**Methamphetamine increases HIV infectivity in neural progenitor cells**

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HIV-1 infection and methamphetamine (METH) abuse frequently occur simultaneously and may have synergistic pathological effects. Although HIV-positive/active METH users have been shown to have higher HIV viral loads and experience more severe neurological complications than non-users, the direct impact of METH on HIV infection and its link to the development of neurocognitive alternations are still poorly understood. In the present study, we hypothesized that METH impacts HIV infection of neural progenitor cells (NPCs) by a mechanism encompassing NFκB/SP1-mediated HIV LTR activation. Mouse and human NPCs were infected with EcoHIV (modified HIV virus infectious to mice) and HIV, respectively, in the presence or absence of METH (50 or 100 μM). Pretreatment with METH, but not simultaneous exposure, significantly increased HIV production in both mouse and human NPCs. To determine the mechanisms underlying these effects, cells were transfected with different variants of HIV LTR promoters and then exposed to METH. METH treatment induced transcriptional activity of the HIV LTR promoter, an effect that required both NFκB and SP1 signaling. Pretreatment with METH also decreased neuronal differentiation of HIV-infected NPCs in both *in vitro* and *in vivo* settings. Importantly, NPC-derived daughter cells appeared to be latently infected with HIV. This study indicates that METH increases HIV infectivity of NPCs, through the NFκB/SP1-dependent activation of the HIV LTR and with the subsequent alterations of NPC neurogenesis. Such events may underlie METH-exacerbated neurocognitive dysfunction in HIV-infected patients.

Methamphetamine (METH) abuse is becoming an epidemic problem worldwide. It is estimated that there are around 35 million people abusing METH, with over 10 million users in the United States. The prevalence of METH abuse is rising mostly due to its relatively easy production, low costs, and, consequently, high availability. METH exerts a strong and long lasting effect on the CNS, which makes it highly addictive. METH abuse not only creates social problems and has devastating effects on health, generating costs of approximately $24 million/year in the United States (75), but it has also been shown to contribute to increased transmission of infectious diseases, including HIV (1–4). HIV infection and METH abuse co-exist in ~10–15% of HIV-infected patients (5).

METH abuse and HIV infection are independently associated with neuropathological changes in the brain resulting in neuropsychiatric disturbances and cognitive disorders. Despite widespread use of highly active antiretroviral therapy, HIV persists within the CNS, causing cell damage that results in ~69% of HIV-infected individuals diagnosed with neurocognitive disorders (6). Similarly, METH abuse itself is linked to alterations in brain structure and dopaminergic parameters, as well as decline of cognitive function (7). When comorbidity of both HIV and METH occurs, additive deleterious effects on neuropsychological functions can be observed. Although potential mechanisms underlying such interaction have not been fully explored, impairment of the blood–brain barrier (8, 9), decreased viability and functions of dopaminergic neurons and neuroglia (10), and neuroinflammation (11) have been proposed to be involved in this phenomenon. In addition, recent reports suggest that METH may contribute to increased HIV infectivity. It is suggested that METH might enhance transcriptional activity of the HIV LTR (12, 13) or augment HIV entry into the cells through up-regulation of HIV co-receptors CXCR4 and CCR5 (14, 15). Data derived from animal and human studies support these observations by demonstrating that METH abuse is associated with higher viral loads (12, 16–20).

In the present study, we focus on the interaction between METH and HIV involving neural progenitor cells (NPCs). Evi-
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Results

Pretreatment with METH increases HIV infectivity in mouse and human NPC

Mouse or human NPCs (mNPCs or hNPCs, respectively) were treated with METH simultaneously or 24 h before HIV infection. mNPCs were infected with EcoHIV (Fig. 1, A and B), whereas hNPCs were infected with HIV (Fig. 1, C and D). HIV production was measured by the assessment of p24 antigen (HIV capsid protein), a sensitive and useful test for the detection of active HIV generation as p24 is produced during viral replication. All groups were treated with the same amount of virus, and the initial levels of p24 were not significantly different between groups (Fig. 1, A–D). All infections of NPCs were performed by adding virus for 12 h to cell culture medium. Then the virus was washed out, fresh medium was added, and aliquots of culture medium were collected over time to measure the p24 level.

Both mNPCs and hNPCs were susceptible to infection; however, the levels of p24 were higher in hNPCs compared with mNPCs, indicating more productive infection. Exposure to METH at the time of HIV infection resulted in a transient increase in p24 levels in mNPCs, which was observed 24 and 48 h postinfection, as compared with the HIV only group (Fig. 1A). However, a simultaneous treatment with METH at the
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Figure 2. Integration of HIV DNA with the host NPC genome. Mouse (NE4C cells) and human (ReNcell VM cells) NPCs were pretreated with 100 μM METH for 24 h and infected with EcoHIV or HIV, respectively, as in Fig. 1. HIV DNA integrated into the host genome was measured by digital droplet PCR. Results are mean ± S.D. (error bars) and expressed as number of copies/100,000 cells; n = 3. *, p < 0.05, HIV versus 100 μM METH + HIV; A, 72 h: p = 0.0225, EcoHIV versus 100 μM METH + EcoHIV; B, 48 h: p = 0.0007, HIV versus 100 μM METH + HIV; 72 h: p = 0.0075, HIV versus 100 μM METH + HIV.

METH induces HIV replication by NFκB/SP1-mediated stimulation of the HIV LTR

Whereas the changes in METH- and/or EcoHIV-induced activation of NFκB and SP1 were not robust (although significant), the significance of these results was next addressed in functional studies by transfection of mNPCs with the plasmid pXP1 LTR WT, containing the complete HIV LTR sequence upstream of the luciferase reporter gene (nucleotides −644 to +77; Fig. 4A), along with the control plasmid pR-SV40. The transfected cells were treated with vehicle and/or with METH in the presence or absence of EcoHIV. Then luciferase activity was measured as a marker of HIV replication. As indicated in Fig. 4B, exposure to METH significantly enhanced the transcriptional activity of the HIV LTR.

