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Methamphetamine enhances cell-associated feline immunodeficiency virus replication in astrocytes

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Human immunodeficiency virus (HIV) infection among substance abusers is on the rise worldwide. Psychostimulants, and in particular methamphetamine (METH), have detrimental effects on the immune system as well as causing a progressive neurodegeneration, similar to HIV infection. Many Lentivirinae, including feline immunodeficiency virus (FIV), penetrate into the central nervous system early in the course of infection with astrocytes serving as a reservoir of chronic brain infection. We demonstrate that the FIV-Maryland isolate infects feline primary and cell line (G355-5)-cultured astrocytes only under cell-associated conditions. Infected astrocytes yielded a new astrocytotropic isolate, capable of cell-free infection (termed FIV-MD-A). This isolate contained four amino acid substitutions in the envelope polyprotein resulting in a change in net charge as compared to FIV-MD. Infection for both isolates was dependent upon a functional astrocyte CXCR4 receptor. Methamphetamine increased significantly FIV replication in feline astrocytes for cell-associated infection only, with no effect on peripheral blood mononuclear cells or astrocytes infected with FIV-MD-A. This viral replication was related to proviral copy number, suggesting the effect of METH is at the viral entry or integration into host genome levels, but not at the translational level. Thus, lentiviral infection of the brain in the presence of the psychostimulant METH may result in enhanced astrocyte viral replication, producing a more rapid and increased brain viral load. *Journal of NeuroVirology* (2002) **8,** 240–249.

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Introduction

Human immunodeficiency virus-1 (HIV-1) induces HIV-associated dementia (HAD) in approximately one third of adults and one half of pediatric patients (Belman, 1994; Glass and Johnson, 1996). Epidemiological studies show a worldwide increase in HIV infection among drug abusers (Gorman, 1996; WHO, 2000). In particular, methamphetamine (METH) use, a mood-elevating, positively reinforcing drug of high abuse potential, in humans is on the rise (Centers for Disease Control, 1995). Continuous use of METH can lead to a number of adverse psychological, immunological, and permanent neurotoxicological effects. Species sensitivity to the neurotoxicological effects vary, but are uniform in creating degeneration of serotonergic and dopaminergic nerve terminals of subcortical structures (e.g. basal ganglia) (Levine *et al*, 1980; Chapman *et al*, 2001).

The synergistic effects of chronic METH abuse and HIV-1 infection are presently poorly understood. Both METH and cocaine accelerated rapidly HAD despite treatment with highly active antiretroviral therapy in a patient (Nath *et al*, 2001). The high risk behavior in this group of people not only

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jeopardizes their own health status, but may advance the spread of HIV through rapid viral replication and dissemination, as both conditions have detrimental affects on the immune system and cause a progressive, subcortical-mediated neurodegeneration (Ellison and Switzer, 1993; Schmued and Bowyer, 1997; McArthur and Grant, 1998). In particular, psychostimulants alter CD8-mediated cytotoxic T-lymphocyte function (House *et al*, 1994), the primary immune cell responsible for early lentiviral replication suppression (Bagasra and Pomerantz, 1993b). Opiates, cocaine, and norepinephrine enhance lentiviral replication in peripheral blood mononuclear cells (Bagasra and Pomerantz, 1993a; Peterson *et al*, 1993; Cole *et al*, 1998). A recent report indicated a similar effect of METH in chronically infected feline astrocytes (Phillips *et al*, 2000).

The ability of feline immunodeficiency virus (FIV) to rapidly and persistently infect the central nervous system (CNS) of cats offers an excellent experimental model to investigate the temporal course of pathogenesis of neuroAIDS (Podell *et al*, 2000). Similar to HIV-1, FIV-induced encephalopathy is viral strain dependent, results in progressive immunodeficiency, increasing early peripheral but stable brain viral load, preferentially affects the developing nervous system, produces quantifiable behavioral and neurophysiologic impairment that is not directly linked to neuronal infectivity, and induces neuronal injury and loss both *in vivo* and *in vitro* (for review, see Podell *et al*, 2000).

