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HIV-1 Alters Neural and Glial Progenitor Cell Dynamics in the CNS: Coordinated Response to Opiates during Maturation

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Abstract

HIV-associated neurocognitive disorders (HAND) are common sequelae of HIV infection, even when viral titers are well controlled by anti-retroviral therapy. Evidence in patients and animal models suggests that neurologic deficits are increased during chronic opiate exposure. We have hypothesized that CNS progenitor cells in both adult and developing CNS are affected by HIV infection, and that opiates exacerbate these effects. To examine this question, neural progenitors were exposed to HIV-1 $Tat_{1\text{-}86}$ in the developing brain of inducible transgenic mice and in vitro. We examined whether Tat affected the proliferation or balance of progenitor populations expressing nestin, Sox2, and Olig2. Disease relevance was further tested by exposing human-derived progenitors to supernatant from HIV-1 infected monocytes. Studies concentrated on striatum, a region preferentially targeted by HIV and opiates. Results were similar among experimental paradigms. Tat or HIV exposure reduced the proliferation of undifferentiated ($Sox2^+$) progenitors and oligodendroglial ($Olig2^+$) progenitors. Co-exposure to morphine exacerbated the effects of Tat or HIV-1_{SF162} supernatant, but partially reversed HIV-1_{IIIB} supernatant effects. Populations of $Sox2^+$ and $Olig2^+$ cells were also reduced by Tat exposure, although progenitor survival was unaffected. In rare instances, p24 immunolabeling was detected in viable human progenitors by confocal imaging. The vulnerability of progenitors is likely to distort the dynamic balance among neuron/glial populations as the brain matures, perhaps contributing to reports that neurologic disease is especially prevalent in pediatric HIV patients. Pediatric disease is atypical in developed regions, but remains a serious concern in resource-limited areas where infection occurs commonly at birth and through breast-feeding.

Keywords

striatum; neuroAIDs; morphine; Tat; HIV-1; precursor

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INTRODUCTION

Morbidity and mortality due to human immunodeficiency virus-1 (HIV-1) infection has been significantly decreased by combined anti-retroviral therapy (cART). cART treatment has also changed the landscape of central nervous system (CNS) disorders. Fewer patients exhibit HIV associated dementia (HAD), but with increased life expectancy and the potential toxic effects of long-term anti-retroviral exposure (Apostolova et al. 2011; Clifford 2002), there is increased prevalence of asymptomatic and mild-to-moderate HIV-associated neurocognitive disorders (HAND) among patients with absence of peripheral viremia (Gonzalez-Scarano and Martin-Garcia 2005; Sacktor 2002; Simioni et al. 2010). Additionally, many HIV therapeutics do not readily penetrate the blood brain barrier, leaving a reservoir of HIV in the CNS.

Microglia are the principal source of active HIV infection in the brain; astroglia can be infected but tend not to produce new virus (Gonzalez-Scarano and Martin-Garcia 2005; Jordan et al. 1991; Kaul 2008). Several studies suggest that CNS neural progenitor cells (NPCs), the undifferentiated precursors of both neurons and glia, are also susceptible to HIV-1 infection (Lawrence et al. 2004; Rothenaigner et al. 2007; Schwartz et al. 2007). Even if NPCs are not readily infected, they may be affected by HIV-1 proteins directly (Buch et al. 2007; Hahn et al. 2010; Krathwohl and Kaiser 2004; Lee et al. 2011; Mishra et al. 2010; Okamoto et al. 2007; Peng et al. 2008), or secondarily by extracellular changes. NPCs are widely distributed in germinative zones during development. Neurons in most brain regions are formed prenatally, but production of astroglia and oligodendroglia continues postnatally in both rodents and humans (Chan et al. 2002; Geha et al. 2010; Lee et al. 2000; Skoff 1990; Skoff and Knapp 1991). NPCs also are present in adult CNS, where neurogenesis occurs in specific regions, including the hippocampal dentate gyrus subgranular zone and the subventricular zone of the lateral ventricles (Doetsch et al. 1997; Eriksson et al. 1998; Kaplan and Hinds 1977; Kornack and Rakic 1999). Glial progenitors throughout the adult CNS undergo constant, slow turnover (Imamoto and Leblond 1978; Kornack and Rakic 1999; Kornack and Rakic 2001; Messier et al. 1958; Sturrock 1979); more active gliogenesis occurs during injury/disease (Burns et al. 2009; Rola et al. 2006; Romanko et al. 2004).

To test whether HIV-1 infection affects the dynamics of cell production during development, we used stage-specific markers to follow changes in the proliferation and populations of NPCs (nestin$^+$ and Sox2$^+$ [SRY (sex determining region Y)-related HMG (high mobility group)-;box gene2]) and their glial progeny both in culture and in intact brains during the perinatal period. NPCs and glial progenitors express functional opioid receptors in adult and neonatal rodent and human CNS (Buch et al. 2007; Hauser et al. 2009; Persson et al. 2003; Reznikov et al. 1999; Rius et al. 1991; Tripathi et al. 2008). Since co-exposure to opiate drugs of abuse enhances HIV-related CNS deficits in patients and experimental models (Anthony and Bell 2008; Bell et al. 2006; Burdo et al. 2006; Byrd et al. 2011; Hauser et al. 2007; Kopnisky et al. 2007), we examined interactive opiate effects. HIV-1 Tat was used to model HIV exposure both in vitro and in vivo. Progenitor responses were tested in a more clinically relevant model by exposing human NPCs (hNPCs) to supernatant from HIV-infected monocytes ± opiates. Tat or morphine alone reduced NPC

and glial progenitor proliferation and/or altered the population dynamics of murine progenitors *in vitro* and *in vivo*. In certain progenitors, co-exposure to morphine accelerated the timing and extent of Tat effects. Similarly, proliferation of hNPCs was reduced by exposure to Tat, R5 HIV-1_{SF162}, or X4 HIV-1_{IIIB}, and there were interactive opiate-virus effects. We speculate that by affecting expansion of specific CNS progenitors, HIV ± opiate effects might alter the balance of glia and neurons in the CNS of pediatric HIV patients. Since adult progenitors share certain characteristics of neonatal progenitors, our findings may also relate to adult HIV patients. At either age, an altered progenitor pool is predicted to adversely affect CNS function.

METHODS

Protocols conformed to Virginia Commonwealth University Institutional Animal Care and Use Committee and national (PHS) guidelines on the care and ethical use of animals. Experiments were designed to minimize total animals used and their discomfort.

Murine neural progenitor cell cultures

Murine NPCs were cultured from striata of embryonic day 15 (E15) mice (ICR strain; Charles River, Boston, MA) using modifications of published methods (Khurdayan et al. 2004). In brief, striata from 10–12 embryos were dissected from the cortex, minced, and incubated with DNase (0.015 mg/mL, Sigma-Aldrich Co., St. Louis, MO) in Dulbecco's Modified Eagle's Medium (DMEM) (15 min, 37°C). Tissue was triturated, centrifuged (5 min, 1,000 rpm), and resuspended in NPC maintenance medium consisting of 1:1 DMEM/F12 (Invitrogen Corp., Carlsbad, CA), 10% Knockout® SR (Invitrogen; a serum replacement product), insulin (25 μg/mL), transferrin (100 μg/mL), progesterone (20 nM), putresceine (60 μM), Na-HCO_3 (6 mM), and sodium selenite (30 nM) (all from Sigma-Aldrich). Cells were filtered through a 70-μm nylon cell strainer (BD Falcon, Bedford, MA), centrifuged, and resuspended in the same medium. A total of 5×10^4 cells were plated on poly-L-lysine coated 15-mm coverslips, and medium was refreshed every 2–3 d until treatment. Cells were grown at 37° C in a humidified atmosphere of 5% CO_2. After 5 d, cells were treated with $Tat_{1\text{-}86}$ (Clade B; 100 nM; ImmunoDiagnostics, Woburn, MA), ± morphine sulphate (500 nM; NIDA Drug Supply System, Rockville, MD), ± naloxone (1.5 μM; Sigma-Aldrich) for 12–48 h. Morphine, the major bioactive product of heroin in the brain, is a potent mu-opioid receptor (MOR) agonist; naloxone is a broad spectrum opioid receptor antagonist. These concentrations of $Tat_{1\text{-}86}$ and morphine have been established to elicit functional effects in glia and CNS progenitors in earlier studies (Buch et al. 2007; El-Hage et al. 2005; Hauser et al. 2009; Khurdayan et al. 2004). Naloxone was applied 30 min prior to other treatments, which were added simultaneously in pre-warmed medium. Lastly, 5-bromo-2′-deoxyuridine (10 μM; BrdU, Sigma-Aldrich) was added to medium at 12 h prior to harvest to detect proliferating cells in S phase.

Opiate delivery to prenatal and postnatal Tat transgenic mice *in vivo*

In vivo, perinatal studies utilized transgenic mice engineered to express the HIV-1 $Tat_{1\text{-}86}$ protein (clade B) under control of a glial fibrillary acidic protein (GFAP) promoter in a doxycycline-inducible manner. As previously described (Bruce-Keller et al. 2008; Fitting et

al. 2010; Hauser et al. 2009), mice expressing the *tat* gene under control of a *tet* responsive element (TRE) in the pTREX vector (Clonetech, Mountain View, CA) were crossed with mice expressing the reverse tetracycline transactivator (RTTA) driven by the pGFALac-1 human GFAP promoter. Inducible Tat^+ transgenic mice express both GFAP-RTTA and TRE-Tat genes; Tat^- mice express only the GFAP-RTTA gene. To induce Tat production during the pre- and early postnatal period, pregnant dams were fed chow containing doxycycline (Harlan, Indianapolis, IN; 6 mg/g) from gestational day 15–16 until postnatal day 7. Doxycycline, a water-soluble tetracycline derivative, crosses the placenta and is secreted in breast milk, and reliably induces transgene expression in embryos and neonates via the *tet*-on system (Fedorov et al. 2001; Perl et al. 2002a; Perl et al. 2002b; Shin et al. 1999). All dams received the same chow, to control for effects of doxycycline unrelated to activation of the *tat* transgene. Standard PCR procedures were performed on genomic DNA isolated from ear clip samples to confirm genotype. Morphine was delivered by subcutaneous (*s.c.*) injection at a dose of 2 mg/kg in a 20 μl volume of saline, based on the average daily litter weight. This dose reportedly gave circulating morphine levels approximating those measured in human preterm infants given intermittent or continuous intravenous morphine infusion (Vien et al. 2009). Male, P4 to P7 Tat^+ mice received a morphine injection twice daily at 10:00 am and 6:00 pm. Sham pups received 20 μl of saline *s.c.* twice daily. For BrdU labeling *in vivo*, 50 μl of 10 mg/ml BrdU (32.5 mM) was injected *s.c.* in sterile saline twice at 2 h intervals immediately prior to perfusion. There was <5% mortality; death was not related to genotype or administration of morphine or doxycycline. For immunohistochemistry, pups were deeply anaesthetized with isoflurane (Baxter, Deerfield, IL) prior to perfusion with 4% paraformaldehyde. Brains were postfixed overnight, hemisected, rinsed overnight in 15 ml changes of phosphate-buffered saline (PBS), cryopreserved through graded sucrose solutions (10 and 25 %), embedded in Tissue Tek OCT compound (Sacura Finetek, Torrance, CA), and stored at −80°C. 10 μm frozen sections containing striatum were thaw-mounted on SuperFrost Plus slides (VWR Scientific, West Chester, PA).