To analyze the functional role of the SP1 and/or κB enhancer in METH-induced activation of the HIV LTR, variants of pXP1 LTR containing mutations in the SP1-binding site and/or deletion of the κB enhancer were employed (Fig. 4A). Cells were co-transfected with one of these reporter constructs, along with a control plasmid, followed by a luciferase activity assay. The time of HIV infection had no impact on p24 levels in hNPCs (Fig. 1C). In contrast, a 24-h pretreatment with METH significantly increased HIV production in both mouse and human NPCs as compared with HIV alone (Fig. 1, B and D, respectively). In mNPCs, pretreatment with METH elevated p24 levels in cell culture medium by ~33% 72 h postinfection. In hNPCs, pretreatment with METH enhanced p24 levels by ~15% at 24 h and ~30% at 48 and 72 h postinfection. Pretreatment with METH at 50 and 100 μM was equally effective in stimulation of HIV production.

We next analyzed HIV DNA integration with the host genome. mNPCs and hNPCs were pretreated 24 h with 100 μM METH and infected with EcoHIV and HIV, respectively. Cells were collected at three different time points post-infection (0, 48, and 72 h) and analyzed by digital droplet PCR (ddPCR). METH induced a ~24% increase in the integrated HIV DNA at 72 h in mNPCs as compared with EcoHIV alone (Fig. 2A). METH-induced impact on HIV DNA integration in hNPCs was much higher, reaching a ~3-fold increase at 48 h postinfection (Fig. 2B). Cells used for HIV DNA integration experiments were undifferentiated and were maintained in proliferating condition. Typically, only a small ratio of cultured NPCs is infected with HIV; however, the entire NPC population proliferates. Therefore, a decrease in detected HIV DNA at 72 h postinfection as compared with 48 h may stem from the fast proliferation of the entire NPC culture and “diluting” HIV DNA within the total genomic DNA.

METH activates NFκB and SP1 in NPCs

To address the mechanisms of increased p24 production in METH-pretreated HIV-infected cells, we focused on transcriptional regulation of HIV replication. NFκB and SP1 are considered the main regulatory elements of the HIV LTR, and binding sites for both transcription factors are present in the core of the HIV enhancer. Therefore, we analyzed the impact of METH on activation of NFκB and SP1 in mNPCs. It has been reported previously that cellular effects of METH involve impaired regulation of these transcription factors (32, 33); however, not much is known about activation of these factors in NPCs. Because acetylation at Lys-310 is required for full transcriptional activity of NFκB, acetylated NFκB/p65 was analyzed as a marker for NFκB activation. Regarding SP1, phosphorylation was shown to play a critical role in transcriptional activity and stability of this transcription factor (34). Therefore, we assessed phosphorylated SP1 as the indicator of its activation.

METH exposure (100 μM) induced translocation of acetylated NFκB and phosphorylated SP1 into the nuclei. Whereas acetylated NFκB was equally distributed throughout the nuclei, phosphorylated SP1 was forming foci. Representative images illustrating these changes are presented in the top panels, and the quantitative data in the form of bar graphs are shown in the bottom panels of Fig. 3 (A and B). NFκB was activated by a 1-h exposure to METH and EcoHIV by 38 and 33%, respectively. When added together, METH plus EcoHIV increased the intensity of acetylated NFκB fluorescence in the nuclei by 52% (Fig. 3A). A similar pattern of changes was observed with activation of SP1. METH and EcoHIV treatment for 30 min increased the number of foci corresponding to phosphorylated SP1 in the nuclei by 48 and 41%, respectively. METH and Eco-HIV added together increased the number of foci by 58% (Fig. 3B).

We also analyzed NFκB and SP1 activation in hNPCs to confirm that similar mechanistic events take place in human and mouse NPCs. Fig. S1 demonstrates that both NFκB and SP1 were activated by METH and/or HIV treatment in hNPC.
Figure 3. METH- and/or HIV-mediated activation of NFκB and SP1 in NPCs. mNPCs (NE-4C cells) were pretreated with METH (100 μM) for 1 h (NFκB experiments) or 0.5 h (SP1 experiments) and/or infected with HIV as in Fig. 1. NFκB activation was evaluated by measuring the fluorescence intensities of NFκB acetyl-Lys-310 staining (A) and activation of SP1 by phospho-Thr-453 foci (B) in cell nuclei. A confocal laser-scanning microscope was used to obtain fluorescence imaging. Approximately 300 randomly chosen cells were analyzed. Images show representative results, and the bar graphs reflect quantitative results. Results are mean ± S.D. (error bars), expressed as a percentage of control, n = 3 (3 independent experiments). *, p < 0.05 versus control. A, p = 0.0026, control versus EcoHIV; p < 0.0001, control versus 100 μM METH; p < 0.0001, control versus 100 μM METH + EcoHIV. B, p = 0.0004, control versus EcoHIV; p < 0.0001, control versus 100 μM METH; p < 0.0001, control versus 100 μM METH + EcoHIV.

