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Substance P enhances HIV-1 infection in human fetal brain cell cultures expressing full-length neurokinin-1 receptor

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Abstract

The associations between the neurokinin-1 receptor (NK-1R), substance P (SP), and HIV-1 were investigated in neurosphere-derived cultures of microglial-depleted human fetal brain cells (HFBC). Full-length NK-1R was identified in HFBC cultures. SP treatment of the HFBC increased intracellular calcium mobilization and decreased electrical impedance, both of which were blocked by the NK-1R antagonist aprepitant. SP treatment of HIV-1-infected HFBC up-regulated HIV-1 expression. These data show that human neural cells grown from neurospheres express functional full length NK-1R that is responsive to SP, and that SP enhanced HIV-1 infection in HFBC.

Keywords

Substance P; human fetal brain cells; neurokinin-1 receptor; HIV-1; aprepitant

Introduction

Infants, children and adolescents with perinatally acquired HIV-1 disease (PA-HIV+) are at increased risk for clinically significant neurologic and psychiatric impairment (Jeremy et al. 2005; Brackis-Cott et al. 2009; Mellins et al. 2009). Combination antiretroviral therapies (cART) do not eliminate HIV-1 from CNS reservoirs, and thus do not provide full protection against the neurotoxic effects of viral reactivation. Brain growth and neural network maturation in PA-HIV+ occur in a perturbed neural milieu, in which neural inflammation is

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COMPETING INTERESTS JHW holds patents on human neural stem cells.

significant. Cells critical for normal brain development include multi-potential neural progenitors (NPCs) that express the intracellular protein nestin.

A major mediator of neural inflammation and immune-neural interactions that is linked to HIV-1 disease is Substance P (SP). SP is a potent undecapeptide tachykinin mediator of inflammatory responses to injury (Amadesi et al. 2012), infection (Chauhan et al. 2008), and immune cell responses to HIV-1 (Ho and Douglas 2004). SP is widely distributed throughout the immune and nervous systems (Severini et al. 2002; Douglas and Leeman 2011). Elevated plasma concentrations of SP are found in PA-HIV+ children (Azzari et al. 1992) and adults (Douglas et al. 2001; Douglas et al. 2008).

The preferred receptor for SP is the neurokinin-1-receptor (NK-1R), which is a glycosylated, guanine nucleotide-binding protein receptor. Two isoforms of NK-1R are present in the human brain: 1) a full-length (FL) 407 amino acid; and 2) a truncated (Tr) (“tail-less”) isoform lacking 96 amino acid residues at the intracellular C-terminus (Caberlotto et al. 2003; Lai et al. 2008a; Tuluc et al. 2009). The FL NK-1R is approximately ten-fold more sensitive to SP than the truncated isoform. SP interaction with FL NK-1R leads to calcium mobilization and downstream transcriptional modification (Lai et al. 2008b). SP immunoreactivity and NK-1R gene expression are present in human fetal microglia (Lai et al. 2000) and in placental-derived blood stem cells (Li et al. 2000). SP interaction with NK-1R is linked to brain inflammation in Rhesus macaques infected with simian immunodeficiency virus (SIV) (Vinet-Oliphant et al. 2010). Antagonists to NK-1R inhibit HIV-1 in human monocytes (Lai et al. 2001; Wang et al. 2007; Wang et al. 2008). The NK-1R antagonist aprepitant can cross the blood-brain barrier and has significant anti-HIV activity against both R5 and X4 strains of HIV (Manak et al. 2010).

Relatively little is known about SP effects on neural cells. Responsiveness to SP (1 μ M) has been observed in mouse and rat neural progenitor cells (Park et al. 2007; Kärkkäinen et al. 2009), which are permissive for HIV-1 infection (Lawrence et al. 2004; Rothenaigner et al. 2007; Schwartz et al. 2007; Hahn et al. 2012). However, the effect of SP on HIV-1 expression from these cells, a potential reservoir in the CNS, has not been examined. The present study investigated the associations between SP, NK-1R and HIV-1 in neurosphere-derived human fetal brain cell (HFBC) cultures processed to deplete microglia.