Lentivirus CNS penetration occurs early in the course of infection, with resident microglia serving as the primary cell of infection and viral replication (Kaul *et al*, 2001). Astrocytes, however, are the most abundant CNS cells, serving as an integral component in brain protection from injury and infection. Although astrocytes are susceptible to lentiviral infection *in vitro* and *in vivo*, this infection is often characterized as non-replicative (Brack-Werner, 1999a). Thus, astrocytes may serve as a potential reservoir of chronic HIV-1 infection in the brain. The consequences of this chronic infection are not well elucidated in terms of the functional effects and reactivation to a replicative state of virus propagation in the face of physiologic and psychostimulant influences. One possible scenario is activation of latent provirus in the astrocytes resulting in virus replication and increased viral load in the brain. Additional effects on astrocyte function may also occur. One important function is to scavenge extracellular glutamate, thus preventing glutamate accumulation that could lead to the phenomenon of excitotoxic-induced neuronal death (Blumberg *et al*, 1994; Epstein and Gelbard, 1999). Excitotoxicity appears to be a common mechanism of neuronal death in METH abuse, HAD, and FIV-induced encephalopathy (Cubells *et al*, 1994; Gruol *et al*, 1998; Kaul *et al*, 2001). Moreover, HIV and FIV can reduce astrocytic glutamate uptake *in vitro*, and thus, propagate neuronal injury/loss (Yu *et al*, 1998).

The objectives of this experiment were to characterize receptor-mediated tropism of the Maryland (MD) isolate of FIV and the effect of METH on virus replication in feline primary astrocytes and an established feline astrocyte cell line (G355-5) under both cell-free and cell-associated infection conditions. We have demonstrated that peripheral blood mononuclear cell (PBMC)-associated FIV-MD infection of astrocytes produces an astrocytotropic isolate (FIV-MD-A) capable of cell-free astrocyte infection. Furthermore, METH increased markedly cell-associated, but not cell-free, virus replication in primary feline astrocytes and the established feline astrocyte cell line G355-5.

Results

Cell-associated virus is necessary for wild-type FIV-MD infection of feline astrocytes

Primary feline astrocytes or the feline astrocyte cell line G355-5 (ATCC; CRL-2033) were incubated with wild-type, cell-free FIV-MD for 2 h to 21 days. FIV-MD is known to infect PBMCs in culture and cause progressive neurological disorders in cats after being intravenously injected (Podell *et al*, 1993; Podell *et al*, 1997). Cell-free exposure of wild-type FIV-MD failed to infect astrocytes even at a concentration of 10000 $TCID_{50}$ up to 21 days after coculture as confirmed by p24 antigen assay (Figure 1C), reverse transcriptase (RT) activity, and PCR analysis. Both PBMC and monocyte-derived macrophages (MDM), however, were infected by cell-free FIV-MD at 100 $TCID_{50}$ or less by 2 h of virus exposure. In the course of the study we found that productive infection of astrocytes occurred only after coculture with FIV-MD infected PBMC. This cell-associated virus infection of astrocytes (CAVIA) was confirmed by p24-core protein immunocytochemistry staining (Figure 1B) and ELISA, FLOW-cytometry (Figure 2), RT activity of cell-free cultured media (Figure 3), and PCR (Figure 4). Cell passage of FIV-MD in astrocytes yielded a new astrocytotropic variant, FIV-MD-A, that was shown to infect G355-5 cells and feline primary astrocytes in cell-free conditions (Figure 1D) with a 10-fold higher $TCID_{50}$ as compared to PBMC (data not shown).

Envelope gene for both viral isolates was cloned and sequenced (Figure 5). We found four amino acid substitutions in FIV-MD-A envelope polyprotein as compared to FIV-MD (GenBank accession numbers are AF 452126 and AF452127 for FIV-MD and FIV-MD-A, respectively). All mutations were within polar hydrophilic residues and were different only in charge. Positively charged lysine and arginine in FIV-MD envelope were substituted to neutral asparagine

