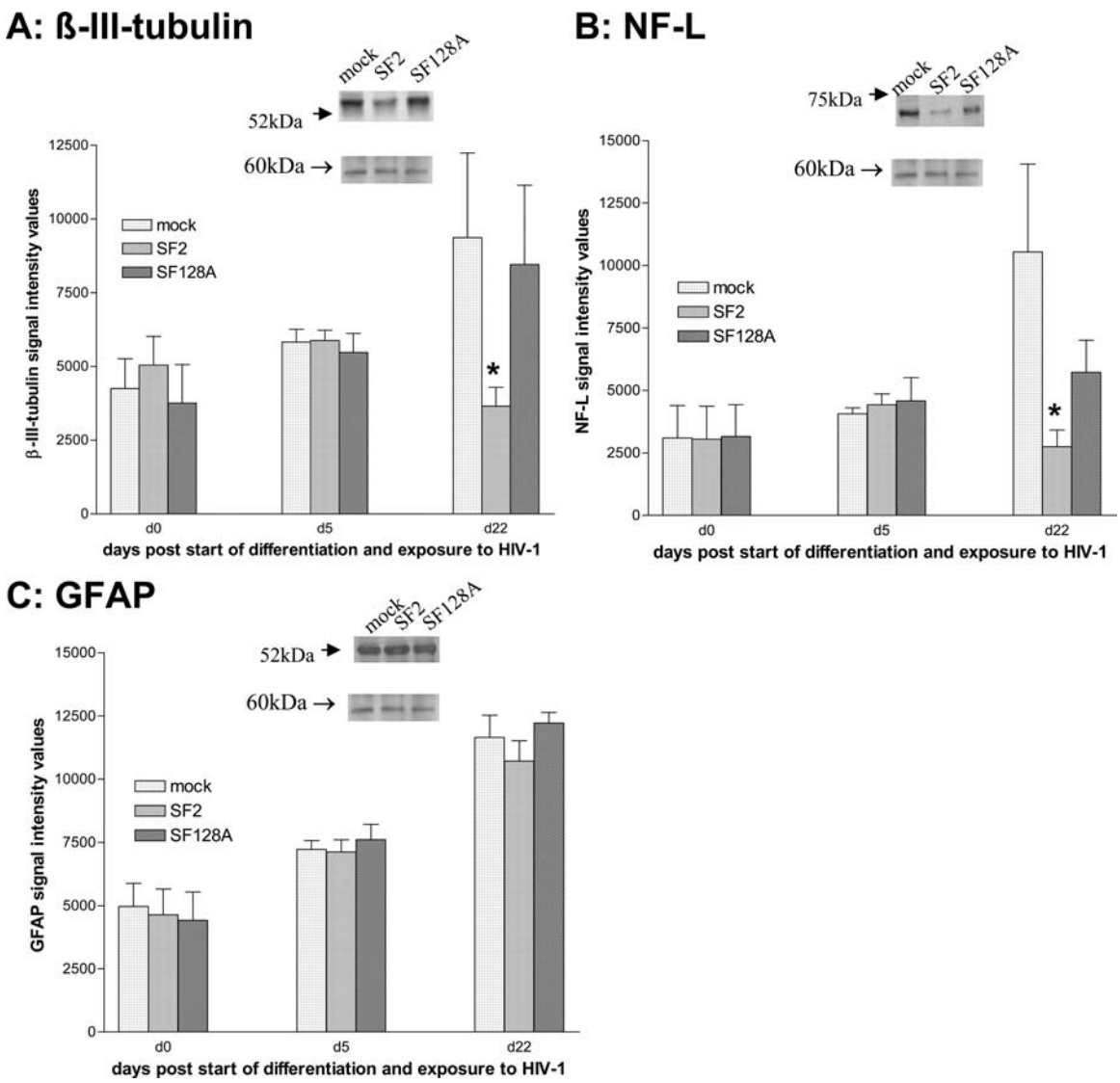


Figure 2 Quantitative determination of cell antigen expression by differentiating human NEP-derived cells exposed to HIV-1(SF2) or HIV-1(SF128A), or mock-exposed as described in Materials and Methods. Adherent neurosphere-derived cells were harvested after 3 h of culture in differentiating medium (d0), or 5 (d5) or 22 (d22) days of culture. Data are the mean of scaled signal intensity values ± standard error from 4 independent experiments. Immunoblot images are from individual representative cultures harvested at day 22 and include 60-kDa internal standard α-tubulin (Kusdra *et al*, 2002), and 52- and 75-kDa molecular weight marker positions. **A**: β-III-tubulin; **B**: NF-L; **C**: GFAP. *Values were significantly lower in HIV-1 (SF2)-exposed cultures when compared to mock-exposed cultures ($P \leq .05$ by unpaired t-test).

Neuronal cell numbers

Virus exposure could result in fewer neurons, either through failure of neurogenesis or diminished neuronal survival, or both. To examine this question, the population of neuronal lineage-committed cells defined by concomitant expression of nuclear Hu and cytoplasmic β-III-tubulin antigens was counted in digitally recorded, multiply stained immunofluorescent images. This cell population should represent late mitotic or postmitotic neurons derived from NEP cells (Marusich *et al*, 1994; Okano and Darnell, 1997). HIV-1–exposed cultures increased their total cell numbers by approximately 35% to 45% comparing day 22 to day 5, whereas mock-exposed cultures showed a 40% decrease in total cell numbers comparing day 22 to day 5 (Figure 4B). Nevertheless, the fractional number of Hu/β-III-tubulin–expressing neurons was similar among mock-exposed and virus-exposed cultures at days 5, 11, and 22 (Figure 4C). The neuronal fraction decreased by approximately 10% during that interval in mock-exposed as well as virus-exposed cultures, as postmitotic neurons ceased proliferation while astrocytes continued to proliferate. Two-way ANOVA analysis confirmed that time but not culture treatment (mock or virus exposure) had a significant impact on the fractional number of Hu/β-III-tubulin neurons ($P = .0017$). Consistent with this was the finding that virus exposure

