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Short Communication

Evidence of human immunodeficiency virus type 1 infection of nestin-positive neural progenitors in archival pediatric brain tissue

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Human immunodeficiency virus type 1 (HIV-1) central nervous system (CNS) infection in children is associated with impaired brain growth and neurodevelopmental delays. Neural progenitors are critical for neurogenesis. Human multipotential neural progenitors grown in culture are permissive for HIV-1 infection, but it is not known if infection of these cells occurs *in vivo*. Brain tissue from pre–highly active antiretroviral therapy (HAART) era pediatric acquired immunodeficiency syndrome (AIDS) patients was examined for evidence of HIV-1 infection of nestin-positive neural progenitors by *in situ* hybridization; or after laser microdissection harvest, DNA extraction, and polymerase chain reaction (PCR). HIV-1 or viral DNA was identified in nestin-positive cells in four of seven HIV-1–infected children, suggesting *in vivo* infection of neural progenitors. *Journal of NeuroVirology* (2007) **13,** 274–283.

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Human immunodeficiency virus type 1 (HIV-1) is a neurotropic virus shown to be present in the central nervous system (CNS) of infected pediatric and adult patients, and in the developing fetal brain (Kure *et al*, 1991; Lyman *et al*, 1990). The clinical manifestations of HIV-associated CNS disease in children are distinct from those seen in adults (Mintz, 1994). HIV-1–associated progressive encephalopathy (PE) is a syndrome of clinically significant developmental delays, poor brain growth, neurocognitive impairment, and neuropsychiatric dysfunction observed in pediatric patients infected perinatally, or early in postnatal life (Belman *et al*, 1985; Epstein *et al*, 1986). More recently described in perinatally infected children and also in adolescents approaching adulthood is "HIV-related CNS compromise," characterized by a constellation of language, memory, and other subtle neurocognitive deficits. Although far less severe than PE, this syndrome negatively impacts school performance, and the capacity

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to progress to a fully independent life (Tamula *et al*, 2003).

The underlying mechanisms of neuropathology in pediatric acquired immunodeficiency syndrome (AIDS) are incompletely understood. Although productively infected microglia are likely the predominant source of new virus and multiple proinflammatory molecules, the involvement of additional neural cell populations has also been demonstrated. For example, latent infection of astrocytes (Tornatore *et al*, 1991; Churchill *et al*, 2006) and glial activation in the presence of virus-associated proteins have been shown *in vitro*, and are associated with the astrocytic production of multiple proinflammatory and neurotoxic molecules (Lawrence and Major, 2002; Deshpande *et al*, 2005; Wang *et al*, 2004). The clinical relevance of these findings is supported by postmortem identification of virus within astrocytes of pediatric (Tornatore *et al*, 1994; Saito *et al*, 1994) and adult brain (Brack-Werner, 1999; Ranki *et al*, 1995), and a growing body of literature suggesting an association between a diagnosis of HIV-1 CNS disease and the presence of the astrocyte-associated proinflammatory chemokine monocyte chemoattractant protein-1 (MCP-1/CCL2) (Conant *et al*, 1998). In contrast, although restricted infection of neuronal cell lines has been demonstrated *in vitro* (Li *et al*, 1990; Hsia *et al*, 1997) and viral DNA detected in neurons harvested by laser capture microdissection from the brain tissue of HIV-1–infected adults and children (Torres-Muñoz *et al*, 2001; Trillo-Pazos *et al*, 2003), there is insufficient clinical evidence that infection of neurons contributes significantly to the cerebral atrophy seen in adult and pediatric neuroAIDS, or to the poor brain growth and developmental delays of PE.

Neural progenitor cells are an additional population of cells to consider in the neuropathology of HIV-1, especially in pediatric neuroAIDS, due to their central and critical role in neurogenesis (Eriksson *et al*, 1998), support of hippocampal and dentate function (Van Praag *et al*, 2002; Schwartz and Major, 2006), and response to brain injury (Holmin *et al*, 1997; Frisen *et al*, 1995; Nakatomi *et al*, 2002). Multipotential mammalian, including human, neural progenitors express nestin, a 220-kDa class VI intermediate filament protein first described in radial glia (Hockfield and McKay, 1985; Lendahl *et al*, 1990). Nestin-positive cells have been identified in early gestational human fetal brain (Tohyama *et al*, 1992; Messam *et al*, 2002), the subventricular (SVZ) and subgranular zones of the dentate gyrus region of the hippocampal formation (Blümcke *et al*, 2001), human subcortical white matter (Nunes *et al*, 2003), cerebellum (Yachnis *et al*, 1993), and adult hippocampal and forebrain tissue (Gu *et al*, 2002). A possible relationship between regions with high nestin expression and increased HIV-1 viral load is suggested by quantitative evidence of increased viral mRNA in the hippocampus (Wiley *et al*, 1998), but the data are otherwise inconclusive given the generally inconsistent correlation between clinical signs and symptoms of neuroAIDS and viral load in the CNS (Glass and Wesselingh, 2001).

We had shown previously in cell culture that multipotential human fetal brain-derived, nestin-positive progenitors are permissive for HIV-1 infection (Lawrence *et al*, 2004). In this study, we examined archived pediatric brain tissue from a series of HIV-1–infected infants and children who came to autopsy in the pre-HAART era, seeking *in vivo* evidence of HIV-1 infection of nestin-positive cells. Two techniques were used after immunohistochemical identification of nestin positivity: *in situ* hybridization (ISH) or polymerase chain reaction (PCR) amplification of DNA extracted from laser-microdissected pressure catapult (LMPC)-harvested cells.

