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Human immunodeficiency virus type 1 Tat modulates proliferation and differentiation of human neural precursor cells: implication in NeuroAIDS

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Human immunodeficiency virus type 1 (HIV-1) and viral proteins affect neuronal survival and neuron-glial cell interactions, which culminate in neurological disorders. HIV-1 infects regions of neurogenesis in human adult and pediatric brain. However, little is known about the effect of HIV-1 or viral proteins on the properties of human neural precursor cells (hNPCs), particularly neurogenesis, hence a detailed investigation on these lines is warranted. Human neural precursor cells were cultured in presence and absence of HIV-1B transactivating protein Tat to investigate if HIV-1 viral protein alters the properties of human neural precursor cells. Cellular and molecular approaches were adopted to study the effect of HIV-1B transactivating protein Tat on proliferation and differentiation potential of human fetal brain–derived NPCs. Cell proliferation assays such as BrdU and Ki67 staining and pathway-specific cDNA and protein arrays were used in the study. Data reveal that HIV-1B Tat protein severely affects proliferation of hNPCs, as evident by lower incorporation of BrdU and Ki67 staining as well as neurosphere assay. HIV-1 Tat substantially attenuated neurogenesis, as evident by the smaller numbers of Tuj-1– and doublecortin-positive cells differentiated from hNPCs, without affecting their viability. These data suggest that HIV-1 Tat alters the properties of human neural precursor cells via attenuation of the cell cycle regulatory unit cyclin D1 and the mitogen-activated protein kinase (MAPK) pathway, particularly extracellular signal-related kinase 1/2 (ERK1/2). The study provides new insights into cellular and molecular mechanisms that may modulate human neural precursor cell properties in HIV/AIDS (acquired immunodeficiency syndrome) individuals. Validation with autopsy brain samples is necessary to further substantiate these important observations. *Journal of NeuroVirology* (2010) **16,** 355–367.

Keywords: HIV-1–associated dementia (HAD); HIV-1–associated neurocognitive disorder (HAND); MAPK pathway; neural precursor cell; neurodegeneration; neurogenesis; Tat

Introduction

Central nervous system (CNS) infection with human immunodeficiency virus type 1 (HIV-1) and subsequent development of acquired immunodeficiency syndrome (AIDS) are often accompanied with debilitating neurological problems, collectively referred to as HIV-1–associated neurocognitive

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disorders (HANDs). The neuropathogenesis of AIDS involves irreversible damage to neurons through apoptotic pathways, mediated via "virotoxins" released from the infected cells (Gonzalez-Scarano and Martin-Garcia, 2005; Nath, 2002). Until recently, HIV-1 was believed to be affecting only astrocytes and microglial cells in brain, as neurons are rarely infected (Gonzalez-Scarano and Martin-Garcia, 2005). Interestingly, HIV-1 can also infect neural precursor cells in culture (Lawrence *et al,* 2004; Rothenaigner *et al,* 2007). It was recently reported that HIV-1 is present in nestin-positive cells in areas of neurogenesis in archival autopsy brain sections from pediatric AIDS patients, suggesting human neural stem/precursor cells harbor HIV-1 (Schwartz *et al,* 2007). Presence of HIV-1 in neural precursor cells raises concern about the consequence of harboring of HIV-1 in hNPCs. Further, the number of adult neural precursor cells is far smaller in HIV patients with neurological deficits as compared to their age matched noninfected controls, or from HIV-1–infected but nondemented individuals (Krathwohl and Kaiser, 2004b). Detailed investigation into the effect of HIV-1 and its proteins on the properties of neural stem cells/precursor cells, particularly neurogenesis, is lacking.

HIV-1 surface protein gp120 and transactivating protein Tat are directly implicated in HIV dementia patients because these proteins cause neuronal apoptosis (Kaul *et al,* 2001; Kaul and Lipton, 2006). However, it is not clearly understood if these two viral proteins affect neural precursor cells in a similar manner. According to a recent study, the number of proliferating neural precursor cells is low in HIV/gp120 transgenic mice compared to wild-types, and gp120 protein seems to affect proliferation of neural precursor cells without affecting their viability (Okamoto *et al,* 2007). Few other studies show that HIV and its proteins may affect the neural stem cells (Krathwohl and Kaiser, 2004b; van Marle *et al,* 2005); however, the details are poorly defined.

Of the two major neurotoxic viral proteins, gp120 and Tat, the effect of viral coat protein gp120 on neural precursor cells has been studied recently. However, the modulation by soluble HIV-1 transactivating protein Tat, which actually affects multiple genes in the infected as well as neighboring cells and has a profound effect on functions of neuron and glial cells, has not been studied so far in human neural precursor cells. It is hence important to understand the effect of HIV-1 transactivating protein Tat on proliferation and differentiation properties of human neural stem/precursor cells in detail. In this paper, we report for the first time that HIV-1 Tat attenuates the growth, proliferation, and differentiation capabilities of human fetal brain–derived neural stem/precursor cells. Furthermore, our data provide evidence that Tat attenuates proliferation of human neural precursor cells (hNPCs) via down-regulation of cyclin D1, the regulatory unit of cyclin-dependent kinases (CDKs), and formation of new neurons or neurogenesis by modulating several neural stem cell and neurogenesis-related genes and extracellular signal-regulated kinases 1 and 2 (ERK 1/2) of the mitogen-activated protein kinase (MAPK) pathways.

