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Productive infection of human neural progenitor cells by R5 tropic HIV-1: opiate co-exposure heightens infectivity and functional vulnerability

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Abstract

Objective—HIV type-1 (HIV-1) causes a spectrum of central nervous system (CNS) complications; many are worsened by opiate co-exposure. Human neural progenitor cells (hNPCs) give rise to all CNS neurons and macroglia. We tested the hypothesis that hNPC maturation and fate are altered by HIV and opiates, contributing to HIV-1-related neuropathology. Reports of hNPC infection remain controversial. We rigorously examined this question, testing whether hNPC propagated infection and whether HIV affected hNPCs absent their infection.

Design and methods—Primary hNPCs were characterized over multiple passages. Following R5 HIV-1BaL exposure, p24, Nef, and tat assays monitored infection; a serial dilution approach tested infection transfer to naive hNPCs. Bromodeoxyuridine uptake, population doubling time, and immunostaining assessed proliferation and differentiation. Morphine co-exposure assessed opiate interactions. Supernatant from HIV-1BaL-infected PBMCs (HIV_{sup}), HIV-1BaL, and ultraviolet light-inactivated HIV_{sup} were compared to test effects of inflammatory milieu versus virus or infection *per se*.

Results—The hNPCs (CD4_/CD8_/Iba_/CXC3CL1_/CD11b_) were infectable and could transfer infection to naive hNPCs. Infection was partly blocked by maraviroc, implicating CCR5. HIV_{sup} reduced hNPC proliferation and caused premature differentiation into neurons/astroglia. Effects on proliferation were due to soluble factors/viral proteins, not infection per se. Morphine co-exposure exacerbated certain functional consequences of HIV_{sup}, and sustained the infection of hNPCs.

Conclusion—hNPCs can be infected and propagate virus in vitro. hNPCs or their progeny may represent an underappreciated viral reservoir. Factors from infected cells alter hNPC proliferation and neural cell maturation which likely compromises CNS structure and function. Morphine–HIV interactions may worsen dysfunction and sustain infection.

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Introduction

CNS complications occur in 40–50% of people living with HIV/AIDS, even those receiving combination antiretroviral therapy (cART) [1, 2]. Collectively known as HIV-1-associated neurocognitive disorders (HAND), these impairments manifest largely as asymptomatic or mild disease. More severe CNS disease, including HIV-associated dementia, has been diminished by cART but remains problematic in more resource-limited settings [3]. HIV-1 invades the CNS soon after systemic infection, predominantly infecting perivascular macrophages, microglia, and less frequently astrocytes, within the brain parenchyma [4, 5]. It has been occasionally reported that human neural progenitor cells (hNPCs), which generate all CNS neurons and macroglia, can harbor infection [6–8], although this remains controversial. The cumulative response of infected CNS cells promotes inflammatory and toxic conditions, the combination of which leads to neuronal injury and synaptic dysfunction [9, 10]. Sublethal damage to the integrity of dendrites and synapses is considered the basis of neurological impairments associated with HAND [11].

Substance abuse is a major co-morbidity associated with HIV-1 disease progression [12, 13], as highlighted by the recent heroin crisis and concurrent outbreaks of HIV infection among new injection drug abusers in the US [14, 15]. Studies have established strong links between opiate abuse and HIV-1 neuropathogenesis [16–20]. Morphine, the major metabolite of heroin, reportedly potentiates CNS viral replication [21, 22], and enhances the neurodegenerative effect of HIV-1 via reverberating inflammatory/neurotoxic signaling between infected and/or activated CNS cells [23, 24]. Opiates associated with abuse liability modulate HIV-1 neuropathogenesis through actions primarily on μ-opioid receptors (MOR). Although MORs are expressed by both neurons and glia, neuropathological effects of opiates appear to largely be mediated by glia [25]. Despite this, current understanding of how opiates augment HIV-1 neuropathology remains incomplete.

Because NPCs express MORs and are reportedly targeted by both HIV-1 [26–30] and opiates [26, 28, 31–33], we examined their contribution to HIV-1 and opiate-mediated neuropathogenesis. We reevaluated the possibility that hNPCs may be infected using CCR5-tropic (R5) HIV-1_{BaL} and a primary hNPC culture system, and systematically confirmed that HIV-1_{BaL} infects hNPCs. A serial dilution and infection approach demonstrated that hNPCs propagate viral infection; morphine interacted to sustain productive infection. In addition, we explored the effects of HIV-1 on hNPC function. HIV_{sup} exposure reduced proliferation and induced premature differentiation of hNPCs into both astrocytes and neurons; effects were significantly enhanced by morphine co-exposure. UV-inactivation showed that infection was not required for HIV/morphine-dependent alterations in proliferation, which were likely due to factors in the infectious supernatant.

We speculate that HIV and opiate-mediated disruptions in the genesis of new CNS cells may create imbalance in overall neuronal and glial populations. This may be critical in the developing brain of children and adolescents, and for maintaining neuroplasticity in the adult brain. Importantly, infection of hNPCs and perhaps their progeny may create an additional viral reservoir in the CNS compartment. The recent escalation in opioid

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dependence and misuse, which includes adolescents and women of child-bearing age, underscores the potential clinical importance of our findings.