Figure 4. Transcriptional regulation of METH-induced modulation of HIV replication. A, schematic representation of the HIV LTR constructs used in the experiment. Mutated SP1-binding sites are represented by crossed circles. B, HIV LTR activities were detected by measuring the luciferase activity in the cell lysates. NPCs were transfected with construct from B for 24 h. Cells were left untreated or stimulated with 100 μM METH with or without infection with EcoHIV for 12 h, followed by a luciferase assay. Results are mean ± S.D. (error bars) from 3–5 cell culture replicates, and each experiment was done in quadruplicates. *, p < 0.05 (p = 0.0003, control versus METH; p < 0.0001, control versus METH + EcoHIV). SP1 mut, mutated SP1 binding site; ΔNFκB, deleted NFκB enhancer.
removal of functional SP1- or NFkB-binding sites decreased the basal expression of HIV LTR transcriptional activity and completely prevented METH-induced effects. These results indicate that both NFkB and SP1 sites are necessary for stimulation of HIV replication by METH.

**METH and HIV decrease differentiation of NPCs**

We next assessed whether METH-induced HIV infectivity affects differentiation of NPCs. Primary mNPCs were treated with 100 μM METH in the presence or absence of EcoHIV under cell culture conditions that induce differentiation (i.e. without growth factors and in restricted availability of serum). Neurogenesis was monitored by counting doublecortin (DCX)- and neuronal-specific nuclear protein (NeuN)-positive cells at different time points throughout the differentiation process for up to 10 days. DCX is a marker of immature neurons, and NeuN is a marker for mature neurons. At day 10 after initiation of differentiation, the numbers of both DCX- and NeuN-positive cells were significantly decreased in the METH plus EcoHIV group, when compared with control (Fig. 5). Representative images illustrating these changes are presented in the top panels, and the quantitative data from these experiments are shown in the form of bar graphs (bottom panels).

Further, we analyzed and compared the morphology of newly differentiated neurons from different experimental groups. The ratio of multipolar neurons was decreased, whereas the pool of anaxonic neurons was increased in the HIV, METH, and METH + HIV groups when compared with control (Fig. 6A). Furthermore, a decrease in complexity of multipolar neurons in all of the above groups was observed (Fig. 6B).

In addition, we identified neuronal and glia cell population derived from differentiated mNPCs at day 14 by FACS based on the cell surface signatures specific to distinct cell types (35, 36). CD184 and CD44 are two major markers on which the distinction between differentiated glia and neurons was based. In detached cells that form a single cell suspension, CD184 content is lost from the vast majority of the neurons (35), whereas CD44 has been shown to be a marker of both glial progenitors and astrocytes. In addition, CD24 was used to confirm the differentiation processes.

Cells were first examined for live/dead aqua staining. Then the CD24+ population was identified, which appeared to be
almost 100% of the total cell population. CD24+ cells were next separated based on distinct patterns of CD184 and CD44 immunoreactivity: CD184+/CD44+ cells were predicted to be glial cells, whereas CD184−/CD44− cells were predicted to be neurons. The sorting procedure is illustrated in Fig. 7 (A and B), and the quantitative results are reflected in Fig. 7 (C and D). METH treatment in both noninfected and HIV-infected cells caused a significant ~3-fold decrease in glial (i.e. CD24+/CD184+ /CD44+ ) cells as compared with controls (Fig. 6C). In contrast, neurons (i.e. CD24+/CD184−/CD44− cells) decreased only in the METH plus HIV group (Fig. 6D). It is noteworthy that the decreased number of neurons and glial cells was not associated with a loss in cell viability (Fig. S3). Therefore, most likely it was an outcome of impaired/delayed neurogenesis and differentiation processes.

To assess the purity of isolated cells, CD24+/CD184+/CD44+ and CD24−/CD184+/CD44− cells were plated after sorting, cultured for 5 days, and then co-stained for neuronal (Tuj-1) and astrocytic (GFAP) markers. As shown in Fig. 7E, the obtained cell populations were pure, and no cross-contamination was observed.

**Integrated HIV DNA is preserved in neurons and astrocytes derived from HIV-infected NPCs**

Sorted populations of differentiated neurons and glial cells derived from infected mNPCs were next analyzed for integrated HIV DNA. Interestingly, a high number of copies of integrated HIV DNA was detected in both cell populations. As many as 37,775 ± 8,019 copies/100,000 cells were detected in neurons, and 46,981 ± 4,269 copies/100,000 cells were identified in glial cells (Fig. 8A). Despite the fact that NPC-derived daughter cells carry a substantial number of integrated HIV DNA copies, only trace levels of extracellular HIV p24 were detected in these cultures (~100 pg/ml).

To determine whether differentiated mNPCs were latently infected, we performed HIV reactivation studies. mNPCs were differentiated for 2 weeks as in Fig. 8A; then we attempted to reacti-

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*Figure 6. METH and/or HIV affects morphology of newly differentiated neurons.* Primary mouse NPCs were induced to differentiate in the presence or absence of 100 μM METH with or without EcoHIV. Cells were immunostained for MAP2 to assess changes in morphology. A, the percentage ratio of different types of neurons was analyzed and compared between the groups. Results are mean ± S.D. (error bars), n = 3. For multipolar neurons: *, p < 0.05 (p = 0.0034, control versus EcoHIV; p = 0.001, control versus METH; p = 0.0016, control versus METH + EcoHIV); for anaxonic neurons: #, p < 0.05 (p = 0.0103, control versus EcoHIV, p = 0.0065, control versus METH; p = 0.0039, control versus METH + EcoHIV). B, multipolar neurons were next subjected to Scholl analysis. Branching complexity and axonic and dendritic lengths were analyzed. Results are mean ± S.E. (error bars); n = 14–19. *, p < 0.05 (for point 0; p = 0.0083, control versus EcoHIV; p = 0.0038, control versus METH; p = 0.0126, control versus METH + EcoHIV; for point 10; p = 0.0001, control versus EcoHIV; p < 0.0001, control versus METH; p = 0.0037, control versus METH + EcoHIV).
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A

B

C

D

E

CD24+/CD44+/CD184+

GFAP/Tuj-1

CD24+/CD44+/CD184+

GFAP/Tuj-1

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Note that there is no cross-contamination in sorted cell populations.