Results

HIV-1 Tat decreases proliferation of human fetal brain–derived neural precursor cells without affecting their viability

HIV-1 Tat exposure causes significant neuronal cell death via apoptosis. To check whether neurotoxic concentrations of HIV-1 Tat cause similar effects on hNPCs, we treated the adherent (monolayer) and nonadherent (neurospheres) with 50 and 100 ng/ml concentrations of Tat for 24, 48, and 72 h and studied its effect on cell viability by TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) assay as described earlier (Mishra *et al,* 2008). At 24 h, where 50 and 100 ng/ml of Tat cause 25% and 60% cell death in human neurons, similar concentrations did not cause major cell death in hNPCs. At longer time points, i.e., 48 and 72 h, although most of the neurons were dead (65.17% ± 3.45%), there was comparatively lower cell death in neural precursors (9.98% ± 0.97%) as assessed by TUNEL assay, suggesting hNPCs remain unaffected by HIV-1 Tat even after longer exposure. Similar results were observed by a cell viability assay, the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (data not presented). We extended these studies to day 5 post treatment and observed similar results.

Human neural precursor cells exhibit self-renewing and proliferative property and form neurospheres when grown as nonadherent cultures. In order to investigate the effect of HIV-1 Tat on proliferation of hNPCs, we exploited the high proliferative ability of these cells. We assessed alterations in size of growing neurospheres with time and compared them with neurospheres cultured in presence of various concentrations of HIV-1 Tat. Neurospheres cultured in presence of HIV-1 Tat were significantly smaller in size as compared to untreated neurospheres. The differences in size of neurospheres appeared as early as 36 h following exposure of Tat and were highest at 72 h (data not presented). A quantitative assessment of neurospheres categorized into small and large neurospheres on basis of their size revealed that smaller neurospheres were mostly abundant in cultures where Tat was present, as compared to the control group. The same is represented as percentage of small and large neurospheres in each treatment group (Figure 1A). In addition to this, we also counted the number of cells from control neurospheres and Tat-treated

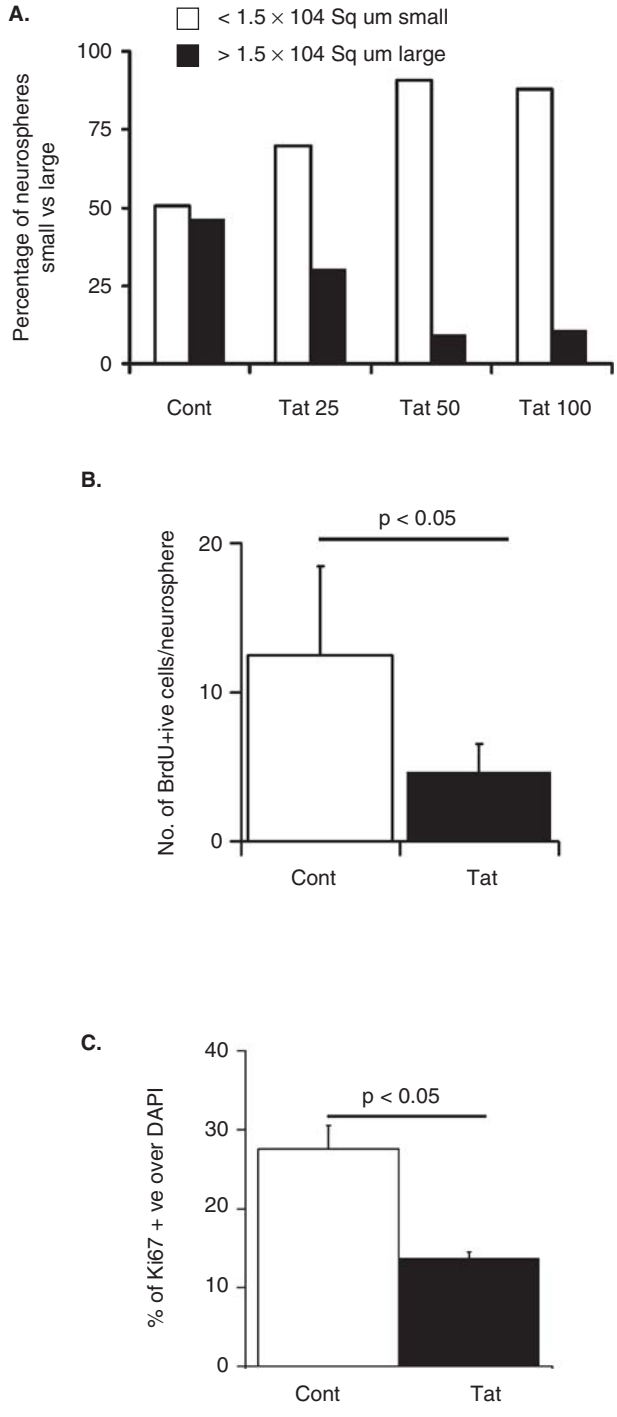

Figure 1 HIV-1 Tat attenuates proliferation of human neural precursor cells (hNPCs). (**A**) Represent quantitative assessment of human neurospheres cultured in absence (control) and presence (25, 50, and 100 ng/ml) of HIV-1 Tat. Growth/proliferation of the neurospheres were assayed by measuring the area of neurospheres using Image-J software and neurospheres were categorized into small and large categories as described under experimental protocols. Percentages of both small- and large-category neurospheres were calculated and were represented in the graph. The percentage of smaller spheres was higher in the Tat-treated groups (50 and 100 ng/ml) as compared to the same of larger neurospheres. The control group had more or less equal proportion of both small and large neurospheres. HIV-1 Tat decreases proliferation of human neurospheres as assessed (**B**) by BrdU incorporation and (**C**) by Ki67 staining in human neurospheres. The number of BrdU-positive cells or Ki67-immunopositive cells and the total number of DAPI-stained nuclei were counted in images taken from at least five random fields in each group. The percentages of Ki67-positive cells over DAPI were calculated and plotted. HIV-1 Tat treatment resulted in a statistically significant decrease in the number of proliferating cells. Data presented here are from three different fetal brain samples.

neurospheres, after making single-cell preparations of the neurospheres and observed that exposure of Tat (100 ng/ml) resulted in fewer cells ($2.54 \pm 0.18 \times 10^5$ cells) as compared to the control or untreated group ($3.45 \pm 0.19 \times 10^5$ cells), which was statistically significant ($P < .002$).