Results

1. Characterization of primary hNPC cultures

Human cells may more accurately reproduce disease features involving species-dependent interactions. Human NPCs were derived from 8–10-week gestational brain tissues. To minimize variability, samples were carefully matched for age, sex, and passage number, and cell composition was characterized at each passage. Results showed that more than 90% expressed NPC markers nestin and/or SOX2, with smaller percentages expressing the astrocytic marker GFAP (<20%) or the neuronal marker MAP2 (<5–10%). Within populations expressing GFAP and MAP2, 80–90% also expressed SOX2, indicating maintenance of an immature phenotype (Fig 1a–1b). PCR findings demonstrated that cells expressed the NPC genes *PAX3* and *NOTCH1,* the astrocyte *ALDH1L1* and the neuronal gene *dlg4a* that encodes human postsynaptic density protein 95 (PSD-95) (Fig 1c). We did not detect the expression of oligodendrocyte gene, myelin basic protein (MBP). This is likely due to the immaturity of the tissue, as MBP gene expression is not detected in CNS tissues except spinal cord prior to 12–15-week gestation [34–36]. The sex of each culture was determined by the presence of Y- and X-chromosome related genes (Fig 1c). CNS tissue at this early embryonic stage is assumed to be largely sexually undifferentiated and bipotent [37]. Nevertheless, to avoid any sex differences, which have been noted in glial populations [38, 39], we only used male cultures.

2. Sustained and productive infection of hNPCs by R5 HIV-1_{BaL}

To determine the contribution of hNPCs to combined HIV-1 and opiate neuropathogenesis, we first reassessed prior reports that hNPCs could be infected by HIV-1 [6, 8, 40]. Infection was confirmed by monitoring production of HIV-1 transcripts and proteins using multiple methods. PCR analysis indicated expression of HIV-1 *tat* in hNPCs 72 h post-infection at all HIV-1_{BaL} concentrations (Fig 2a). Robust HIV-1 Nef immunoreactivity was also observed in 5–8% of HIV-1_{BaL}-treated hNPCs (Fig 2b). The presence of microglia, macrophages and T-cells was examined to rule out potential contributions to infectivity results. The microglia/macrophage specific Iba-1 protein and transcript were not detected (Fig 2b, 2c); nor were other human microglia/macrophage genes including *CX3CR1* and *CD11b* and T cell genes *CD4* and *CD8* (Fig 2c, 2d). Furthermore, earlier reports [6, 7] of p24 production in the culture supernatant of infected hNPCs were confirmed by ELISA (Fig 2g). CCR5 inhibitor maraviroc, but not CXCR4 inhibitor AMD3100, effectively suppressed p24 production in hNPC cultures infected with 100 and 10 ng/mL HIV_{BaL} (Fig 2e).

To test whether the infection was productive, we adapted a serial dilution and passaging approach. Initial infections were performed by treating hNPCs with purified HIV-1_{BaL}; infection was confirmed 3 d post-wash by ELISA. Supernatant was removed and diluted (1:100), then placed on naïve hNPCs for 3 d. Three serial dilutions were performed and p24 was measured at the end of each infection period (Fig 2f). In the absence of new infection, the p24 level is expected to decline at each passage. Instead, p24 levels were sustained over

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time, suggesting that the infection was productive and transferable (Fig 2g). Morphine did not augment the infectivity of hNPCs even when the infection period was extended to 5 d (not shown). The findings from these multiple experimental approaches show that R5 HIV-1 can infect primary hNPCs, and also that hNPCs are capable of propagating *de novo* infection *in vitro.*

3. Morphine sustains productive R5 infection of hNPCs

The effect of morphine on infection was further analyzed at later stages. hNPCs were initially infected with purified HIV-1_{BaL} (1.0–100 ng/ml p24), then monitored for p24 production in cultures ± morphine for up to 15 d. A gradual increase in p24 production was measured irrespective of the initial HIV titer, was sustained for up to 12 d, and eventually plateaued after 15 d. Morphine enhanced p24 levels at 12 and 15 days after infection when hNPCs initially infected at 100 ng/ml p24 (Fig 2h). Morphine also increased *tat* mRNA levels at 9 d post-infection, a period preceding the morphine interaction on p24 (Fig 2i). The difference in p24 production and *Tat* expression was not due to death of hNPCs exposed to HIV-1_{BaL} ± morphine; since no differences in DNA content between groups was not observed (Fig 2j).

4. HIV-1 and morphine affect DNA synthesis and doubling time

We next tested whether HIV-1 and morphine co-exposure affected hNPCs proliferation, and doubling time. To model inflammatory conditions associated with HIV infection, we treated hNPCs with HIV_{sup} at multiple p24 levels. Soluble factors contained in HIV_{sup} and uninfected supernatant (UNF_{sup}) were compared using human cytokine arrays (Supplemental Fig 1). Bromodeoxyuridine (BrdU) was used to label cells entering the S-phase of DNA synthesis. BrdU immunoreactivity was significantly decreased versus media control at 24 h in hNPCs treated with higher HIV_{sup} concentrations (50 and 500 pg/ml p24), and in all tested HIV_{sup} concentrations at 48 h. Significant differences between the HIV_{sup} and the UNF_{sup} controls at 24 h (500 pg/ml p24) and 48 h (50–500 pg/ml p24) indicated a specific response to the infection *per se* (Fig 3a). Morphine enhanced this effect, as evidenced by further reduction of BrdU labeling in hNPCs treated with combined HIV_{sup} and morphine (Fig 3b, 3d).

Changes in DNA synthesis were reflected in hNPC replication. hNPCs were treated for 5 d with HIV_{sup} at concentrations where interactive effects on BrdU incorporation were observed. Cell density was measured every 24 h to calculate the population doubling time. Co-exposure to morphine at higher HIV_{sup} concentrations significantly prolonged doubling time (Table 1 Supplementary Data). Effects on proliferation and cell division were not attributable to changes in cell death (Fig 3c).