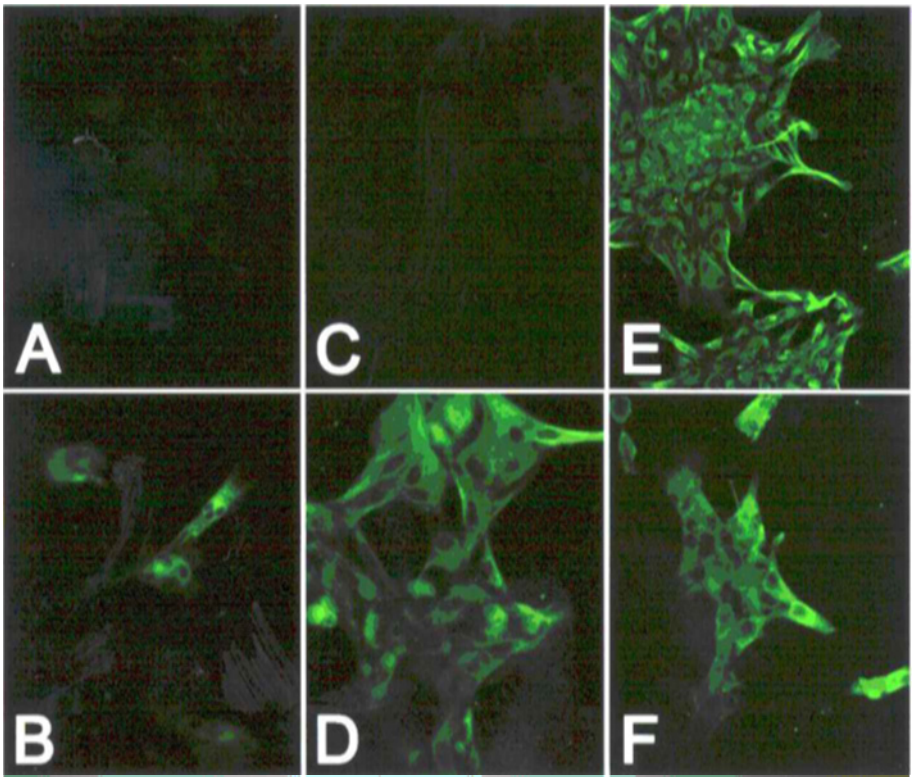


Figure 1 Immunofluorescent analysis of feline astrocytes demonstrates productive feline astrocyte infection (FITC conjugated FIV-p24 antigen immunofluorescence) by PBMC-associated virus (**B**) compared to the inability of cell-free FIV-MD virus to infect astrocytes (**C**) and to uninfected control astrocytes (**A**). Productive cell-free infection of astrocytes with FIV-MD-A is shown in (**D**). Feline G355-5 astrocytes are CXCR4 (**E**) and CCR5 (**F**) immunoreactive.

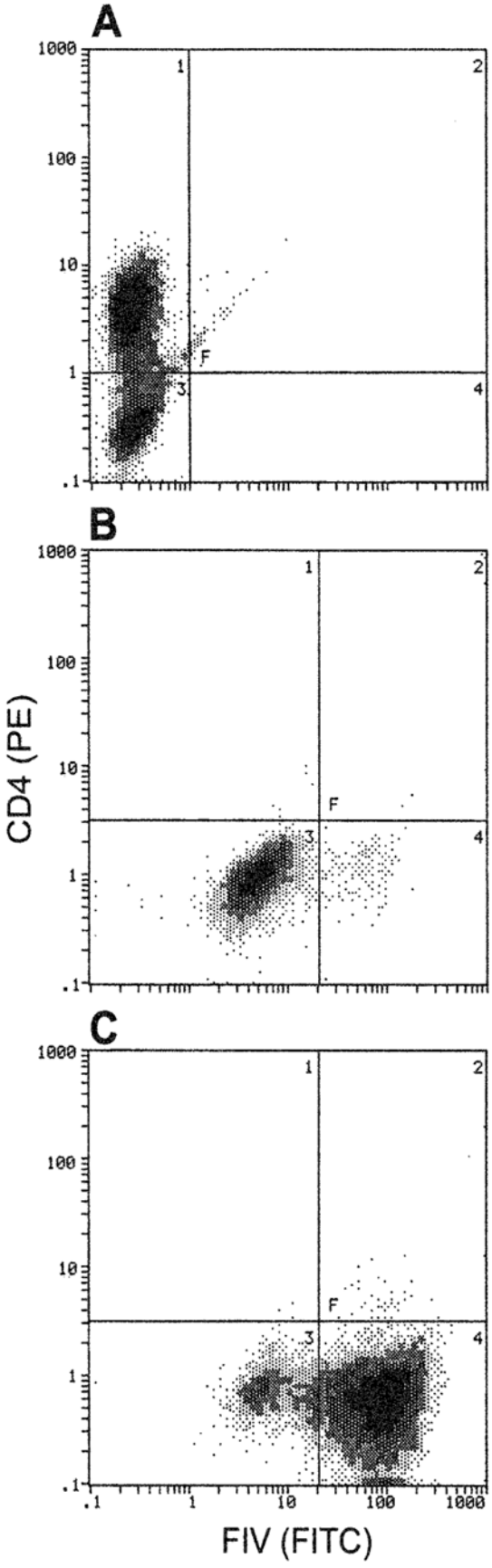
and glycine in FIV-MD-A, neutral serine was mutated to neutral glycine, and negatively charged glutamic acid was converted to a positive lysine residue in FIV-MD-A.