Methods

Cell cultures

Human fetal brain tissue (9-16-week post-conception) was obtained from the Birth Defects Research Laboratory of the University of Washington, with approval from the Children’s Hospital of Philadelphia (CHOP) IRB for the Protection of Human Subjects, and in full compliance with NIH guidelines. Normal tissue is provided based on maternal history and inspection of the material. After dissection, dissociation and antibiotic washes, tissue was transferred to poly-dlysine-coated plasticware and maintained in 5% CO₂ and air at 37°C in serum-free Neurobasal medium (Gibco Life Technologies, Grand Island, NY) supplemented with bovine albumin (Sigma-Aldrich, St. Louis, MO), Neural Survival Factor-1 (Lonza, Basel, Switzerland), N-2 components (Gibco), basic Fibroblast Growth Factor and Epidermal Growth Factor, both from PeproTech (Rocky Hill, NJ), L-glutamine and gentamicin, both from Quality Biologicals (Gaithersburg, MD), and Fungizone from Invitrogen (Life Technologies, Grand Island, NY).

Neurospheres were allowed to form as described (Messam et al. 2000; Messam et al. 2003), and the human fetal brain cells (HFBC) cells growing out from neurospheres were propagated to 60% confluence (passage stage 0: P0). Unattached cells were then removed by

vigorous shaking, and the residual adherent cells treated $\times 24$ hours with 7.5mM, L-leucine-methyl ester (LLME) (Sigma-Aldrich) to accomplish microglial depletion. This step was performed because primary cell cultures from human brain may contain contaminating microglia (Saura 2007); and microglia express NK-1R (Lai et al. 2000). Although a specific microglial-depleting treatment with LLME may not be necessary for primary HFBC propagated in 10% FBS (Crocker et al. 2008) (as were our cells after passage 1-2), LLME was applied at passage 0-1 in order to decrease the risk that contaminating microglia would affect serial assays for NK-1R expression in early passage number cells. Expression of the microglial marker Iba-1 was negative in the culture (data not shown). After vigorous washes, cells were maintained in Minimum Essential Medium (MEM) with Earle's salts and L-glutamine (Mediatech, Inc., Manassas, VA) supplemented with 10% heat inactivated Fetal Bovine Serum (FBS) (Atlanta Biologicals, Lawrenceville, GA), gentamicin and Fungizone, and subsequently passaged at 70% confluence using either trypsin or papain. Tissues from individual fetal brains were independently processed and utilized. Nestin expression in proliferating cells was determined by reverse transcriptase-quantitative polymerase chain reaction (RT-PCR) and immunocytochemistry (ICC). Expression of the astrocytic marker glial fibrillary acidic protein (GFAP) was monitored by ICC.

Positive control human astrocytoma cells (U373) (ATCC: Manassas, VA) and HEK 293 cells expressing FL NK-1R (Lai et al. 2008b; Meshki et al. 2009) were grown in 5% CO₂ at 37°C in DMEM containing 4.5 g/L glucose, 584 mg/L L-glutamine, 110 mg/L sodium pyruvate (Mediatech) supplemented 10% FBS and antibiotics. Cell viability and proliferation after exposure to experimental treatment or blocking reagents were determined using the colorimetric MTT (-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cell growth assay kit (EMD Millipore).

Immunocytochemistry and microscopy

The reagents used for immunocytochemistry were: mouse monoclonal to human nestin (1:100) (MAB5326; EMD Millipore, Billerica, MA); rabbit antiserum to human glial fibrillary acidic protein (GFAP) (1:500) (AB5804; Millipore); mouse polyclonal antiserum against the human neurokinin-1 receptor (NK-1R) (1:250) N-terminus (H00006869-A01), or a rabbit polyclonal also directed against the N-terminus (1:100) (NB300-119) (Novus Biologicals, Littleton, CO); and rabbit polyclonal antiserum against the NK-1R C-terminus (1:250) (SC15323; Santa Cruz Biotechnology, Santa Cruz, CA). Cells grown on sterilized glass coverslips were fixed in freshly prepared 4% paraformaldehyde, permeabilized with 0.3% Triton X-100 (Sigma-Aldrich) when antibody access to intracellular antigens (e.g. nestin, GFAP and C-terminus NK-1R) was required, serum blocked against nonspecific antigens, and incubated overnight at 4°C with the primary antibody. Visualization was accomplished using rhodamine- or fluorescein-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA). Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (Calbiochem® EMD Millipore). Negative controls included the omission of primary and/or secondary antibody. The coverslips were mounted on glass slides with Vectashield medium (Vector Laboratories, Burlingame, CA) and visualized on an Olympus BX61 fluorescent microscopy platform (Olympus Center Valley, PA) with an automated stage, Hamamatsu ORCA-ER digital camera (Hamamatsu, Bridgewater, NJ), and Volocity (version 4.3) software (Improvision, Perkin-Elmer, Waltham, MA). A minimum of three high-powered (20 $\times$) fields in three slides per condition were examined to compute the proportion of visualized cells positive for nestin or GFAP.