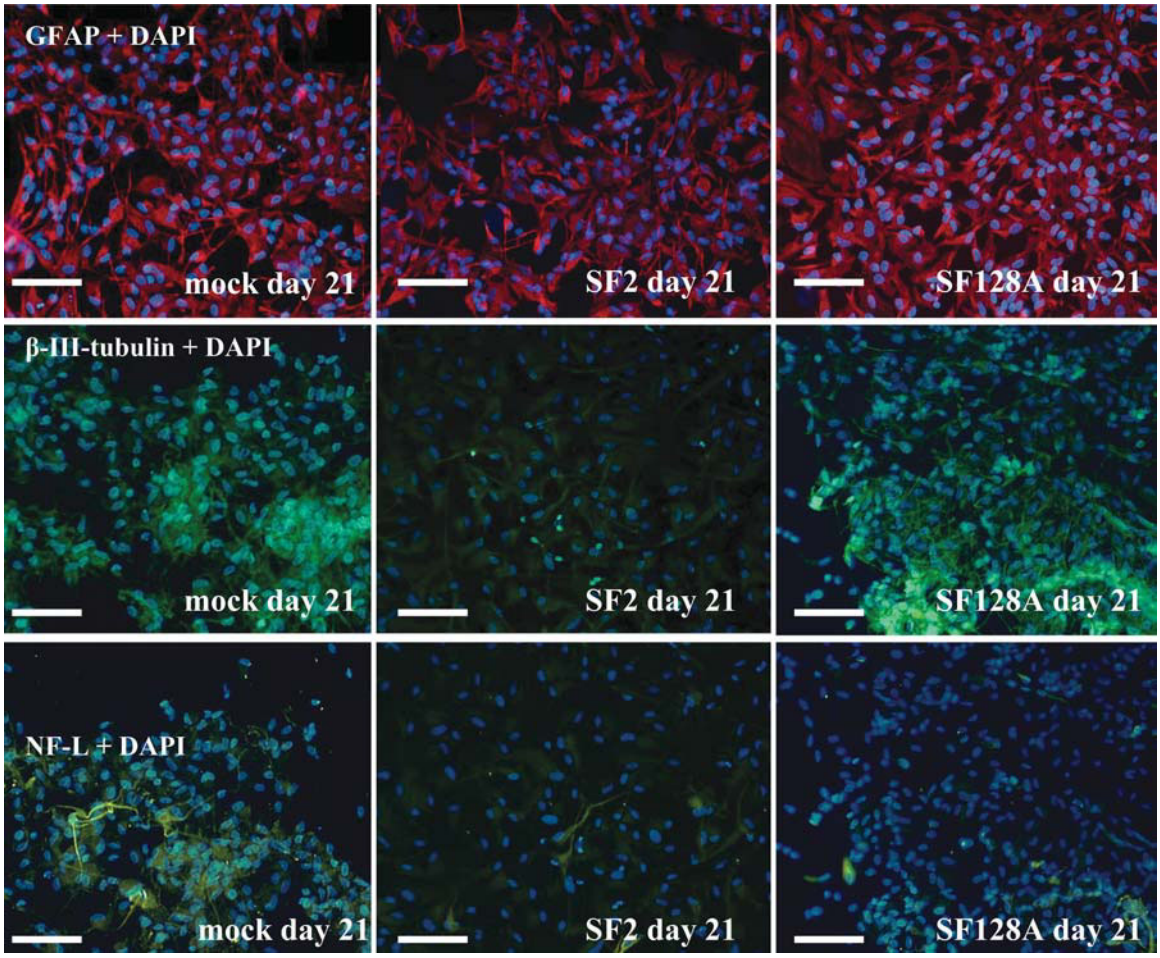


Figure 3 Indirect immunofluorescent antibody staining for cell antigen expression by differentiating human NEP exposed to HIV-1(SF2) or HIV-1(SF128A), or mock-exposed as described in Materials and Methods. Monolayers differentiated from adherent human neurospheres were fixed and stained after 21 days of culture in differentiation medium with low serum. All cultures were counterstained with DAPI (*blue-ultraviolet*) to detect cell nuclei. Upper panel depicts GFAP-expressing astrocytes (*red*; rhodamine). Middle panel depicts neuronal lineage-committed cells expressing β-III-tubulin (*yellow-green*; fluorescein). Lower panel depicts NF-L–expressing postmitotic neurons (*yellow-red*; Texas red). Magnification bars = 100 μm.

produced only transient, modest (1.5-fold) increases in caspase-3 activity in differentiating cultures between days 5 and 22 (Figure 5A). Activated caspases colocalized with GFAP-expressing cells, not with NF-L–expressing cells, when dually stained cultures were examined by fluorescent microscopy (Figure 5B).

Assay for HIV-1 DNA in virus-exposed, differentiating NEP-derived cells

To determine whether differentiating NEP-derived cells may have been directly infected by virus exposure conditions, the cultures were tested for p24 production and viral DNA expression. Differentiating, NEP-derived cultures, were continuously exposed to HIV-1(SF2) or HIV-1(SF128A) for 5 days, then washed and returned to differentiation medium without added virus supernatant. Cultures did not secrete p24 over the subsequent 10 days of culture (Figure 6). Moreover, viral *env* DNA sequences were not detected after nested polymerase chain reaction (PCR) of cellular DNA extracted from these virus-exposed cells (Figure 6). By contrast, when neurospheres were adhered and immediately adsorbed with HIV-1(SF2) or HIV-1(SF128A) for 3 h, then washed free of virus inoculum, differentiating cultures showed limited p24 production over 3 weeks. Viral *env* DNA sequences were detected at day 16 even though p24 production had declined. This is consistent with "restricted" HIV-1 infection previously described in human NEP cells (Lawrence *et al*, 2004) and in human fetal astrocytes (Tornatore *et al*, 1991; Blumberg *et al*, 1994; McCarthy *et al*, 1998).

Effect of antioxidant-enriched culture medium on cell antigen expression

Depression of NF-L expression was partially overcome by differentiating virus-exposed NEP-derived

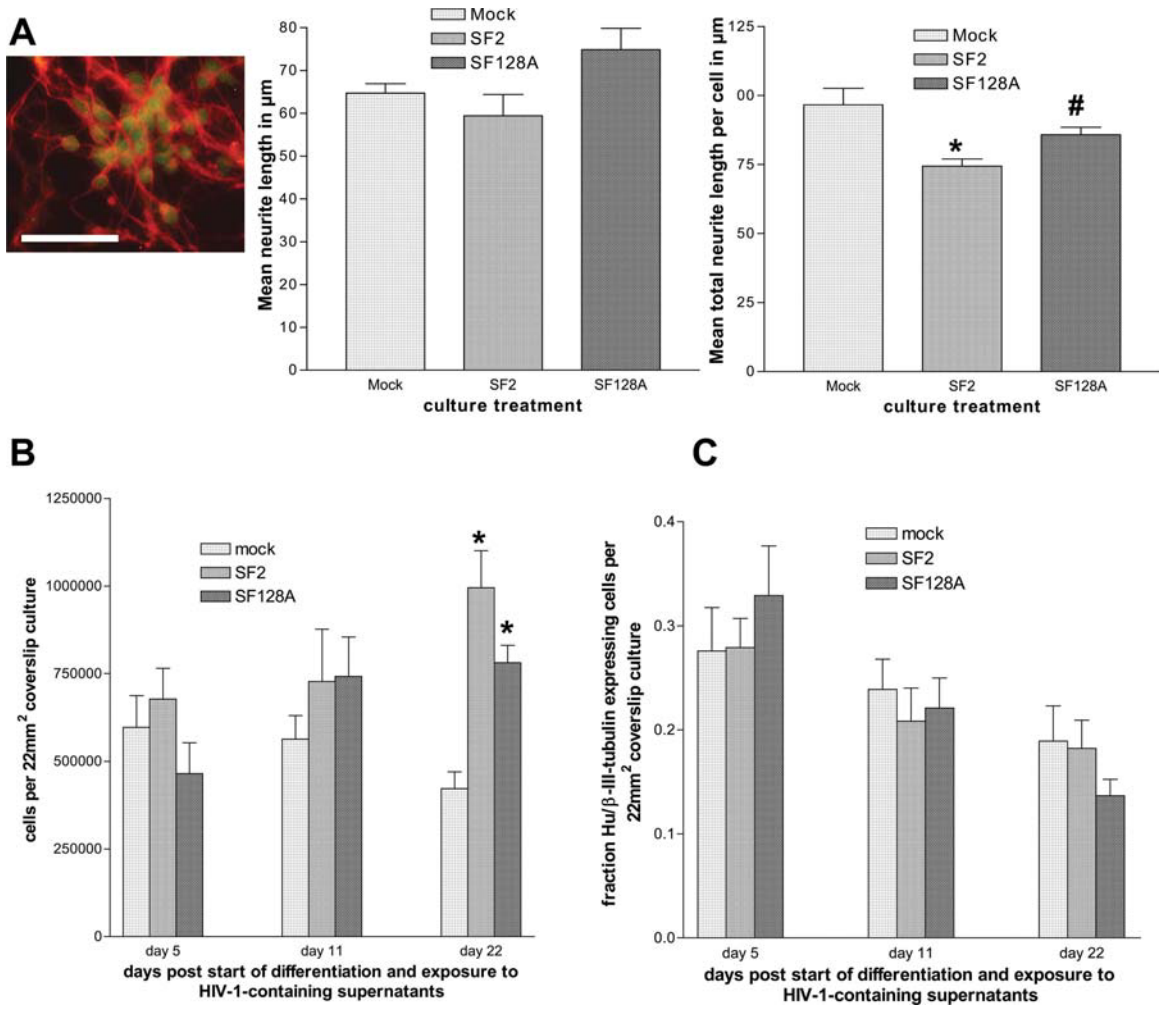
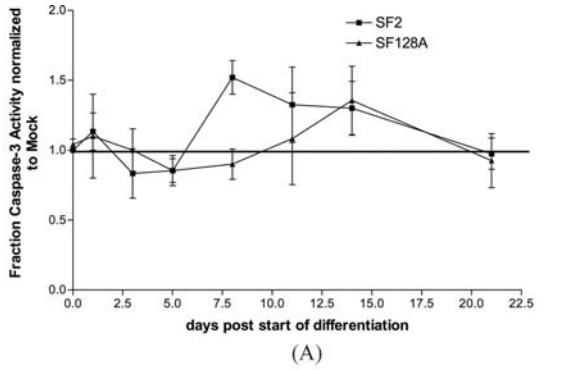
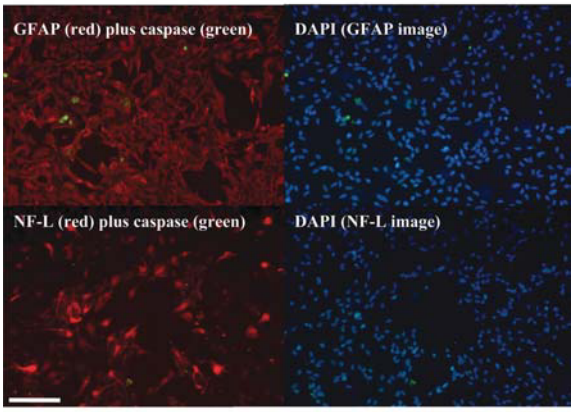