5. HIV-1 and morphine affect human neural progenitor differentiation

Human NPCs were treated with HIV_{sup} (50 and 500 pg/ml p24) ± morphine for 12 d, and immunolabeled with antibodies to nestin, SOX2, GFAP, and MAP2. Compared to controls, cultures treated with both HIV_{sup} concentrations had significantly higher percentages of both $GFAP^+$ and $MAP2^+$ cells. HIV-1 and morphine interactions were observed on neuronal and astroglial differentiation, evidenced by increased percentages of $MAP2^+$ cells in

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response to morphine and HIV_{sup} at 50 and 500 pg/ml p24, and $GFAP^+$ cells in response to morphine and HIV_{sup} at 500 pg/ml p24. The ratio of neurons to astroglia was unchanged by any treatment (data not shown). It is notable that despite the increased expression of MAP2 and GFAP upon HIV_{sup} ± morphine treatment, the percentage of nestin- and SOX2-expressing cells was unchanged (Fig 4a, b).

6. HIV-1 infection is not required for effects on proliferation

As hNPCs were productively infected by HIV-1, we tested whether infection underlies the functional responses of hNPCs by comparing the effects of UV-inactivated HIV_{sup} and concentrated HIV-1 virions. UV irradiation inactivates virions in infective supernatant without eliminating inflammatory or other deleterious factors. Inactivation was confirmed by lack of p24 production by PBMCs after exposure to UV-irradiated HIV (Fig 5a). Analysis showed that 48 h exposure to concentrated $HIV\text{-}1_{BaL}$ did not change the percentage of $BrdU^+$ hNPCs relative to control. However, a significant decrease of BrdU incorporation was observed in hNPCs treated with UV-inactivated supernatant (Fig 5b). Thus, changes in hNPC proliferation are not due to infection *per se*, but rather mediated by the combined effect of inactive virions, HIV proteins, and inflammatory conditions created by infected cells.

Discussion

We demonstrated that hNPCs are targets for HIV-1 infection at two levels. Conditions created by HIV-1 infection significantly restrict hNPC proliferation and alter the dynamics of their differentiation. Also, hNPCs can be infected and can propagate infection, at least under certain conditions *in vitro*. Many outcomes were exacerbated by opiate co-exposure. Injection drug abuse is a major vector for HIV transmission, and opiate abuse is re-emerging as a major public health crisis, underscoring the likely clinical importance of HIV and opiate interactions.

NPCs are present throughout the developing brain, and persist in the dentate gyrus and subventricular zone of the adult brain [41]. They are highly mitotic and migratory during development and while adult NPCs are more quiescent, they can be activated to proliferate, particularly in response to insult/injury [42]. Aberrant NPC behavior can perturb the balance of CNS populations, and the consequences of hNPC dysfunction have been implicated in various psychiatric [43, 44] and neurodegenerative diseases [45], as well as in injuries caused by inflammation [46, 47], stroke/ischemia [42], or epilepsy [48]. However, responses of fetal hNPCs to HIV may of course differ from those in the adult CNS.

The concept that hNPCs may be infected by HIV-1 has remained controversial despite studies showing limited p24 production by fetal-derived hNPCs exposed to $HIV\text{-}1_{IIIB}$ and $HIV\text{-}1_{NL4\text{-}3}$ [7], and the presence of pro-viral DNA in a human neural stem cell line treated with $HIV\text{-}1_{IIIB}$ [6]. The presence of viral DNA was also noted in $nestin^+$ hNPCs microdissected from the hippocampus of pediatric AIDS patients [8]. We hoped to clarify this controversy by systematically examining the capacity of hNPCs from multiple independent samples to be infected and to propagate infection. Infection resulted in detection of *tat*, Nef, and p24. PCR confirmed that microglia, monocytes/macrophages, and

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T cells were absent from cultures and could not contribute to infection. Confident that a small percentage of hNPCs were infected, we tested whether infection could be transferred to naïve cells. The p24 titer was sustained even after three dilution/infection cycles, providing compelling evidence that hNPCs can actively propagate HIV-1 infection. No evidence of *CD4* expression was observed, suggesting a CD4-independent mechanism for infection. Given that CCR5 and CXCR4 are expressed in immortalized hNPCs [30], we tested whether these HIV co-receptors were involved in hNPC infection. Using CCR5 and CXCR4 inhibitors, we determined that infection was partly mediated by CCR5. Other receptors/modes of entry are likely as p24 production was not completely blocked by maraviroc, and there was considerable variability in the response to maraviroc.

Human NPCs were only infected at relatively high viral titers, suggesting that NPCs in close proximity to HIV-infected microglia/macrophages may be most vulnerable. In adults, this likely occurs in NPC-rich brain regions such as the hippocampus, where a high density of infected microglia and macrophages has been demonstrated [49]. Viral tropism may also influence the capacity for hNPC infection. Studies here used R5 HIV-1_{BaL}, but hNPCs might also be susceptible to infection by other strains, perhaps requiring lower viral titers to achieve infection. Two studies reported evidence of p24 production in progenitor cultures exposed to X4-tropic HIV-1_{IIIB} [6, 7]. While R5 viruses represent the predominant virus population early after infection, X4 viruses are generally associated with heightened virulence, rapid $CD4^+$ T cell-decline, CNS injury, and accelerated AIDS progression [50–54]. The likelihood of hNPC infection is increased if they are vulnerable to multiple strains.