S.A.H.A. (10\(^{-10}\)) with vorinostat (suberanilohydroxamic acid (SAHA) at both 5 and 10\(\mu\)M) added together with TNF-\(\alpha\) (10 ng/ml) for 3 days. The effect of treatments on p24 production (\(B\)) and HIV RNA expression (\(C\)) was examined. Results are mean \(\pm\) S.D. (error bars) from three independent experiments. *, \(p = 0.0035\), EcoHIV versus EcoHIV + 5 \(\mu\)M SAHA; **, \(p = 0.0476\), EcoHIV + 10 \(\mu\)M SAHA; ***, \(p < 0.0001\), EcoHIV versus EcoHIV + TNF (10 ng/ml) + SAHA (5 \(\mu\)M) and EcoHIV versus EcoHIV + TNF (10 ng/ml) + SAHA (10 \(\mu\)M), \(C\), \(n = 6 - 9\), from three independent experiments. *, \(p = 0.0054\), EcoHIV versus SAHA (10 \(\mu\)M). **, \(p < 0.0001\) EcoHIV versus EcoHIV + TNF (10 ng/ml) + SAHA (5 \(\mu\)M) and EcoHIV + TNF (10 ng/ml) + SAHA (10 \(\mu\)M).

METH and EcoHIV impair neurogenesis in mouse hippocampus

Because NPCs are located in the hippocampal dentate gyrus, we analyzed effects of METH with or without EcoHIV on neurogenesis in this particular brain region. BrdU was used to detect proliferating cells. BrdU incorporation was performed during METH and EcoHIV treatment; therefore, the number of NeuN/BrdU double-positive cells represent newly formed neurons that differentiated from progenitors specifically within 2 weeks of METH and EcoHIV exposure. The number of NeuN\(^+/\)BrdU\(^+\) cells was significantly reduced in METH and METH + EcoHIV-treated animals (Fig. 9), a finding that supports the in vitro finding from Figs. 5–7.

Figure 7. METH acting together with HIV decreases NPC differentiation to glial cells and neurons. Primary mNPCs were differentiated for 2 weeks in the presence or absence of 100 \(\mu\)M METH and/or EcoHIV, followed by FACS analysis. \(A\), sequential cell sorting strategy of differentiated NPCs based on live/dead sorting (left two images), followed by CD24, CD184, and CD44. The obtained population of CD24\(^+/\)CD184\(^-/\)/CD44\(^+\) cells is considered to be glial cells, and the CD24\(^-/\)/CD184\(^+/\)/CD44\(^-\) population is considered to be neurons. \(B\), examples of intensity distributions of cell cultures stained with surface markers defined for isolation of NPC-derived neuronal and glia populations. \(C\) and \(D\), quantitative data from the sorting procedure for glial and neuronal populations, respectively. Results are mean \(\pm\) S.D. (error bars) from three independent experiments. *, \(p < 0.05\) versus control. \(C\), \(p = 0.008\), control versus 100 \(\mu\)M METH; \(p = 0.0177\), control versus 100 \(\mu\)M METH + EcoHIV. \(D\), \(p = 0.0125\), control versus 100 \(\mu\)M METH + EcoHIV. \(E\), sorted cells were plated, cultured for 5 days, and stained for GFAP (marker of astrocytes) and Tuj-1 (marker of neurons). \(L\)eft, CD24\(^+/\)/CD184\(^-\)/CD44\(^+\) cells positive for GFAP; \(r\)ight, CD24\(^-/\)/CD184\(^+/\)/CD44\(^+\) cells positive for Tuj-1. Note that there is no cross-contamination in sorted cell populations.

Figure 8. Latent infection of differentiated mNPCs. \(A\), integrated HIV DNA in NPC-derived daughter neuronal and glial populations. Primary mouse NPCs, not infected or infected with EcoHIV, were differentiated for 2 weeks, followed by sorting for neuronal and glial population as in Fig. 7. After sorting, both cell populations were collected, and HIV DNA integrated into the host genome was assessed. Results are mean \(\pm\) S.D. (error bars) and expressed as number of copies/100,000 cells. \(B\) and \(C\), reactivation of EcoHIV from latently infected differentiated mNPCs. Primary mNPCs were infected with EcoHIV and differentiated for 2 weeks. Then cells were treated with potential latency-reversing agents: METH (100 and 300 \(\mu\)M), and/or SAHA (5 and 10 \(\mu\)M) alone or in combinations (as indicated) for up to 3 days. The effect of treatments on p24 production (\(B\)) and HIV RNA expression (\(C\)) is examined. Results are mean \(\pm\) S.D. \(B\), \(n = 6 - 16\), from four independent experiments. *, \(p = 0.0335\), EcoHIV versus EcoHIV + 5 \(\mu\)M SAHA; **, \(p = 0.0476\), EcoHIV + 10 \(\mu\)M SAHA; ***, \(p < 0.0001\), EcoHIV versus EcoHIV + TNF (10 ng/ml) + SAHA (5 \(\mu\)M) and EcoHIV versus EcoHIV + TNF (10 ng/ml) + SAHA (10 \(\mu\)M), \(C\), \(n = 6 - 9\), from three independent experiments. *, \(p = 0.0054\), EcoHIV versus SAHA (10 \(\mu\)M). **, \(p < 0.0001\) EcoHIV versus EcoHIV + TNF (10 ng/ml) + SAHA (5 \(\mu\)M) and EcoHIV + TNF (10 ng/ml) + SAHA (10 \(\mu\)M).
Discussion