Figure 4 Image analysis of immunofluorescent stained monolayers differentiated from adherent human neurospheres in differentiation medium with low serum and with mock or viral exposure to HIV-1(SF2) or HIV-1(SF128A) as described in Materials and Methods. **A** Cells were fixed and stained after 22 days of culture, and neurons coexpressing β-III-tubulin (*red*; rhodamine) and nuclear Hu (*yellow-green*; fluorescein) antigens were scored for mean neurite length (*left graph*) or mean total neurite length per cell (*right graph*) ± standard error. Data from three independent experiments. Magnification bar = 100 μm. *Value was significantly lower in HIV-1(SF2)–exposed neurons when compared to mock-exposed neurons (74 versus 97 μm $P = .0001$ by unpaired t-test). #Value for HIV-1(SF128A)–exposed neurons was significantly higher when compared to HIV-1(SF2)–exposed neurons (86 versus 74 μm $P = .003$ by unpaired *t* test). **B**: Mean number of cells per 22-mm^2 culture ± standard error after indicated days of culture with mock or viral exposure to HIV-1(SF2) or HIV-1(SF128A) as described in Materials and Methods. *Values for HIV-1–exposed cultures were significantly higher than that for the mock-exposed culture at day 22 (one-way ANOVA at day 22 $P = .0094$ with Bonferonni's post tests, significant for mock versus SF2 [$P < .001$], mock versus SF128A ($P < 0.05$]). **C**: Number of neurons coexpressing β-III-tubulin and Hu expressed as mean fraction ± standard error of total cells per 22 mm^2 culture after indicated days of culture with mock or viral exposure. **B** and **C** include data from three independent experiments.

cells in an alternative differentiation medium, Neurobasal medium with B27 supplement (Gibco Invitrogen, Carlsbad, CA) and 2.5% fbs (Figure 7). Neurobasal with B27 supplement was developed to enhance neuronal survival (Brewer *et al*, 1993), and the B27 supplement contains corticosterone, triodothyronine, and antioxidants ("AO"), including glutathione, α-tocopherol, and superoxide dismutase. At day 22, the expression of NF-L was increased 1½- to 2-fold in virus-exposed cultures differentiated in Neurobasal/B27 with AO plus 2.5% fbs compared to virus-exposed cultures differentiated in MDM plus 2.5% fbs (Figure 7). This difference is statistically significant for HIV-1(SF2)–exposed cultures ($P \leq .05$, unpaired *t* test) and close to significant ($P = .0824$, unpaired *t* test) for HIV-1(SF128A)–exposed cultures. One-way ANOVA analysis of NF-L expression at day 22 found significant group variation among mock versus HIV-1(SF2)–versus HIV-1(SF128A)–exposed cells differentiating in MDM plus 2.5% fbs ($P = .0429$), but not in Neurobasal/B27 with AO plus 2.5% fbs ($P = .274$). There was still a trend, though not statistically signficant, to lower NF-L expression in the HIV-1–exposed versus mock-exposed cultures



(A)



(B)

Figure 5 (**A**) Temporal pattern of caspase-3 activity in lysates from differentiating NEP-derived cultures continuously exposed to HIV-1 strains. Data are the mean ± standard error of activity in virus-exposed cultures normalized to corresponding mock-exposed culture at each time point. Data are from five independent experiments. (**B**) Fluorescent microscopy to localize activated caspases in HIV-1(SF2)–exposed, differentiating NEP-derived cultures after 14 days. Live cultures were incubated for 30 min with fluorescein-conjugated VAD-FMK for intracellular localization of caspase activity (*green*; fluorescein). Then cultures were fixed for immunofluorescent staining with antibody to GFAP or NF-L. Images illustrate colocalization of activated caspases in astrocyte cell bodies. Magnification bar = 100 μm.