Exposure to HIV_{sup} at p24 levels measured in infected brains [55, 56] affected critical hNPC functions. HIV_{sup} dramatically decreased BrdU incorporation and prolonged hNPC doubling-time. Similar effects on proliferation were previously observed with HIV-1 proteins [26, 27, 29, 57], and on immortalized hNPCs [26]. Hippocampal tissue from autopsied HIV-1 dementia patients showed reduced Ki-67^+ cells [27], which would be consistent with reduced NPC proliferation. In contrast, supernatant from lipopolysaccharide (LPS)-stimulated, HIV-infected macrophages increased hNPC proliferation [30], although the proliferative effects of acute LPS posed a confound [58]. Morphine co-exposure accentuated the effect of HIV_{sup} on hNPC proliferation, paralleling interactions previously reported between morphine and HIV-1 Tat on fetal hNPCs [28], and morphine and HIV-1 Tat or HIV_{sup} on immortalized hNPCs [26]. Effects were unrelated to hNPC survival, which was unchanged by any treatment. Morphine by itself did not alter hNPC proliferation, although this was reported in rodent NPCs [31, 33] and hNPCs [28], perhaps reflecting species or timing differences.

We also found that HIV_{sup} exposure resulted in premature differentiation of hNPCs into both neurons and astrocytes. Morphine co-exposure significantly enhanced this effect. HIV_{sup} ± morphine effects on proliferation and differentiation suggest that extended exposure will alter the balance/size of $MAP2^+$ and $GFAP^+$ cell populations. Meaningful, *in vivo* assessments of this will require stereological analysis of potentially affected brain regions.

Are these *in vitro* results relevant to human disease? NPCs are present in both developing and adult systems, albeit in different regions. Adult and immature NPCs also have unique

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characteristics that may influence their response to HIV infection and to opiate exposure. An accurate balance between NPC proliferation and differentiation is necessary for establishing and maintaining cell populations, and perturbations to these regulated processes can influence overall CNS architecture and function [59–61]. Cortical atrophy is a common feature of pediatric neuroAIDS, especially in cART-naïve children [62, 63]. The reduced NPC production and early differentiation described here may contribute to cortical atrophy and other neurological manifestations in children and adolescents with HIV-1 [40, 64, 65]. Although cART has greatly reduced HAND incidence in children, currently 2.6 million children worldwide are infected with HIV-1, and children represent 1–2% of cases in the US (http://www.who.int/hiv/en/).

Unlike NPCs in the developing brain, adult NPCs are restricted to the subventricular zone and subgranular zone of the hippocampal dentate gyrus [41, 66]. There is a strong correlation between hippocampal atrophy/dysfunction and reduced spatial recognition and learning/memory ability, both in HIV experimental models [67–71] and in patients [72–74]. The hippocampus of HIV-1 infected adults exhibits high viral load [49] and often shows significant atrophy [74, 75], suggesting that NPCs may have limited expansion. Interestingly, the proliferation of hippocampal NPCs from adult subjects with HIV-1 dementia was significantly reduced when compared to infected subjects without dementia, also suggesting that hippocampal impairments might involve failure to generate normal numbers of adult NPCs [27].

There could be multiple clinical consequences of productive hNPC infection. First, infected hNPCs might augment overall CNS disease by increasing the levels of virus, viral proteins, and inflammation in the brain. The ability of HIV-1 infection to disrupt hNPC proliferation without affecting viability might promote HIV neurovirulence, similar to other cells with active viral replication. The virus may be able to maintain persistent infection by switching host DNA synthesis to viral DNA synthesis in infected cells [76], as has been shown for hNPCs infected with human cytomegalovirus (HCMV) [77]. Secondly, infected hNPCs may pass the infection to long-lived progeny, generating new viral reservoirs and new barriers for eradication. In support of this concept, inflammatory mediators such as TNF-α [7] and IL-1β [78] were found to reactivate viral production in cultured NPC-derived astrocytes. The ability of NPCs to pass infection to progeny might also explain reports of infected, immature neurons [79, 80].

Morphine exacerbated the effect of HIV-1 on proliferation and differentiation, and sustained *de novo* viral production *in vitro*. If these results can be extrapolated to the human brain, it suggests that the extent of dysfunction and infection in both adult and young NPCs will increase with opiate co-exposure. These findings are especially concerning given the current opiate drug crisis. Heroin use and overdose-related hospitalizations have markedly increased over the past 10 years [81, 82]; the number of heroin users nearly tripled between 2005–2012 [83]. A major, coincident, consequence of increased opiate abuse has been the rising outbreak of HIV infection outside urban communities, where new infections occur frequently in young adults, almost half of whom are women [14, 15]. Although perinatal HIV transmission has been significantly decreased by cART, the Centers of Disease Control (CDC) reported 174 new cases of HIV in children in the US in 2014, and 1,999 total

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children living with perinatal HIV. Furthermore, the CDC estimated that 9,131 adults and adolescents were living with HIV acquired perinatally (http://www.cdc.gov/hiv/group/gender/pregnantwomen/). Importantly, maternal drug use is strongly associated with increased risk of perinatal transmission [84, 85]. A high percentage of children and young adults with HIV/AIDS also receive opiates for pain intervention, as pain prevalence among this group is reportedly as high as 60% [86].

Our study shows that hNPCs are capable of sustaining and propagating HIV-1 infection. The infective milieu also causes functional deficits in uninfected hNPCs. Dysregulation of these proliferative, multipotent cells by HIV-mediated inflammatory/toxic insults may intensify neuropathology, and may especially affect the developing brains of HIV-infected children and adolescents. Infected hNPCs may also serve as an additional CNS viral reservoir. The significant interactions between morphine and HIV-1 suggest that HIV patients with a history of opiate abuse or exposure may be particularly vulnerable to HIV-related cognitive and motor deficits.