Archived formalin-fixed, paraffin-embedded pediatric brain tissues collected in the pre-HAART era (1986 to 1995) from children diagnosed as HIV-1 infected ($N = 7$) and additional negative-control pediatric patients ($N = 2$) not diagnosed or suspected as being HIV-1 infected or exposed, were obtained from the Department of Pathology, Children's National Medical Center, Washington, DC (Table 1). Four- to 6-μm slices from the periventricular region, hippocampus, deep frontal white matter, and cerebellum were adhered to silane-treated glass slides, deparaffinized, rehydrated, then subjected to microwave irradiation antigen retrieval (Citra Plus, BioGenex, San Ramon, CA) diluted 1:10 in glycerol (Invitrogen, Carlsbad, CA). After permeabilization and treatment to block nonspecific binding and endogenous tissue peroxidases, nestin-positive cells were identified using a rabbit polyclonal antibody raised against a 150–amino acid sequence of human nestin (hNestin-331B) (Messam *et al*, 2000). Chromogenic visualization was accomplished using a biotinylated secondary antibody followed by ABC reagent detected with DAB (Vector Laboratories, Burlingame CA). Fluorescence visualization of nestin-positive cells was achieved using tyramide signal amplification (TSA detection kit; Molecular Probes, Eugene, OR) and nuclear counterstaining with bisbenzamide (1:500) (Calbiochem Biosciences, La Jolla, CA). Nestin expression was observed in multiple areas, including periventricular, hippocampal, cerebellar, deep frontal white matter, and temporal region tissue. This finding is consistent with previously published reports of nestin expression and neural progenitors in the human brains.

Of interest, the numbers of nestin-positive cells per slide identified by fluorescence IHC in hippocampal tissue from HIV-1–infected cases (cases 6 to 8) were notably fewer than numbers seen in hippocampal tissue from the negative-control case (case 9). Possible explanations include the discrepancy in the ages and CNS development between HIV-1–infected patients and the negative-control case, time to autopsy after death, and/or the substantial difference in duration of archival storage between the

Table 1 Description of the HIV-1 patients and controls studied post mortem

Case no.	Diagnosis	Mode of transmission	Age at death	Gender	Neuropathology	Cause of death	Tissue Examined	Method	No. nestin$^+$ cells	Results
1	AIDS	Vertical	3 mo	F	Periventricular white matter gliosis; neuronal necrosis	PCP	BG, H, TL, CB, FWM	ISH	NA	ISH negative
2	AIDS	Unknown	11 yr	F	No specific histological abnormalities	Sepsis, bronchop-neumonia	BG, TL, H, PV	ISH	NA	ISH negative
3	AIDS	Vertical	22 mo	F	Brain infarct, BG calcifications	CNS infarct	BG, CC, PV	ISH	NA	ISH negative
4	AIDS	Vertical Premature birth s/p transfu-sions	4 yr	F	Microcephaly, gliosis, periventricular leukomalacia	FTT	PV	ISH	66	1/66 ISH positive
5	Brain tumor	Not identified as HIV exposed or infected	4 yr	F	Ependymal tumor	Brain tumor	CC, FWM, PV, TL	ISH	NA	ISH negative
6	AIDS	Vertical	4.5 mo	M	Glial nodules, infarct, neuronal necrosis	Sepsis PCP	BG, PV, TL, CB	ISH PCR	80	ISH negative, PCR positive
7	AIDS	Vertical	15 mo	M	Gliosis, BG calcifications	Sepsis,	H, TL, PV, CB,	PCR	251	PCR positive
8	AIDS	Vertical	19 mo	F	Glial nodules, perivascular inflammation, microcephaly	Myocarditis, sepsis	H	PCR	410	PCR positive
9	Premature delivery at 26 wk GA	Not identified as HIV exposed or infected	28.3 weeks post conception	F	Germinal matrix hemorrhage	IVH	H	PCR	>800	PCR negative

Note. Paraffin-embedded, formalin-fixed tissue was obtained from the Children's National Medical Center Department of Pathology archives from seven HIV-1–infected pediatric patients and two children not diagnosed as HIV-1 infected or exposed. Time from death to autopsy, when available upon review of the medical record, was within 3 days post mortem. Nestin-positive cells were identified by immunohistochemistry (IHC), followed by *in situ* hybridization (ISH) detection of HIV-1 with a ^{35}S-probe encompassing approximately 90% of the HIV-1 genome; or laser microdissection pressure catapult cell harvest, DNA extraction, and PCR amplification with primers directed against *gag*. Four of seven HIV-1–infected patients were found to have evidence of cells double positive for nestin and HIV-1.
BG: basal ganglia; H: hippocampus; TL: temporal lobe; PV: periventricular region; CB: cerebellum; CC: cerebral cortex; FWM: deep frontal lobe white matter.
ISH: *in situ* hybridization; PCR: DNA extracted from LMPC nestin$^+$ cells; NA: not applicable or assessed.
PCP: pneumocystis carnii; IVH: intraventricular hemorrhage.

three HIV-1–infected cases (all died in 1989) and the negative-control infant (2004). Alternatively, it has been shown that HIV-1–associated inflammatory chemokines activating CXCR4 (SDF-1) or CCR3 (MCP-4, eotaxin, RANTES) coreceptors for HIV-1 reversibly inhibit neural progenitor cell proliferation (Krathwohl and Kaiser, 2004a). Additionally, the same authors reported decreased numbers of proliferation marker Ki 67–positive cells in hippocampal tissue harvested from autopsied HIV-1–infected adults with a diagnosis of AIDS dementia complex (ADC), compared to HIV-1–infected patients without ADC, and uninfected controls (Krathwohl and Kaiser, 2004b). These data suggest the possibility of decreased neural progenitors in neuroAIDS, and offer a rationale for future quantitative investigations comparing neural progenitor cell density in brain tissue from age- and gender-matched HIV-1–infected, uninfected but exposed, and unexposed patients.

ISH was accomplished after chromogenic nestin detection as previously described (Haase *et al*, 1996). Briefly, slides were washed, acetylated in 0.35% acetic anhydride ×10 min, dehydrated through graded alcohols, and incubated ×24 h at 45°C with hybridization solution containing ^{35}S-labeled hydrolyzed antisense (or sense control) riboprobe representative of more than 90% of the full-length HIV-1 genome. Slides were then washed and digested at 37°C with ribonucleases A (25 μg/ml) and T1 (25 units/ml) ×30 min, followed by additional washes and dehydration through graded alcohols containing 0.3 M ammonium acetate. Slides were next coated with Kodak NTB-1 emulsion, autoradiographically exposed for 10 days at 4°C, developed, counterstained with hematoxylin, and analyzed visually under 400x× microscopic magnification. Cells were scored positive when the silver grain count over an individual cell differed from the Poisson distribution of average background signal by $P > .95$. Sixty-six HIV-1–positive cells were identified in a total of 50 slides.