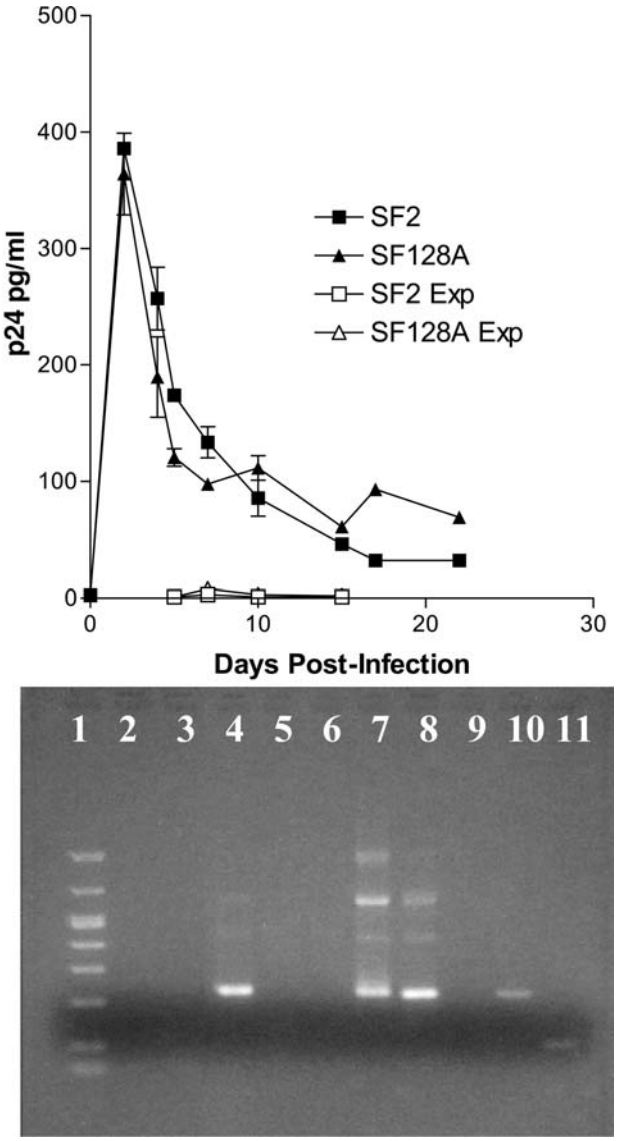


Figure 6 *Upper panel*: Time course of p24 production by cultures of differentiating human NEP-derived cells adsorbed with (*filled symbols*) or exposed to (*open symbols*) HIV-1(SF2) or HIV-1(SF128A) as described in Materials and Methods. Time points are the mean ± standard error of p24 values from two independent specimens. *Lower panel*: Attempted amplification of the 245-bp *env* DNA sequence from HIV-1 by nested PCR of cellular DNA extracted after 16 days of culture from NEP-derived monolayers adsorbed with or exposed to HIV-1(SF2) or HIV-1(SF128A). Lane 1, DNA molecular weight markers from 50–1000 bp; lane 2, negative control (water, no amplification); lane 3, negative control (nested PCR amplification using the negative control sample from the first reaction); Lane 4, nested PCR amplification of DNA from NEP-derived cultures adsorbed with HIV-1 and producing p24; lane 5, nested PCR amplification of DNA from SF2-exposed NEP-derived cultures not producing p24; lane 6, nested PCR amplification of DNA from SF128A-exposed NEP-derived cultures not producing p24; lanes 7, 8, positive controls (lane 7, nested PCR of DNA from SF2-infected PBMCs; lane 8, nested PCR of DNA from SF128A-infected PBMCs); lane 9, negative control (nested PCR of DNA from mock-infected PBMCs); lane 10, product of a single round of PCR using the env-3, env-4 primers with DNA from SF2-infected PBMCs; lane 11, 115-bp *gag* gene sequence amplified from the GeneAmplimer positive control DNA and HIV-1 SK38 and SK39 primers (Applied Biosystems, Foster City, CA).

incubated in Neurobasal/B27with AO plus 2.5% fbs. β-III-Tubulin expression was not consistently higher in cells differentiated in Neurobasal/B27 with AO plus 2.5% fbs, so no conclusions could be drawn about neuroprotection with respect to this neuronal microtubule protein. GFAP expression was equivalent in both differentiation media (data not shown).

To specifically test the contribution of the AO components in B27 supplement to the enhancement of neuronal survival, virus-exposed NEP-derived cells were also differentiated in medium using B27 formulated without the antioxidants ("B27 minus AO"; Gibco-Invitrogen). NEP-derived cultures differentiated in Neurobasal/B27 minus AO plus 2.5% fbs showed reduced and more variable levels of NF-L compared to those differentiated in Neurobasal/B27 with AO plus 2.5% fbs (Figure 7), and this effect was seen in mock- as well as virus-exposed cultures.

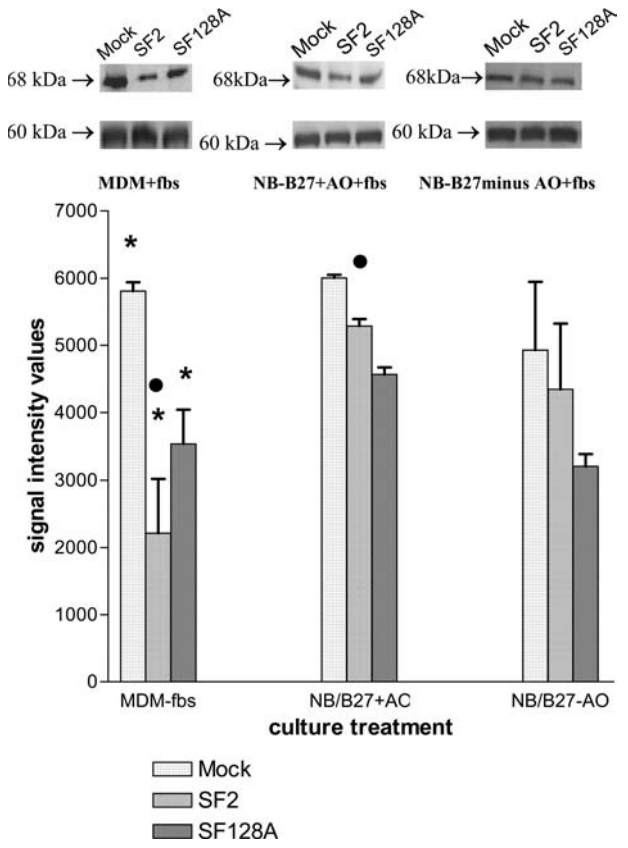


Figure 7 Quantitative NF-L expression at day 22 in NEP-derived cultures differentiated in MDM plus 2.5% fbs (MDM-fbs) versus Neurobasal (NB) medium containing B27 supplement with antioxidants (NB/B27 + AO) plus 2.5% fbs or B27 minus anti oxidants (NB/B27 − AO) plus 2.5% fbs. Data include the mean ± standard error of signal intensity values for mock-exposed or virus-exposed cultures. *Cultures differentiated in MDM-2.5% fbs show a significant group variation at day 22 (one-way ANOVA, $P = .0429$; mock versus SF2, $P < .05$ by Bonferonni post tests for select pairs). There is no significant group variation for the cultures differentiated in the Neurobasal-B27 medium, either with or without antioxidants. •HIV-1(SF2)-exposed cultures differentiated in NB/B27+AO plus 2.5% fbs had significantly higher levels of NF-L expression than cultures differentiated in MDM-fbs. Immunoblot images are from single representative experiments with cultures differentiated as indicated.

Both mock-exposed and (HIV-1)SF2–exposed cells showed approximately 20% reduction in NF-L, whereas (HIV-1)SF128A–exposed cells showed 30% reduction in NF-L when AO were not included in the B27 supplement. Because of this, NF-L expression in virus-exposed cultures differentiated in Neurobasal/B27 minus AO plus 2.5% fbs was not significantly increased over NF-L expression in virus-exposed cultures differentiated in MDM plus 2.5% fbs. Although there was a trend to lower NF-L expression in the HIV-1–exposed versus mock-exposed cultures differentiated in Neurobasal/B27 minus AO plus 2.5% fbs, one-way ANOVA analysis of NF-L expression at day 22 found no significant group variation ($P = .430$).

Cell antigen expression by differentiating NEP-derived cells exposed to HIV-1 gp120

Differentiating NEP-derived cultures were incubated with recombinant HIV-1(SF2) envelope glycoprotein gp120 to determine whether gp120 exposure alone would decrease neuronal antigen expression. Cultures incubated with rgp120 at 200 pM or 2 nM showed 25% to 40% less NF-L expression than mock-exposed cultures at day 22 (Figure 8). Depression of NF-L was reflected in the diminished intensity and density of NF-L-stained processes in immunofluoresent images from gp120(SF2)-exposed cultures (Figure 8), though measured mean neurite lengths were not significantly lower (data not shown). Expression of GFAP or β-III-tubulin was not significantly affected by gp120(SF2) exposure through day 22 (data not shown). Thus the effects of both HIV-1 and gp120 exposure are selective for neurons, but the effects could result from injury to developing neurons and/or impairment of the neuronal developmental process itself. The data do not exclude either mechanism.

Discussion

Exposure to lymphotropic or macrophage-tropic HIV-1 did *not* inhibit the emergence of astrocytes or neurons from human fetal-derived neuroepithelial progenitor and precursor cells that were stimulated to differentiate in low serum conditions. With HIV-1 added to the differentiation medium, adherent neurospheres gave rise to cell populations that included GFAP-expressing astrocytes, and neurons expressing postmitotic nuclear (Hu) and cytoskeletal (NF-L) antigens. The emergence of postmitotic neuronal antigens suggests that virus-exposed, neuronal-restricted precursor cells were able to mature into terminally differentiated neurons. However, with continued exposure to HIV-1, neurons "failed to thrive," and signs of neuronal injury emerged, particularly with exposure to lymphotrophic HIV-1(SF2). Between days 5 and 22 of culture, the expression of neuronal cytoskeletal antigens in cultures exposed to HIV-1(SF2) declined to less than half that of control cultures (Figure 2). Although prolonged exposure to HIV-1 (up to 22 days) did not significantly alter the overall morphology of astrocytes or neurons differentiated from neurospheres, immunofluorescent images showed decreased intensity of neuronal β-III-tubulin (microtubule) or NF-L (intermediate filament) immunoreactivity (Figure 3). Moreover, image analysis showed that the mean total neurite length per cell in postmitotic (Hu-expressing) neurons was significantly decreased in cultures exposed to either HIV-1 strain (Figure 4). Neither astrocyte morphology, immunofluorescent GFAP staining intensity, or quantitative GFAP expression was affected by HIV-1 exposure. Thus the neuronal-lineage cells in these differentiating NEP-derived cultures were selectively vulnerable to HIV-1 exposure. Moreover, the signs of