Materials and Methods

Ethics Statement

All human tissues were purchased from biological supply vendors, were anonymized, and not classified as human subjects research.

Primary human NPC culture

Human NPCs were derived from anonymized 8–10 week-old brain samples (Advanced Bioscience Resources, Alameda CA), a developmental stage, where mature, ramified microglia are generally absent [87]. Supplemental Data describes isolation, characterization, and culture.

HIV-1 propagation

Peripheral blood mononuclear cells (PBMCs) isolated from peripheral blood Leuko Paks (ZenBio, Research Triangle Park, NC) via ficoll gradient centrifugation were activated with phytohemagglutinin (1 mg/ml, PHA), then infected with HIV-1_{BaL} (Advanced Biotechnology, Eldersburg, MD; 1 ng/mL p24) for 72 h. Infection was assayed using p24 Antigen ELISA kit (Advanced Bioscience, Rockville, MD).

hNPC infection

hNPCs were exposed to purified HIV-1_{BaL} for 24 h, washed thoroughly to remove residual virus and incubated in NPC media.

Treatments for proliferation/differentiation assays

Human NPCs were treated with supernatant from HIV-infected PBMCs (HIV_{sup}) at 0.5–500 pg/mL p24. Controls included media only, and equal volumes of supernatant from un-infected, PHA-activated PBMCs at the same initial cell density. Morphine (500 nM) was added coincident with supernatant. For UV-inactivation, a sample of HIV_{sup} was UV-crosslinked (1200 j/sm^2, 5 min).

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Immunostaining/BrdU

Fixation, processing, and antibodies are detailed in Supplemental Data. Samples were imaged using a Zeiss LSM 700 laser scanning confocal microscope (20/63x magnification). Images were collected using ZEN 2009 Light Edition software (Carl Zeiss, NY).

RT-PCR

Target genes were amplified from hNPC cDNA via PCR using Bioline MangoMix™ or Roche FastStart DNA Master SYBR Green and gene-specific primer sets. Supplemental Data contains primer sequences.

Cell growth and doubling time

Doubling-time was calculated using the equation $D(t) = T \ln 2/\ln(X_e/X_b)$ (ATCC Animal Cell Culture Guide). Supplemental Data describes cell density determination.

Statistics

Analyses were performed using GraphPad Prism 5 software (GraphPad Software Inc, La Jolla CA). Unless indicated, data were analyzed by one-way ANOVA followed by Bonferroni post-hoc testing. $p<0.05$ was considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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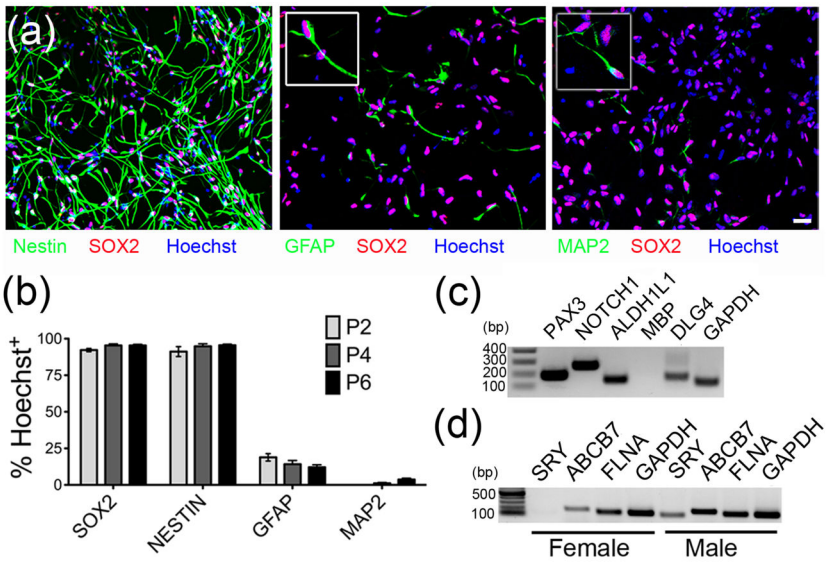

Figure 1. Detailed characterization of hNPC cultures

(a) Representative fluorescent images of hNPCs double-immunostained for nestin (green), glial fibrillary acidic protein (GFAP) (green), microtubule associated protein 2 (MAP2) (green) and sex-determining region Y-box2 (SOX2) (red). Hoechst 33342 (blue) staining indicates cell nuclei. Scale bar represents 50 μM. (b) The percentage of hNPCs expressing nestin, SOX2, GFAP, and MAP2 at passage 2, 4, and 6 (P2, P4, P6) were calculated from a population of >200 Hoechst^{+} cells. Error bars show mean ± SEM from four studies (*n=4*), each using hNPCs derived from independent tissue samples. (c) 1% agarose gel showing PCR products for NPC genes [paired box 3 (*PAX3*), Notch homology 1 (*NOTCH1*)], glial genes [aldehyde dehydrogenase 1 family, member L1 (*ALDH1L*), myelin basic protein (*MBP*)], and a neuronal gene (*DLG4*) that codes for human PSD95, amplified from purified RNA of cultured hNPCs. (d) Sex of individual hNPC cultures was determined via RT-PCR using primers specific for the Y-chromosome genes, sex determining region Y (*SRY*), and primers specific for the X-chromosome genes, ATP-binding cassette sub-family B member 7 (*ABCB7*) and filamin A (*FLNA*).