Immunohistochemistry and quantification

For *in vitro* studies, NPCs on coverslips were immunolabeled after 12–48 h treatment using antibodies to cell and lineage-specific markers including nestin (1:5000, Aves Labs, Inc., Tigard, OR), Olig2 (oligodendrocyte transcription factor 2; 1:100, Immuno-Biological Laboratories, Minneapolis, MN), and Sox2 (1:200, Millipore, Temecula, CA). Cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. Double-staining was performed with anti-BrdU (1:200, Abcam, Cambridge, MA) and anti-Sox2 or anti-Olig2 to detect proliferating progenitors. Coverslips were first treated with 1 N HCl in PBS to expose incorporated BrdU. Immunohistochemistry with precursor and BrdU antibodies was done sequentially; all primary antibodies were applied overnight at 4°C and visualized with appropriate fluorescent-conjugated second antibodies (Invitrogen). Immunolabeled coverslips were finally incubated with Hoechst 33342 dye (0.5 μg/ml, 8 min) to identify nuclei (Molecular Probes/Invitrogen), then mounted in ProLong Gold anti-fade reagent (Molecular Probes/Invitrogen). To assess the proportion of different progenitors in cultures, 300–350 $Hoechst^+$ cells were selected randomly per coverslip and assessed for nestin, Sox2, and Olig2 immunoreactivity (n = 6) under oil immersion at 63× using a Zeiss

AxioObserver system with integrated MRm camera system (Carl Zeiss, Inc., Thornwood, NY). Cell proliferation was estimated in the overall NPC population by determining the proportion of 300–350 Hoechst$^+$ cells that incorporated BrdU. Cell proliferation was assessed in each progenitor population by counting 200 nestin$^+$, Sox2$^+$, or Olig2$^+$ cells and scoring the proportion of cells with co-localized BrdU. Similar immunodetection methods were applied to 10 μm tissue sections from neonatal brains. The proportion of different cell types in vivo was estimated by averaging the percentage of Sox2$^+$ or Olig2$^+$ cells among 250–350 randomly selected Hoechst$^+$ cells per striatum (left side) in two sections, in n = 6 mice. Proliferation was estimated by scoring BrdU incorporation in 200 cells expressing each marker in two sections per mouse. Double-immunolabeling was performed to assess whether hNPCs expressed the μ opioid receptor (MOR) and C-C chemokine receptor type 5 (CCR5) or C-X-C chemokine receptor type 4 (CCR4) in vitro. MOR (1:250, Antibodies Incorporated, Davis, CA), CCR5 (1:200, BD Pharmingen, Mountain View, CA), and CXCR4 (1:100; BD Pharmingen) antibodies were applied to fixed hNPCs and incubated overnight at 4°C, then visualized as described above, prior to labeling with NPC markers and Hoechst 33342. p24 was detected on hNPCs after exposure to supernatants from HIV-1 infected monocytes using an antibody commonly used to study infective processes (1:50, Dako, Carpinteria, CA) (Eugenin et al. 2011; Hahn et al. 2008; Lawrence et al. 2004). Images were taken under oil immersion at 63×.

Viability assay

Mouse NPCs were cultured on poly-L-lysine coated 48 well plates and treated at 5 d after plating with Tat, morphine and naloxone either alone or in combination as described earlier. After 12–48 h, medium was replaced with PBS containing 4 μM ethidium homodimer-1 (EthD-1) and 5 μg/mL Hoechst 33342 (15 min, at 37°C). The intensity of EthD-1 and Hoechst signals was measured using a PHERAstar FS plate reader (BMG LABTECH, Ortenberg, Germany) in n = 8–10 cultures using appropriate excitation/emission filters (EthD-1: 495/635 nM; Hoechst: 350/510 nM).

RNA Extraction and Reverse transcription PCR

RT-PCR was performed to confirm expression of Tat mRNA in the striatum of 7 d postnatal pups receiving doxycycline through the placenta and lactation as described. Pups were deeply anaesthetized with isoflurane. Striata were rapidly dissected from brains, and stored at −80°C. Total RNA was isolated using TRIzol reagent (Invitrogen). RNA samples were cleaned using GenElute™ Mammalian Total RNA kit (Sigma-Aldrich), after RNase-free DNase I treatment to eliminate genomic DNA. cDNA was synthesized from 2 μg of total RNA using the High-Capacity cDNA Archive Kit (Applied Biosystems, Warrington, UK). PCR was performed to detect Tat mRNA expression using primers previously described (Bruce-Keller et al. 2008; Hauser et al. 2009) with 18S mRNA as a control (Chauhan et al. 2003). PCR products were separated by electrophoresis on 1.5% agarose gels, stained with ethidium bromide.

Viral stock preparation

CCR5- and CXCR4-preferring strains of HIV (HIV-1_{SF162} and HIV-1_{IIIB}, respectively) were used to test strain specific effects on some outcome measures. The following viral

stocks were obtained through the NIH AIDS Research and Reference Reagent Program: HIV-1_{SF162} from Dr. Jay Levy (Cheng-Mayer and Levy 1988); HIV-1_{IIIB} from Dr. Robert Gallo (Ratner et al. 1985). R5 and X4 strains were propagated in a U937 monocyte cell line (ATCC, Chicago, IL) activated with IL-2 (10 U/ml) and phytohaemagglutinin (5 ng/ml) for 2 d in RPMI with 10% fetal calf serum. Viral stocks containing 500 pg HIV-1 p24, measured by ELISA, were added to U937 cells. The culture supernatant was collected at the peak of infection, passed through a 0.2 μm filter, and stored at −80°C. For FACS analysis, an aliquot of medium containing 500 pg p24 was added to hNPCs grown to 75–80% confluence in each well of 6-well plates, with a final volume of 1.5 ml per well. Viral exposure time was 12 h.

Human progenitor cell line culture and CCR5/CXCR4 blocking assays

These studies used the human neural progenitor ReNcell VM cell line, derived from 14-wk gestation ventral mesencephalon (Millipore). Cells are ≥ 95% $Sox2^+$ and $nestin^+$, and have the potential to produce dopaminergic neurons and glia (Donato et al. 2007). All experiments used cells from passage 4. Frozen cells were thawed and expanded on laminin-coated T75 cm^2 culture flasks in ReNcell NSC Maintenance Medium (Millipore) supplemented with epidermal growth factor (20 ng/ml; Millipore) and basic fibroblast growth factor-2 (20 ng/ml; Millipore). Three to four days after plating, at < 80% confluence, cells were passaged by detachment with accutase (Millipore), centrifuged at 300 × g for 5 min and resuspended in fresh maintenance media containing growth factors. For FACS analysis, cells were replated in laminin-coated 6-well plates (30,000 cells/well). Three to four days after plating, cells were treated for 12 h with Tat or HIV^+ supernatant ± morphine ± naloxone. For the blocking experiments, non-signaling monoclonal antibodies to CCR5 (2D7; 2 μg/ml; BD Pharmingen) (Lee et al. 1999) and CXCR4 (12G5, 2 μg/ml; BD Pharmingen) (Bleul et al. 1997; Endres et al. 1996), or mouse IgG (2 μg/ml; BD Pharmingen) were applied 1 h prior to treatment. hNPCs were treated with BrdU (10 μM) 2 h prior to harvesting. For p24 immunolabeling, 5,000 cells per well were replated on laminin-coated glass coverslips in 24-well plates and treated with HIV-1 supernatant prior to reaching 80% confluence as described above. p24 immunolabeling was localized in cells fixed at 12 h post-inoculation using a Zeiss LSM 700 confocal laser scanning module configured to an Axio Observer Z1 inverted microscope and 63x (1.40 NA) oil immersion objective. To optimize detection of Alexa 594-labeled anti-human p24 antibodies, 555-nm laser excitation was filtered through a dichroic beam-splitter set at 585 nm. Optical sections were acquired from a single Z plane with acquisition parameters, including the scan step (0.315 μm) and pinhole size (34 μm), set to optimize X-, Y-, and especially Z-plane resolution (Zen 2010 software; Zeiss).

FACS analysis of hNPC proliferation and p24 expression

The APC BrdU Flow Kit (BD Pharmingen) was used to determine the proportion of S-phase hNPCs. Cells were incubated with BrdU (10 μM) for the final 2 h of a 12 h exposure to Tat or to supernatant from HIV-1_{SF162} or HIV-1_{IIIB} infected monocytes ± morphine. Cells were detached using enzyme-free cell dissociation buffer (Sigma-Aldrich), fixed with BD Cytofix/Cytoperm buffer and treated with DNase to expose incorporated BrdU. An APC-conjugated anti-BrdU antibody and 7-amino-actinomycin D (7-AAD) were used to

determine cell cycle phases (G_0/G_1, S, G_2+M). Fluorescent signals for APC (Ex: 650 nm; Em: 615 nm) and 7-AAD/DNA complexes (Ex: 546; Em: 647) were detected on a FACSCanto (BD Biosciences, San Jose, CA) and analyzed using the DIVA FACS data program (BD Biosciences). Single and intact hNPCs were first gated by cell area and width. APC-BrdU fluorescence intensity was assessed after gating on approximately 3×10^5, 7-AAD$^+$ cells. G_0/G_1 phase cells (APC-BrdU$^-$ and 7-AAD$^+$) were defined using appropriate negative (non-specific IgG followed by APC-conjugated secondary antibody only); and positive (7-AAD only; APC-anti-BrdU antibody only) controls. S-phase cells were defined as being APC-BrdU$^+$ and 7-AAD$^+$.