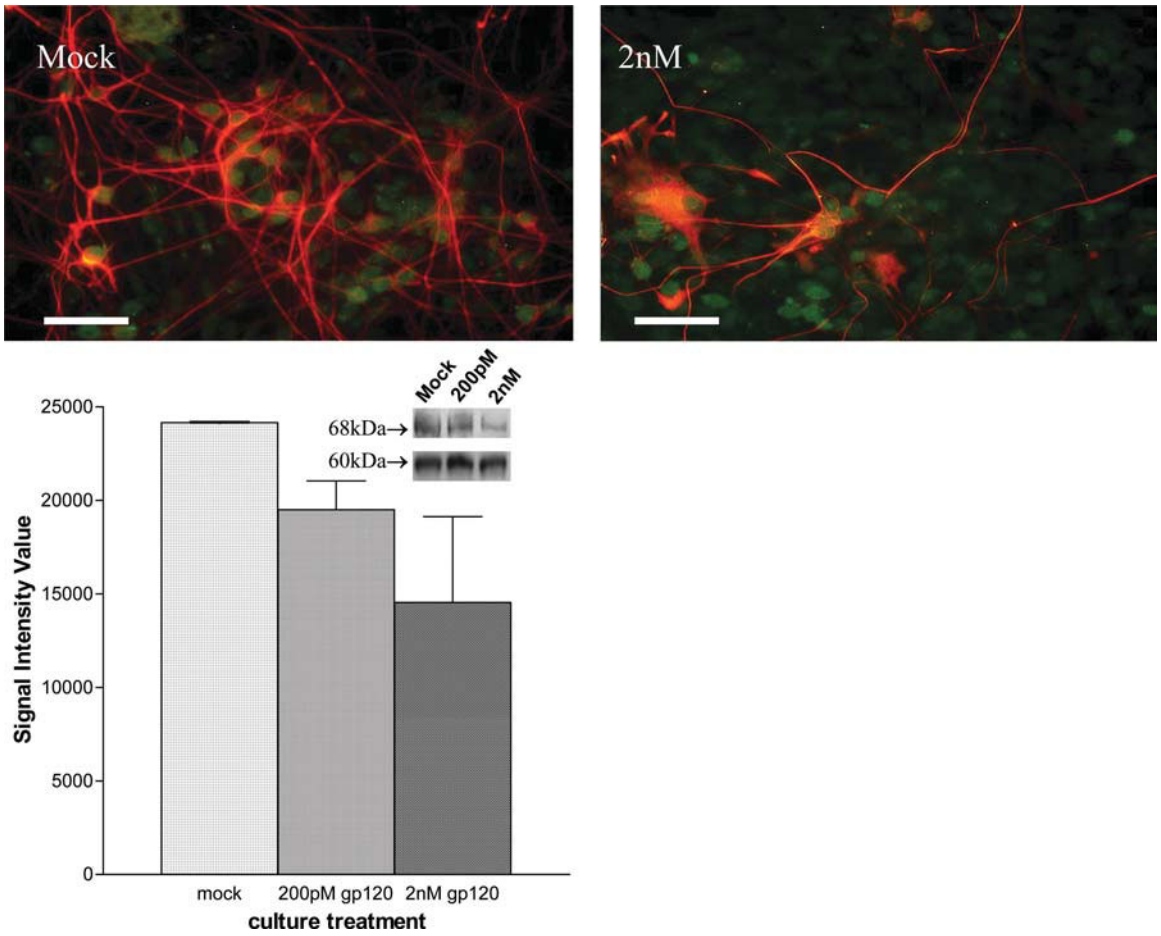


Figure 8 NF-L expression in differentiating human NEP-derived cultures exposed to $gp120_{SF2}$ for 22 days. Micrographs depict mock-exposed cultures and cultures exposed to 2nM $gp120_{SF2}$. Neurons coexpressed NF-L in cell processes (*red*; rhodamine) and Hu in cell nuclei (*yellow-green*; fluorescein). Magnification bar = 50 μm. Mean NF-L expression (*graph*) at day 22 was decreased in cultures exposed to either 200 pM or 2 nM $gp120_{SF2}$, though group or pairwise differences did not reach statistical significance. Data are mean ± standard error from two independent specimens.

neuronal injury, particularly the decline in neuronal cytoskeletal antigen expression, emerged between days 5 and 22 of culture, when quantitative NF-L expression in control (mock supernatant–exposed) cultures doubled. In that NF-L is an early postmitotic neuronal antigen, this timing suggests that HIV-1 exposure disproportionately affected terminally differentiated neurons withdrawing from the cell cycle. As suggested by prior pathological (Masliah *et al*, 1997; Everall *et al*, 1999) and *in vitro* studies (Iskander *et al*, 2004), these data indicate that HIV-1 exposure affects neuronal cell processes, and are consistent with the hypothesis that changes in the proteins found in neuronal cell processes may reflect early primary neuronal injury (Iskander *et al*, 2004).

The decline in neuronal antigen expression was not due to selective neuronal cell loss. There was no significant change in the fractional number of β-III-tubulin/Hu–expressing neurons in differentiating cultures continuously exposed to HIV-1, and no evidence of neuronal apoptosis. After approximately 11 days of culture, the neuronal fraction declined, but equally so in mock- and HIV-1–exposed cultures, and was due to a relative increase in astrocytes. From days 5 to 22 of culture, total cell numbers increased approximately 35% to 45% in HIV-1–exposed versus mock-exposed cultures, whereas the neuronal fraction remained constant (Figures 4B, C). This suggests that HIV-1 exposure did not inhibit proliferation of neuronal or astroglial lineage restricted precursors in differentiating NEP-derived cultures. Because extracellular signal-regulated kinase (ERK)1 (p44) or ERK2 (p42) signaling can subserve cell proliferation or differentiation (Schaeffer and Weber, 1999), we used immunoblotting to assess ERK and phosphorylated ERK expression in differentiating NEP-derived cultures (data not shown). Although there was a trend to higher levels of phosphorylated ERK in virus-exposed cultures at day 22, the differences were not statistically significant and therefore not conclusive. However, the trend to higher levels of phosphorylated ERK at day 22 is consistent with previous

studies (Lannuzel *et al*, 1997) and consistent with the finding of greater cell proliferation in virus-exposed cultures.

We did not observe inhibition of NEP adherence or NEP proliferation with either HIV-1 or gp120 exposure, as was reported by Krathwohl and Kaiser (2004). However, the culture conditions used in this study emphasized HIV-1 exposure during differentiation conditions, and our data do not rule out an inhibitory effect of HIV-1 or gp120 exposure on NEP proliferation under conditions that favor progenitor self-renewal.

The observed effects of HIV-1 exposure were independent of HIV-1 infection of differentiating NEP-derived cells. After prolonged exposure to HIV-1, cultures did not secrete p24 and did not express viral DNA. Lawrence *et al* (2004) first reported that multipotent NEP cells are permissive for "restricted" HIV-1 infection, with limited p24 production that could be enhanced by cytokine exposure or directed differentiation of NEP to the astrocyte phenotype. We also observed limited p24 production and viral *env* gene expression in differentiated cultures under conditions in which multipotent NEP were first adsorbed with lymphotropic or macrophage-tropic HIV-1 inoculum, then washed extensively prior to addition of differentiation medium (Figure 6). However, the experimental design used to "expose" NEP-derived cells to HIV-1 in this study (dilution of viral stock into differentiation medium) did not facilitate infection of these cells.