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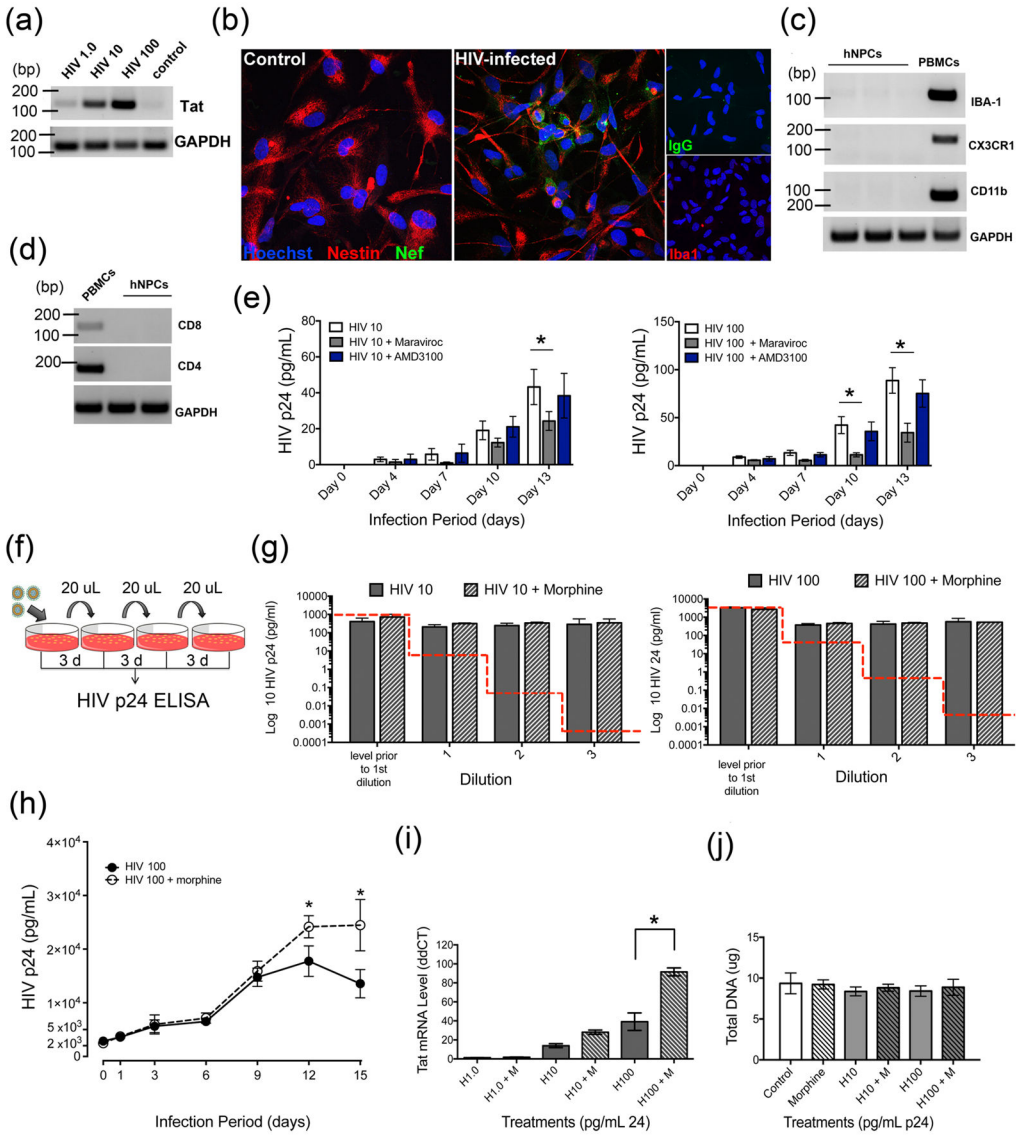

Figure 2. Morphine co-exposure prolonged the productive infection of hNPCs by R5-tropic HIV-1$_{BaL}$

Productive infection of hNPCs treated with HIV-1$_{BaL}$ at varying concentrations (1.0, 10, 100 ng/ml HIV p24) for 24 h then thoroughly washed to remove residual virus. Infection was monitored post-wash. (a) Agarose gel containing PCR product for HIV trans-activator of transcription (*tat*) amplified from hNPCs infected with purified HIV-1$_{BaL}$ at 1.0, (HIV 1.0), 10 (HIV 10), and 100 ng/ml p24 (HIV 100) for 72 h. Control represents cultured hNPCs not exposed to HIV-1$_{BaL}$. (b) Representative confocal images of HIV negative regulatory factor (Nef) (green) and nestin (red), IgG isotype control (green), and ionized calcium-binding adapter molecule-1 (Iba-1) (red) immunostaining of uninfected (control) and HIV-1$_{BaL}$-infected (HIV-infected) hNPCs. (c) mRNA expression analysis of *Iba-1,* fractalkine receptor 1 (*CXC3R1*), and integrin alpha M (*CD11b*) from hNPC and PBMC cultures. (d) mRNA expression analysis of T-cell surface glycoproteins, cluster of differentiation 8 (*CD8*) and cluster of differentiation 4 (*CD4*) in cultured hNPCs and PBMCs. The PCR results from (a),