METH use and HIV-1 infection often coexist, mostly because of the association of METH use with engagement in high-risk behaviors. This coexistence presents an important problem, as recent studies suggest that METH abuse exacerbates damaging effects of HIV infection in the brain. Although the mechanisms responsible for this phenomenon still need to be explored in more detail, one possible explanation may be METH-enhanced HIV production by cells in the CNS. In this study, we focus on NPCs as the critical cells involved in memory processes as well as regeneration after neuronal loss in a variety of neurodegenerative diseases. Importantly, NPCs are susceptible to HIV infection (25, 26), a process that can impair NPC differentiation, leading to acceleration of HIV-associated cognitive deficits. The neurogenic niches are localized around the brain microvasculature (37, 38). METH is known to affect the integrity of the brain microvasculature, directly affecting NPC biology (39), which further supports the studies on the link between METH, HIV, and NPC neurogenesis.

NPCs mostly occupy the subventricular zone and the dentate gyrus (DG) areas in the adult brain. Whereas they constantly produce new populations of cells, NPCs can be additionally activated in response to exercise, injury, inflammation, or other stimuli to form mature neurons that, in turn, can be integrated into neuronal networks (40–42). The role of NPCs in proper brain function is especially emphasized by the fact that their aberrant proliferation and differentiation can contribute to a variety of neurodegenerative disorders, including Parkinson’s, Huntington’s, and Alzheimer’s diseases (43–47).

Results of the present study indicate that pretreatment (but not simultaneous exposure) with METH significantly increases HIV replication in both mouse and human NPCs. These findings are in agreement with other reports showing enhanced HIV infection after METH exposure. In human peripheral blood mononuclear cells infected with a monocyte-tropic strain of HIV-1 (HIVADA), METH significantly increases HIV production in a dose-dependent manner (12). Similar observations are made in human monocyte-derived dendritic cells (14) and macrophages (15, 48). In contrast, METH at concentrations higher than 100 μM is shown to inhibit HIV replication and up-regulate the anti-HIV miRNAs in CD4+ T cells (49).

Effects of METH treatment have also been examined in rhesus macaques infected with simian immunodeficiency virus (16) and in an HIV transgenic mouse model (12). In both cases, administration of METH increases virus production when compared with control animals. Specifically, METH increases HIV viremia by ~6-fold in a transgenic mice model (12), and higher viral loads are observed in the brains in the rhesus macaque model (16). Finally, METH use is significantly associated with higher plasma viral loads when compared with patients not abusing METH. These effects are independent from antiretroviral therapy (17–20).

Upon HIV infection, the viral RNA genome is reverse-transcribed into a proviral DNA that integrates into the genome of the host cell. The double-stranded DNA proviral genome is flanked by LTRs, of which the 5’-LTR acts as a promoter for the synthesis of viral RNA. Important regulatory elements of the LTRs are the highly conserved NFκB and SP1 sites. Therefore, to explain the mechanisms of METH-induced potentiation of HIV infectivity in NPCs, we focus on transcriptional regulation related to activation of the HIV LTR via NFκB and SP1. We observe that treatment of NPCs with METH induces activation of the HIV LTR that is dependent on the NFκB- and SP1-mediated pathway. Indeed, removing binding sites for any of these
factors from HIV LTR completely attenuates the effects of METH. At the same time, treatment with METH activates both NFκB and SP1 in NPCs. These findings are consistent with other studies showing that METH stimulates HIV gene expression through activation of NF-κB in peripheral monocytes (12) and microglial cells (13). In contrast to NFκB, contribution of SP1 activation to METH-mediated cellular effects and stimulation of HIV replication has been less explored. It is demonstrated in an indirect study that an inhibitor of SP1, mithramycin, attenuates METH neurotoxicity in mice. However, this impact is observed only when METH is administered repeatedly (33), suggesting that SP1 activation requires repeated doses of the drug. Consistent with these observations, we maintain METH levels in cell culture medium by adding a new dose of the drug every 24 h for the duration of experiments. A single dose of METH is ineffective in stimulating HIV infectivity and/or altering NPC biology.

In addition to stimulating HIV LTR, other mechanisms are also proposed to have an impact on METH-induced HIV infectivity. For example, METH was shown to induce up-regulation of HIV co-receptors CXCR4 and CCR5 (14, 15) and the dopamine receptors D1 and D2, with the subsequent activation of the MAPK pathway in human monocyte-derived dendritic cells (14). Our experiments examining the role of CCR5 and CXCR4 fail to provide any evidence that METH stimulates expression of these receptors in NPCs (Fig. S2), suggesting that this mechanism is not involved in METH-enhanced HIV infection in these cells.

In addition to evaluating the impact of METH on HIV infection of NPCs, our study also aimed to examine NPC differentiation and neurogenesis using two independent methods, namely immunofluorescence and FACS. The number of newly differentiated neurons was significantly decreased in infected cells exposed to METH as compared with control, whereas those that differentiated showed impaired characteristics; the complexity of multipolar neurons was reduced, and the overall ratio of multipolar to anaxonic neurons was greatly decreased in METH and METH+HIV groups.

In addition, FACS analysis indicates that differentiation of NPCs to the glial population is significantly reduced upon METH exposure. These results support previous findings that HIV can affect survival characteristics and impair differentiation process (25, 28). On the other hand, studies have also indicated that HIV infection may change NPC fate toward production of glia and astroglia at the expense of neurons (25, 50, 51), tendencies that are not observed in our experiments.