After 12 h exposure to HIV$^+$ supernatant, p24 expression on hNPCs was determined by FACS analysis. Cells were fixed and immunolabeled with p24 antibody and Alexa Fluor 488-conjugated second antibody, followed by incubation in 7-AAD. Single and intact hNPCs were gated as above. p24 immunolabeling intensity was assessed on approximately 3×10^4 intact, 7-AAD$^+$ cells. Single laser excitation (488 nm) and dual fluorescence emission (585/42 band pass; 530/30 band pass) analysis was used to compensate for autofluorescence. Controls included p24 labeling in untreated cells and non-specific IgG followed by second antibody in HIV-treated cells.

Statistical analysis

Significance was assessed by analysis of variance (ANOVA) followed by Duncan's post-hoc testing if indicated, or main effect testing, using StatSoft software (Statistica, Tulsa, OK).

RESULTS

Effect of HIV-1 Tat and morphine on murine neural progenitors *in vitro*

To determine independent or interactive effects of Tat_{1-86} and morphine on maturation, NPCs from embryonic mouse striata were treated with 100 nM Tat_{1-86} ± morphine (500 nM) over a period of 48 h, with BrdU added for the final 12 h. Double immunostaining for BrdU and lineage specific markers was performed following 12 h, 24 h and 48 h treatments. Nestin and Sox2 are markers of uncommitted NPCs (Episkopou 2005; Gu et al. 2002; Nunes et al. 2003; Qu and Shi 2009), while Olig2 is a transcription factor expressed in more differentiated cells that develop largely into oligodendrocytes (OLs) and motor neurons (Masahira et al. 2006; Takebayashi et al. 2002). Treatment with Tat ± morphine for 12 h did not alter either the proportion of nestin$^+$ (nestin$^+$/Hoechst$^+$) or Olig2$^+$ (Olig2$^+$/Hoechst$^+$) cells, or the percentage of nestin$^+$ or Olig2$^+$ cells expressing BrdU (Fig. 1A, D vs C, F). There were, however, significant effects on the subset of NPCs expressing Sox2. While neither morphine nor Tat alone had any effect, both the Sox2$^+$/BrdU$^+$ cells and the total Sox2$^+$ cell population were reduced in cells exposed concurrently to Tat and morphine (Fig. 1B, E). Since only Sox2$^+$ cells were affected after 12 h, we hypothesized that the sensitivity of NPCs to Tat ± morphine might depend on differentiation, and that changes in Sox2+ cells might affect their progeny. Experiments were extended to 24 h and 48 h using markers for Olig2 (Fig. 2), and at both times the total Olig2$^+$ population was reduced by Tat alone. The proliferating (Olig2$^+$/BrdU$^+$) population at 24 h was unaffected by either Tat or morphine

alone, but Tat and morphine interacted to reduce BrdU uptake (Fig. 2A). By 48 h, Tat alone had reduced the $Olig2^+/BrdU^+$ population. Tat and morphine interacted at this time point to further decrease BrdU uptake (Fig. 2B). Thus, interactive effects of Tat and morphine were only observed on proliferative behavior of $Olig2^+$ cells, while Tat by itself affected cell populations. Naloxone partially reversed interactive effects of morphine in proliferating cells, as the groups receiving Tat, morphine, and naloxone were not different from control, but also not different from the group co-exposed to Tat and morphine. Naloxone also appeared to reduce overall $Olig2^+$ cells when given concurrently with Tat and morphine. We conducted the experiments described below to understand these findings.

Effect of naloxone on murine progenitors *in vitro*

Since naloxone unexpectedly decreased the percentage of $Olig2^+$ cells when combined with morphine and Tat ("a" in Fig. 2C, D), we treated murine NPCs with naloxone alone for 12–48 h and compared the proportion of $Olig2^+$ cells in untreated control and naloxone treated groups. There was no significant reduction in the $Olig2^+$ population (Fig. 2E), suggesting that naloxone by itself is not toxic to $Olig2^+$ cells or their immediate progenitors. Instead, naloxone might interfere with endogenous opioid signals resulting from Tat and morphine treatment. Autocrine opiate signaling pathways are known to play pivotal roles in OL survival (Buch et al. 2007; Hauser et al. 2009; Khurdayan et al. 2004; Knapp et al. 2001) and overall glial cell fate (Gorodinsky et al. 1995; Kim et al. 2006; Persson et al. 2003).

Effect of HIV-1 Tat and morphine on murine neural progenitors: proliferation vs. viability

To determine whether cell death might contribute to observed reductions in proliferating or total NPCs, we examined NPC viability at up to 48 h of Tat ± morphine exposure. There was no cell death in response to Tat ± morphine treatment at any time period measured by EthD-1 fluorescence intensity (Fig. 3).

Effects of HIV-1 Tat and morphine on neural progenitors in striatum

Transgenic mice with inducible expression of HIV-1 Tat in the CNS were used to assess Tat and morphine effects on NPCs *in vivo*. Detection of Tat mRNA and protein in the brains of these mice was previously reported by our group (Duncan et al. 2008; Fitting et al. 2010; Hauser et al. 2009) and also shown here (Fig. 4A–C). Pregnant dams received chow containing doxycycline from gestational day 15–16 through postnatal day 7 to activate the HIV-1 Tat transgene in fetal and neonatal mice. Reverse transcription PCR confirmed that doxycycline delivered to dams induced Tat mRNA expression in the cortex and striatum of Tat^+, but not Tat^-, neonatal mice (Fig. 4C).

All mice used in experiments were male. Some mice received morphine *s.c.* at postnatal days 4–7 to assess whether co-exposure to HIV-1 Tat and morphine caused effects on NPCs *in vivo* similar to those in culture. There were several significant effects on proliferating cell populations. Morphine exposure and Tat induction independently reduced BrdU uptake in both $Sox2^+$ and $Olig2^+$ cells in the striatum, and in the overall population of striatal NPCs (Fig. 4D). An interactive effect of morphine and induced Tat was seen both in the $Sox2^+/BrdU^+$ population and in the overall population of $BrdU^+$ cells (Fig. 4D). We examined whether this might lead to changes in the overall populations of $Sox2^+$ and $Olig2^+$ cells.

Morphine and Tat by themselves each reduced the percentage of the population that was $Sox2^+$ or $Olig2^+$ (Fig. 4E); there was no interactive effect. Additionally, Tat alone, but not morphine alone, reduced the percentage of the population that was $Olig2^+$; again there was no Tat and morphine interaction.

Effects of HIV-1 Tat, HIV, and morphine on proliferation of a human NPC line

To validate our results in a more clinically relevant system, we exposed human NPCs to HIV-1 $Tat_{1\text{-}86}$, or to supernatant from monocytes infected with R5-tropic HIV-1_{SF162}, or X4-tropic HIV-1_{IIIB} with or without concurrent morphine. Before conducting experiments, we briefly characterized the hNPC cell line by co-localizing MOR, nestin, and/or Sox2 antigenicity. In accordance with previous reports (Breier et al. 2008), more than 95% of hNPCs expressed nestin and Sox2. Approximately 95% of hNPC also showed MOR immunoreactivity (Fig. 5A, B); Olig2 immunofluorescence was not detected (not shown). Since we used both R5-tropic and X4-tropic HIV strains, that respectively use CCR5 or CXCR4 as preferred co-receptors for viral entry, CCR5 and CXCR4 immunoreactivity was co-localized in $Sox2^+$ hNPCs to verify expression (Fig. 5C, D).

FACS analysis was performed at 12 h following exposure to Tat ± morphine or to supernatant from infected U937 cells ± morphine to assess proliferation (Fig. 6). Due to their rapid cell cycle (Breier et al. 2008), hNPCs were treated with BrdU 2 h prior to harvest. FACS analysis showed that both Tat and morphine independently caused significant reductions in the proportion of S-phase cells as compared to control, with no synergistic or additive effect of combining Tat and morphine (Fig. 6B). Interestingly, hNPCs exposed to supernatant from HIV-1_{SF162} or HIV-1_{IIIB} infected cells also showed significantly reduced proliferation (Fig. 6C, E). There was an additional reduction in proliferation rate when HIV-1_{SF162} supernatant was combined with morphine exposure (Fig. 6C). The opposite effect was seen for HIV-1_{IIIB} supernatant, whose effects were partially reversed by morphine co-exposure (Fig. 6E). Specific, non-signaling, CCR5 (2D7) and CXCR4 (12G5) blocking antibodies were applied 1 h before exposure to viral supernatant to test whether some effects might be due to viral entry, or to viral proteins binding to hNPCs. Compared to treatment with control mouse IgG, 2D7 immunoblockade partially reversed the anti-proliferative effect of HIV-1_{SF162} supernatant (Fig. 6D) while 12G5 immunoblockade was completely ineffective (Fig. 6F).

p24 expression in hNPCs by FACS analysis and immunolabeling

hNPC infection by HIV has been reported by other investigators (Lawrence et al. 2004; Mishra et al. 2010; Schwartz et al. 2007). We assessed hNPCs in the present study for the presence of p24 antigen. hNPCs were exposed for 12 h to HIV-1_{SF162} or HIV-1_{IIIb} infective supernatant, then fixed and either detached from the plate, immunolabeled with p24 antibody and Alexa Fluor 488–conjugated second antibody, stained with 7-AAD, and subjected to FACS analysis (Fig. 7A–C), or immunolabeled for confocal microscopic analysis of p24 within individual cells (Fig. 7D–F). Immunoreactivity was observed in sporadic hNPCs in cultures with both R5- and X4-infective supernatant, but never in control cultures. $p24^+$ staining appeared as numerous, small foci found throughout the cytoplasm that were not localized in the nucleus (Fig. 7D, E). In FACS studies, approximately 1×10^4

intact, 7-AAD$^+$ cells were assayed using single laser excitation (488 nm) and dual emission (585/42 band pass; 530/30 band pass) analysis to compensate for spectral overlap between p24 and autofluorescence signals. Neither control nor experimental groups showed any evidence of autofluorescence (Fig. 7A–C). A p24$^+$ signal was detected in 0.34% of cells treated with HIV-1_{SF162} infective supernatant (Fig. 7A). p24 was detected in 0.1% and 0.001% of control populations, respectively.