We assessed BrdU incorporation and Ki67 staining, two well accepted markers for cell proliferation, in hNPCs cultured with and without HIV-1 Tat (Kee *et al,* 2002). The HIV-1 Tat group had a remarkable decrease in the number of BrdU-stained cells compared to neurospheres cultured for 72 h in the control/untreated group (Figure 1B). Similarly there was a clear reduction in the number of Ki67-positive cells in the Tat-treated group (Figure 1C). The decrease in BrdU- as well as in Ki67-positive cells in Tat-exposed neurospheres confirmed decrease in cell proliferation ability of human neurospheres (Figure 1B and C). Later, we also carried out few experiments where mutant Tat was also used in addition to Tat; interestingly, there was no significant decrease in the number of Ki67-positive cells/DAPI (4′,6-diamidino-2-phenylindole) in the mutant Tat groups (31.48% ± 7.72%) of human neurospheres in comparison to the control or untreated group (30.83% ± 8.56%), although in the Tat group the number of Ki67-positive cells were significantly low (12.84% ± 4.17%; $P < .05$, $n = 3$), indicating specificity of the effect of HIV-1 Tat protein on proliferation of hNPCs.

HIV-1 Tat modulates proliferation by attenuating cyclin D1 in human neural precursor cells

In search of the mechanism for attenuation of proliferation and growth of neurospheres, we looked into involvement of cyclin D1, one of the important components that drive cell cycle via interaction with cyclin-dependent kinases 4 and 6 (CDK4 and CDK6) (Swanton, 2004). Cyclin D1 is hence an essential factor that needs to be investigated in early events of cell proliferation, which was the objective of this study. We focused on cyclin D1 because recently its role has been demonstrated in proliferation and differentiation of neural stem cells (Ma *et al,* 2010). Our assessment of cyclin D1 levels by immunocytochemistry (Figure 2A) and Western blotting (Figure 2B) revealed that HIV-1 Tat (100 ng/ml), but not mutant Tat (100 ng/ml), down-regulated cyclin D1 in hNPCs (Figure 2C; $P < .003$), suggesting that HIV-1 Tat affects the cell cycle component cyclin D1.

HIV-1 Tat alters the differentiation of human neural precursor cells

We investigated effect of HIV-1 Tat on neurogenesis or their ability to differentiate into neurons. hNPCs undergoing neurogenesis in presence of 25, 50, or 100 ng/ml of HIV-1 Tat for 1, 3, or 5 days falter in their ability to differentiate into new neurons, as seen by substantial decrease in the expression of the early neuronal marker Tuj-1 (Figure 3A). In addition to this, an investigation into the temporal kinetics of cell-specific marker nestin (labeled red) and Tuj-1 (labeled green) was performed for hNPCs and new neurons, respectively. HIV-1 Tat reduced differentiation of Tuj-1–positive cells in hNPC cultures undergoing neurogenesis, as compared to that in the control or untreated group, in a dose- and time-dependent manner, with maximum effect observed on day 5 and at 100 ng/ml concentration (Figure 3B). We intended to extend the study to longer time points, but as differentiation of hNPCs were extraordinarily low in Tat-containing cultures, it was difficult. At later time points, unlike as seen in undifferentiating hNPCs, Tat resulted in modest increase in cell death in hNPCs undergoing neurogenesis, as assessed with TUNEL assay.

In addition to the immature neuronal marker Tuj-1, we also looked at expression levels of doublecortin (DCX), a well-accepted marker for early events of neurogenesis (Couillard-Despres *et al,* 2005), in differentiating human neurons, under similar treatment and differentiation conditions at days 1, 3, and 5. We observed that similar to Tuj-1 expression, DCX expression increased with time in the control or untreated group consistently till day 5 (maximum duration studied by us), whereas the presence of Tat protein at 100 ng/ml in the medium resulted in a significant decrease in DCX expression in comparison to respective controls, as seen by immunocytochemistry at the three time points studied by us. Noticeably, there were not many differences among the data from three different fetuses as indicated by shorter error bars in the assay (Figure 3C). More precise data of different fetal samples have been provided in a separate table (Table S1), which shows that there was not much difference between the three fetal samples within respective treatment groups.

Further, the generation of new neurons from neurospheres was aberrant and uneven in hNPC cultures undergoing neurogenesis in presence of 50 and 100 ng/ml HIV-1 Tat (Figure 3A).

HIV-1 Tat down-regulates genes related to neural stem cells and neurogenesis

To identify genes affected by HIV-1 Tat in hNPCs, we carried out detailed review of the expression profile of genes crucial for human neural stem cells and neurogenesis, using a pathway-specific cDNA array containing 263 known genes. Total RNA isolated from hNPCs undergoing differentiation/neurogenesis in presence and absence of HIV-1 Tat for 5 days were used. At day 5 post differentiation, Tat resulted in down-regulation of genes crucial for regulation of cell differentiation (ASCL1, JAG1), cell signaling genes involved in neurogenesis (JAG1), cell