One cell from periventricular tissue from a 4-year-old female with severe progressive encephalopathy (Case 4) was double positive for both HIV-1 RNA and nestin (1/66 or 1.5%) (Figure 1). One additional HIV-1–positive cell was seen in a nestin-rich area of periventricular tissue from this same patient, and another in nestin-positive basal ganglia tissue from case 6. However, in both instances, the underlying cellular morphology was obscured by the intense positive signal of the HIV-1 probe.

Due to low copy number of virus, the technical difficulty of discerning nestin expression in cells containing strong ^{35}S-HIV-1 hybridization signal, and the need to preserve as much archived tissue as possible for future studies, we next turned to tyramide signal amplification (TSA) immunofluorescence (Strappe *et al*, 1997; Wang *et al*, 1999) to identify nestin-positive cells (representative results: Figure 2), which were then LMPC harvested (Zeiss PALM Microlaser Technologies, Germany) into the caps of autoclave-sterilized 0.5-ml thin-walled PCR tubes (Brinkman Instruments, Westbury, NY)) containing lysis buffer prepared according to the manufacturers written instructions (PicoPure DNA extraction kit; Arcturus Bioscience, Mountain View, CA). DNA extraction followed the same manufacturers written protocol, with storage at 4°C or −20°C pending PCR amplification of viral *gag* sequences.

Prior to LMPC, 100× magnification was used as necessary to confirm that the nuclei targeted were within nestin-positive cells. Optimal laser settings for power and focus were determined for each slide, and slides were visually monitored during laser microdissection (40× magnification) and after harvest to confirm minimal apparent capture of material outside of the targeted nuclear area (representative image: Figure 3). All nestin-positive cells identified in any one individual slide of tissue from patients known to be HIV-1 infected were microdissected and laser catapulted into a single cap and tube. The number of nestin-positive cells identified varied in number, with a range of 8 to 42 nestin-positive cells per 4- to 6-micron tissue section slide prepared from HIV-1–infected patients. Table 1 gives the total number of cells harvested and examined by PCR per HIV-1–infected patient. Comparable numbers of nestin-positive cells from the negative-control tissue (case 9) were used per PCR reaction tube. "Buffer only" negative-control material was obtained by moving caps into position, but not capturing cells from slides.

Amplification reactions were run on a single GeneAmp PCR System 2400 (Perkin-Elmer) or 9700 (Applied Biosystems, Branchburg, NJ) thermal cycler. DNA integrity in lysates was confirmed by comparison with positive-control template after amplification with primers previously described (forward: 5′-CCATGGAGAAGGCTGGGG-3′; reverse: 5′-CAAAGTTGTCATGGATGACC-3′) directed against *glyceraldehydes-3 phosphate dehydrogenase (GAPDH)* (Rollison *et al*, 2005) reacted with HotStar Taq DNA polymerase Master Mix (Qiagen, Valencia, CA). PCR parameters for *GAPDH* amplification (50-μl reaction) after enzyme activation (95°C × 15 min) were 40 cycles of denaturation at 95°C × 30 s, annealing at 55°C × 30 s, and extension at 72°C ×1 min, followed by 72°C × 7 min.

Two PCR approaches were utilized. Initial PCR for HIV-1 *gag* was performed using 20 μl of template with primers SK38 (forward: 5′-ATAATCCACCTATCCCAGTAGGAGAAAT-3′) /SK39 (reverse: 5′-CATTCTGGACATAAGACAAGGACCAA A-3) directed against a 115–base pair segment of HIV-1 *gag* (GeneAmplimer HIV-1 Control Reagents; Applied Biosystems). Negative placental DNA (10 μg/ml) and positive (range 2 to 1000 copies HIV-1 DNA/20 μl) controls and tissue lysates were amplified in a 100-μl reaction with HotStar Taq Master Mix after enzyme activation (95°C × 15 min) as follows: 40 cycles of denaturation at 95°C × 1 min,