Our results on METH-induced aberrant neurogenesis are supported by the observation that the formation of new glia and/or neurons (29, 30) and adult hippocampal neurogenesis (52, 53) are reduced upon exposure to this drug. Results of our in vivo experiments are in line with in vitro findings. Hippocampal neurogenesis in the DG area is reduced upon METH and EcoHIV treatment. Nevertheless, there are also contradictory studies showing the opposite impact. For example, increased NPC proliferation has been reported upon intermittent exposure to METH, whereas daily exposure does not have any effect (54). The current study also evaluated the impact of METH on latent HIV infections in NPCs and their daughter cells. Such studies are consistent with the notion that the brain is an important reservoir for latent HIV infection. Although combined antiretroviral therapy can reduce plasma HIV to undetectable levels, the infection is frequently preserved in a latent form in the brain, which is poorly penetrated by antiretroviral therapeutics. Indeed, HIV DNA is found to be present in 55% of brains analyzed at autopsy from combined antiretroviral therapy–treated HIV patients who had undetectable viral loads in other tissues. Additionally, all tested brain tissues are identified with some degree of pathological changes (55).

NPCs are known for their ability to develop persistent HIV infection that can last for an extensive period. For example, HIV-infected NPC lines were found to produce HIV proteins for over 60 days, with proviral HIV DNA being detected for over 100 days postinfection (56). These observations suggest that these cells may act as a potential reservoir for HIV in the brain (26, 56). A long-term persistence of virus in NPCs can lead to a number of alterations, including morphological changes, proliferation arrest, inhibition of differentiation, or even cell death. In the current study, we report on another potentially important consequence of this process, which is the formation of new HIV reservoirs in NPC-derived daughter cells. Indeed, a high number of copies of integrated HIV DNA are found both in glial and neuronal populations derived from infected NPCs. This finding is particularly important regarding astrocytes, as it has been shown that latently infected astrocytes are capable of transmitting the virus to other cell populations, such as monocyctic cells and T cells, inducing fully productive infection (57, 58). In addition, the uptake and release of the virus by astrocytes does not always require virus replication. Instead, astrocytes can harbor virus within intracellular vesicles and transmit it without replication, possibly for as long as 4 or 5 months postinfection (57).

Interestingly, integrated HIV DNA is also detected in neurons derived from infected NPCs. Although there is evidence of neuronal abnormalities resulting from HIV infection that contribute to HIV-associated cognitive and motor disorders, mature neurons are considered not to be susceptible to HIV infection. In fact, there is no evidence for productive neuronal infection in vivo. Neuronal injury is believed to occur indirectly due to neurotoxins or cytotoxic viral proteins released by activated and infected immune and glial cells (59, 60). However, integrated HIV DNA has been reported in neurons of pediatric patients or the hippocampal regions, specifically the CA3, and is strictly correlated with HIV-associated dementia and neurodegeneration (61–63). Thus, latent infection of neurons derived from HIV-infected NPCs might be another potential mechanism for neuronal damage.

In conclusion, this study shows that exposure to METH increases HIV infectivity in NPCs through the NFκB/SP1-dependent activation of the HIV LTR. Infection of NPCs might have other long-lasting consequences, such as abnormal differentiation and generation of new potential cellular sites carrying latent HIV infection in the CNS. Such events are likely to underlie METH-induced progression of HIV-associated neurodegeneration.
**HIV infectivity in neural progenitor cells**

**Experimental procedures**

**Cell cultures**

- **mNPCs**—The neuroepithelial cell line NE-4C (CRL-2925), established from the cerebral vesicles of a 9-day-old mouse, was purchased from ATCC (Manassas, VA). These cells were shown to have the potential to differentiate into neurons and astrocytes in both *in vitro* and *in vivo* conditions (64). Cells were cultured on poly-d-lysine (100 μg/ml; Sigma)- and laminin (1 μg/ml; Sigma)-coated dishes. Neurospheres were allowed to expand to 100–150 μm in diameter, followed by passage or harvesting. The cells were used in less than fifth passage.

- Primary mNPCs, derived from cells isolated from embryonic day 14 mouse cortex, were purchased from Stemcell Technologies (Vancouver, Canada) and cultured according to the technical manual provided by the company on poly-d-lysine (100 μg/ml; Sigma)- and laminin (1 μg/ml; Sigma)-coated dishes. Neurospheres were allowed to expand to 100–150 μm in diameter, followed by passage or harvesting. The cells were used in less than fifth passage.

To maintain mNPCs in an undifferentiated/proliferating state, Complete Proliferating Medium (Stemcell Technologies), containing 20 ng/ml recombinant human epidermal growth factor (rhEGF), was used. To differentiate NPCs, the medium was switched to Complete Differentiation Medium (Stemcell Technologies), and cells were allowed to differentiate for 1, 5, 10, or 14 days, depending on the experimental protocol.

- *hNPCs*—An immortalized hNPC line ReNcell VM, derived from 10-week human ventral mesencephalon, was obtained from Millipore and cultured according to the manufacturer’s protocols. The cells were validated for high expression of Sox2 and nestin, as well as for their self-renewal and differentiation capacity. Cells were grown on laminin-coated tissue culture dishes in a maintenance medium (Millipore) containing 20 ng/ml FGF-2 and 20 ng/ml rhEGF. Cells were used for experiments at <80% confluence, 3–4 days after plating.

**METH exposure**

NPCs were treated with METH at 50 or 100 μM simultaneously or 24 h before HIV infection. This concentration is consistent with human abuse. For example, the majority of chronic METH abusers use the drug more than 20 days/month at a frequency of 1–3 doses/day (65, 66). A binge pattern of abuse can produce METH blood levels up to 2.5 mg/liter (~17 μM) (67). Taking into consideration that the blood/brain concentration ratio of METH is ~1.9 (68, 69), such concentration in the blood would result in up to 150 μM METH in the brain. In the present study, METH was added to cell culture medium each day in 24-h intervals for the duration of individual experiments. The preliminary studies revealed that METH at a single dose of 50 or 100 μM did not alter HIV infectivity (data not shown).