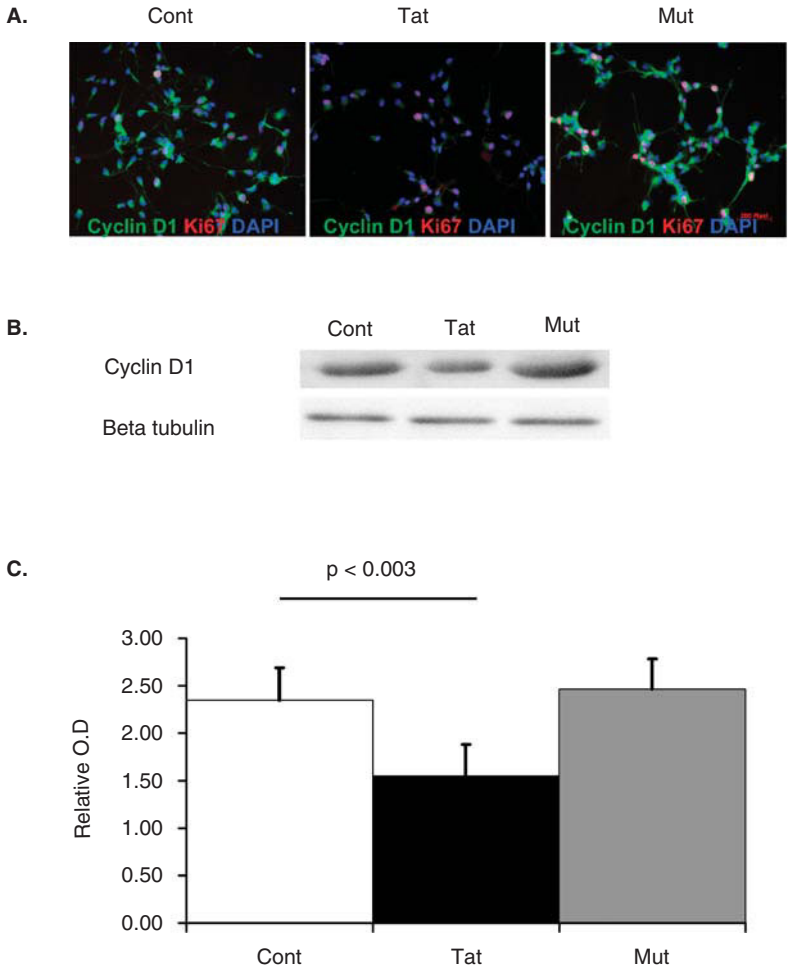

Figure 2 Cyclin D1 expression is down-regulated by HIV-1 Tat exposure. Human neural precursor cells were exposed to HIV-1 Tat B as well as mutant Tat protein (both 100 ng/ml) for 24 h. Cells from control, Tat-treated, and mutant Tat–treated groups were fixed and immunostained for cyclin D1 along with the proliferation marker Ki67 (**A**). From parallel cultures, cell lysates were prepared and immunoblotted with cyclin D1 antibody (**B**). The blots were reprobed with β-tubulin. (**C**) Densitometric analysis of immunoblots representing the relative intensity of the cyclin D1–immunoreactive bands, normalized with β-tubulin levels that served as loading control. HIV-1 Tat, but not mutant Tat, induced statistically significant decrease of cyclin D1 level ($P < .003$, $n = 6$; in case of mutant Tat, $n = 3$).

proliferation (JAG1, S100 beta, PTN, SPOCK1), regulation for cell motility and migration (KAL1 and MTSS1), cell adhesion (KAL1, MTSS1, SPOCK1, PCDHB-2, -5, -14, -15, and -16, and ROBO2), synaptic function (S100 beta and PCDHB-2, -5, -14, and -16), regulation of transcription (ASCL1, CHD6, and PBX1), cell cycle regulation (MTSS, PTN, KAL-1, and SPOCK1), and growth factors and cytokines (CSPG5, FGF13, JAG1, NRG1, and PTN) (Figure 4). Most of these were validated by reverse transcriptase–polymerase chain reaction (RT-PCR) (Figure S1).

HIV-1 Tat protein affects proliferation and neurogenesis via ERK-1/2 of the MAPK pathway

Extracellular signal-related kinases (ERK1/2) of the mitogen-activated protein kinase (MAPK) pathway have been broadly linked to processes of growth, proliferation, and differentiation, hence we investigated whether HIV-1 Tat effects are mediated via the MAPK pathway. Effect of HIV-1 Tat was studied on a battery of MAPK components, including ERK1/2, JNK, Akt, GSK, and p38. The expression of phospho-ERK1/2 was significantly elevated in hNPCs undergoing neurogenesis ($P < .05$), in comparison to undifferentiating hNPCs. However in presence of HIV-1 Tat, the expression of phospho-ERK1/2 was inhibited considerably in hNPCs undergoing neurogenesis (Figure 5A, B). In addition to phospho-ERK1/2, we noticed marginal but not statistically significant alterations in p38 levels. The modulation by HIV-1 Tat of other MAPK pathways molecules was minimal and hence not studied further. We validated our protein array findings of phospho-ERK1/2 by