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(c) and (d) are representative of *n=3* independent cultures. (e) Quantification of supernatant p24 from hNPCs infected with 10–100 ng/mL HIV_{BaL} and treated with CCR5 inhibitor maraviroc (1 uM) or CXCR4 inhibitor AMD3100 (1 uM) for 13 d. Data are from *n=2* cultures derived from independent tissue samples (f) Schematic diagram of the method for serial dilution and infection of hNPCs. (g) p24 measurements from cultured hNPCs with or without morphine co-exposure after each 3 d incubation with diluted culture supernatant. Initial p24 level before dilution in hNPC supernatants ranged between 263.50 ± 78.51 and 412.90 ± 71.11 pg/mL. Data are presented as log10 of p24 concentration (pg/mL) measured at each dilution. Dotted red line represents the expected p24 concentration after each dilution without new virus production. (h) Infection was monitored for up to 15 d via p24 ELISA in cultured hNPCs initially infected with 100 (HIV 100) ng/mL HIV-1_{BaL} alone or with morphine. Data are from *n=4* cultures derived from independent tissue samples. (*) $P<0.05$ vs. HIV 100 alone. (i) Quantification of *Tat* mRNA expression in hNPCs exposed to HIV-1_{BaL} at concentrations of 1.0 ng/mL (H1.0), 10 ng/mL (H10), or 100 ng/mL (H100) p24 alone or with morphine (M) for 9 d. (j) Spectrophotometric measurement of total DNA content from hNPC cultures exposed to morphine, purified HIV-1_{BaL} at 10 ng/ml p24 (H10) or 100 ng/ml p24 (H100) alone or with morphine (M) for 15 d. Unless noted, error bars show mean ± SEM from *n=3* hNPC cultures derived from independent tissue samples. * $P<0.05$.

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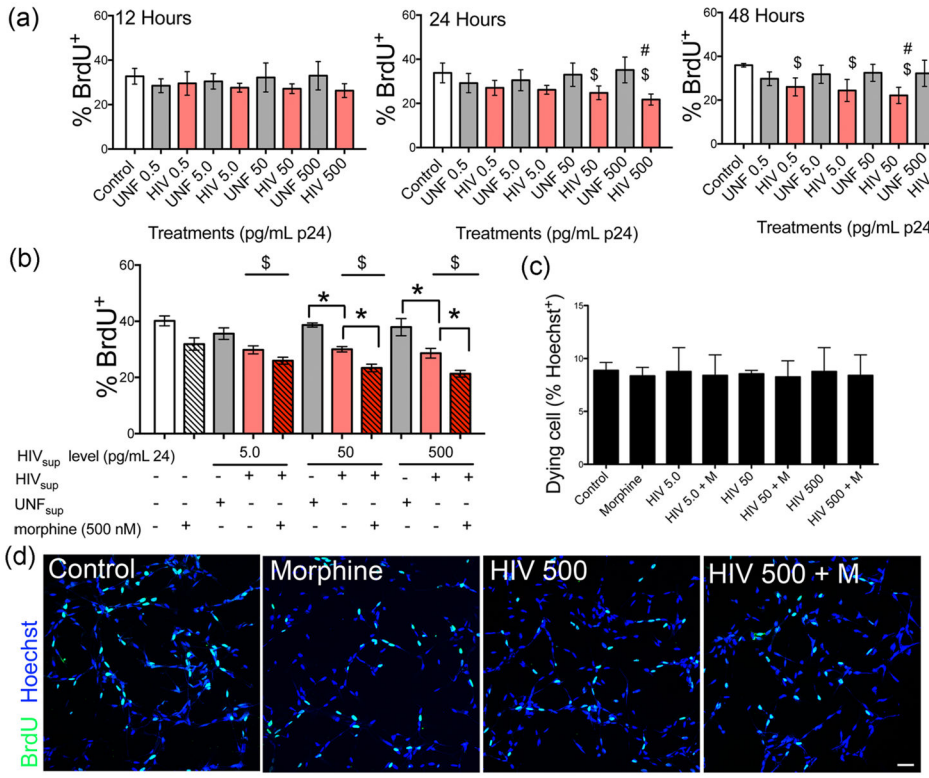

Figure 3. HIV-1 ± morphine co-exposure modulates DNA synthesis and doubling time of hNPCs

(a) BrdU immunostaining analysis on hNPCs treated with HIV_{sup} at 0.5 - 500 pg/ml HIV p24 (HIV 0.5, HIV 5.0, HIV 50, HIV 500) for 12, 24, and 48 h. Cells were pulsed with BrdU (1mM) 6 h prior to the end of treatments. Controls included NPC media only (control), and equal volumes of uninfected supernatant (UNF 0.5 – UNF 500) for each HIV_{sup} level. (b) BrdU analysis on hNPCs treated with HIV_{sup} at 5.0 – 500 pg/ml HIV p24 ± morphine (500 nM), equal volumes of uninfected supernatant (UNF_{sup}), and media only (control) for 48 h. Controls included NPC media only (control) and equal volumes of uninfected supernatant (UNF_{sup}) for each HIV_{sup} level. (c) The LIVE/DEAD viability assay (Thermo Fisher Scientific) was used to analyze cell death rates in hNPCs treated with morphine, HIV_{sup} at 5.0–500 pg/ml HIV p24 (HIV 5.0, HIV 50, HIV 500) ± morphine (M) for 48 h. Data were derived from >200 hNPCs, and presented as percentage of dying (ethidium homodimer-1-positive) positive cells relative to total cells. (d) Representative images showing BrdU (green) immunostaining of hNPCs treated with 500 pg/ml p24 HIV_{sup} (HIV 500) alone or with morphine (HIV 500 + M). Data for (a) and (b) were derived from >200 $Hoechst^+$ hNPCs, and presented as percentage of $BrdU^+$ cells relative to the total cells. For (a), (b) and (c), error bars show mean ± SEM from *n=4* studies, each using hNPCs derived from independent tissue samples. Significance in (b) was determined by two-way ANOVA with Bonferroni post-hoc testing. ($) $P<0.05$ vs. control, (*) $P<0.05$.