Infection of wild-type and mutant virus is dependent upon CXCR4 chemokine receptor

We have shown that primary and G355-5 feline astrocytes infected with either PBMC-associated (CAVIA) FIV-MD or cell-adapted FIV-MD-A possess the chemokine receptors CXCR4 (Figure 1E) and CCR5 (Figure 1F). Both PBMC and MDM, cells that are susceptible to FIV-MD and FIV-MD-A infection, are also highly CXCR4 and CCR5 positive. However, dual staining of G355-5 astrocytes and CAVIA with FITC-labeled anti-FIV and PE-labeled anti-CD4 antibodies showed that FIV-infected astrocytes, in contrast to PBMC, are CD4 negative (Figure 2). Selective blocking of the chemokine receptors CCR5 and CXCR4 on astrocytes with RANTES and SDF-1α, respectively, demonstrated the primary role of the CXCR4 receptor for infection of astrocytes with cell-free FIV-MD-A as well as CAVIA (Figure 3). Only SDF-1α, the CXCR4 receptor ligand, significantly ($P < 0.05$) abolished CAVIA infection in a dose-dependent manner. PCR analysis also failed to detect integrated provirus in DNA of CAVIA infected in the presence of 1 μg/ml SDF-1α and detected no change after treatment with 1 μg/ml RANTES (Figure 4).

Methamphetamine enhancement of virus replication in astrocytes

Cell-associated virus infection of astrocytes with FIV-MD in the presence of METH at concentrations equivalent to blood levels reported for human drug abusers (10^{-6}–10^{-10} M) (Driscoll *et al*, 1971; Stewart and Meeker, 1997) resulted in a progressive significant rise of viral core protein p24 and RT activity ($P < 0.01$) in cell culture media over the time period studied, as compared to FIV-MD infected, untreated astrocytes (Figure 6). This phenomenon was observed only with CAVIA of G355-5 and feline primary astrocytes and was absent for cell-free FIV-MD-A infection of astrocytes and FIV-MD infection of PBMC and MDM. Proviral copy number in DNA from CAVIA was increased markedly after METH treatment (Figure 4), which appeared to correlate with RT activity of cell media. SDF-1α also attenuated the enhanced effect of METH on FIV-MD replication (Figure 3).



Discussion

We report that feline astrocytes were infected with the wild type FIV-MD isolate only after association with infected PBMC. Infected astrocytes yielded a new astrocytotropic isolate, FIV-MD-A, capable of cell-free infection. Methamphetamine enhanced viral replication in feline astrocytes for cell-associated infection only. This viral replication was related to proviral copy number, suggesting the effect of METH is at the viral entry or integration into host genome levels, but not at the translational level.

CXCR4 but not CCR5 is the critical co-receptor for FIV infection of astrocytes

Our results agree with previous reports that feline astrocytes are highly CCR5 and CXCR4 (Koirala *et al*, 2000) positive, comparable with PBMC (Willett and Hosie, 1999) or MDM, but are CD4 negative. CXCR4 is a major receptor for FIV infection (Nakagaki *et al*, 2001; Willett and Hosie, 1999). Indeed, soluble ligand blocking of virus binding to certain chemokine receptors indicated the critical role of CXCR4 receptor in FIV-MD and FIV-MD-A entry into the astrocytes in this study. The inhibition of astrocyte infection by PBMC-associated FIV-MD and cell-free FIV-MD-A was dose- and time-dependent upon SDF-1α concentration in the culture media. RANTES, a ligand of the CCR5 receptor, did not prevent infection of astrocytes.

Similar to HIV, invasion of the CNS by FIV occurs early after primary infection and the virus resides primarily in microglia and astrocytes (Dow *et al*, 1992; Billaud *et al*, 2000). Despite detection of provirus in the brain of FIV-MD infected cats (Podell *et al*, 1999), wild-type FIV-MD failed to infect CXCR4-positive G355-5 and feline primary astrocytes under cell-free conditions. Productive infection could be achieved only after coculture with virus-infected PBMC. Similar findings were shown for FIV-PPR and FIV-Petaluma (Lerner and Elder, 2000; de Parseval and Elder, 2001). We now report that a new mutated variant, FIV-MD-A, can infect astrocytes under cell-free conditions. The observed difference of infection of CXCR4-positive cells by two FIV isolates and the ability of CXCR4 ligand SDF-1α to inhibit the astrocyte infection by the same two FIV isolates, support the notion that CXCR4 serves as one of the coreceptors in a postulated two-receptor model system (de Parseval and Elder, 2001).

Figure 2 FIV-MD infection of G355-5 astrocytes is CD4 independent. FITC-labeled anti-FIV antibody *vs* PE-labeled CD4 antibody is plotted. (**A**) PBL are CD4 positive; (**B**) uninfected G355-5 cells are CD4 and FIV negative; and (**C**) Infected astrocytes are CD4 negative but FIV positive. Quadrant 1 = PE-labeled CD4-positive cells; quadrant 2 = double positive (PE-labeled CD4-positive cells and FITC-labeled FIV-positive cells); quandrant 3 = double negative (PE-CD4 *vs* FITC-FIV); and quadrant 4 = FITC-labeled FIV-positive cells.