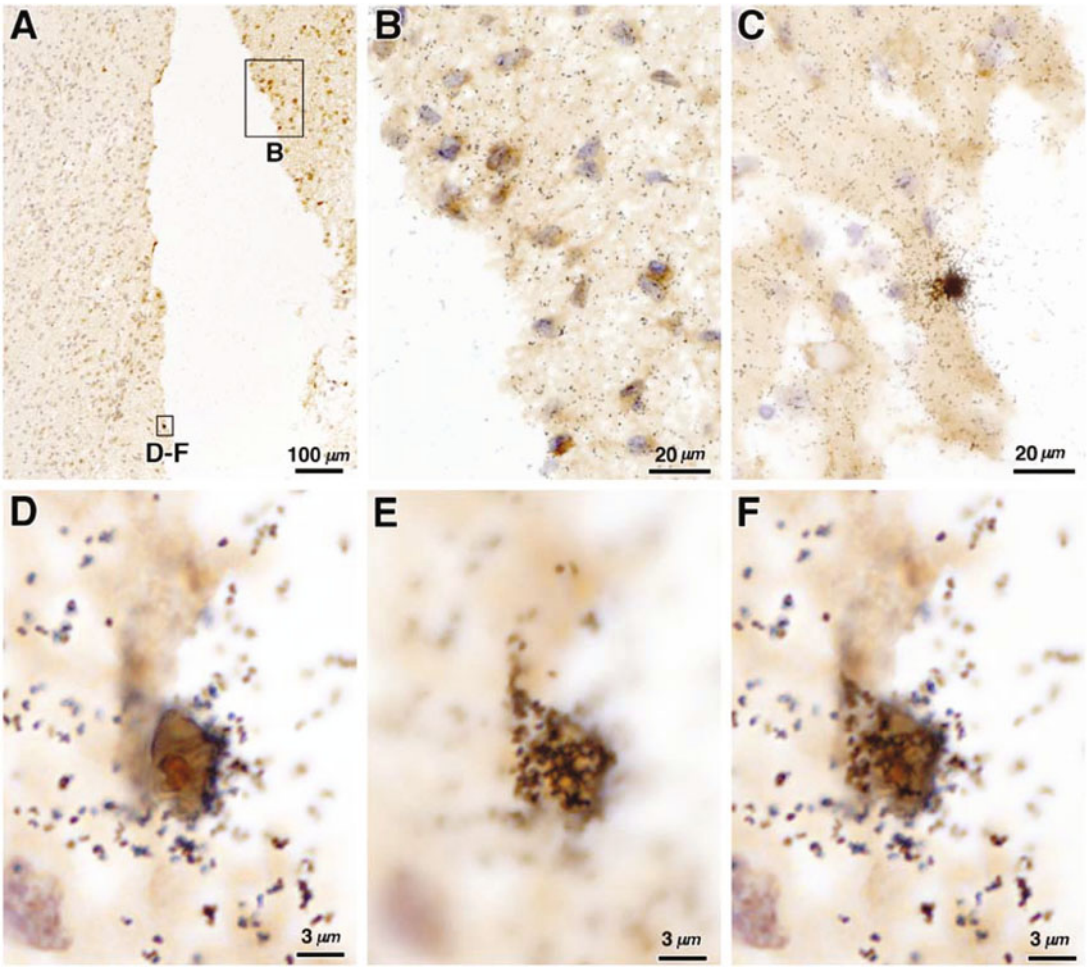

Figure 1 Cellular colocalization of nestin and HIV-1 in periventricular tissue from a 4-year old female with severe neuroAIDS. Chromogenic immunohistochemistry to detect nestin expression was performed utilizing a rabbit polyclonal anti-human nestin antibody (1:200) raised against human nestin. A biotinylated secondary antibody followed by ABC reagent was detected with DAB (nestin = brown intracellular staining). HIV-1 was detected by ISH with ^{35}S-labeled hydrolyzed antisense riboprobe representative of more than 90% of the full length HIV-1 genome. After autoradiographic exposure, slides were developed and counterstained with hematoxylin and analyzed visually under 400× microscopic magnification. Cells were scored positive when the silver grain count over an individual cell differed from the Poisson distribution of overage background signal by $P > .95$. (**A**) 10x magnification: (**B**) 63× magnification: nestin-positive cells identified within periventricular tissue. (**C**) 63× magnification: HIV-1 positive cell with intense signal obscuring the underlying cell. (**D**) 100× magnification: nestin-positive cell in focus. (**E**) 100× magnification: darkened silver grains of HIV-1–hybridized probe in focus. (**F**) Merged images of (**D**) and (**E**).

annealing at 55°C ×1 min, followed by extension at 72°C ×7 min and storage at 4°C pending gel electrophoresis. Subsequent PCR for HIV-1 *gag* were performed using 20 μl of lysate template, "zero copy" DNA (NIH AIDS Research and Reference Reagent Program), as well as positive- and negative-control DNA, with primers directed against a 324–base pair segment of *gag* (forward: 5′-CAATGATGGAGAGAACAACTGCT-3′/reverse: 5′-CAGGCTCAGATCTGGTCCAACCA-3′) (Torres-Muñoz *et al*, 2001). All controls and tissue lysates were amplified in a 100-μl reaction with Takara PrimeStar HS (Takara, Japan) after enzyme activation (95°C ×3 min) as follows: 50 cycles; denaturation at 95°C ×12–13 s, annealing at 64°C ×10 s, followed by extension at 72°C ×8 min and storage at 4°C pending product separation by gel electrophoresis in 2% Ultrapure agarose (Invitrogen, Carlsbad, CA) ethidium bromide (Invitrogen) gels with Tris acetate EDTA [TAE] 1× (KD Medical Columbia, MD), visualized under ultraviolet (UV) illumination, and photographed. cDNA products from the HIV-1 *gag* amplification were purified (MinElute PCR Purification kit; Qiagen), or excised and purified from the agarose gel (Qiagen kit for gel extraction), and reacted on a PE 9700 thermal cycler using BDT3.1 and the BDT cycling profile (Millipore, Billerica, MA). The reaction was then purified and analyzed (Applied Biosystems 3100). Results were displayed (Four Peaks, Mekentosj), and searched against a reference database (basic local alignment search tool [BLAST], National Center for Biotechnology Information [NCBI], National Library of Medicine, National Institutes of Health).

PCR amplification using SK38/SK39 primers and GeneAmplimer reagents identified a 115–base pair