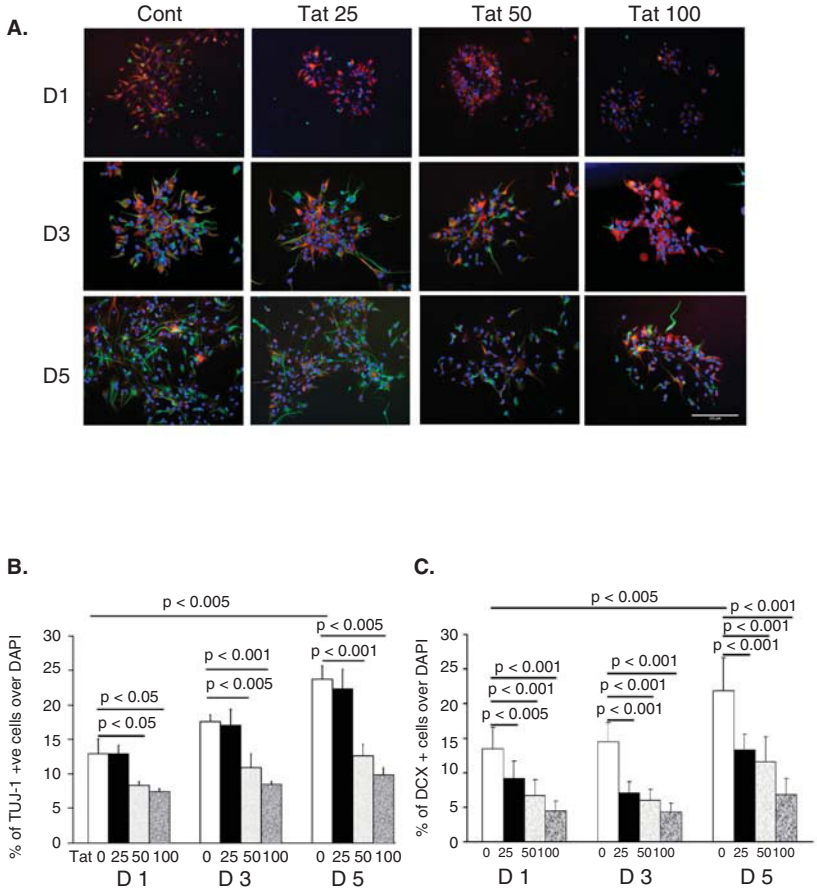

Figure 3 HIV-1 Tat impairs neurogenesis of human neural precursor cells (hNPCs). (**A** and **B**) Differentiation of hNPCs was initiated into neuronal lineage in presence or absence of HIV-1 Tat (25, 50, and 100 ng/ml) for varied time points (days 1, 3, and 5). Cells were fixed at respective time points and immunostained for neuronal marker Tuj-1. Expression pattern of Tuj-1 was assessed by immunofluorescence and images were captured from five random fields. Tuj-1–positive cells (*green*) were counted along with DAPI (*blue*) and nestin (*red*). Tuj-1 expression (*green*) increased considerably in human neurospheres undergoing neuronal differentiation in the control groups from day 1 (D1) to day 5 (D5) and nestin the precursor cell marker decreased as the neurons were formed. Level of Tuj-1 expression (*green*) in hNPCs undergoing neurogenesis was decreased at higher doses of HIV-1 Tat at most time points, particularly at day 3 and day 5 ($n = 5$). (**C**) Immunocytochemistry was performed on hNPCs undergoing neurogenesis with and without Tat (25, 50, and 100 ng/ml) at days 1, 3, and 5 to study the expression of a neurogenesis marker, doublecortin (DCX). Similar to Tuj-1, DCX levels increased with from D1 to D5 in the control groups, suggesting that the hNPCs are undergoing neurogenesis. Compared to the control groups, DCX levels were significantly lower in the Tat-treated groups, the maximum effect of Tat was at 100 ng/ml concentration ($n = 9$). Scale bar represents 200 μm. ANOVA was used to assess statistical significance of the effects of Tat on hNPCs undergoing neurogenesis.

Western blotting and observed similar results (Figure 5C, D).

Discussion

Until recently it was not known that human neural stem/precursor cells were infected by HIV-1. Recent evidence for presence of HIV-1 in nestin-positive neural precursor cells in adult and pediatric brain added a new dimension to the current understanding of neuropathology of HIV-1. In fact they are now looked upon as cells that may harbor HIV-1 as latent infections or reservoirs. It has raised an important question of whether presence of HIV-1 and its proteins affect the properties of human neural stem/precursor cells, and if yes, to what extent. Initial studies suggest that HIV-1 may affect the very existence of neural precursor cells or may force them into quiescence; however, such studies either involved nonhuman models or were rather exploratory, lacking detailed mechanistic explanations for this important observation (Kitayama *et al,* 2008; Krathwohl and Kaiser, 2004a, 2004b; McCarthy *et al,* 2006; van Marle *et al,* 2005). A recent study using gp120 transgenic mice suggests that HIV-1 gp120 can affect proliferation of neural stem cells through delay in G1 phase of the cell cycle via activation of the p38 MAPK pathway (Okamoto *et al,* 2007).