DISCUSSION

NPCs are present throughout development and in adult brains, although the population and their distribution is substantially reduced with age. Using murine and human embryonic cultures, and an in vivo perinatal model, we determined that HIV-1 Tat or viral exposure dysregulates NPC proliferation and affects cell populations in developing systems. Exposure to Tat, or to infective HIV$^+$ supernatant, both reduced progenitor proliferation. Morphine interacted with both treatments to cause further dysregulation, although developmental stage and HIV strain were critical variables. Results suggest that HIV exposure and opiate co-exposure elicit fundamental changes in the types of NPCs produced that likely contribute to neurologic impairment in both pediatric and adult patients. Morphine by itself affected NPCs in mice exposed during the perinatal period; this may be relevant to children exposed to morphine or heroin before birth.

HIV-1 Tat and opiates alter proliferation/population dynamics of murine NPCs *in vitro*

Previous studies showed that HIV-1 Tat affects expansion and viability of fetal human or murine NPCs or glial-restricted progenitors in culture, without determining if cells at particular stages of maturation are selectively affected (Buch et al. 2007; Khurdayan et al. 2004; Mishra et al. 2010). We also questioned whether opiate exposure might enhance Tat effects, since opiates can amplify CNS dysfunction in experimental HIV models (Bokhari et al. 2011; Bruce-Keller et al. 2008; Fitting et al. 2010; Hauser et al. 2006; Hu et al. 2005; Kumar et al. 2006; Zou et al. 2011) and in patients (Anthony et al. 2005; Bell et al. 2002; Byrd et al. 2011). We examined striatum, an area that is rich in opiate receptors and extremely vulnerable to HIV-1-induced neuropathology (Berger and Nath 1997; Mirsattari et al. 1998; Nath et al. 2000; Nath et al. 2002). Murine NPCs derived from E14 striatum have been characterized as a mixture of progenitors and very immature cells (Hahn et al. 2010); most proliferate and express MOR (Fig. 5) (Hauser et al. 2009).

We distinguished between cells expressing nestin or Sox2, markers of relatively undifferentiated NPCs, and Olig2, a marker typical of developing OLs. Tat and/or morphine had effects at specific stages of differentiation. As early as 12 h after co-exposure to morphine and Tat, proliferation of Sox2$^+$ cells was reduced and the total Sox2$^+$ population was diminished (Fig. 1B, E). With increasing time, Olig2$^+$ cell proliferation was also affected, by both Tat alone and by Tat-morphine interactions. The gradually increased response of immature cells to Tat ± morphine during the exposure period suggests an increased receptivity of the cells during maturation. By itself, Tat did not affect cell replication until 48 h, and did not affect cell viability at any time studied; however, Tat did reduce the percentage of cells expressing Olig2 at both 24 and 48 h. A potential unexplored

explanation is that Tat may influence NPC lineage decisions, perhaps by stalling cells at the $Sox2^+$ stage, or by increasing production of $GFAP^+$ or $NG2^+$ cells. In keeping with this idea, an increase in $GFAP^+$ cell production was noted in human progenitors exposed to HIV-1 (Peng et al. 2008).

Treatment with naloxone partially reversed morphine-Tat interactions in most studies. However, in the Olig2 population studies, naloxone treatment decreased the percentage of $Hoechst^+$ cells expressing Olig2 ("a" in Fig. 2). This effect only occurred for $Olig2^+$ cells, and not for $nestin^+$ or $Sox2^+$ cells. The results are reminiscent of *in vivo* studies with another non-selective opioid receptor antagonist, naltrexone, which was shown to modulate NPC proliferation in adult rodent hippocampus (Holmes and Galea 2002; Ra et al. 2002) and developing rat brain (Schmahl et al. 1989). However, we found that naloxone did not directly affect the $Olig2^+$ population (Fig. 2E). We previously suggested that naloxone or naltrexone may interfere with endogenous opiate signaling pathways that modify survival and development of OL lineage cells (Hauser et al. 2009; Knapp et al. 2001; Knapp et al. 1998). The present study suggests a more complex phenomenon, since naloxone reversal was only partial, and particularly impaired in the presence of combined Tat and morphine. CCR5, an HIV co-receptor, has well documented interactions with MOR, leading to bi-directional, heterologous cross-desensitization (Chen et al. 2004; Chen et al. 2007; Suzuki et al. 2002; Szabo et al. 2002; Szabo et al. 2003). Tat can elevate β-chemokine ligands that activate CCR5 (RANTES, MIP-1α, and MIP-1β) in multiple cell types including NPCs (Hahn et al. 2010), which may interfere with naloxone activity through the above mechanisms.

HIV-1 Tat and opiates alter proliferation/population dynamics of murine NPCs *in vivo*

We used inducible HIV-1 $Tat_{1\text{-}86}$ transgenic mice to model Tat and opiate exposure in vivo. While all animal models of a human-specific disease have limitations, these mice have been extensively characterized and shown to exhibit neurologic abnormalities associated with chronic HIV-1 exposure, including glial activation (Bruce-Keller et al. 2008), dendritic damage and reduced spine density in striatal neurons (Fitting et al. 2010), abnormal OLs (Hauser et al. 2009), circadian rhythm (Duncan et al. 2008) and behavioral (Fitting et al. 2012) abnormalities. Since our culture experiments used E14 tissue grown for 7 d in vitro, we performed *in vivo* studies at similar times. Results may therefore have particular relevance to pediatric HIV patients, but may also pertain to unique progenitor populations in adult brain parenchyma.

We focused on $Sox2^+$ and $Olig2^+$ cells, which are relatively abundant in the striatum compared to $nestin^+$ cells. The *in vivo* results showed declines that paralleled culture studies in both the proportion of progenitors replicating DNA and the relative number of progenitors, but there were notable differences. Importantly, morphine exposure by itself (Fig. 4D, E) reduced BrdU incorporation in both $Sox2^+$ and $Olig2^+$ cells, as well as in the $Sox2^+$ and overall $Hoechst^+$ populations. This finding, similar to work in vivo by Kornblum et al. (1987), infers that progenitor dysfunction may drive cell population changes in children of opiate-exposed women. Morphine did not affect these parameters in vitro, also in agreement with Kornblum et al. (1987), who postulated an indirect mechanism for

morphine-induced changes in progenitor dynamics. Previous findings that morphine reduces neuron generation in neonatal (Hauser 1992; Kornblum et al. 1987; Seatriz and Hammer 1993) and adult rodent brain (Arguello et al. 2008; Eisch et al. 2000; Kornblum et al. 1987) may reflect progenitor dysregulation.

Tat exposure also decreased BrdU uptake into $Sox2^+$ and $Olig2^+$ cells, and within the entire NPC population (Fig. 4D). The greater effect of Tat on BrdU uptake in vivo may reflect the longer Tat exposure time (12 d); BrdU uptake in cultured $Olig2^+$ cells was reduced after 48 h but not earlier. Both $Sox2^+$ and $Olig2^+$ populations were reduced by Tat alone (Fig. 4E), probably due in part to decreased proliferation. As was true in culture, the only interactive effect of morphine and Tat was on BrdU uptake. It seems counter-intuitive that morphine-Tat interactions did not reduce total populations. Neonatal progenitors are a transitory population and it may be more instructive to examine whether their progeny are modified. Small changes in early proliferative rates can translate into biologically significant changes in cell populations over time. To determine if the observed effects of Tat and/or morphine on progenitors adjusts the balance of glia and neurons in the CNS requires a stereological approach. Glial populations may be preferential targets, since their production continues well into the postnatal period in both rodents and humans (Altman 1966; Chan et al. 2002; Price 1994; Skoff and Knapp 1991; Skoff et al. 1976; Sturrock 1979). The present studies predict that OLs are quite vulnerable to HIV-1 Tat, as was observed in the striatum of mature, Tat-transgenic mice, especially with co-exposure to morphine (Hauser et al. 2009).

Tat, HIV-1, and morphine alter proliferation of hNPCs

Findings were re-evaluated in a human NPC line generated from 14-wk gestation human ventral mesencephalon. Over 95% of cells are immunopositive for nestin and/or Sox2, and many also express MOR, CCR5, and CXCR4 (Fig. 5). Tat, HIV-1_{SF162}- or HIV-1_{IIIB}-infective supernatant, and morphine all reduced proliferation after 12 h, with viral supernatant and morphine displaying interactive effects (Fig. 6). In general, these results are in accord with a previous report that 25–100 ng/ml Tat decreased human fetal progenitor proliferation (Mishra et al. 2010). Isolated Tat effects were greater in that study, perhaps reflecting cell heterogeneity, an important consideration, since all $nestin^+$ or $Sox2^+$ cells do not necessarily express equivalent amounts of functional MOR and may not express equivalent MOR splice variants (Dever et al. 2012). Furthermore, $nestin^+$ or $Sox2^+$ cells may not uniformly express molecular targets for Tat, such as $\alpha_v\beta_5$-integrin (Vogel et al. 1993), mannose (Liu et al. 2004) or NMDA receptors (Li et al. 2008), or the low-density lipoprotein receptor-related protein (Liu et al. 2000). Interestingly, another study showed that supernatant from monocyte-derived macrophages infected with R5 virus and co-stimulated with LPS could occasionally increase progenitor production (Peng et al. 2011; Peng et al. 2008). The presence of LPS makes it difficult to compare these results. Different proliferation assessments may also affect results. In our study, BrdU uptake after 2 h gave an immediate picture of DNA synthesis in individual cells. Exposure to infected supernatant was suggested to cause re-direction of progenitors towards an astroglial fate (Peng et al. 2011; Peng et al. 2008), a possibility also suggested by our findings and by others (Mishra et al. 2010). Although concurrent morphine exposure did not alter Tat effects on hNPCs, there were interactive effects of morphine and HIV-1 supernatant (Fig. 6). Morphine enhanced the

anti-proliferative effect of HIV_{SF162}, but unexpectedly reduced the anti-proliferative effect of HIV_{IIIB}. Strain-specific, HIV effects have been observed on other outcomes (El-Hage et al. 2011; Podhaizer et al. 2012). They may be more prominent in complex models, and perhaps especially when viral effects are indirect. Supernatants in the present studies contain virions, viral proteins, and secreted factors from infected cells that may vary with viral strain. Multiple interactions between these factors and morphine may both enhance and reduce viral effects.