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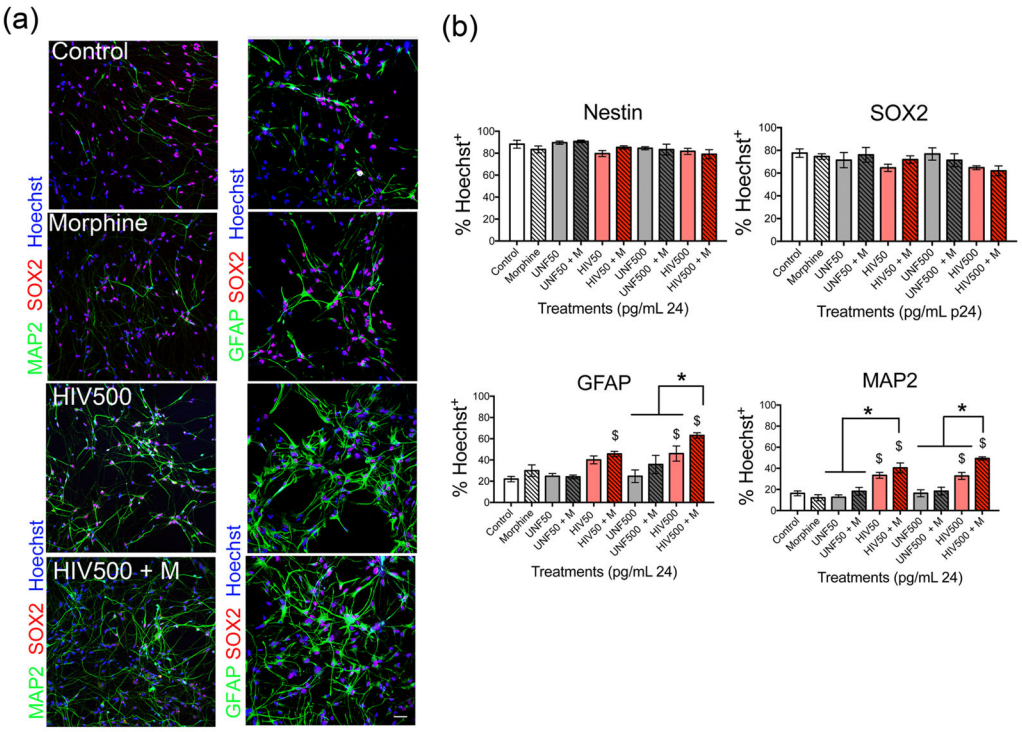

Figure 4. HIV-1 ± morphine co-exposure promotes neuronal and astrocytic differentiation of hNPCs

(a) Representative immunostaining of SOX2 (red), GFAP (green), and MAP2 (green) immunostaining of hNPCs treated with morphine (500 nM), HIV_{sup} at 500 pg/ml p24 alone (HIV 500) or with morphine (HIV 500 + M) in differentiation medium for 12 d. (b) Analysis of immunostaining in hNPCs treated with morphine, HIV_{sup} at 50 pg/ml p24 (HIV 50), 500 pg/ml p24 (HIV 500) alone or with morphine (M), and equal dilutions of supernatant from uninfected PBMCs (UNF50 or UNF500) alone or with morphine (M). The percent of hNPCs expressing nestin, SOX2, MAP2, and GFAP from each treatment group was calculated from >200 $Hoechst^+$ cells. Error bars show mean ± SEM from *n=5* studies each using hNPCs derived from independent tissue samples. ($) $P<0.05$ vs. control, (*) $P<0.05$.

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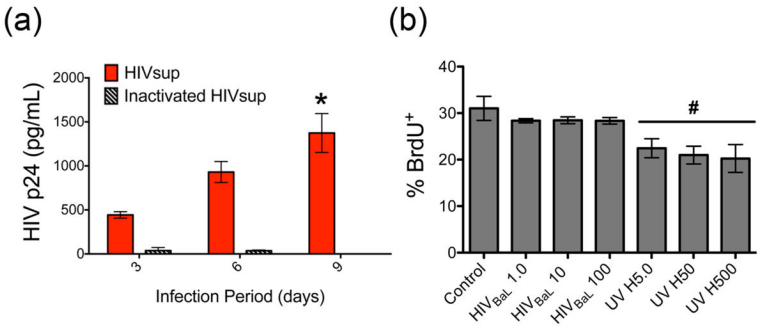

Figure 5. Anti-proliferative effects are a response to the infective milieu, not to purified virus or hNPC infection

(a) p24 measurements in cultured PBMCs treated with HIV_{sup} (1.0 ng/mL p24) or an equal volume of UV-inactivated HIV_{sup} for 3, 6, and 9 d. (b) BrdU immunostaining analysis in hNPCs treated with purified HIV-1_{BaL} at concentrations of 1.0 (HIV_{BaL} 1.0), 10 (HIV_{BaL} 10), and 100 (HIV_{BaL} 100) ng/mL p24 or UV-inactivated HIV_{sup} derived from (a) at concentrations of 5 (UV H5.0), 50 (UV H50), or 500 (UV H500) pg/mL p24 for 48 h. Error bars show mean ± SEM from *n=3* hNPC cultures derived from independent tissue samples. (#) $P < 0.05$ vs. control, and all purified HIV_{BaL} exposure levels.