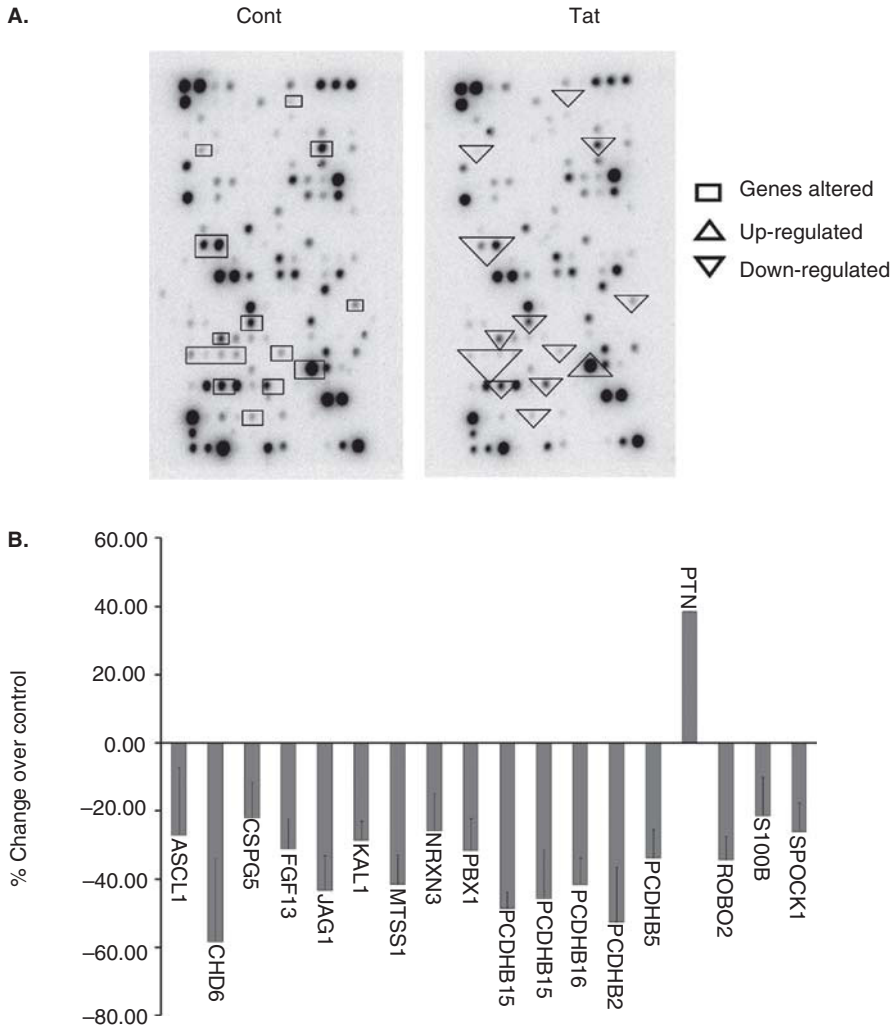

Figure 4 HIV-1 Tat modulates the expression profile of genes for human neurogenesis and neural stem cells in pathway-specific cDNA array. (**A**) Tat-treated hNPCs show deceased expression levels of most of the neurogenesis and neural stem cell genes (indicated with downward arrowhead). Total RNA was isolated from hNPCs undergoing neurogenesis at day 5 in presence or absence of HIV-1 Tat. The cDNA array spotted with 263 known genes was hybridized with cRNA samples prepared from control and Tat-treated samples. Intensity of the spot corresponds to the expression pattern of particular gene. (**B**) Intensity of the expressed genes was analyzed along with positive control normalized with house keeping genes. Spots were identified using GE Array Expression Analysis Suite 2.0 software. The values plotted as mean ± SD of percent change from the respective control group. Genes with alterations of 20% or more over respective control were considered, validated, and analyzed further. Alterations in gene expression from the control groups were shown as either upward or downward arrows.

HIV-1 gp120 and transactivating protein Tat have been detected in CNS of HIV/AIDS patients (Hudson *et al,* 2000) and cause apoptosis in human neurons. HIV-1 gp120 has been the focus of investigations in studies on neurogenesis so far; however, given the fact that HIV-1 Tat affects numerous functions and genes in host cell and virus during the course of neuropathogenesis (Li *et al,* 2009), detailed investigations into effect of HIV-1 Tat on neurogenesis cannot be ignored. HIV enters the brain early in the course of the illness, and antiretroviral drugs are not initiated until CD4 cells counts fall below 500 cells/mm^3, which is usually several years after the initial infection. This presumably allows for substantial infection to occur in the brain. Hence when the cocktail of antiretroviral drugs are started at this stage, even though it may prevent new cell from getting infected, the cells that were already infected by the virus would still be capable of producing Tat protein, since these drugs would have no impact on Tat production by already infected brain cells. Also, because protease inhibitors are important components of the antiretroviral therapy, they may have little effect on Tat production, since Tat is the first protein to be formed once the viral DNA is integrated. Furthermore, because the protease inhibitors act only on the later stages of viral protein production, there is a possibility that the

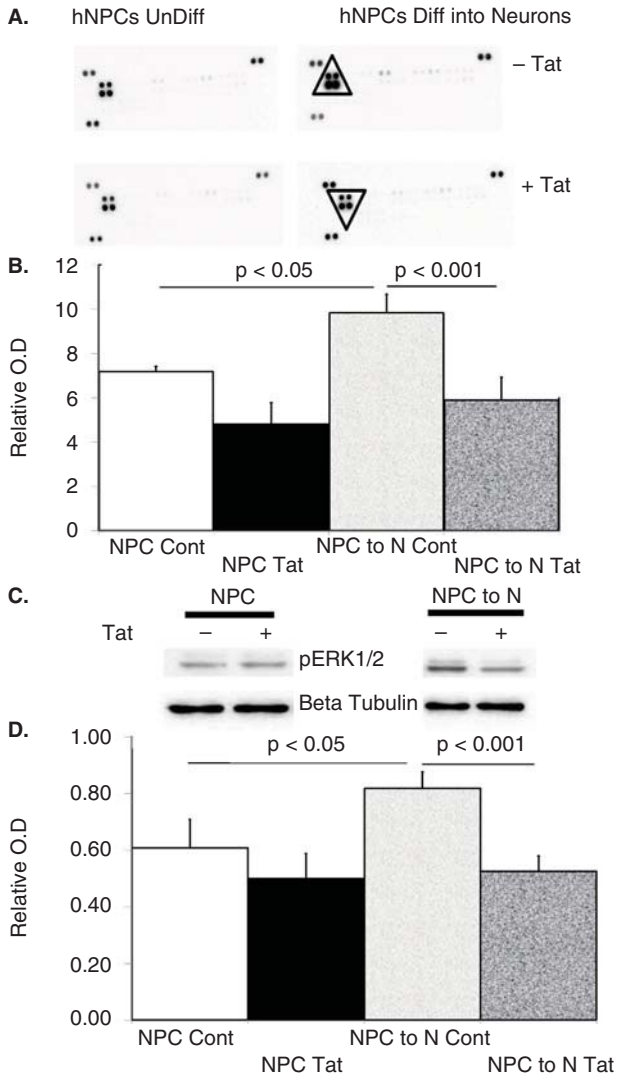

Figure 5 HIV-1 Tat decreases levels of pERK1/2 in hNPCs undergoing neurogenesis. (**A**) Represents MAPK array and (**C**) Western blot (WB) analysis of cell lysates prepared from hNPCs undergoing neurogenesis for 24 h in presence/absence of HIV-1 Tat. For MAPK array 300 μg of samples were used and 30 μg of protein for Western blot analysis. Spot (MAPK array) and band intensity (WB) was measured for pERK1/2 and β-tubulin and normalized. Quantitative assessment of relative optical density represented as mean ± SD. values and plotted for pERK1/2 for MAPK array (**B**). Same samples were validated by classical Western blotting assay (**D**) and exhibit attenuation in the Tat groups. Both assays indicate there was a statistically significant decrease of pERK1/2 levels in the HIV-1 Tat group as compared to control ($P < .001$). pERK1/2 levels increased following differentiation into neurons in the control group ($P < .05$).

transcription of Tat protein would remain unaffected. It was hence mandatory to investigate effect of HIV-1 transactivating protein Tat on the properties of human neural stem/precursor cells, particularly neurogenesis.