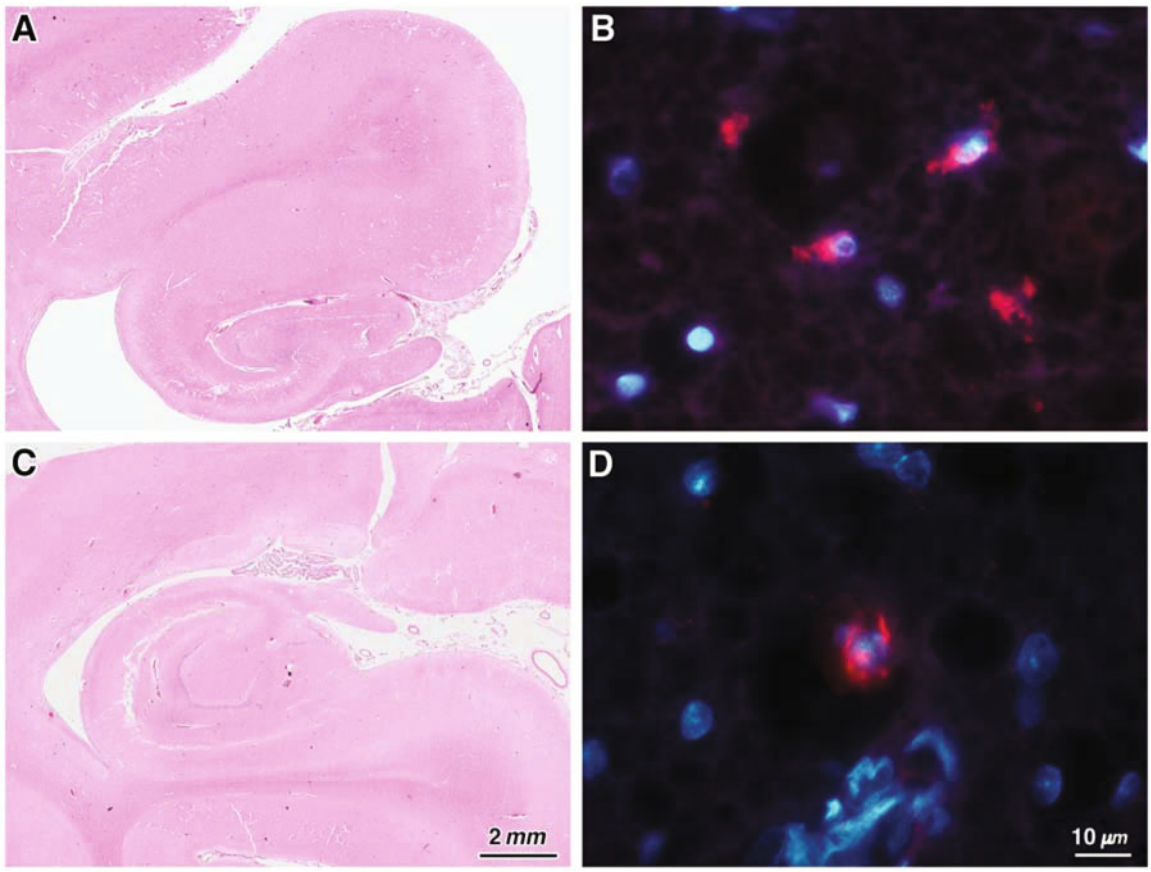

Figure 2 Immunohistochemical detection of nestin-positive cells within pediatric hippocampal tissue. (**A** and **C)** Hematoxylin and eosin–stained slides from archived hippocampal formation tissue blocks of two HIV-1–infected children (cases 7 and 8, respectively). Nestin-positive cells (**B**) and (**D**) were identified in tissue from these blocks after overnight incubation at 4°C in rabbit polyclonal anti-human nestin antibody (1:5000) in 5% serum, followed by a secondary goat anti-rabbit horseradish peroxidase conjugate (1:200) and a brief incubation in Alexa-Fluor 568–labeled tyramide (1:200) diluted in 0.0015% hydrogen peroxide amplification buffer. Nuclei were counterstained with bisbenzamide (1:500). Red cytoplasmic fluorescence identifies nestin surrounding the blue immunostained nuclei; 100× magnification.

cDNA PCR product in DNA extracted from a pool of 25 laser captured nestin-positive cells from the hippocampal tissue of a 19-month-old vertically infected female with neuroAIDS (case 8). Subsequent sequencing and BLAST analysis confirmed a 94% identity with HIV-1 *gag* when queried against a reference database (maximum E-value 9e-25). Two reaction tubes from an additional patient (case 7) run at the same time were negative for the 115-bp product. Negative-control tissues and cells included in each run were negative for the 115-bp product and negative for other HIV-1 sequences. Low–base pair primer dimerization prompted us to perform subsequent amplifications with primers directed against a 324–base pair product reacted with a highly sensitive DNA polymerase (Takara PrimeStar HS). HIV-1 *gag* sequences were found in hippocampal (case 7) and cerebellar (cases 6, 7) tissue, with maximum identities by BLAST query of 97% to 99%, and E-values 2e-97 to 2e-137.

Performing two-step PCR in cases where gel electrophoresis was negative for a desired product after single-round PCR did not improve sensitivity or yield. The potential risk of subject-subject PCR contamination was addressed by frequent change of gloves between handling negative, unknown, and positive samples, and repeated cleansing of work surfaces (RNase Away; Molecular BioProducts, San Diego, CA). At all steps, negative and unknown samples were processed prior to handling known positive tissue and control DNA. The possibility of false-positive results from laboratory strain HIV-1 contamination was excluded by querying each positive sequence against the published HIV NL4-3 *gag* sequence (accession number M19921) (Adachi *et al*, 1986): no significant similarity was found in any case.

ISH studies identified a single HIV-1$^+$/nestin copositive cell (1/66) in the periventricular region of a 4-year-old female with progressive encephalopathy, suggesting an estimated frequency of nestin-positive neural progenitor infection of 1.5%. Taking this as a preliminary estimate, we next turned to laser microdissection pressure catapult technology to identify and harvest several hundred tyramide signal amplified nestin-positive cells per patient (Table 1). Assuming not more than one infected cell per tube of

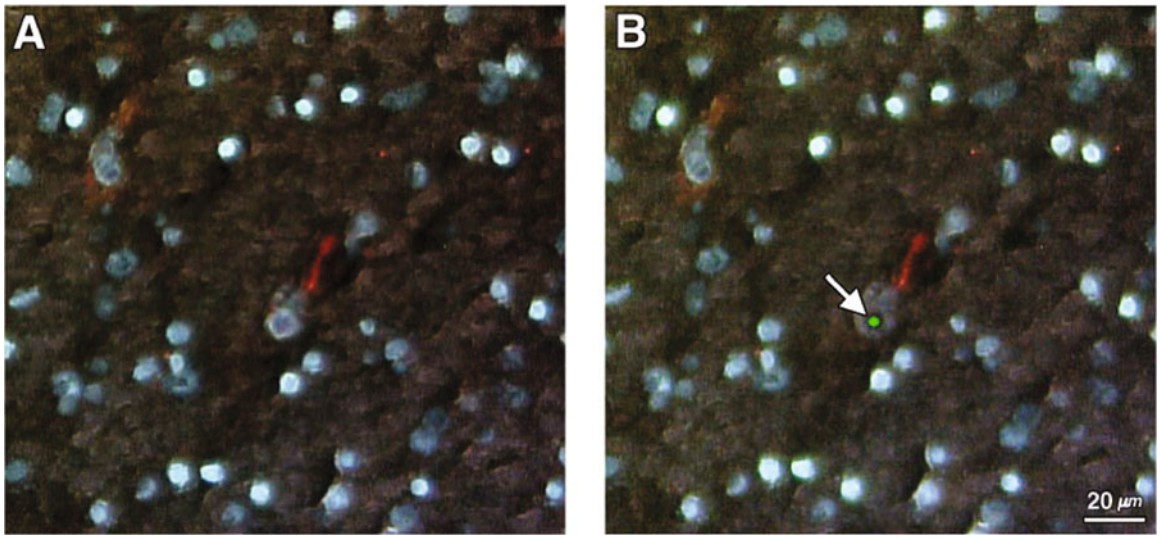

Figure 3 Pre- and post–laser microdissection pressure catapult harvest of a nestin-positive neural progenitor cells within archived pediatric brain tissue. Nestin-positive neural progenitor cell from the temporal lobe/periventricular region of a 4.5-month-old, vertically infected male with neuroAIDS; 40× magnification. Fluorescence immunohistochemistry to detect nestin expression was performed utilizing a rabbit polyclonal anti-human nestin antibody raised against a 150–amino acid sequence of human nestin (1/5000), a secondary goat anti-rabbit horseradish peroxidase conjugate (1/200), and Alexa-Fluor 568–labeled tyramide diluted (1/200) in 0.0015% hydrogen peroxide amplification buffer. Nuclei were counterstained with bisbenzamide (1/500). Nestin-positive cells were identified under triple filter fluorescence microscopy, laser microdissected, and pressure catapulted into the caps of autoclave-sterilized 0.5-ml thin-walled PCR tubes containing lysis buffer. All harvests were performed at 40× magnification after optimal laser settings for power and focus were determined for each slide. Nestin = red; nuclei = blue. (**A**) Nestin-positive cell prior to microdissection; (**B**) the same cell after laser microdissection pressure catapult harvest of targeted nuclear material within the identified nestin-positive cell.