Enzyme-linked immunosorbent assay (ELISA)

Cell-free supernatants were collected and stored at -20°C. HIV-1 p24 antigen was detected using a commercial kit [(Z7040001; BioChain, Hayward, CA) or (0801002; ZeptoMetrix,

Buffalo, NY)]. Concentrations were normalized to the original seeded cell number as determined by trypan blue exclusion. The results were calculated as pg/mL based on the standard linear regression line. The lower level of detection (LLD) was calculated from the mean optical density (OD) of replicate negative control wells plus 2 standard deviations (SD) from the mean. Samples with an OD less than the LLD were assigned a p24 value of 0 for statistical analysis. Supernatant concentrations were calculated from the slope-intercept equation of the linear regression line.

Polymerase chain reaction

Total RNA was isolated from cells using the RNeasy mini kit (Qiagen, Valencia, CA), followed by treatment with RNase-free DNase to remove DNA contaminants. cDNA was synthesized from 1.0 µg of total RNA using Affinity Script qPCR cDNA kit (Agilent, Santa Clara, CA), then stored at -20°C pending utilization. Quantitative PCR was performed on an iCycler iQ Real-Time Detection System (Bio-Rad) using iQ or SYBR Green Supermix reagents. Specific primers and probes for the genes of interest were designed using the Web Primer 3 program (<http://frodo.wi.mit.edu>) (IDT: Coralville, IA). Primer and probe sets for the FL and Tr NK-1R and GAPDH were as detailed previously (Lai et al. 2006). Primer pair sequences for nestin were: forward, 5'-AGGTGGCCACGTACAGGA-3'; reverse, 5'-CTCTGGGGTCCTAGGGAATT-3'; probe, 5'-CTG AGAACTCCCGGCTGCAAAC-3'. Copy numbers of mRNA were calculated based on internal cDNA standards for each gene. Unknown samples were analyzed in duplicate or triplicate, and results averaged for each condition. Data are expressed as the relative fold-difference in mRNA copies normalized to the GAPDH mRNA copy number and multiplied $\times 10,000$. All sets had >85% efficiencies in qPCR.

Calcium mobilization

The mobilization of intracellular calcium in response to SP (Sigma-Aldrich) was measured in cells loaded with fura-2 (Molecular Probes® Life Technologies, Invitrogen), as described (Meshki et al. 2009). Cells were seeded at a density of 5×10^3 into a 96-well plate, allowed to adhere overnight in a 5% CO₂ incubator at 37°C, incubated for 45-minutes at room temperature in medium containing 4µM fura 2-AM (#F-1221: Invitrogen) and 0.01% Pluronic F-127 (Molecular Probes, Eugene, OR), and washed in Hank's balanced salt solution (HBSS) containing 1mM calcium chloride. Cells were visualized on an Olympus IX51 inverted microscope platform with a Hamamatsu Orca camera. The effect of SP (100-1000nM) with or without the NK-1R antagonist aprepitant (1µM) (Emend®: Merck & Co., Whitehouse Station, NJ, purified by chromatography as described [Chernova et al., 2009]) on the mobilization of intracellular calcium was measured by recording fluorescence in individual cells using MetaFluor Olympus fluorescence ratio imaging software (version 7.7: Molecular Devices, Sunnyvale, CA). Fluorescence intensity recordings were made at excitation wavelengths 340 and 380 nm, and emitted light was measured at 510 nm. Results are reported as the 340/380 nm ratio. SP treated U373 MG cells served as a positive control (Meshki et al., 2011). Negative controls were HBSS treated cells. Background levels of intracellular calcium mobilization were determined by baseline recordings in each cell 30 seconds before adding SP to medium. Aprepitant treatment was applied 15 minutes before SP. Viable cells were assessed by calcium mobilization after treatment with ATP (1 µM).