We chose 25, 50, and 100 ng/ml doses of HIV-1 Tat for most of our experiments related to proliferation and differentiation, and highest dose (100 ng/ml) for subsequent studies to delineate the mechanisms of experimental observations. Although it has been very difficult to accurately assess the concentration of HIV-1 Tat in brain of HIV-1/AIDS patients, the doses selected for our studies were in accordance with research literature on the subject (Conant *et al,* 1998; Eugenin *et al,* 2010; Mishra *et al,* 2008; Pu *et al,* 2003). Use of recombinant protein has some obvious drawbacks. Even though our experimental approach is consistent with the published literature, it is well known that the Tat protein becomes easily oxidized and polymerized in experimental conditions. Hence only a small portion of the recombinant Tat protein is biologically active. In a paper by Li et al (Li *et al,* 2008), it was estimated that the Tat protein produced by mammalian cells was at least 5000-fold more potent in causing neurotoxicity compared to the recombinant protein. Hence it is likely that similar concentrations would be relevant to its effects on neural progenitor cells.

Due to specificity of HIV-1 for human cells, use of primary cultures of human brain cells remains a feasible choice to investigate HIV/AIDS neuropathogenesis (Seth and Major, 2005). We employed neurosphere assay to assess if HIV-1 Tat interferes with proliferative property of hNPCs using BrdU incorporation and Ki67 staining. HIV-1 Tat substantially reduced the rate of proliferation of hNPCs in both assays. In addition to these experiments, we used a mutant form of HIV-1 Tat that was devoid of its neurotoxic region (amino acids 31 to 61) to confirm that the effect of Tat on hNPC properties was specific to HIV-1 Tat, and not due to a component that may have introduced during Tat induction or purification.

The other important function attributed to hNPCs is their exit from cell cycle to undergo differentiation into postmitotic neural cells by process of neurogenesis. Neurogenesis is affected in several neurodegenerative diseases, including HIV-1, that involve inflammation (Whitney *et al,* 2009). Neurogenesis is often assessed by the ability of hNPCs to differentiate into cells positive for the early neuronal cell-specific marker Tuj-1, and decrease in the level of neural precursor cell marker nestin. Immunostaining reveals a statistically significant decrease in the number of neurons differentiated from hNPCs. We followed the expression of Tuj-1, an early neuronal marker, instead of microtubule-associated protein-2, because the latter is a mature neuronal marker that would be expressed only after 2 weeks of neurogenesis, as seen previously in our hands. In addition to Tuj-1, we studied the expression of doublecortin, another marker for early events of neurogenesis (Couillard-Despres *et al,* 2005), especially because the main focus of the study was to investigate the effect of HIV-1 Tat on early events of neurogenesis in human neural precursor cells and observed that HIV-1 Tat attenuates doublecortin expression in hNPCs undergoing neurogenesis.

The major steps involved in process of regulation of neural precursor cells and neurogenesis are proliferation, migration, differentiation, and axonal guidance. We used a pathway-specific cDNA microarray comprising 263 genes specifically related to these functions rather than a global gene expression profile. Microarray experiments revealed that several genes were significantly down-regulated by HIV-1 Tat in differentiating hNPCs. This further strengthens the fact that HIV-1 Tat has far profound effect on neural stem cell properties of hNPCs than originally envisaged. The role of HIV-1 Tat in modulating neural stem cells proliferation and neurogenesis genes have not been studied in detail, hence we explored the intricate events of a rather complex cell cycle pathway that regulates proliferation very closely. Cyclin D1 is an important component of the cell cycle that links with cyclin-dependent kinases 4 and 6 during cell cycle progression, forming a complex that inactivates pRb (phospho-retinoblastoma) through phosphorylation, resulting in activating a series of events that allow entry into S phase and cell division (Swanton, 2004). Furthermore, cyclin D1 has been shown to be important for regulation of proliferation of neural stem cells in a knockout study (Ma *et al,* 2010). We investigated if cyclin D1 expression levels were altered in hNPCs due to HIV-1 Tat with help of immunostaining and western blotting techniques. We found Tat significantly down-regulates cyclin D1 levels. These effects of Tat treatment on cyclin D1 expression and Ki67 staining were seen only with Tat and not mutant Tat, clearly suggesting specificity of HIV-1 Tat protein. Furthermore, we used a protein array comprising a wide range of MAPK pathway components, including ERK1/2, to understand molecular events involved in modulation of hNPC properties. It was rather intriguing to find that HIV-1 Tat could negatively modulate proliferation and differentiation of hNPCs via the ERK1/2 involvement of the MAPK pathway. Detailed experiments with MAPK inhibitors are in progress in another study in our laboratory, though preliminary observations suggest that MAPK inhibitors block the effects of Tat on hNPCs. Interestingly, the effect of HIV-1 Tat on the properties of hNPCs occurs without any effect on viability of these cells, this may be explained based on findings of a recent study that suggests the importance of the Tat-LRP-PSD-95-NMDAR macromolecular complex formation within neurons. The formation of this macromolecular complex may be lacking in undifferentiated hNPCs as well as immature human neurons (Eugenin *et al,* 2007, 2010). This possibility is being further investigated in hNPCs in our laboratory. Recently, a study involving an another neurotropic virus reported that the Japanese encephalitis virus falters the proliferation of rodent neural progenitor cells without affecting their viability (Das and Basu, 2008).