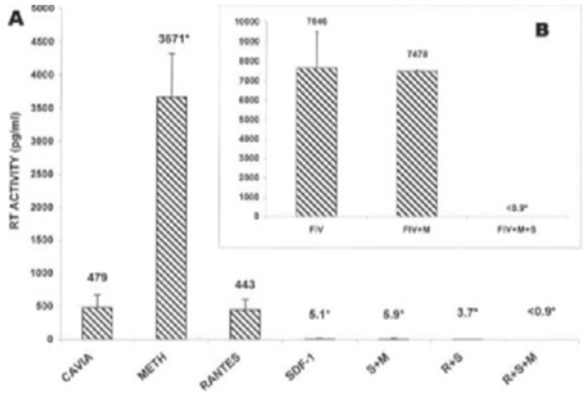


Figure 3 (**A**) Cell-associated FIV-MD infection of astrocytes (CAVIA) is CXCR4-dependent and CCR5-independent. The CXCR4 ligand, SDF-1α, inhibits completely cell culture supernatant reverse transcriptase (RT) activity and abolishes the enhancing effect of METH on virus replication in feline astrocytes, whereas CCR5 ligand RANTES does not. METH (M) (10^{-8} M), SDF-1α (S) (1 μg/ml), and RANTES (R) (1 μg/ml) were present in CAVIA media during infection and incubation. (**B**) Cell-free FIV-MD-A infection of astrocytes is also CXCR4 dependent. Triplicate results are reported as the mean and standard deviation. * = $P < 0.05$.

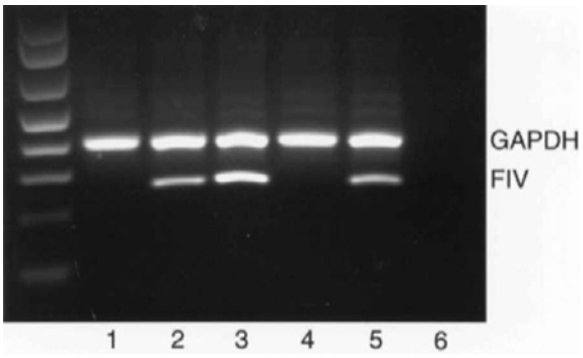


Figure 4 FIV-MD provirus is absent in G355-5 cells infected with wild type cell-free FIV-MD (lane 1) compared to infection of CAVIA (lane 2) in the presence of 10^{-6} M of METH (lane 3), 1 μg/ml SDF-1α (lane 4), 1 μg/ml RANTES (lane 5), or H_2O instead of DNA template (lane 6). FIV-specific amplificate (266 bp) was cloned and sequenced. Equal DNA loading was verified with an internal control, GAPDH.

Cell-associated viral infection is necessary for FIV-MD infection of astrocytes

The new astrocytotropic viral isolate, FIV-MD-A, exhibited a higher efficiency for astrocyte infection, as compared to PBMC, suggesting a cell-selective mutational effect. This adapted FIV-MD-A isolate infected G355-5 cells in cell-free conditions with similar CXCR4 expression as CAVIA. Primary retroviral isolates can be adapted to propagate in a wide range of cells after several *in vitro* passages (Kolchinsky *et al*, 1999; Richardson *et al*, 1999; de Parseval and Elder, 2001). As seen in this study, prior mutations for a newly acquired FIV phenotype have been mapped to variable regions of the FIV env gene (Verschoor *et al*, 1995; Hohdatsu *et al*, 1996; Vahlenkamp *et al*, 1999; Lerner and Elder, 2000). Moreover, the described phenotypic diversity is confirmed by genotypic differences in variable regions of the envelope polyprotein. Net charge may play a critical role in the change of envelope polyprotein conformation due to the amino acid substitution found in the FIV-MD-A variant. de Parseval and Elder (2001) reported that adaptation of FIV-PPR for propagation in CrFK cells resulted in an increase of the net charge due to a change of glutamate to lysine.

Similarly, we found a glutamate to lysine substitution at position 259 for FIV-MD-A. Thus, it appears that the motif between 252 and 259 amino acids seems to be very critical for these observed mutations. Specifically, the arginine to glycine substitution greatly reduces the residue weight by 100 Dalton while eliminating the highly restricted rotation around the α-carbon, the glutamate-to-lysine change significantly reverts the charge, and the two arginine and two tryptophan amino acids remain within the mutated residues. Overall, three of the four amino acid substitutions lead to the change of residue charge, one decreases molecular weight and size, possibly leading to an alteration in envelope conformation and the ability to bind to specific receptors. We plan to investigate further the structure-function differences between these two FIV isolates.