Real time cell electronic sensing assay

A real-time cell electronic sensing assay (RT-CES) was used to measure a change in electrical impedance, as described previously (Meshki et al. 2009). After SP treatment, cells were grown as monolayers in gold electrode-embedded wells seeded at a density of 4000 cells/well in 100µl of medium. After seeding, the plate and associated analyzer were placed in a standard, humidified cell culture incubator at 37°C and 5% carbon dioxide and air

overnight to allow cells to adhere. Cell impedance recordings were taken before and after the addition of treatment reagents to the medium. Integrated software (Acea Biosciences, Inc., San Diego, CA) was used to record and analyze data. Results were expressed as a normalized cell index.

Infectivity studies

Cell-free stocks of Bal-1 and IIIB virus of known p24 protein concentration were provided by the Center for AIDS Research (CFAR) at the University of Pennsylvania. HFBC were plated at identical densities in sterile culture plates (day 0), allowed to adhere, and then incubated in enriched culture medium, with or without SP (100 nM) for 6 days. The medium was replenished with fresh SP every 72 hours. The rationale for pre-treatment with SP before HIV-1 exposure was to mimic a more persistent SP-associated inflammatory milieu, such as might be present in a neural environment containing SP-expressing activated microglia and transmuting monocytes.

Infectious viral stock containing a mixture of the M-tropic HIV-1 strain Bal-1 (5 ng / 2×10^5 cells: 5×10^7 viral particles) and T-tropic IIIB (2 ng / 2×10^5 cells: 2×10^7 viral particles) was added to the medium on day 7. Negative controls were cells unexposed to the viral inoculum. The 5 ng concentration of Bal-1 applied was slightly less than the amount (30 ng / 10^6 cells) used previously to infect microglia/macrophage cultures (Wang et al. 2008). The concentration of IIIB virus applied was higher than the primary isolate exposure dose (1-2 ng/mL p24 antigen / 2×10^6 cells) used by Sabri (Sabri et al. 1999), but less than the 10-11.6 ng amount applied with Polybrene by Lawrence (Lawrence et al. 2004). Cells were incubated in virus-containing medium for 48 hours rather than 12-24 hours used previously (Sabri et al. 1999; Rothenaigner et al. 2007) in order to maximize the opportunity for viral entry. The focus of this work was to test SP effects on infectivity in general. Therefore, a mixture of CCR5 and CXCR4-co-receptor using viral isolates was used because the existing literature is conflicted as to whether HIV-1 entry into HFBC is chemokine co-receptor dependent. Two reports suggest not (Sabri et al. 1999; Boutet et al. 2001), whereas others using human neural progenitor cell lines do not exclude chemokine receptor utilization (Rothenaigner et al. 2007; Hahn et al. 2012).

The viral inoculum was removed on day 9 (post infection day 2). After vigorous wash steps, the cells were maintained in fresh medium supplemented with SP for 5 additional days. Supernatants were then harvested on post-infection day 7 (day 14 of the experiment) for ELISA p24 protein determination. Based on the data presented by Lawrence (Lawrence et al. 2004), post-infection day 7 was selected as the point furthest from inoculation where we might reasonably expect to detect measurable quantities of supernatant p24 antigen by ELISA. At that point, the cells would have been vigorously washed twice: once to remove the initial inoculum, and again on post-infection day 4, thereby minimizing the chance that cultures or plasticware would shed contaminating residuals of the initial viral stock. A limitation of this approach is that potential peak levels of p24 antigen production that might have occurred on earlier post-infection days would go undetected.

Statistical analysis

Three to seven independent experiments were performed for each condition. Data were displayed using Excel for Mac v12.3.2 (Microsoft). Statistical analysis was carried out with GraphPad Prism v6.0 (GraphPad Software, La Jolla, CA). Data were analyzed using Student's unpaired *t* test, or one-way analysis of variance (ANOVA) for normally distributed data, or by the nonparametric Mann-Whitney or Kruskal-Wallis tests. A *p*-value 0.05 was considered statistically significant.

Results

The associations between SP, NK-1R and HIV-1 infectivity of HFBC were examined by first determining NK-1R expression and responsiveness to SP in HFBC cultures subjected to early (P0) microglial depletion. NK-1R expression was assessed by RT-qPCR in HFBC across serial passages (P). The FL NK-1R mRNA was expressed in P 9-15 HFBC (Fig 1) and the copy numbers were not statistically different ($p>0.5$). The truncated NK-1R mRNA was only detectable at the lowest limits of the assay sensitivity (4-5 copies) and treatment with SP (100-1000 nM) had no effect on the Tr-NK-1R isoform mRNA copy number. The addition of gp120 had a <2-fold effect on FL NK-1R message and no detectable effect on the Tr-NK-1R mRNA (data not shown).