**Viral stock preparation and NPC infection**

EcoHIV/NL4-3-GFP and EcoHIV/NDK (abbreviated here as EcoHIV) were constructed by replacing the gp120 envelope protein in HIV NL4-3 with the gp80 envelope protein of murine leukemia virus (70, 71). This change altered species susceptibil-
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Cell transfection and luciferase assay

The reporter constructs pXP1-LTR-kB-SP1wt-Luc (wild type), pXP1-LTR-kBwt-SP1mut-Luc (SP1 sites mutated only), pXP1-LTR-SP1wt-Luc (deleted kB enhancer), and pXP1-LTR-SP1mut-Luc (mutated SP1 sites, deleted kB enhancer) were constructed as described (73) and kindly provided by Dr. Patrick Holz (Translational Research Center, Erlangen, Germany). pR-SV40 reporter plasmid (Promega) was used as an internal control.

mNPCs (NE-4C cells) were seeded in 24-well plates and transiently transfected with 1.5 μg of plasmid DNA (pXP-1-Luc plasmids and pRL-SV40 control vector; 1:1000) in the presence of 3 μl of Lipofectamine (Lipofectamine 2000, Thermo Fisher Scientific) in a total volume of 500 μl/well for 4 h. Cells were allowed to recover for 24 h, followed by treatment with 100 μM METH and/or infection with EcoHIV (20 ng of p24/ml) for 12 h. Samples were then lysed with a lysis buffer (250 μl/well) for 15 min, and lysates were transferred to the Eppendorf tubes and centrifuged for 2 min at 12,000 rpm to remove cell debris. Luciferase activity in the supernatants was estimated using the Dual-Luciferase Reporter Assay System (Promega) in a Luminometer, Promega). Results were expressed as relative luciferase activity, calculated as the ratio of firefly luciferase activity to renilla luciferase activity, to normalize for variability of transfection efficiency.

NFκB and SP1 activation

NF-κB and SP1 activation was assessed by the fluorescence intensity of acetylated NF-κB/p65 staining and the number of phosphorylated SP1 foci per cell nucleus, respectively. mNPCs (NE-4C cells) were cultured on glass coverslips placed in 24-well plates and exposed to 100 μM METH and/or EcoHIV NL/4-3 (20 ng/ml) for 0.5 h (to assess SP1 activation) or 1 h (to measure NFκB activation) 48 h after seeding. Then cells were fixed with ice-cold methanol/acetone (1:1), and nonspecific binding sites were blocked with BlockAid blocking solution (Life Technologies) for 2 h at room temperature. Cells were immunolabeled with antibodies against acetylated NFκB/p65 (rabbit anti-NFκB p65 AcK310, Abcam) or phosphorylated SP1 (anti-SP1 phospho-Thr-453), 1:200, overnight at 4 °C, followed by incubation with secondary antibody Alexa Fluor 568 (1:200) or anti-mouse-Alexa Fluor 488 (1:200; Invitrogen) as secondary antibodies. Afterward, coverslips were washed three times with PBS, counterstained with DRAQ5 (1:500, 15 min). Cell fluorescence was visualized by a confocal laser-scanning microscope, and fluorescence intensity was assessed using ImageJ software. Experiments were performed in triplicates, and four randomly chosen fields were scanned per coverslip. Then, for NFκB analysis, intensity of fluorescence was measured in ~100 randomly chosen cells and analyzed per picture. For SP1 activation analysis, a Foci picker 3D plug-in was employed to detect and count all SP1 foci; then an average number of SP1 foci/cell nucleus was calculated.

Protein isolation and immunoblotting

Cells were washed with PBS, homogenized, and incubated at 4 °C with radioimmune precipitation assay lysis buffer containing protease inhibitors (Santa Cruz Biotechnology, Inc.). The homogenates were centrifuged for 10 min at 12,000 × g and 4 °C, and supernatants were used for further analysis. Proteins (20 μg) were separated on SDS-PAGE and then transferred onto nitrocellulose membrane (Bio-Rad). Blots were blocked for 1.5 h at room temperature with 5% BSA in TBS-T buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.1% Tween 20) and incubated overnight at 4 °C with antibodies against CD4 (anti-human, 1:500, Abcam; or anti-mouse, 1:500, Thermo Fisher), CCR5 (anti-human, 1:500; or anti-mouse, 1:500; both from R&D Systems), CXCR4 (anti-human/anti-mouse, 1:1000, Abcam), and β-tubulin (anti-human/anti-mouse, 1:10,000, Sigma), followed by a 1-h incubation with secondary antibodies diluted at 1:20,000 (anti-rat 680RD, anti-rabbit 800CW, anti-rabbit 680RD, anti-mouse 800CW, and/or anti-mouse 800RD, all from LI-COR) for detection by the LI-COR CLX imaging system. The signal quantification was performed using Image Studio version 4.0 software (LI-COR).