In the process of virus spreading between various cell types, cell-cell interaction occurs. One explanation for the need of cell-associated infection is that wild-type FIV requires at least a two-step binding process (de Parseval and Elder, 2001): CXCR4 and an additional coreceptor, which could be deficient on astrocytes. In experiments with binding of recombinant

	Selective positional alignment of envelope amino acid sequences																	
Isolate	131			252								259			505			
FIV-MD	Q	**K**	E		G	**R**	I	W	R	R	W	N	**E**	T		D	**S**	F
FIV-MD-A	•	**N**	•		•	**G**	•	•	•	•	•	•	**K**	•		•	**G**	•

Figure 5 Amino acid sequences of envelope polyprotein of wild-type FIV-MD and astrocytotropic FIV-MD-A isolates demonstrating four mutations at positions 131, 252, 259, and 505. Lysine (K) and arginine (R) are positively charged, glutamic acid (E) is negatively charged, yet asparagine (N), glycine (G), and serine (S) are neutral.

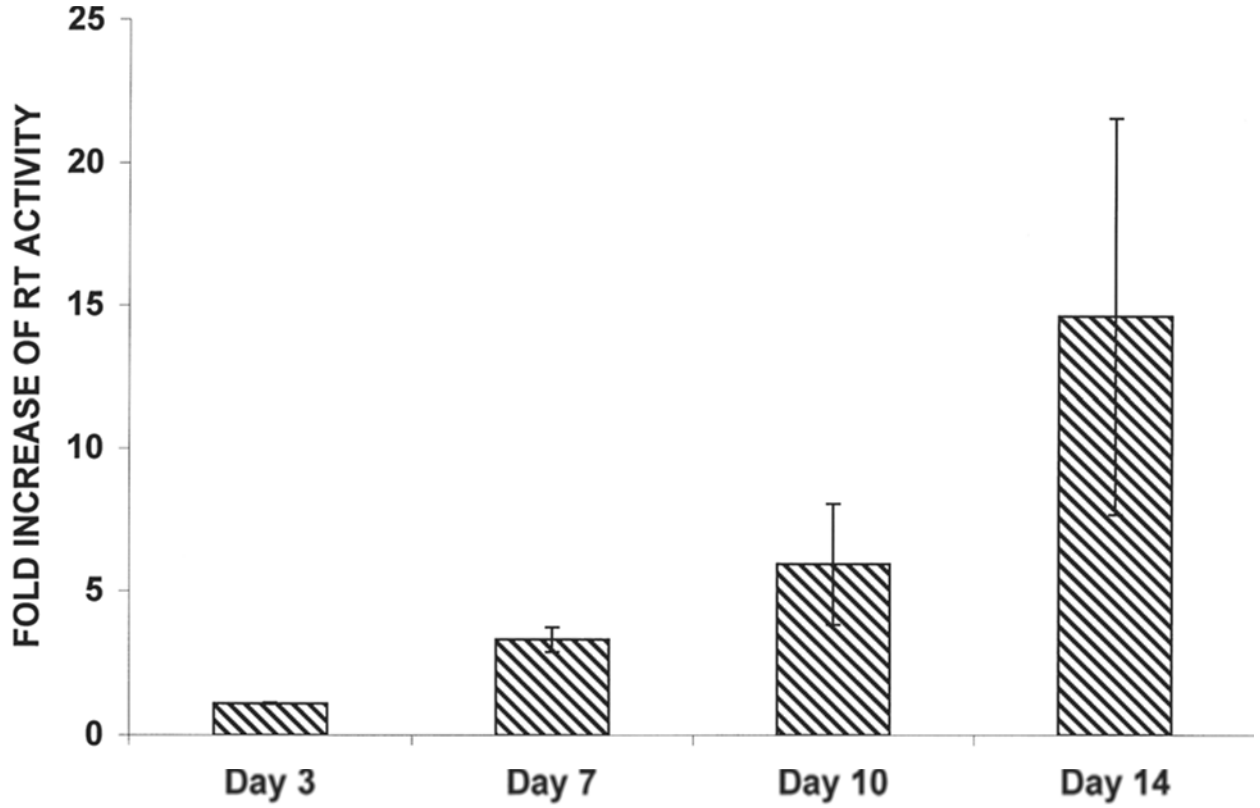


Figure 6 Methamphetamine (10^{-6}–10^{-10} M) enhances viral replication in feline G355-5 astrocytes up to 15 fold at day 14 postexposure to PBMC-associated FIV-MD as compared to non-METH treated FIV-MD infected G355-5 astrocyte cultures for each time point. Mean and standard deviation are shown from a total of nine replicate experiments.