FACS analyses after 12 h exposure to infective supernatant showed that < 0.34% of intact hNPCs were $p24^+$ in R5-treated cultures (Fig. 7A), and we very occasionally detected cells exposed to R5 and X4 supernatants that appeared $p24^+$ by immunocytochemistry in 0.32 μM optical sections (Fig. 7E, F). Our experience supports the concept that hNPCs can be infected by HIV-1 (Lawrence et al. 2004; Rothenaigner et al. 2007; Schwartz et al. 2007), but it was not a common event. Most progenitors in the brain appear to respond as bystanders to events triggered by infected cells.

Relationship to HIV Pathology in Humans

Prior to the advent of cART, the prevalence, rate of onset, and severity of HIV-1-associated CNS disease in children was quite high compared to adult patients (Mintz 1994; Schwartz et al. 2007; Schwartz and Major 2006). This is not surprising, since much of CNS development occurs after birth, and the immature brains of children or adolescents exposed to HIV are highly vulnerable to pathologic agents. CNS disease in pediatric HIV patients manifested as static or progressive encephalopathy, with classic symptoms including delay or loss of major motor and mental milestones, and atrophy of cortical/subcortical regions and other abnormalities on CT scans (Drotar et al. 1997; Epstein et al. 1988; Schwartz and Major 2006; Tahan et al. 2006; Ultmann et al. 1985; Van Rie et al. 2007). In developed countries, rates of HIV-1 vertical transmission have been reduced to 1–2%, from 40% in 1995 (Buchholz et al. 2010; Van Dyke 2011). Treatment with CNS-penetrating drugs has reduced the incidence of pediatric and adolescent HIV-associated encephalopathy by ≥ 50%, especially in cases of perinatal acquisition (Patel et al. 2009). It is currently estimated that 10% of HIV^+ children on cART therapy will show signs of CNS disease (Chiriboga et al. 2005). The situation in resource-limited settings is quite different. In 2008, an estimated 1,600 children per day became HIV-infected worldwide, 90% of them in sub-Saharan Africa (Joint United Nations Programme on HIV/AIDS. 2009; Joint United Nations Programme on HIV/AIDS. and World Health Organization. 2008). In such regions, mother-to-child transmission via breastfeeding is common in infants not infected at birth, and HIV infection remains a major source of infant mortality (Becquet et al. 2009; Meyers et al. 2007).

In the context of pediatric HIV, reduced production of $Sox2^+$ progenitors is expected to impact populations of neurons and glia that derive from $Sox2^+$ cells (Fig. 8). We find that immature, $Olig2^+$ OLs are reduced in vivo, and others have reported lower neuron production, possibly through altered cyclin D1 and MAPK signaling (Mishra et al. 2010; Okamoto et al. 2007). Although the hypothesis that Tat exposure can alter lineage decisions remains to be fully tested, a proportional increase in astroglia might enhance interactions between astroglia and HIV-1 infected macrophages/microglia that are deleterious for

developing neurons and neural circuitry (Blumberg et al. 1994). Our finding of Tat-morphine and HIV-1-morphine interactive effects is clinically relevant, given the high rate of opioid abuse in HIV patients, and that morphine may initially be used to treat neonatal abstinence syndrome in children of opioid-maintained or non-maintained mothers (Bandstra et al. 2010; Colombini et al. 2008; Ebner et al. 2007; Jansson et al. 2009). (Fig. 8)

If the observed effects also occur on progenitors in the adult CNS, the predicted outcome would be fewer NPCs and a tendency towards increased astroglia at the expense of OL production (Fig. 8). Importantly, neural precursors were also reduced in human hippocampal slice cultures by HIV coat proteins and patient CSF (Krathwohl and Kaiser 2004) and in gp120 transgenic mice (Lee et al. 2011; Mishra et al. 2010; Okamoto et al. 2007). Neural precursors were also reportedly reduced in HIV patients with neurological deficits as compared to HIV-1 infected individuals without such deficits and non-infected controls (Krathwohl and Kaiser 2004).

SUMMARY

Consistent findings that Tat and HIV-1 reduce NPC production in a variety of model systems that assess maturation are compelling. By disrupting proliferation and subsequent differentiation of NPCs into mature glia and neurons, HIV-1 infection may increase the severity of neurological impairment in pediatric and adolescent patients. Interactions between Tat/HIV and morphine may exacerbate neurologic problems in young HIV-1 patients receiving opiates prescribed for neonatal abstinence syndrome or pain, as well as HIV-1^+ adolescents experimenting with abused opiates. Evidence that some neurologic abnormalities in the pediatric HIV population may be reversible (McCoig et al. 2002) is encouraging from a therapeutic standpoint, and points to the need to fully understand how HIV and opiates individually and interactively affect CNS progenitors. This is especially true in resource-limited environments, where the spectres of perinatal HIV infection and high rates of pediatric disability/mortality have not been diminished.

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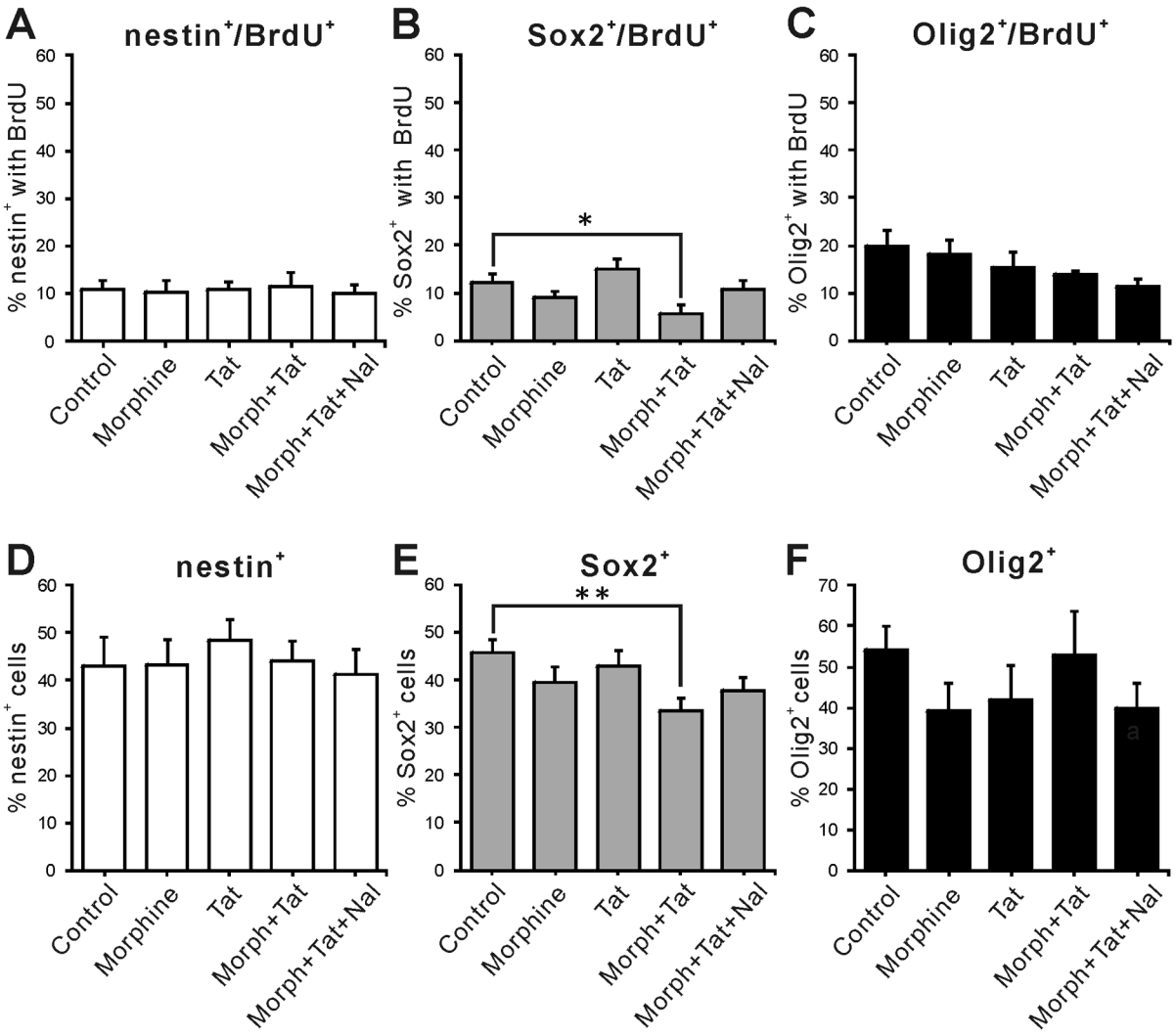

Figure 1.

Co-exposure to HIV-1 Tat and morphine for 12 h alters the proliferation index and population of $Sox2^+$ cells in vitro. Neither $nestin^+$ (A, D) nor $Olig2^+$ (C, F) populations were affected by any combination of Tat, morphine, or naloxone treatment at 12 h. However, both the percentage of the population that was $Sox2^+$ and the proliferation of cells at this stage of differentiation were decreased by Tat and morphine co-exposure (B, E). (* $p<0.05$, ** $p<0.01$; with Duncan post-hoc test, n = 4–6, Morph = Morphine (500 nM), Tat (100 nM), Nal = naloxone (1.5 μM).