The neuroprotective effect seen with antioxidant (AO) supplementation of differentiation medium (Figure 7) implies that HIV-1 exposure causes oxidative stress on maturing neurons in NEP-derived cell cultures. After 22 days of culture, NF-L expression was increased by twofold in HIV-1(SF2)–exposed cultures and by approximately 30% in HIV-1(SF128A)–exposed cultures differentiated in Neurobasal/B27 with AO plus 2.5% fbs compared to corresponding cultures differentiated in MDM plus 2.5% fbs. The B27 supplement provides anti-oxidants such as glutathione, α-tocopherol, and superoxide dismutase to the culture medium. Removal of the anti-oxidants from B27 (Figure 7) results in lower and more variable levels of NF-L expression in both mock- and virus-exposed cultures. However, NB/B27 minus AO medium still appears to provide some neuroprotective effect during virus exposure. NF-L expression still trends higher in HIV-1(SF2)–exposed cultures differentiated in NB/B27 minus AO plus 2.5% fbs compared to HIV-1(SF2)–exposed cultures differentiated in MDM plus 2.5% fbs (Figure 7); this difference may be due to the effects of other distinguishing components found in B27, such as L-carnitine or triodothyronine (Brewer *et al*, 1993). A similar neuroprotective effect has been described for the antioxidant ascorbate, a scavenger of reactive nitrogen and oxygen species (Walsh *et al*, 2004). Ascorbate pretreatment suppressed astrocytic hypertrophy and neuronal dendritic injury seen in primary mixed human fetal forebrain cultures exposed to 1 nM $gp120_{SF2}$ for 24 h (Iskander *et al*, 2004; Walsh *et al*, 2004). These cultures have been reported to have a high degree of neuronal differentiation (Pulliam *et al*, 1988; Hammond *et al*, 2002). In these cultures, exposure to $gp120_{SF2}$ upregulated inducible nitric oxide synthase (iNOS) in astrocytes; this up-regulation was attenuated with ascorbate pretreatment. HIV-1 gp120 has also been reported to exert neurotoxic effects by potentiating free radical–based oxidative damage in monocytes (Foga *et al*, 1997). However, with increased iNOS and nitric oxide production by astrocytes, oxidative and nitrosative stress could accumulate in the absence of monocytes or microglia (Hori *et al*, 1999). Our data confirm a role for $gp120_{SF2}$ in suppressing NF-L expression; this could be the result of cumulative oxidative and nitrosative stress induced in differentiating NEP-derived cells continuously exposed to HIV-1. Similarly the HIV transmembrane protein gp41 (Adamson *et al*, 1999) and the transactivating protein Tat (Aksenov *et al*, 2001; Hayman *et al*, 1993) have also been shown to cause increased oxidative and nitrosative stress in brain. In addition, patients with HIV dementia have evidence of large increases in oxidative stress parameters in brain and cerebrospinal fluid (CSF) (Turchan *et al*, 2003).

Moreover, the data also suggest a role for chemokine receptor CXCR4 in mediating the neurotoxic effects of HIV-1 exposure. This chemokine receptor has been detected on the majority of human NEP and NEP-derived precursor cells in culture (Lawrence *et al*, 2004). Signs of neuronal injury to maturing neurons were quantitatively significant after exposure to lymphotrophic strain HIV-1(SF2), which has been characterized as a syncytia-inducing strain that can use CXCR4 or CCR5 as coreceptor (Trkola *et al*, 1998). Exposure to CCR5-using HIV-1(SF128A) did suppress neuronal antigen expression, but to a lesser extent. Krathwohl and Kaiser (2004) reported that CXCR4 but not CCR5 mediated the inhibitory effect of gp120 proteins on NEP proliferation, consistent with a role for CXCR4 in mediating injury to NEP-derived cells. The CXCR4 ligand SDF-1 alpha has also been shown to cause neurotoxicity and has been implicated in the pathogenesis of HIV dementia (Kaul and Lipton, 1999; Rostasy *et al*, 2003; Zhang *et al*, 2003) as well as impairing neurogenesis (Tran and Miller, 2005)

Expression of the 68 to 70-kDa "light" isomer of neurofilament protein (NF-L) was particularly sensitive to HIV-1 exposure. NF-L expression declined relative to controls when NEP-derived cultures were exposed to either strain of HIV-1 or to $gp120_{SF2}$; however, NF-L expression was twofold lower with exposure to lymphotrophic HIV-1(SF2), and this reflected net loss of NF-L in the neuron population. Also, cultures incubated with $gp120_{SF2}$ at 200 pM or 2 nM for 22 days showed 25% to 40% less NF-L

expression than control cultures (Figure 8). This implies that the neurotoxic effects of HIV-1 exposure are mediated at least in part by gp120. Expression of the microtubule antigen β-III-tubulin was more variable with HIV-1 or $gp120_{SF2}$ exposure. However, β-III-tubulin is expressed by both proliferative neuronal restricted precursor cells (Piper *et al*, 2001) as well as Hu-expressing "mature" neurons, and so reflects a heterogeneous and dynamic neuronal lineage-restricted cell population. As an early postmitotic intermediate filament antigen, NF-L may be a sensitive marker for primary events in HIV- or gp120-induced neuronal injury affecting neuronal morphology and the structural integrity of neuronal processes, particularly axons. Nosheny *et al* (2004) found that gp120 from lymphotrophic strain IIIB caused a dramatic and persistent decrease in neurotrophic factor levels when injected into rat striatum; this decrease localized to neuronal processes. Local reduction of neurotrophic factors could lead to reduction in expression of the cytoskeletal proteins that maintain neuronal cell processes, including NF-L, which maintains the core of the neurofilament (Hoffman *et al*, 1987; Shaw, 1991). NF-L has been postulated as a marker for axonal injury in AIDS and other neurological diseases with neurodegenerative and/or inflammatory components, including multiple sclerosis (Malmestrom *et al*, 2003) and amyotrophic lateral sclerosis (ALS) (Rosengren *et al*, 1996). Elevations of CSF NF-L levels have been reported to be more frequent in patients with AIDS (12 of 18 patients) versus asymptomatic HIV-1 infection (3 of 12 patients) (Hagberg *et al*, 2000). CSF NF-L levels were noted to rise after structured anti-retroviral treatment interruption in 3 of 8 patients (Gisslen *et al*, 2005). However, rising CSF NF-L was not clearly associated with CSF markers of inflammation (e.g., leukocyte count) or neurological deterioration (Gisslen *et al*, 2005). Our study demonstrated neuronal loss of NF-L with HIV-1 exposure in the absence of detectable infected or immune activated monocytes or microglia in virus-exposed cultures of astrocytes and neurons. This implies that HIV-1–mediated neuronal injury can occur without the expansion or infiltration of monocyte-lineage cell subpopulations, such as occurs with CNS inflammation that may accompany primary HIV-1 infection or immune reconstitution after antiretroviral therapy (Torre *et al*, 2005). Although we cannot rule out the possibility that trace subpopulations of monocyte-lineage cells contributed to the observed depression of NF-L expression, our data nevertheless provide *in vitro* support for the further study of CSF NF-L levels in all stages of HIV-1 infection.

Neuroepithelial progenitor and precursor cells may be a resilient neural cell population, capable of proliferating and differentiating after HIV-1 exposure (Krathwohl and Kaiser, 2004) or during HIV-1 infection (Lawrence *et al*, 2004). Our study and these recent studies suggest that HIV-1 exposure does not irreversibly block neurogenesis by NEP cells derived from human fetal CNS. However, the data in this study imply that HIV-1 and gp120 exposure may injure developing neurons and/or impair the neuronal developmental process itself. HIV-1 exposure has intrinsic neurotoxicity, likely related to the capacity of viral proteins to induce oxidative and nitrosative stress in populations of astrocytes and neurons. Our study has shown that newly differentiated postmitotic neurons show subtle signs of injury affecting neuronal processes, which suggests that neuronal function will be impaired even as new neurons might arise to replace those lost. Thus, ongoing HIV-1 infection in the brain and the production of viral proteins is less a threat to NEP and the genesis of new neural cells, and more an ongoing threat to the differentiated neuronal population.