FIV surface glycoprotein to feline cells of different lineage, another 40-kDa protein plays an important role in gp95-cell surface interaction (de Parseval and Elder, 2001). The function of this unidentified receptor may be similar to the function of CD4 for HIV. Virus adaptation resulting in a wider cellular tropism could switch from using two receptors (CD4-like unidentified and CXCR4) to only CXCR4. Similarly, both HIV-1 and HIV-2 can adapt to infect CD4 negative cells, and in particular, astrocytes (Reeves *et al*, 1999).

Cell-cell contact appears to be an important step for astrocyte infection that can lead to cell-selective viral adaptation. One intriguing report showed that cells with only CXCR4 or CD4 may cooperate in *trans*, and be infected with HIV-1 (Speck *et al*, 1999). In addition to *trans* cooperation, cell-cell contact is important for the release of microparticles bearing membrane receptors. Transfer of the chemokine receptor CCR5 between cells by membrane-derived microparticles (Mack *et al*, 2000) implicates a new mechanism for cellular lentivirus infection. In contrast to the inability of wild-type isolates of HIV-1 to infect CD4 negative astrocytes, mixed CNS cell cultures (astrocytes, microglia, and neurons) could be infected with HIV-1 and inhibited by anti-CD4 mAb (Boutet *et al*, 2001).

The significance of a mutated FIV (or HIV) isolate capable of cell-free astrocyte infection may be related to an "escape" strategy from immune attack. Infected PBMC may enter the brain via transfer through the blood–brain barrier (Wu *et al*, 2000), bringing cell-associated virus into the brain. Virus-infected PBMC could then infect astrocytes by a number of mechanisms involving direct cell-cell interaction. Mutated virus can then replicate to allow for a progressive infection of astrocytes, independent of immune cell interaction. To prove this concept, we plan to compare sequences of the variable region of the env gene of FIV-MD and FIV-MD-A to the brain and buffy coat isolates derived from FIV-MD and FIV-MD-A infected cats.

Methamphetamine enhances proviral copy number and RT activity of CAVIA

The second major observation was the phenomena that METH greatly enhanced virus replication in CAVIA. Little is known about lentivirus-METH interaction *in vivo*. Phillips *et al* (2000) showed that METH increased FIV/34TF10 replication in chronically infected G355-5 cells. We observed, however, that METH enhances FIV replication only in the CAVIA experiments after cell-cell contact in both G355-5 and feline primary

astrocytes. This enhancement still occurred in the presence of RANTES, but was abrogated with the addition of SDF-1α. No effect of METH on FIV-MD replication in PBMC, MDM, or cell-free FIV-MD-A replication in astrocytes was seen. These results suggest that METH influences the first step of virus-cell interaction during cell-to-cell transmission of virus. Norepinephrine, one of the major stress factors and structurally related to METH, accelerates HIV-1 replication in PBMC (Cole *et al*, 1998) via G-protein adrenoreceptor signaling pathway and protein kinase A dependent suppression of cytokine production. Norepinephrine also binds to $G_{\alpha S}$ protein that activates adenylate cyclase and formation of cAMP. Chronic use of METH and heroin in humans significantly decreases concentration of $G_{\alpha i}$, protein that inhibits adenylate cyclase in the nucleus accumbens (McLeman *et al*, 2000). Cyclic AMP is able to up-regulate CXCR4 (Cole *et al*, 1999), thus implying a possible mechanism of virus-drug interaction and pathogenesis.

METH may also directly interact with the nucleus of brain cells to activate transcription factors AP-1 and cAMP response element-binding protein (CREB) (Asanuma *et al*, 2000). cDNA array profile of mouse brain showed that METH administration was characterized by an upregulation of transcriptional factors, growth factors, cytokines, and chemokines (Cadet *et al*, 2001). We also cannot exclude the direct effect of METH on properties of viral promoter to drive transcription of integrated provirus differentially in glial versus blood cells (Brack-Werner, 1999a; Brack-Werner, 1999b; Krebs *et al*, 1998). Morphine, for example, was shown to induce transactivation of HIV-1 LTR in human neuroblastoma cells (Squinto *et al*, 1990).

Our experiments show that FIV infection of astrocytes is dependent upon a functional CXCR4 receptor. Cell-cell contact is important for initial wild-type FIV-MD productive infection of astrocytes. Virus can mutate to an isolate form that does not require cell-associated infection. METH significantly enhances astrocyte infectivity by only cell-associated virus, suggesting the effect of METH is likely at the viral entry or integration levels. Future work will be directed at elucidating mechanisms of these reported phenomena.