The function of FL NK-1R was determined by measuring intracellular calcium mobilization after exposure to SP. Increased intracellular Ca^{++} was observed in P10-13 HFBC exposed to 500 nM SP, which is a SP concentration that activates the FL NK-1R (Meshki et al. 2011). Data from P12 cells are shown in Fig 2, but similar calcium mobilization responses to 500 nM SP were seen in P10 and P13 HFBC. Calcium mobilization was also evident at these passages after treatment with 100 nM or 1000 nM SP (data not shown). Pre-treatment with the NK-1R antagonist, aprepitant suppressed SP-induced calcium mobilization (Fig 2a) but had no effect on calcium mobilization after ATP (not shown), thus demonstrating NK-1R specificity. Real-time cell electronic sensing (RT-CES) recordings provided a second measure of FL NK-1R responsiveness to SP. SP treatment (500 nM) induced a change in cell shape, and thus a drop in electrical impedance. This response was also inhibited by aprepitant pre-treatment (Fig 2b).

Nestin mRNA transcription levels were assessed by RT-qPCR in passage 9-15 HFBC (Fig 3) and the differences between these passages were not significant. Staining for nestin in P10 cells showed 58% (597/1035) were nestin+ (Fig 3b). By P16 there were still a substantial number of nestin + cells in the culture, but the cells were transitioning to a more astrocytic phenotype (Fig 4). Most of the cells were either nestin+ or GFAP+ with only a small number being double-positive.

The effect of SP on HIV-infection was tested in P12 HFBC (Table 1). Pre-treatment of the HFBC with 100 nM SP resulted in an 11-fold higher expression of HIV-1 p24 antigen in supernatant compared to untreated cells at day 7 post-infection ($p=0.02$). Supernatant p24 antigen was not detected in negative controls unexposed to virus.

Discussion

Neurocognitive and neuropsychiatric complications of HIV-1 disease are an ongoing challenge for PA-HIV+ survivors (Jeremy et al. 2005; Mellins et al. 2009), diminishing quality of life and the potential for independent adulthood, despite otherwise successful suppression of systemic viral proliferation with cART. Nestin+ neural progenitors are cells critical for normal brain development; and a growing literature suggests that they are vulnerable to HIV-1 infection (Lawrence et al. 2004; Schwartz et al. 2007; Rothenaigner et al. 2007; Hahn et al. 2012). A potential neuroprotective strategy would be antiviral therapies targeting neural progenitors.

The present study shows that human neural progenitor cells have a functional full-length NK-1R and are responsive to SP. Since the HFBC were established from neurospheres grown in neural stem cell medium (including EGF and FGF-2), they maintained a neural progenitor cell phenotype (nestin +) for at least 15 passages even though propagated in a growth medium. Microglial-depleting treatment using LLME treatment was applied in addition to propagation in growth medium because monocyte lineage cells express NK-1R

and are a known reservoir of HIV in the brain. SP was shown to act through NK-1R in the calcium mobilization and impedance assays by blocking the effect with an NK-1R antagonist.

SP enhanced HIV-1 infectivity in HFBC that were infected with a mixture of T- and M-tropic HIV-1. Mechanisms by which SP enhances HIV-1 entry and replication have been described in other cell types. SP interacting with the FL NK-1R leads to downstream up-regulation of NF- κ B dependent gene products (Lai et al. 2008b). NF- κ B transacting factors enhance HIV/SIV expression in lymphoid and monocytes (Palmieri et al. 2004), and reactivate latent HIV in hematopoietic progenitor cells (McNamara et al. 2012). Since NK-1R antagonists can block the effects of SP, and aprepitant can cross the blood-brain barrier, it may be useful as an antiviral strategy to reduce viral burden in the CNS (Tebas et al. 2011, Douglas and Leeman 2011).

Conclusion

Expression of functional full-length NK-1R was present in HFBC cultures and SP enhanced HIV-1 infection in these cells. This finding raises the possibility that the pro-HIV effect of SP in non-monocytic brain cells could be ameliorated by NK-1R antagonism.