LMPC-harvested nestin-positive cells, the calculated frequency of nestin-positive/HIV-1 *gag* copositive cells ranges from 0.23% to 1.25%. The discrepancy between ISH and PCR data may reflect the fact that the HIV-1 riboprobe spanned approximately 90% of the HIV-1 genome, including regions coding for *nef*, whereas our PCR primers were directed against *gag* alone. Future studies with primers directed against *nef* might therefore identify additional, latently infected, nestin-positive neural progenitors, as has been described previously in pediatric brain astrocytes (Saito *et al*, 1994) and hippocampal neurons (Torres-Muñoz *et al*, 2001).

A mechanism for HIV-1 entry into nestin-positive neural progenitors remains to be determined. CD4 expression has not been described for neural progenitors. It has been shown that human fetal astrocytes can be infected by primary HIV-1 isolates through otherwise undefined CD4- and major chemokine receptor- independent mechanisms (Sabri *et al*, 1999), but parallel studies have not been done on nestin+ progenitors. CD4-independent viral entry via the human mannose receptor has been demonstrated in astrocytes (Liu *et al*, 2004), but mannose receptor expression in neural human progenitors has not been described. Additional possibilities would include entry via galactosyl ceremide or heparan sulfate proteoglycan mediated pathways (Bhat *et al*, 1991; Kumar *et al*, 2006).

Multiple chemokine receptors are constitutively expressed in human brain, including several associated with viral entry and HIV-1 infection of the CNS (CCR1, CCR2, CCR3, CCR5, CXCR3, CXCR4, CX3CR1, and the promiscuous CC chemokine D6; Biber *et al*, 2006; Neil *et al*, 2005; van der Meer *et al*, 2000). CXCR4 and CCR5 HIV-1 coreceptors have been identified in the brains of children with AIDS (Vallat *et al*, 1998). Several studies have presented evidence of chemokine receptor expression, including CXCR4 and CCR5, in neurogenic regions of the mammalian brain (Tran *et al*, 2007), and in neural progenitors grown as neurospheres (Tran *et al*, 2004; Ji *et al*, 2004), or in cell culture (Ni *et al*, 2004). Taken together, these studies support the possibility of chemokine-mediated viral entry into neural progenitors; but even if present and functional, it is also possible that susceptibility studies might show that these receptors do not play a role in cell infection, as demonstrated in studies on isolated human astrocytes (Boutet *et al*, 2001).

How HIV-1 infection of nestin+ neural progenitors might participate in the neuropathogenesis of the pediatric syndromes of progressive HIV-1 encephalopathy and HIV-related CNS compromise syndrome, or adult HIV-1 associated dementia (HAD), is speculative at this point. One possibility would be that neural progenitors are an additional brain reservoir for HIV. Our finding of *gag* DNA in nuclear material harvested from nestin+ cells suggests that these cells might be permissive for new virus production, but confirmation of this possibility would be dependent upon demonstrating proviral integration, as has been done in astrocytes (Churchill *et al*, 2006). Care was taken to harvest nuclear material rather than entire cells,

but it is possible that the DNA amplimers identified were present as intranuclear or even cytoplasmic fragments, not integrated material. A second possibility is that HIV-1 infection, or perhaps altered CXCR4 receptor–mediated signaling in the presence of virus, HIV-1–associated viral proteins, or HIV-1–associated neuroinflammatory molecules, affect neural progenitor distributions and/or critical functions such as migration, dentate gyrus development and neurogenesis, or repair of brain injury (reviewed in Schwartz and Major, 2006).

In conclusion, this study of archived pediatric brain from HIV-1–infected children is significant in that it supports the clinical relevance of previously reported cell culture data, which showed that neural progenitors are permissive for HIV-1 infection. Directions for future studies would include examination of mechanisms of viral entry into neural progenitors, the relationship between viral tropism and infection, and potential functional implications of infection of these cells in the developing pediatric brain.

References

Adachi A, Gendelman HE, Koenig S, Folks T, Willey R, Rabson A, Martin MA (1986). Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone. *J Virol* **59:** 284–291.

Belman Al, Ultmann MH, Horoupian D, Novick B, Spiro AJ, Rubinstein A, Kurtzberg D, Cone-Wesson B (1985). Neurological complications in infants and children with acquired immunodeficiency syndrome. *Ann Neurol* **18:** 560–566.

Bhat S, Spitalnik SL, Gonzalez-Scarano F, Silberberg DH (1991). Galactosyl ceremide or a derivative is an essential component of the neural receptor for human immunodeficiency virus type 1 envelope glycoprotein gp120. *Proc Natl Acad Sci U S A* **88:** 7131–7134.

Biber K, de Jong EK, van Weering HRJ, Boddeke HWGM (2006). Chemokines and their receptors in central nervous system disease. *Curr Drug Targets* **7:** 29–46.

Blümcke I, Schewe J-C, Normann S, Brüstle O, Schramm J, Elger CE, Weistler OD (2001). Increase in nestin-immunoreactive neural precursor cells in the dentate gyrus of pediatric patients with early-onset temporal lobe epilepsy. *Hippocampus* **11:** 311–321.

Boutet A, Salim H, Taoufik Y, Lledo P-M, Vincent J-D, Delfraissy J-F, Tardieu M (2001). Isolated human astrocytes are not susceptible to infection by M-and T-tropic strains despite functional expression of the chemokine receptors CCR5 and CXCR4. *Glia* **34:** 165–177.

Brack-Werner R (1999). Astrocytes: HIV cellular reservoirs and important participants in neuropathogenesis. *AIDS* **13:** 1–22.