Materials and methods

Cell infection with virus

Primary cells (PBMC and astrocytes) were purified from specific pathogen-free, FIV-tested negative adult cats under approval of the University Laboratory Animal Care and Use Committee according to the NIH Care and Use of Laboratory Animals Guideline. Feline PBMC, stimulated with concanavalin A (7.5 μg/ml) and IL-2 (20 U/ml) for 5 days, were infected with 100 $TCID_{50}$ of FIV-MD per 5×10^4 cells, and maintained 5–7 days prior to coculture, as described (Podell et al, 2001). Twenty-four hours before coculture with infected PBMC, 5×10^4 G355-5 cells were seeded into a 12 well-plate, the medium was changed and equal numbers of FIV-MD infected PBMC were added to the astrocytes. PBMC were washed off astrocyte monolayers after overnight incubation. Removal of PBMC was over 99.5%, as visually confirmed by green fluorescent dye CFSE staining of PBMC prior to coculture. METH (10^{-6}–10^{-10} M) was present in the medium during infection and replaced every time medium was changed. In experiments with selective ligand blocking, certain concentrations of SDF-1α and RANTES were added to G355-5 cells 1 h before starting infection and replaced with medium changing.

Immunofluorescence

G355-5 cells and feline primary astrocytes were maintained in McCoy medium containing 10% fetal bovine serum and antibiotics. PBMC were isolated by Ficoll-Percoll two-density (1.077–1.056) gradient followed by monocyte separation by adherence to plastic as previously described (Gavrilin *et al*, 2000). Monocyte-derived macrophages were maintained in RPMI-1640 with 5% fetal bovine serum and antibiotics in the absence of IL-2. Macrophages developed typical macrophage morphology within 7 days including lamellipodia and foamy cytoplasm. Cells were grown in 4-well glass chamber slides (Lab-Tek) until 50–75% confluence. After media was removed, cells were washed with washing buffer (PBS containing 2% FBS), fixed in methanol-acetone (50:50) for 5–10 min at room temperature, and washed twice with washing buffer. FIV-p24 antigen was detected with feline polyclonal antiserum (obtained from FIV-infected cats) diluted 1:500 and revealed with 1:200 anti-feline IgG-FITC (Calbiochem). Uninfected astrocyte cultures and omission of primary antibody served as controls. CXCR4 staining was performed with monoclonal CXCR4 antibody (R&D, clone 44716.111) at 1:1000 dilution and detected with anti-murine IgG FITC (Sigma). CCR5 staining was performed with CCR5 antibody (R&D, clone 45549.111) at 1:200 dilution. Photomicrographs were obtained with a SPOT camera (Diagnostic Instruments) using a BH-2 Olympus microscope with a mercury light source. For each antibody, photographic exposure was optimized on a field of positive cells, with all subsequent photos using the same exposure setting.

Flow cytometry

Adherent G355-5 cells and feline primary astrocytes were harvested mechanically without trypsin treatment, and stained in solution with PE-labeled

antibody for CD4 (Southern Biotechnology) or with secondary FITC-labeled antibody followed by staining with primary antibody for FIV antigen, CXCR4 (R&D), and CCR5 (PharMingen, clone 3A9). Negative cell controls were G355-5 cells, determined by fluorescence in the absence of primary antibody. Cells were counted on a COULTER flow cytometer with 10000 cells counted for each determination.

Reverse transcriptase activity
Cell culture media was collected at different time points as indicated, spun, and the particle-free supernatant was transferred to fresh tubes and stored at −80°C until use. Reverse transcriptase activity was measured with the Lenti-RT Activity Assay kit (Cavidi Tech Uppsala, Sweden). Briefly, the RT in the sample synthesizes a DNA strand. BrdUTP in the reaction mixture is incorporated into the immobilized template/primer construct and is quantified colorimetrically by BrdU binding antibody conjugated to alkaline phosphatase. Standard curve and samples were read at A_{405} within the linear measurable range of the plate reader. Limit of detection was 0.9 pg/ml.

DNA isolation and PCR
Cell DNA was isolated with Puregene DNA isolation kit (Gentra Systems). Then, 100–500 ng of total DNA was subjected to PCR with the primers for gag polyprotein gene of the integrated FIV-MD and FIV-MD-A provirus: forward 5′-GAC CCA AAA ATG GTG TCC-3′ and reverse 5′-CCT ATT CCC ATA ATC TCT GC-3′. Both FIV isolates (MD and MD-A) had complete homology for the selected gene region. To confirm, 266-bp amplified product was cloned into pCR4 plasmid (Invitrogen) and sequenced. To verify an equal amount of DNA used for amplification, GAPDH was used as an internal control with the set of primers: forward 5′-CCT TCA TTG ACC TCA ACT ACA-3′ and reverse 5′-CCA AAG TTG TCA TGG ATG ACC-3′.

Statistics
Descriptive statistics were calculated for RT activity for astrocyte cell infection experiments (JMP Statistical Software, SAS Institute, Cary, NC). A Wilcoxon 2-way test was used to compare differences in median values between groups. Significance was set at $P < 0.05$.

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