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Fig 1. Full-length NK-1R expression in HFBC

(a) The relative copy number of mRNA transcripts for the full-length neurokinin-1 receptor (NK-1R) (mean 141 ± 176 , median: 42, range: 14-475) compared to the truncated NK-1R isoform was assessed by RT-qPCR in passage (P) 8-15 HFBC. Few truncated transcripts were detected. (b) In the representative P8 image set shown, the N-terminus common to both isoforms is visualized using a rat polyclonal antibody against extracellular amino acid residues 1-17 and a rhodamine-conjugated secondary antibody (1:500), thus showing that NK-1R is expressed. An intracellular C-terminus “tail” indicating expression of the full-length isoform was identified with a rabbit polyclonal antibody directed against intracellular amino acid residues 325-407, and visualized with a rhodamine conjugated secondary. The specificity of the antibodies has been demonstrated (Lai et al 2006). Cell nuclei (blue) were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (1:500)

Fig 2. SP induced intracellular calcium mobilization and impedance changes in HFBC are blocked by aprepitant

(a) The calcium-mobilizing effect of SP was measured by fluorescence intensity recordings at wavelengths 340 and 380 nm in individual fura-2 loaded cells. Cells were seeded into a 96-well plate at 5×10^3 per well, and incubated over night at 37°C in 5% CO_2 . Measurements were taken on 16-20 cells per well, with 8 wells per condition (negative control, SP, or aprepitant + SP) $\times 3$ for each well plate. The addition of SP (500 nM) to medium is indicated by an *arrow*. Negative controls were: baseline recordings $\times 30$ seconds before SP treatment; and substituting Hanks buffered salt solution (HBSS) for SP. U373 cells served as a positive control. The data shown are from passage 12 HFBC, and are representative of separate experiments at passages 10 and 13. SP-induced calcium mobilization was inhibited, but not completely blocked by the NK-1R antagonist aprepitant ($1\mu\text{M}$) applied 15 minutes before SP. (b) Representative real time cell electronic sensing (RT-CES) recordings in P12 HFBC show a drop in impedance after SP (500 nM) treatment (*arrow*) that was partially inhibited by aprepitant ($1\mu\text{M}$) in HFBC and U373 controls

Fig 3. Nestin-positive cells cultured from human fetal brain tissue

(a) Nestin expression was determined by RT-qPCR in passage 9-15 cultures of HFBC. Values are expressed as the relative copy number normalized to GAPDH. Although the number of nestin mRNA transcripts was highest at P12 (mean: 5584 ± 356 , range: 5332-5836), the differences between copy numbers at P9-15 were not significant ($p=0.33$).

(b) Nestin expression at P10 is visualized using a mouse monoclonal antibody (1:100) against the human intracellular nestin protein, and a fluorescein-conjugated secondary antibody. Nuclei were counterstained with DAPI. 58% of cells visualized at passage 10 were nestin-positive. Representative image shown: 20×

Fig 4. Cultures of HFBC expressed nestin and glial fibrillary acidic protein positive at passage 16
 Expression of the astrocytic marker glial fibrillary acidic protein (GFAP) and neural stem cell marker nestin were monitored across serial culture passages by immunocytochemistry. Nuclei were counterstained with DAPI. Although GFAP-positive cells were evident soon after the introduction of serum-supplemented culture medium, conversion to >95% GFAP+ status had not occurred by passage 16, as shown in this representative image (a), and nestin immunoreactivity was still evident at this same passage point (b). Images shown: 20×

Table 1
Effect of SP on HIV-1 infectivity in passage 12 human fetal brain cells

Passage 12 human fetal brain cells (HFBC) were incubated in plain or SP-enriched (100 nM) medium for 6 days. HIV-1 Bal-1 (5 ng of p24 antigen) and IIIB (2 ng of p24) were added on day 7. Negative controls were wells unexposed to HIV-1. The virus-containing medium was removed on day 9. SP treatment continued throughout. Cell supernatants were collected on day 14 (postinfection day 7) and p24 antigen concentration determined by ELISA. All negative control samples were below the lower levels of detection. Data were analyzed by unpaired t-test, taking unequal variances into account.

	No SP No HIV	100 nM SP No HIV	No SP + HIV-1	100 nM SP + HIV-1	Fold change	Significance HIV+ No SP vs 100 nM SP
Supernatant P24 mean pg/mL $\pm$ SD (range) median	0.0 $\pm$ 0.0 (0-0) 0.0	0.0 $\pm$ 0.0 (0-0) 0.0	1.39 $\pm$ 1.28 (0.04-3.77) 1.18	15.83 $\pm$ 10.01 (3.86-27.32) 17.41	11.4	p=0.02