Churchill MJ, Gorry PR, Cowley D, Lal L, Sonza S, Purcell DFJ, Thompson KA, Gabuzda D, McArthur JC, Pardo CA, Wesselingh SL (2006). Use of laser capture microdissection to detect integrated HIV-1 DNA in macrophages and astrocytes from autopsy brain tissues. *J NeuroVirol* **12:** 146–152.

Conant K, Garzino-Demo A, Nath A, McArthur JC, Halliday W, Power C, Gallo RC, Major EO (1998). Induction of monocyte chemoattractant protein -1 in HIV-1 tat-stimulated astrocytes and elevation in AIDS dementia. *Proc Natl Acad Sci U S A* **95:** 3117–3121.

Deshpande M, Zheng J, Borgmann K, Persidsky R, Wu L, Schellpeper C, Ghorpade A (2005). Role of activated astrocytes in neuronal damage: potential links to HIV-1 associated dementia. *Neurotoxicol Res* **7:** 183–192.

Epstein LG, Sharer LR, Oleske JM, Connor EM, Goudsmit J, Bagdon L, Robert-Guroff M, Koenigsberger MR (1986). Neurologic manifestations of human immunodeficiency virus infection in children. *Pediatrics* **78:** 678–687.

Eriksson PS, Perfilieva E, Björk-Eriksson T, Alborn A-M, Nordborg C, Peterson DA, Gage FH (1998). Neurogenesis in the adult hippocampus. *Nat Med* **4:** 1313–1317.

Frisen J, Johansson CB, Török C, Risling M, Lendahl U (1995). Rapid, widespread, and long lasting induction of nestin contributes to the generation of glial scar tissue after CNS injury. *J Cell Biol* **131:** 453–464.

Glass JD, Wesselingh SL (2001). Microglia in HIV-associated neurological diseases. *Microsc Res Tech* **54:** 5–105.

Gu H, Wang S, Messam CA, Yao Z (2002). Distribution of nestin immunoreactivity in normal adult human forebrain. *Brain Res* **943:** 174–180.

Haase AT, Henry K, Zupancic M, Sedgewick G, Faust RA, Melroe H, Cavert W, Gebhard K, Staskus K, Zhang ZQ, Dailey PJ, Balfour HH Jr, Erice A, Perelson AS (1996). Quantitative image analysis of HIV-1 infection in lymphoid tissue. *Science* **274:** 985–989.

Hockfield S, McKay RD (1985). Identification of major cell classes in the developing mammalian nervous system. *J Neurosci* **5:** 3310–3328.

Holmin S, Almqvist P, Lendahl U, Mathiesen T (1997). Adult nestin-expressing subependymal cells differentiate to astrocytes in response to brain injury. *Eur J Neurosci* **9:** 65–75.

Hsia K, Spector DH, Spector SA (1997). Molecular analysis of the differential expression of human immunodeficiency virus type 1 replication in neuronal cell lines. *J Gen Virol* **78:** 3255–3264.

Ji JF, He BP, Dheen ST, Tay SSW (2004). Expression of chemokine receptors CXCR4, CCR2, CCR5, and CX_3CR1 in neural progenitor cells isolated from the subventricular zone of the adult rat brain. *Neurosci Lett* **355:** 236–240.

Krathwohl MD, Kaiser JL (2004a). Chemokines promote quiescence and survival of human neural progenitor cells. *Stem Cells* **22:** 109–118.

Krathwohl MD, Kaiser JL (2004b). HIV-1 promotes quiescence in human neural progenitor cells. *J Infect Dis* **190:** 216–226.

Kumar RB, Maher DM, Herzberg MC, Southern PJ (2006). Expression of HIV receptors, alternate receptors and co-receptors on tonsillar epithelium: implications for HIV binding and primary oral infection. *Virol J* **3:** http://www.virologyj.com/content/3/1/25 (open access): 25–37.

Kure K, Llena JF, Lyman WD, Soeiro R, Weidenheim KM, Hirano A, Dickson DW (1991). Human immunodeficiency virus-1 infection of the central nervous system:

an autopsy study of 268 adult, pediatric and fetal brains. *Hum Pathol* **22:** 700–710.

Lawrence DM, Durham LC, Schwartz L, Seth P, Maric D, Major EO (2004). Human immunodeficiency virus type 1 infection of human brain-derived progenitor cells. *J Virol* **78:** 7319–7328.

Lawrence DM, Major EO (2002). HIV-1 and the brain: connections between HIV-1 associated dementia, neuropathology and Neuroimmunology. *Microb Infect* **4:** 301–308.

Lendahl U, Zimmerman LB, McKay RD (1990). CNS stem cells express a new class of intermediate filament protein. *Cell* **60:** 585–595.

Li XL, Moudgil T, Vinters HV, Ho DD (1990). CD4-independent, productive infection of a neuronal cell line by human immunodeficiency virus type 1. *J Virol* **64:** 1383–1387.

Liu Y, Liu H, Kim BO, Gattone VH, Li J, Nath A, Blum J, He JJ (2004). CD-4 independent infection of astrocytes by human immunodeficiency virus type 1: requirement for the human mannose receptor. *J Virol* **78:** 4120–4133.

Lyman WD, Kress Y, Kure K, Rashbaum WK, Rubinstein A, Soeiro R (1990). Detection of HIV in fetal central nervous system tissue. *AIDS* **4:** 917–920.

Messam CA, Hou J, Berman JW, Major EO (2002). Analysis of the temporal expression of nestin in human fetal brain-derived neuronal and glial progenitor cells. *Dev Brain Res* **134:** 87–92.

Messam CA, Hou J, Major EO (2000). Coexpression of nestin in neural and glial cells in the developing human CNS defined by a human-specific anti-nestin antibody. *Exp Neurol* **161:** 585–596.

Mintz M (1994). Clinical comparison of adult and pediatric neuroAIDS. *Adv Neuroimmunol* **4:** 207–221.

Nakatomi H, Kuriu T, Okabe S, Yamamoto S-i, Hatano O, Kawahara N, Tamura A, Kirino T, Nakafuku M (2002). Regeneration of hippocampal pyramidal neurons after ischemic brain injury by recruitment of endogenous neural progenitors. *Cell* **110:** 429–441.

Neil SJD, Aasa-Chapman MMI, Clapham PR, Nibbs RJ, McKnight A, Weiss RA (2005). The promiscuous CC chemokine receptor D6 is a functional coreceptor for primary isolates of human immunodeficiency virus type 1 (HIV-1) and HIV-2 on astrocytes. *J Virol* **79:** 9618–9624.