Cell differentiation

Primary mNPCs were seeded on poly-d-lysine/laminin-coated glass coverslips (12-mm diameter) and incubated overnight at 37 °C in proliferating culture medium, containing 10 μg/ml rhEGF (Stemcell Technologies). The following day, differentiation was induced by changing the medium to Complete NeuroCult NSC differentiation medium (Stemcell Technologies). Cells were allowed to differentiate for 1, 5, and 10 days, in the presence or absence of METH and/or HIV. At each time point analyzed, cells were fixed (4% paraformaldehyde, 20 min, room temperature), permeabilized (0.2% Triton X-100 in PBS, 30 min, room temperature), and subjected to immunostaining for DCX and NeuN markers to detect neurons at different stages of development. In a separate set of experiments, immunostaining for microtubule-associated protein 2 (MAP2) was performed to analyze morphological changes within newly formed neurons. Nonspecific binding was blocked with 5% BSA in PBS for 2 h. Cells were then incubated overnight at 4 °C with rabbit anti-DCX (Abcam) and mouse anti-NeuN (Millipore) or rabbit anti-MAP2 (Abcam) antibodies, diluted 1:200 in PBS containing 2.5% BSA, followed by three washes with PBS and a 1-h incubation with goat anti-rabbit-Alexa Fluor 568 or goat anti-mouse-Alexa Fluor 488 (1:200; Invitrogen) as secondary antibodies. Afterward, coverslips were washed three times with PBS, counterstained with DRAQ5 (1:500 in PBS) or Hoechst (1:2000 in PBS) to visualize cell nuclei, and mounted onto glass microscope slides with ProLong Gold antifade reagent (Life Technologies). Fluorescence images were acquired through a confocal laser-scanning microscope (Olympus Fluoview 1200) and analyzed using ImageJ software (National Institutes of Health, Bethesda, MD). To assess morphological changes and the complexity of neuronal branching, Scholl analysis was performed using the Simple Neurite Tracer plug-in.
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### HIV DNA integration analysis

Total DNA was extracted from hNPCs and mNPCs using the QIAamp DNA blood minikit (Qiagen) according to the manufacturer’s protocol. Primers and fluorogenic probes were designed to quantitate total HIV-1 clade B genome: Rtotal primer, 5’-CTG CAT CCG GAG TAC TCC AAG ATG TG-3’; C1r primer, 5’-TCC CAG GCT CAG ATC TGG TCT A-3’; 2n4n probe, 5’-AGT GGC GAG CCC TCA GAT GCT GC-3’ (74). All targets were measured by ddPCR using a Bio-Rad QX100 ddPCR droplet digital PCR system. The ddPCR method is based on water-oil emulsion droplet technology, where each sample is fractionated into 20,000 droplets, and PCR amplification of the target occurs in each individual droplet. Such an approach allows for detection of virus at very low levels and discrimination of subtle differences in viral load, exceeding the performance of conventional RT-PCR. The dynamic range of detection for ddPCR is 1–10^10 copies. The single-copy human CCR5 gene was quantified to measure the number of cell equivalents in DNA samples for standardization of human samples (74). In addition, the double-copy murine β-globin (Hbb) gene was used for standardization in mouse cells: forward primer MGBP, 5’-CTG CCT CTG CTA TCA TGG GTA AT-3’; reverse primer MGBR, 5’-TCA CTG AGG CTG GCA AAG GT-3’; probe MGBP, 5’-FAM-TTA ACG ATG GCC TGA ATC-MGB-3’ (71).

### HIV RNA detection

Total RNA was extracted from differentiated mNPCs using the RNeasy minikit (Qiagen) according to the manufacturer’s protocol. Then one-step quantitative RT-PCR was performed using qScript XLT One-Step RT-qPCR ToughMix (Quanta Biosciences), in which the first-strand cDNA synthesis and PCR amplification are carried out in the same tube; 100 ng of RNA per reaction was used. Primers and fluorogenic probe were designed to detect the HIV-1 gag region: gag-Forward primer, 5’-GAC ATA AGA CAG GGA CCA AAG G-3’; gag-Reverse primer, 5’-CTG GGT TTG CAT TTG GGA CC-3’; probe, 5’-AAC TCT AAG AGC CGA GCA AGC TTC AC-3’. Real-time PCR was performed using an Applied Biosystems 7500 system. Changes in gene expression were calculated using ΔΔCt (where Ct is cycle number at threshold). Normalization was conducted based on GAPDH.

### Cell viability test

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was performed. MTT solution (1 mg/ml) was added to the cell cultures for 15 min at 37 °C. Cells were then lysed in DMSO for 15 min, and absorbance was measured spectrophotometrically at 570 nm. Each experimental group was analyzed in quadruplicates, and the mean ± S.D. were calculated. The net absorbance of samples derived from control cells was taken as the 100% viability value.

### Neuron and glial cell sorting from differentiated NPC cultures

Primary mNPCs were induced to differentiate in complete differentiation medium (Neurocult, Stemcell Technologies). After 2 weeks of differentiation, cells were harvested by 20–30-min treatment with Accutase (Stemcell Technologies), and a single cell suspension was obtained by repeated trituration. Accutase was neutralized with culture medium, and cells were centrifuged (1,000 rpm, 3 min) and then resuspended in FACS wash buffer (0.5% FBS in PBS) and stained with Live/Dead Aqua fluorescence dye (1:1000; Live/Dead® Fixable Dead Cell Stain Kit, Life Technologies) for 30 min to differentiate viable from non-viable cells. Cells were washed with FACS wash buffer and stained with fluoroochrome-conjugated antibodies (Table 1) for 20–30 min. Then cells were washed again and resuspended in FACS wash buffer. Samples were adjusted to a concentration of 1 million cells/ml. At least 100,000 cells/sample were acquired, and 20,000 cells were collected to analyze the impact of METH and/or HIV on glial and neuron cell populations. Cells were sorted and analyzed with FACS SORP Aria-IIu (BD Biosciences) with a 75-mm nozzle.

### Protein determination

Protein concentration in cell lysates was determined with the BCA protein assay kit (Thermo Scientific, Pierce, Rockford, IL).

### Statistical analysis

Statistical analysis was performed using two-way analysis of variance, followed by the Tukey comparison test. All analyses were performed with GraphPad Prism software (GraphPad Software).

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