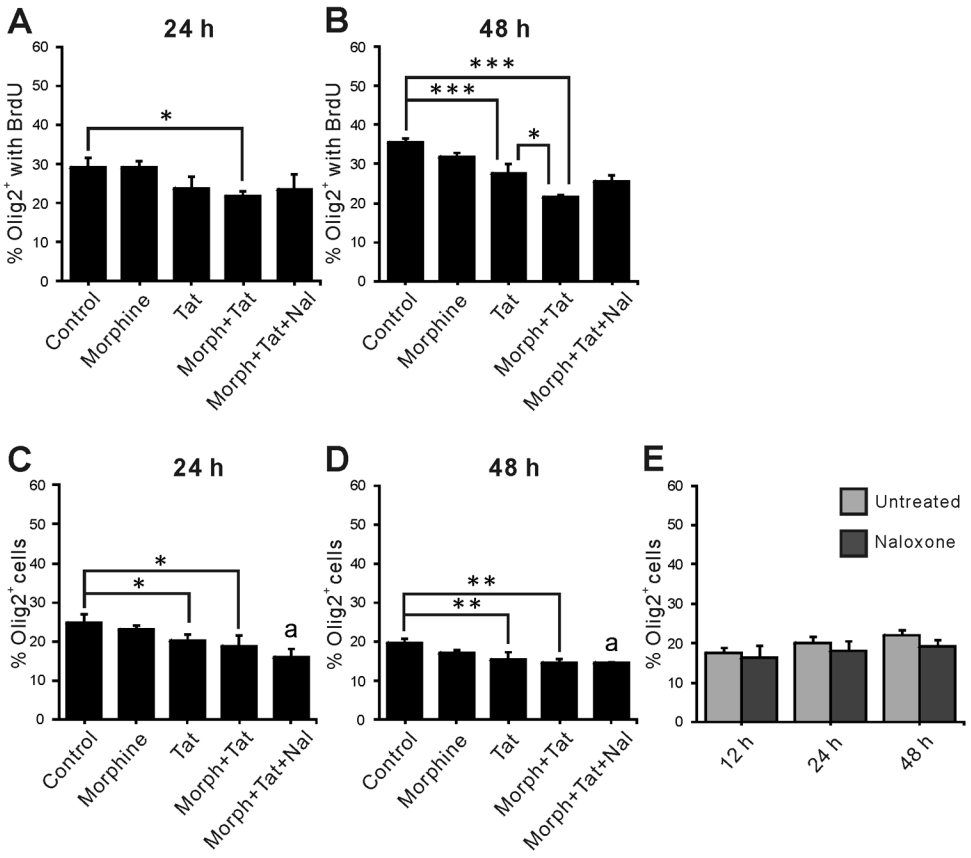

Figure 2.

Effects of HIV-1 Tat ± opioids on proliferation and the relative number of Olig2$^+$ cells in vitro. Interactive effects of morphine and Tat reduced Olig2$^+$ cell proliferation at both 24 and 48 h (A, B); Tat by itself also reduced Olig2$^+$ proliferation at 48 h (B), and reduced the percent of Olig2$^+$ cells in the overall population after both 24 h (C) and 48 h (D); there were no additional effects of morphine co-exposure. Morphine by itself did not affect Olig2$^+$ proliferation or populations. Naloxone partially reversed the effect of morphine in both A and B; the percentage of Olig2$^+$/BrdU$^+$ cells in the Morph+Tat+Nal groups was not different from either control or Morph+Tat treatment groups at the same exposure time. Naloxone did not reverse the interactions between morphine and Tat in C and D (indicated as "a"), suggesting that naloxone might directly affect the Olig2$^+$ population. This was not the case at 12 – 48 h exposure (E). Morph, Morphine (500 nM); Tat, HIV-1 Tat$_{1\text{-}86}$ (100 nM); Nal, naloxone (1.5 μM); * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ^{a} $p < 0.05$ vs. control; ANOVA with Duncan's post-hoc test, n = 4–6.

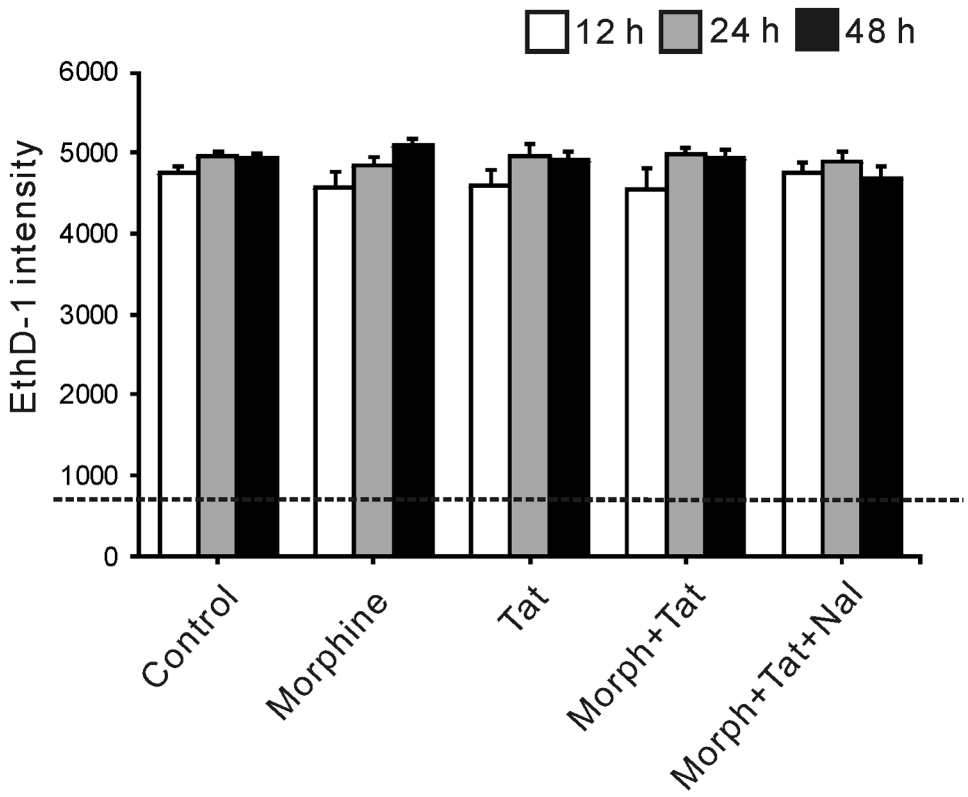

Figure 3.

Neither Tat nor morphine significantly affects NPC death. There was no difference in ethidium homodimer-1 (EthD-1) fluorescence intensity between treatment groups at 12–48 h, indicating no increase in cell death. The dotted line indicates background signal in wells lacking cells. Morph, Morphine (500 nM); Tat, HIV-1 Tat_{1-86} (100 nM); Nal, naloxone (1.5 μM).

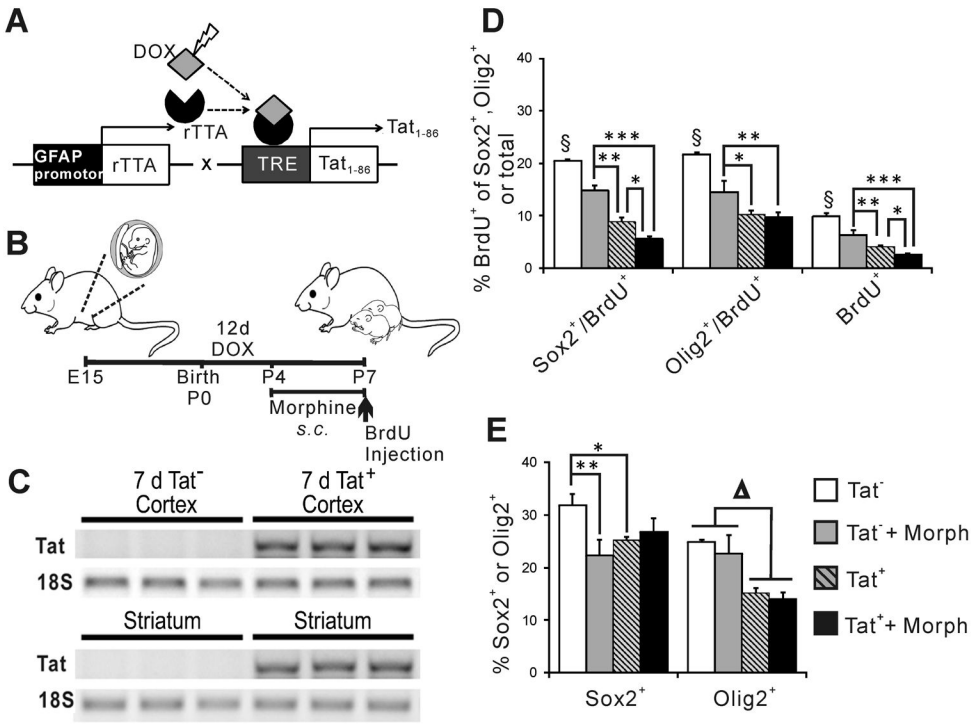

Figure 4.

Proliferation is reduced in neonatal striatum of Tat-transgenic mice. HIV-1 Tat_{1-86} was induced via DOX delivered through placenta and lactation (A, B). All dams received chow containing DOX; Tat mRNA transcription was detected by RT-PCR in cortex and striatum of 7 d Tat^+ pups (C). 12 d Tat induction and 4 d of *s.c.* morphine injections significantly reduced proliferation ($BrdU^+$ cells) overall, and in both $Sox2^+$ and $Olig2^+$ cells. Additive effects of Tat and morphine were observed in $Sox2^+/BrdU^+$ cells and in the overall $BrdU^+$ population (D, * $p < 0.05$; ** $p < 0.01$; § $p < 0.05$, Tat^- vs. all other groups, Duncan's post-hoc test, n = 6–8). Changes in progenitor proliferation appear to affect overall populations of $Sox2^+$ and $Olig2^+$ cells in the neonatal striatum (E, * $p < 0.05$; ** $p < 0.01$ vs. Tat^-, One-way ANOVA, Duncan's post-hoc test, n = 6–8; Δ $p < 0.01$, main effect Tat^- vs Tat^+, n =6–8).