Materials and methods

Culture and differentiation of human neuroepithelial progenitor and precursor cells

Isolation and culture of human NEP cells *in vitro* has been confounded by their very limited capacity to proliferate. However, chronic exposure to epidermal growth factor (EGF) and/or fibroblast growth factor 2 (FGF2) will generate mitotically active NEP cells with the potential to differentiate into lineage-committed precursor cells, which in turn can give rise to terminally differentiated neurons or astroglia (Gage *et al*, 1995; Whittemore and Snyder, 1996; Quinn *et al*, 1999). NEP cells were prepared from mechanically dissociated first trimester (7 to 13 weeks' gestation) fetal human brain as previously described (Quinn *et al*, 1999; McCarthy *et al*, 2000). Cells derived from distinct fetal specimens were cultured separately. Cultures were seeded into 100-mm tissue culture dishes at a density of 5×10^5 cells per milliliter and grown in suspension in either Neural Progenitor Maintenance Medium (NPMM Bullet Kit; Clonetics Division of Cambrex Bioscience, Walkersville, MD) or Neurobasal medium plus B27 supplement (Brewer *et al*, 1993) (Gibco Invitrogen). These defined media were supplemented with epidermal growth factor (EGF) and basic fibroblast growth factor (FGF2). Within a day of seeding, monocyte-lineage cells in the mechanically dissociated cell suspension differentially adhered to the tissue culture dish surface, while NEP cells remained in suspension. Proliferating NEP cell clusters ("neurospheres") developed within 14 to 17 days. Neurospheres were cultured and subcultured in suspension, which further selected against the survival of monocyte lineage cells among the NEP cells. The neurospheres derived from a single fetal specimen typically generated approximately 5×10^7 viable cells.

Neurospheres in suspension maintained an undifferentiated or early differentiated state and expressed nestin (McCarthy *et al*, 2000), an intermediate filament present in NEP (Reynolds and Weiss, 1992) and

reported in human fetal neuronal and glial lineage-committed precursor cell populations (Messam *et al*, 2000). Neurospheres also expressed microtubule protein β-III-tubulin, a cytoskeletal marker that appears in neuronal lineage-committed precursor cells ("immature" neurons) as well as differentiated neurons. To stimulate differentiation, neurospheres were seeded on an adherent substrate (poly-D,L-ornithine plus fibronectin-coated glass or plastic) at 10^6 cells per 0.5 ml minimal culture medium (MDM) per 9 cm^2 surface. MDM consisted of equal volumes Dulbecco's modified Eagle's medium (DMEM) and F12 nutrient mixture (DMEM/F12) supplemented with 1% (*w*/*v*) bovine serum albumin (BSA), glutamine, and N2 supplement (all from Gibco Invitrogen). After 1 h, excess liquid was removed and cultures were established in differentiation medium, consisting of MDM supplemented with 2.5% (*v*/*v*) heat-inactivated fetal bovine serum (fbs). Differentiation in low serum gave rise to monolayer cultures with a mixed population of neuronal lineage- and astroglial lineage-committed cells within 1 day post seeding (McCarthy *et al*, 2000). Astrocyte lineage-committed cells expressed the 52-kDa intermediate filament antigen glial fibrillary acidic protein (GFAP). Neuronal lineage-committed cells expressed the 52- to 54-kDa isoform of β-III-tubulin. Postmitotic neurons were detected by expression of the 68-kDa neurofilament protein (NF-L). Late mitotic and postmitotic neuronal cells expressed nuclear Hu antigens, neuronal RNA-binding proteins (Marusich *et al*, 1994).

Differentiating NEP-derived cultures were monitored for the presence of monocyte-lineage cells, including microglia and macrophages, by two methods. (1) Live cultures seeded on 22-mm^2 glass cover slips were incubated for 4 h with Dil-Ac-LDL (1,1′-dioctadecyl-3,3,3′3′-tetramethylindocarbocyanine perchlorate; Biomedical Technologies, Stoughton, MA), then fixed and counter-stained with 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA) to detect all cell nuclei. This acetylated low density lipoprotein is labeled with a fluorescent probe (Dil) that is visualized with standard rhodamine excitation:emission filter. The lipoprotein is preferentially degraded by lysosomal enzymes so that the Dil accumulates in the intracellular membranes of macrophages, thus specifically distinguishing macrophages from NEP-derived cells. (2) Alternatively, acetone-methanol fixed cultures on 22-mm^2 glass coverslips were subjected to immunofluorescent stain for human CD68 antigen with a mouse monoclonal antibody (DAKO, Carpinteria, CA) and counterstained with DAPI to detect all cell nuclei. In both instances, positive control cultures for monocyte-lineage cell staining included human macrophages or human microglia. Human macrophages were prepared by adhering and differentiating macrophages from human peripheral blood mononuclear cell (PBMC) cultures (McCarthy *et al*, 2002). Human microglia were prepared from human lumbar spinal cord as previously described (McCarthy *et al*, 1998). Immunofluorescent cultures were visualized with epifluoresence optics, and microscopic fields were recorded digitally (detailed methods below). A minimum of 2500 cells were counted and scored for the presence of each monocyte-specific stain. By both methods, no monocyte-lineage cells were detected in differentiating NEP-derived cultures.

Preparation of HIV-1 virus and virus-exposed cell cultures

The lymphotropic strain HIV-1(SF2) or the macrophage-tropic strain HIV-1(SF128A) (Liu *et al*, 1990) were grown in cultures of human PBMCs mitogenically activated for 24 h with 5 μg/ml phytohemaglutinin (PHA) and 10 U/ml interleukin 2 (IL-2; both purchased from Invitrogen) and then maintained in 10 U/ml IL-2 as previously described (McCarthy *et al*, 1998). Control preparations ("mock supernatant") were derived from parallel, matched cultures of PBMCs activated in the same manner and mock infected. Culture supernatants were collected from viral-infected or mock-infected PBMC cultures and clarified by low-speed centrifugation. Infectious titers of viral stocks were determined by limiting dilution assay in PBMC cultures (McCarthy *et al*, 2002); virus stocks were also titered for p24 antigen by enzyme-linked immunosorbent assay (ELISA) (Beckman-Coulter, Miami, FL).

To generate virus-exposed cultures, aliquots of virus supernatant were diluted 40-fold into differentiation medium 1 h after neurospheres were seeded on adherent substrate. The final HIV-1 p24 antigen concentration was 10 to 20 ng p24 per 10^6 cells per 2 ml culture fluid. To generate mock-exposed cultures that would control for virus-specific effects, aliquots of mock supernatant were diluted 40-fold into the differentiation medium of parallel cultures 1 h after neurospheres were seeded. Replicates (duplicates) of viral-exposed or mock-exposed cultures were then incubated for up to 22 days, with change of medium and replenishment of HIV-1 or mock supernatant aliquots every 8 days. For serial determination of experimental parameters, replicate mock and virus-exposed cultures were harvested 3 h after neurospheres were seeded (day 0), and after additional days of culture in differentiation medium. Each experimental paradigm was conducted independently, i.e., with cells derived from distinct tissue specimens, a minimum of three times, excepting the experiments to specifically test the contribution of the AO components in B27 supplement to the enhancement of neuronal survival (Figure 7), which were conducted two times. The following reagent was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: recombinant gp120 derived from HIV-1(SF2) and expressed in chinese hamster ovarian (CHO) cells ($rgp120_{SF2}$) from Dr. Kathelyn Steimer. Soluble $rgp120_{SF2}$ was

diluted with mock supernatant into differentiation medium 1 hour after neurospheres were seeded. One half volume of culture medium and $rgp120_{SF2}$ plus mock supernatant aliquots were replaced weekly.