Ni HT, Hu S, Sheng WS, Olson JM, Cheeran MC-J, Chan ASH, Lokensgard JR, Peterson PK (2004). High-level expression of functional chemokine receptor CXCR4 on human neural precursor cells. *Dev Brain Res* **152:** 159–169.

Nunes MC, Roy NS, Keyoung HM, Goodman RR, McKhann G 2nd, Jiang L, Nedergaard M, Goldman SA (2003). Identification and isolation of multipotential neural progenitor cells from the subcortical white matter of the adult human brain. *Nat Med* **9:** 439–447.

Ranki A, Nyberg M, Ovod V, Haltia M, Elovaara I, Raininko R, Haapasalo H, Krohn K (1995). Abundant expression of HIV nef and rev proteins in brain astrocytes in vivo is associated with dementia. *AIDS* **9:** 1001–1008.

Rollison DEM, Utaipat U, Ryschkewitsch C, Hou J, Goldthwaite P, Daniel R, Helzlsouer KJ, Burger PC, Shah KV, Major EO (2005). Investigation of human brain tumors for the presence of polyomavirus genome sequences by two independent laboratories. *Int J Cancer* **113:** 769–774.

Sabri F, Tresoldi E, Di Stefano M, Polo S, Monaco MC, Verani A, Fiore JR, Lusso P, Major E, Chiodi F, Scarlatti G (1999). Nonproductive human immunodeficiency virus type 1 infection of human fetal astrocytes: independence from CD4 and major chemokine receptors. *Virology* **264:** 370–384.

Saito Y, Sharer LR, Epstein LG, Michaels J, Mintz M, Louder M, Golding BA, Cvetkovich TA, Blumberg BM (1994). Overexpression of *nef* as a marker for restricted HIV-1 infection as astrocytes in postmortem pediatric central nervous tissues. *Neurology* **44:** 474–481.

Schwartz L, Major EO (2006). Neural progenitors and HIV-1 associated central nervous system disease in adults and children. *Curr HIV Res* **4:** 319–327.

Strappe PM, Wang TH, McKenzie CA, Lowrie S, Simmonds P, Bell JE (1997). Enhancement of immunohistochemical detection of HIV-1 p24 antigen in brain by tyramide signal amplification. *J Virol Methods* **67:** 103–112.

Tamula MA, Wolters PL, Walsek C, Zeichner S, Civitello L (2003). Cognitive decline with immunologic and virologic stability in four children with human immunodeficiency virus disease. *Pediatrics 112:* 679–684.

Tohyama T, Lee VM, Rorke LB, Marvin M, McKay RDG, Trojanowski JQ (1992). Nestin expression in embryonic human neuroepithelium and in human neuroepithelial tumor cells. *Lab Invest* **66:** 303–313.

Tornatore C, Chandra R, Berger JR, Major EO (1994). HIV-1 infection of subcortical astrocytes in the pediatric central nervous system. *Neurology* **44**: 481–487.

Tornatore C, Nath A, Amemiya K, Major EO (1991). Persistent human immunodeficiency virus type 1 infection in human fetal glial cells reactivated by T-cell factor(s) or by the cytokines tumor necrosis factor alpha and interleukin-1 beta. *J Virol* **65:** 6094–6100.

Torres-Muñoz J, Stockton P, Tacoronte N, Roberts B, Maronpot RR, Petito CK (2001). Detection of HIV-1 gene sequences in hippocampal neurons isolated from postmortem AIDS brains by laser capture microdissection. *J Neuropathol Exp Neurol* **60:** 885–892.

Tran PB, Banisadr G, Ren D, Chenn A, Miller RJ (2007). Chemokine receptor expression by neural progenitor cells in neurogenic regions of mouse brain. *J Comp Neurol* **500:** 1007–1033.

Tran PB, Ren D, Veldhouse TJ, Miller RJ (2004). Chemokine receptors are expressed widely by embryonic and adult neural progenitor cells. *J Neurosci Res* **76:** 20–34.

Trillo-Pazos G, Diamanturos A, Rislove L, Menza T, Chao W, Belem P, Sadiq S, Morgello S, Sharer L, Volsky DJ (2003). Detection of HIV-1 DNA in microglia/macrophages, astrocytes and neurons isolated from brain tissue with HIV-1 encephalitis by laser capture microdissection. *Brain Pathol* **13:** 144–154.

Vallat A-V, Girolami UD, He J, Mhashilkar A, Marasco W, Shi B, Grey F, Bell J, Keohane C, Smith TW, Gabuzda D (1998). Localization of HIV-1 co-receptors CCR5 and CXCR4 in the brain of children with AIDS. *Am J Pathol* **152:** 167–178.

van der Meer P, Ulrich AM, Gonzalez-Scarano F, Lavi E (2000). Immunohistochemical analysis of CCR2, CCR3, CCR5, and CXCR4 in the human brain: potential mechanisms for HIV dementia. *Exp Mol Pathol* **69:** 192–201.

van Praag H, Schinder AF, Christie BR, Toni N, Palmer TD, Gage FH. (2002). Functional neurogenesis in the adult hippocampus. *Nature* **415:** 1030–1034.

Wang G, Achim CL, Hamilton RL, Wiley CA, Soontornniyomkij V (1999). Tyramide signal amplification

method in multiple-label immunofluorescence confocal microscopy. *Methods* **18:** 459–464.

Wang Z, Trillo-Pazos G, Kim S-Y, Canki M, Morgello S, Sharer LR, Sharer LR, Gelbard HA, Su Z-z, Kang D-c, Brooks AI, Fisher PB, Volsky DJ (2004). Effects of human immunodeficiency virus type 1 on astrocyte gene expression and function; potential role in neuropathogenesis. *J NeuroVirol* **10(Suppl):** 25–32.

Wiley CA, Soontornniyomkij V, Radhakrishnan L, Masliah E, Mellors J, Hermann SA, Dailey P, Achim CL (1998). Distribution of brain HIV load in AIDS. *Brain Pathol* **8:** 277–284.

Yachnis AT, Rorke LB, Lee VM-Y, Trojanowski JQ (1993). Expression of neuronal and glial polypeptides during histogenesis of the human cerebellar cortex including observations on the dentate gyrus. *J Comp Neurol* **334:** 356–369.