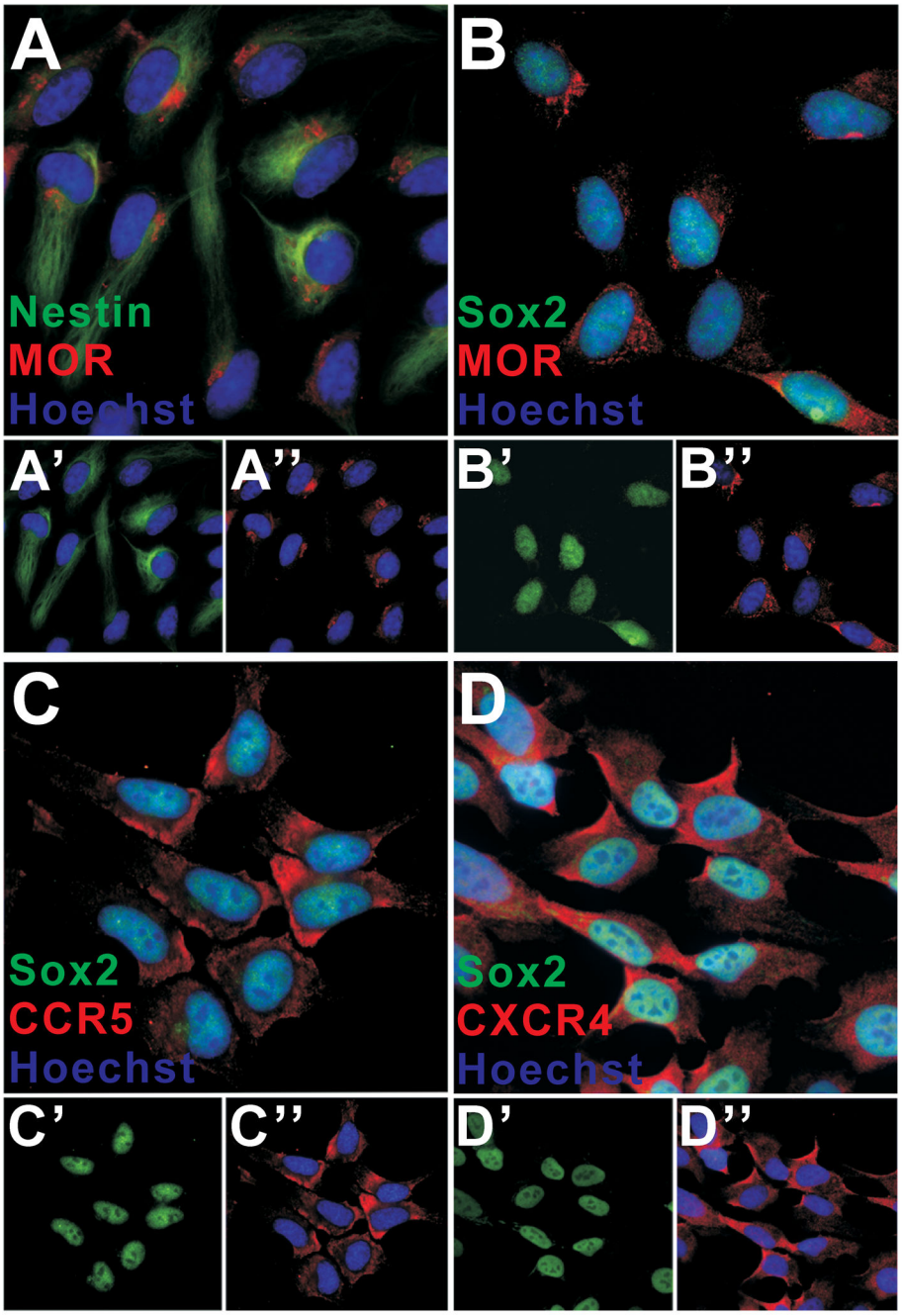

Figure 5.

Immunohistochemical characterization of hNPCs. Double-labeling confirmed that 95% of hNPC were nestin^{+} and Sox2^{+}; 95% also showed immunoreactivity for MOR (A, B). Individual precursor markers are separated in the lower panels (A′: nestin (green) and nuclei (blue); A″: MOR (red) and nuclei (blue); B′: Sox2 (green); B″: MOR (red) and nuclei (blue)). A similar percentage of hNPC express CCR5 (C) and CXCR4 (D). As above, lower panels show individual markers (C′: Sox2 (green); C″: CCR5 (red) and nuclei (blue); D′: Sox2 (green); D″: CXCR4 (red) and nuclei (blue)). The presence of CCR5 and CXCR4 on

hNPCs suggests that effectors such as gp120, β-chemokines (RANTES/CCL5, MIP-1α/CCL3, MIP-1β/CCL4), and the α-chemokine (SDF-1/CXCL12), released in response to Tat or contained in viral supernatant, might affect hNPC behavior.

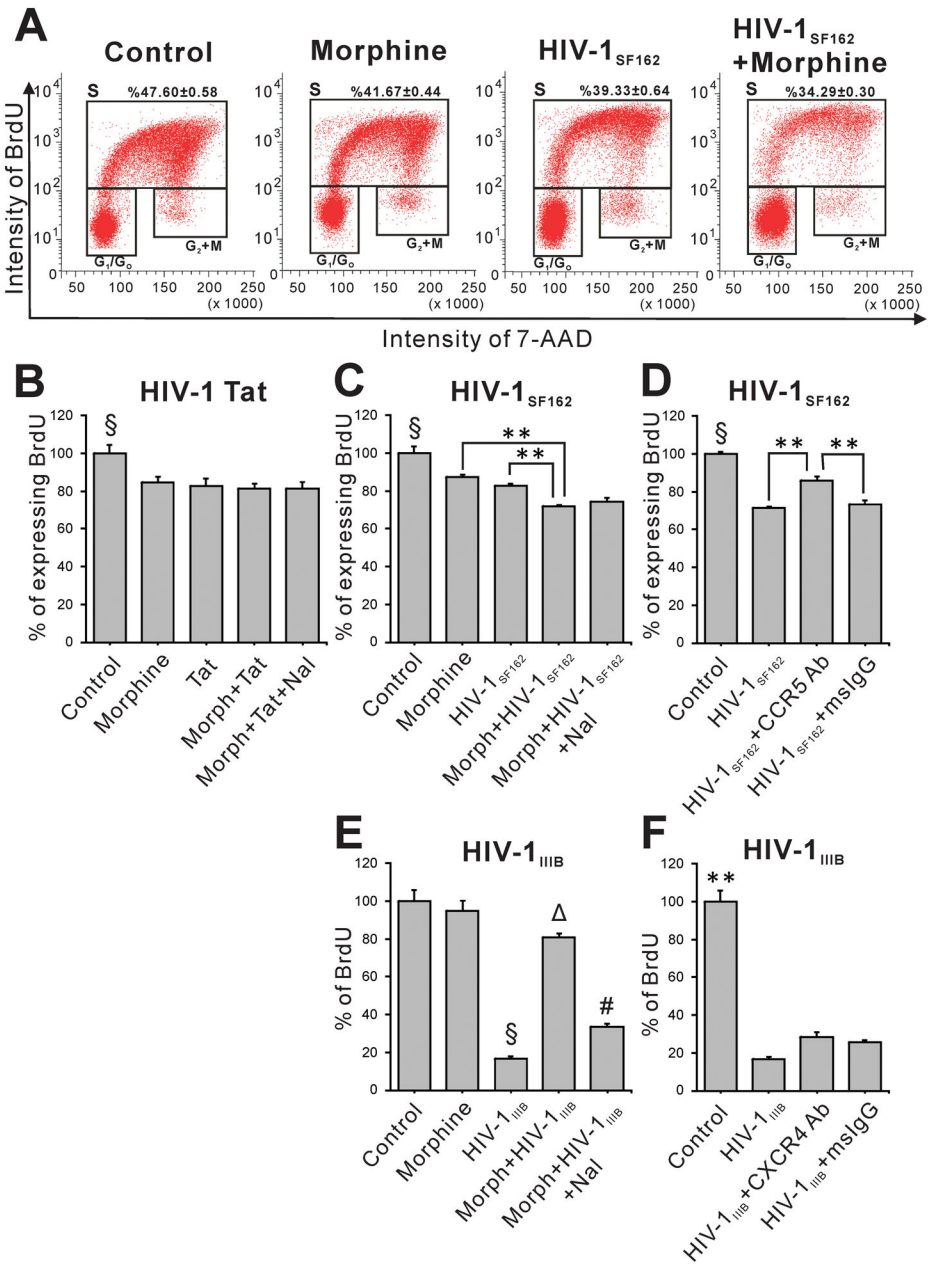

Figure 6.

Effects of Tat, HIV-1 supernatant, and morphine on proliferation of human NPCs. FACS analyses were performed after 12 h treatment with the indicated Tat, virus, or morphine combinations. BrdU was added during the final 2 h to label S-phase cells. Raw FACS data for an HIV-1_{SF162} experiment is shown (A). Histograms in B–F reveal that exposure to Tat (B), and supernatant from either HIV-1_{SF162}- or HIV-1_{IIIB}-infected monocytes (C, E) all reduce ReNcell VM progenitor proliferation. Morphine alone variably reduced proliferation (compare C and E). While there was no interactive effect of Tat and morphine (B), BrdU incorporation was additively reduced in cells treated with combined HIV-1_{SF162} supernatant and morphine (C). A CCR5 blocking antibody (2D7) reduced, but did not abolish, the effect

of HIV-1_{SF162} supernatant (D), suggesting that BrdU uptake was sensitive to multiple factors in the supernatant, only some of which interact with CCR5 (B–D, § $p < 0.05$, compared to all other groups; ** $p < 0.01$, one-way ANOVA, Duncan's post-hoc test, n = 7–8). Surprisingly, morphine reversed HIV-1_{IIIB}-infective supernatant effects, returning proliferation to nearly control values (E) in a naloxone-reversible manner (# $p < 0.05$ compared to all groups). The 12G5 antibody partially blocks CXCR4 but did not block HIV-1_{IIIB}-supernatant effects on proliferation (F), suggesting that factors reducing BrdU uptake in this supernatant do not signal through CXCR4 (E–F, § $p < 0.05$, compared to all other groups; Δ $p < 0.05$, compared to all groups, except morphine; ** $p < 0.01$ compared to all groups, one-way ANOVA, Duncan's post-hoc test, n = 4). Morph, Morphine (500 nM); Tat, HIV-1 Tat_{1-86} (100 nM); Nal, naloxone (1.5 μM); HIV_{SF162}, HIV_{IIIB}, supernatant from U937 monocytes infected with these strains; CCR5 Ab, CCR5 (2D7) antibody (2 μg/ml); CXCR4 Ab, CXCR4 (12G5) antibody (2 μg/ml); msIgG = mouse IgG (2 μg/ml).