Determination of p24 antigen

To assay for any endogenous p24 produced by cells exposed to HIV-1, differentiating cultures were incubated for 5 days with HIV-1(SF2) or HIV-1(SF128A) diluted into differentiation medium as described above. Virus-containing culture fluids were then removed, and the cultures were washed eight times with phosphate-buffered saline (PBS). Cultures were then replenished with fresh differentiation medium without any HIV-1 or mock supernatant aliquots. Culture fluids were subsequently sampled at day 7 and later to assay for p24 (Figure 6). To assay for endogenous p24 produced by adsorbing cells with HIV-1 (Lawrence *et al*, 2004), neurospheres were seeded onto adherent substrate, then inoculated with stock HIV-1 (approximately 50 ng p24 in 50 μl) or mock supernatant (50 μl) diluted in MDM to an inoculum volume of 0.7 ml per 10^6cells per 9 cm^2. After 3 h, unadsorbed virus was removed by washing cultures eight times with PBS. The eighth wash fluid was used to determine the baseline (day 0) p24 value. Washed cultures were then incubated for up to 3 weeks in differentiation medium, with change of medium every 8 days. Supernatant aliquots were removed at various times for soluble p24 assay (Figure 6).

Amplification of HIV-1 proviral DNA from NEP-derived cell cultures

To determine whether HIV-1-exposure would lead to HIV-1 infection of differentiating NEP-derived cells, cellular DNA was assayed for viral sequences by polymerase chain reaction (PCR) amplification. Differentiating cultures were exposed to differentiation medium with HIV-1 or mock supernatants for 5 days, then differentiation medium was removed and cultures were washed eight times with PBS. Cultures were then replenished with fresh differentiation medium without any HIV-1 or mock supernatant aliquots, and incubation was continued for an additional 10 days. Cellular DNA was then harvested after a total of 16 days of culture in differentiation medium. The conditions for PCR were as previously described (McCarthy *et al*, 1998). Nested PCR was performed with the Taq (DNA) polymerase (Invitrogen) using two nested pairs of primers from the *env* gene. (Sequence locations based on HIV-1 HXB2 genome, National Center for Biotechnology Information GenBank database, http://www.ncbi.nlm.nih.gov/, accession number K03455.) Initially a 688-bp product was amplified using oligonucleotide primer pairs env-1 (5′ gtatcaactcaactgctgttaaatggcagt 3′) and env-2 (5'atgagggacaattggagaagtgaattata 3′) located between nucleotides 6987 and 7675 of HXB2. Further amplification of a 245-bp product was obtained using the nested oligonucleotide primers env-3 (5′ cggaattcggcagaagaagaggtagtaattag 3′) and env-4 (5′ cacaatggaataacactctagaaca 3′) located between nucleotides 7010 and 7255 of HXB2. PCR products corresponding to 688 bp for the first reaction and 245 bp for the nested reaction were separated by electrophoresis on 2% low-melting point agarose gels (NuSieve; FMC BioProducts, Rockland, ME), then visualized with ethidium bromide.

Immunocytochemistry

Intracellular expression of cellular structural antigens was studied by indirect immunofluorescent assay as previously described (McCarthy *et al*, 2000). Cultured cells were fixed with 4% (*w*/*v*) paraformaldehyde in neutral phosphate buffer, then permeabilized with 0.2% (*v*/*v*) Triton X-100 in PBS. The intermediate filament protein nestin was detected with a rabbit polyclonal immunoglobulin G (IgG) antibody (Hockfield and McKay, 1985), kindly provided by Dr. Ronald D. G. McKay (National Institutes of Health, Bethesda, MD). The 54-kDa microtubule antigen isoform β-III-tubulin was detected with a mouse monoclonal IgG_1 antibody (TUJ1; Promega, Madison, WI), or with a polyclonal rabbit antibody preparation (Covance, Richmond, CA), which recognizes the same epitope as the monoclonal antibody. The 68 to 70-kDa neurofilament antigen (NF-L) was detected with a mouse monoclonal IgG_1 antibody (clone DA2; Zymed, South San Francisco, CA). Alternatively, the 68-kDa neurofilament antigen, NF-L, was detected with a rabbit polyclonal IgG kindly provided by Dr. John Trojanowski (University of Pennsylvania, Philadelphia, PA). The 52-kDa astrocyte intermediate filament antigen GFAP was detected with a mouse monoclonal IgG_1 antibody (Roche Molecular Biochemicals, Indianapolis, IN), or with a rabbit polyclonal IgG (DAKO). Monoclonal and polyclonal antibody preparations produced superimposable immunofluorescent staining patterns for both anti-GFAP or anti-β-III-tubulin (data not shown). Hu antigens, including Hel-N1 (42 kDa), HuC (33 kDa), and HuD (37 kDa), were detected with a mouse monoclonal IgG_{2b} antibody (clone 16A11; Molecular Bioprobes, Eugene, OR). Nuclear Hu antigen was used to mark late or postmitotic neurons for cell counting or neurite length determinations. Fixed cultures were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA) to detect cell nuclei and facilitate counting of all cells. Each immunostained culture was scanned completely with a Nikon Microphot or Olympus IX70 microscope fitted with epifluoresence optics. Microscopic fields were recorded digitally using an Optronix DEI-750 CCD camera and Image ProPlus 3.0.1 software (Media Cybernetics, Silver Spring, MD). Digitally recorded fields were overlaid to create multiply stained images that depicted all the cell nuclei and colocalized cell antigens, and these images were used to count the total

cells per culture, or the number of cells expressing specific cell marker antigens. Cell counts and neurite length measurements were derived from digital images using Sigma Scan Pro 5 Image Analysis software. The "mean neurite length" (Figure 4) is the mean of all neurite lengths measured in images from mock-exposed or virus-exposed cells. The total neurite length per cell is the sum of the lengths of the neurites measured in each cell. The "mean total neurite length per cell" is the mean of these sums. Statistical analyses were performed with PRISM3 statistical software, GraphPad, San Diego, CA. Total cells per culture is the total number of cells derived from a 22-mm^2 PON-FN–coated glass cover slip seeded with approximately 2×10^5 NEP cells.

Immunoblotting

The total protein content of cell cultures was determined by BCA protein assay (Pierce, Rockford, IL) of culture lysates. Serial quantitative immunoblotting (McCarthy *et al*, 2000) was used to assay neural cell-specific cytoskeletal antigen expression in differentiating NEP-derived cultures exposed to HIV-1, using antibodies described above, with chemiluminescent signal detection. Mitogen-activated protein kinase (MAPK) species were also detected by quantitative immunoblotting. MAPK species included extracellular signal-regulated kinase (ERK)1 (p44) or ERK2 (p42) or MAPK p38. Total (phosphorylated plus nonphosphorylated) species were detected with rabbit polyclonal antibodies directed against phosphorylation-independent proteins. Phosphorylated species were detected with rabbit polyclonal antibodies against human ERK1 (p44) and ERK2 (p42) phosphorylated Thr202/Tyr204 or human MAPK p38 phosphorylated Thr180/Tyr182 (Cell Signaling Technology, Beverly, MA). Immunoblots were scanned in white light using the ChemiImager digital imaging system, and analyzed for molecular weight calculation and densitometry with the AlphaEase software package (Alpha Innotech, San Leandro, CA). To ensure equal loading between gels, α-tubulin was used as an internal control (Kusdra *et al*, 2002). This protein migrates as an approximately 60-kDa band. Chemiluminescent signal detection conditions were such that signal density values from independent immunoblots of the same antigen were linearly related. Independent immunoblots were scaled to a common reference range of signal density values. Statistical analyses of antigen expression were performed with PRISM3 statistical software. Data are presented as mean $\pm$ standard error.

Caspase activity

Caspase-3 activity was determined by colorimetric assay (CaspACE system; Promega, Madison, WI) of lysates prepared from differentiating NEP-derived cultures. Replicate cultures were lysed at serial times post neurosphere adherence and initiation of differentiation. The activity in lysates from HIV-1–exposed cultures was normalized to that from mock-exposed cultures, and expressed as a fraction relative to mock (control). For intracellular localization of activated caspases, live cultures were incubated for 30 min with flourescein-conjugated VAD-FMK (carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone; Promega), an irreversible pan caspase inhibitor that can provide in situ monitoring of caspase activity by fluorescence detection.

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