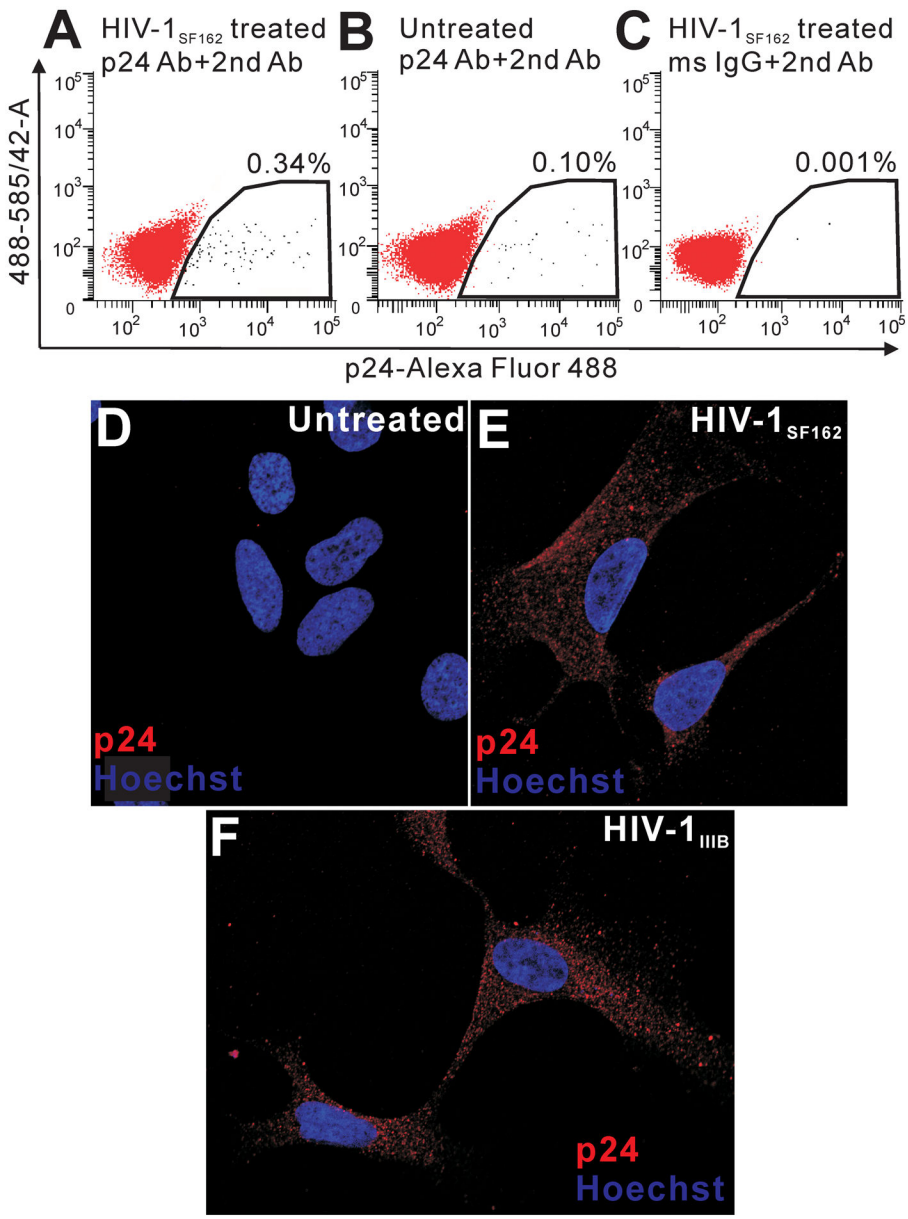

Figure 7.

Detection of p24 on hNPCs by FACS and confocal microscopy. p24 immunolabeling was assessed on 1×10^4, 7-AAD$^+$ cells using a single laser (488 nm) and two color fluorescence emission (585/42 nm band pass; 530/30 hm band pass) FACS approach (A–C). A very small percentage (0.33%) of hNPCs exposed to supernatant from HIV_{SF162}-infected monocytes were p24$^+$ (A). Labeling indices in control groups, including untreated hNPCs immunolabeled for p24 (B) and HIV-treated hNPCs exposed to control IgG and second antibody (C), were 0.1% and 0.001%, respectively, suggesting that some p24 labeling was specifically related to antigen expression. Autofluorescence (585 > 530 signal) was not detected in any sample. FACS diagrams are representative of n=3 experiments. p24 signal was not detected in individual untreated hNPCs (D). p24 immunoreactivity was found

within cytoplasm of cells exposed to both HIV-1_{SF162} (E) and HIV-1_{IIIB} supernatant (F). Images show 0.32 μM optical sections through the nuclear region. Note the absence of p24 immunolabeling in nuclei.

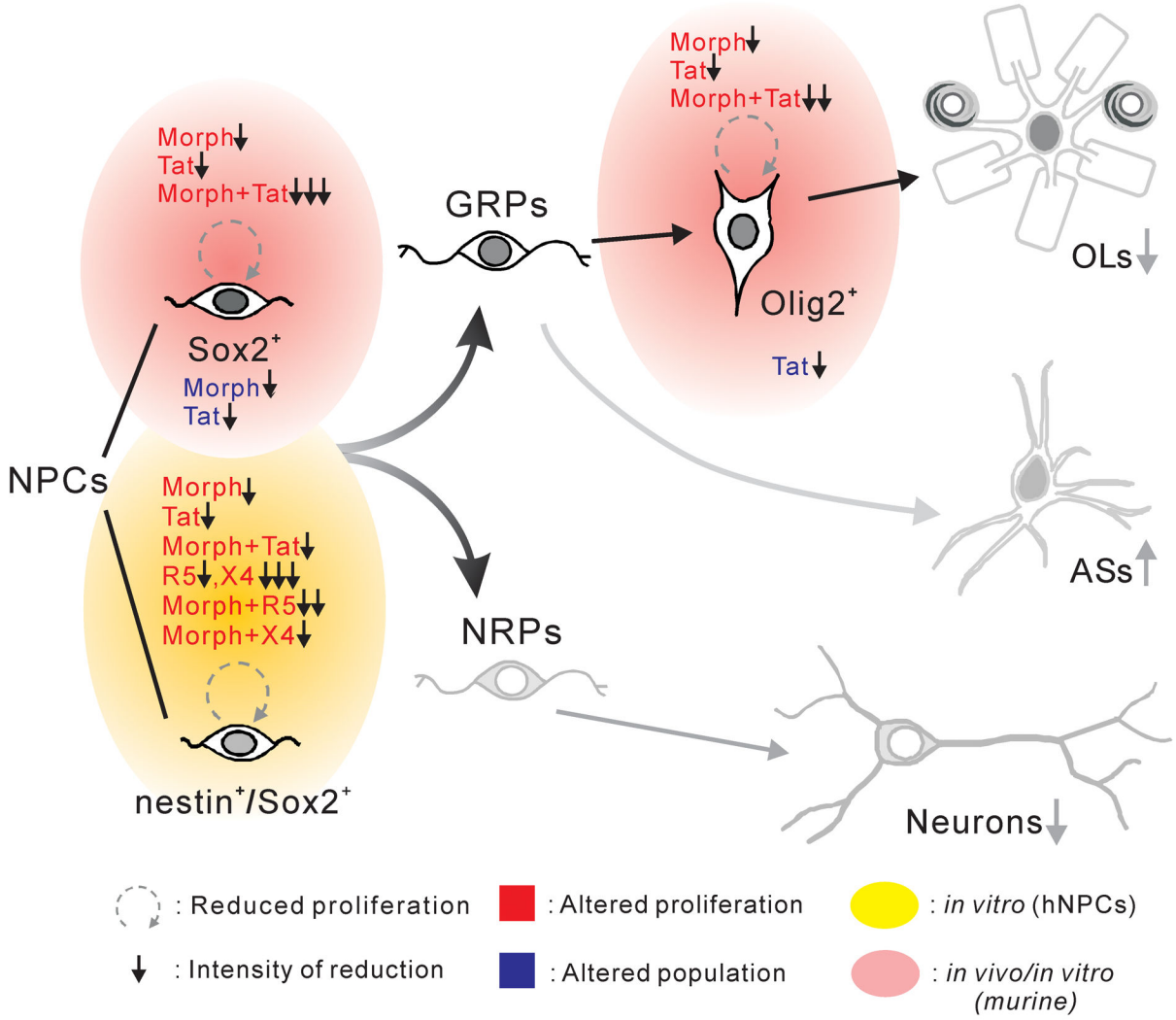

Figure 8.

Proposed effects of HIV-1 Tat or HIV-1, and interactions with morphine, on perinatal NPCs. The intermediate filament nestin and the transcription factor Sox2 identify undifferentiated and self-renewing NPCs in the developing CNS, although nestin and Sox2 expression do not entirely overlap. Sox2 is maintained in immature cells destined to become neuroglia (glial-restricted precursors; GRPs) but lost in immature cells destined to become neurons (neural-restricted precursors; NRPs) (Ellis et al. 2004). GRPs further differentiate into astroglia and oligodendroglia; the transcription factor Olig2 is expressed in precursors that largely become oligodendroglia. HIV-1 Tat, supernatant from HIV-1 infected cells, and morphine, all affect proliferation of progenitor subtypes (red lettering). Morphine or Tat reduce the proliferation of $Sox2^+$ progenitors and immature $Olig2^+$ oligodendrocytes, and have an additive effect that further reduces proliferation. Reduced NPC and GRP proliferation is associated with changes in cell populations (blue lettering). $Sox2^+$ cells are reduced by morphine or Tat exposure; $Olig2^+$ cells are reduced by Tat exposure. Proliferation of human $Sox2^+$/$nestin^+$ NPCs is reduced to variable extents by Tat and by supernatant from cells infected with R5 or X4-preferring strains of HIV. Morphine alone can also marginally reduce proliferation. Co-

exposure to morphine and HIV supernatant results in strain-specific interactions: morphine enhances the effect of HIV-1_{SF162} supernatant, but partially counteracts the effect of HIV-1_{IIIB} supernatant. Our findings support a model where the proliferation of both NPCs and GRPs is significantly reduced by HIV, with morphine interactions that can further compromise progenitor production, depending on viral strain. Tat protein clearly reduces proliferation by itself; effects of HIV supernatant reflect combined actions of multiple viral proteins such as gp120 (Lee et al. 2011) and secreted factors. Reduced proliferation predicts overall reduction in progenitor populations, which was observed. Continued effects on proliferation will likely disturb the balance of glia and neurons in the brain, a prediction that remains to be tested. (Arrows and cartoons in grey represent hypothesized outcomes for which we have no direct experimental evidence.)