We believe that our data on Ki67 staining, BrdU incorporation, and neurosphere assay clearly support our hypothesis that HIV-1 Tat significantly decreases the proliferation of human neural precursor cells. In addition to this, HIV-1 Tat also affects neurogenesis, as assessed by the decrease in the expression of the early neuronal marker Tuj-1 and also doublecortin, in neurons differentiating in the presence of Tat. Interestingly, differentiation of neural stem cells requires the proliferation to be decreased or arrested and hence it may be expected that neurogenesis would increase following the decrease in proliferation; however, that was not seen in our case. Based on the data presented in this study, Tat seems to affect both proliferation and neurogenesis, although it is possible that the effect on neurogenesis may be in part due to decrease in proliferation and warrants in-depth investigation into this aspect.

These studies provide substantial evidence for role of HIV-1 Tat from subtype B in down-regulation

of proliferation and differentiation, particularly neurogenesis, in human neural stem/precursor cells. Our findings provide a new facet to HIV-1 neuropathogenesis and unravel a previously unrecognized role of HIV-1 Tat in the properties of human neural stem cell, including neurogenesis. Our observations may have far-reaching implication in improving current understanding of HIV-1 neuropathogenesis and designing newer drugs against it.

Materials and methods

Isolation of human neural precursor cells
Human fetal brains were obtained from 10- to 14-week-old fetuses from elective medical termination of first-trimester pregnancies. Fetal brain tissue were handled after obtaining the mother's informed consent and processed using protocols approved by the institutional human ethics committee in compliance with recommendations of the Indian Council of Medical Research. Briefly, cells were cultured in neural precursor cell medium comprised of neurobasal (Invitrogen, San Diego, CA, USA) containing basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF) (Sigma-Aldrich, St. Louis, MO, USA) in the presence of neuronal survival factor-1 (Cambrex, Charles City, IA, USA) and N2 supplements (Invitrogen) as described earlier (Messam *et al,* 2003; Mishra *et al,* 2008). hNPCs were isolated, characterized with cell-specific markers such as nestin and the transcription factor SOX-2 in >99% cells, and cultured for 6 to 7 passages during which the hNPCs were positive for nestin and SOX-2 but negative for any mature glial (glial fibrillary acidic protein; GFAP) or neuronal (microtubule associated protein-2; MAP-2) markers. We used hNPCs isolated from three fetuses for the study, since the cells could be cryopreserved and revived for experiments. The hNPCs were differentiated into neuronal lineage using neuronal medium in which the mitogenic factors bFGF and EGF of the neural precursor cell medium were replaced with brain-derived neurotrophic factor (BDNF) and platelet-derived growth factor (PDGF); the rest of the contents remained the same, as described earlier (Messam *et al,* 2003; Mishra *et al,* 2008). The neurons so differentiated were >98% pure, as characterized by presence of Tuj-1 and MAP-2 immunostaining. These neurons were used for cell death studies.

Expression and purification of HIV-1 Tat
A mammalian expression vector of subtype B Tat was prepared from the HIV-1 molecular clone YU-2 (catalog no. M2393; NIH AIDS Research and Reference Reagent Program). The full-length (amino acids 1 to 101) recombinant Tat protein was expressed in the mammalian expression vector pET Tat B with His Tag, using the *Esherichia coli* strain BL 21 (DE3) and was purified using Ni-NTA and Sp-Sepharose chromatography, as described previously (Siddappa *et al,* 2006). In addition to HIV-1 Tat, an inactive form of Tat, devoid of amino acids of the neurotoxic region (amino acids 31 to 61), referred to as the mutant Tat, was also used as a control in some experiments.

Tat treatments and neurogenesis
Human neural precursor cells were plated at a density of 1×10^4 cells/well as a monolayer. Alternatively, 8 to 10 neurospheres/well were plated in chamber slides (Nunc, Kamstrupvej, Denmark). We used 25, 50, and 100 ng/ml of HIV Tat for the neurosphere assay and neurogenesis studies. For proliferation studies, Tat was added to culture medium at the time of cell plating, whereas for differentiation assays, hNPCs were plated first and Tat was added to cultures at the time of initiation of neuronal differentiation.

Neurosphere cultures
Human neural precursor cells were allowed to proliferate as nonadherent cultures to form spherical aggregates called neurospheres. A fixed number of human neural precursor cells (3×10^5) were cultured in non–poly-D-lysine (PDL)-coated 25-cm^2 flask (Tarsons, West Bengal, India) kept upright with 3 ml neural precursor cell medium for 24 h at 37°C and 5% CO_2.

Neurosphere assay
Neurospheres formed independently from three fetuses of ages between 10 and 14 weeks of gestation were seeded in PDL-coated chamber slide (Nunc) in absence or presence of HIV-1 Tat and allowed to attach for 24 h. Images from control and Tat-treated neurospheres were captured from at least five random fields using phase-contrast microscope (Leica DM IL; Leica Microsystems, Germany). Size of neurospheres from each area was measured using the Image-J (version 1.38). Neurospheres were categorized into two groups based on their size (area) as small (less than 1.5×10^4 μm^2) or large (more than 1.5×10^4 μm^2) neurospheres. Neurospheres in small or large category were counted in each group, from more than three separate experiments. At least 50 neurospheres were counted in each group, and represented as percentage of small- and large-neurosphere categories.

Cell proliferation assay and immunocytochemistry
hNPCs were cultured as monolayer cells or neurospheres either in neural precursor cell medium or in neuronal differentiation medium for various time points (day 1, 3, or 5). Cells were incubated with 10 μM bromodeoxyuridine (BrdU; Sigma-Aldrich) for 24 h, washed twice with 1× phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde for 20 min at room temperature. The

immunofluorescence of BrdU-labeled cells was elicited using anti-BrdU antibody (Santa Cruz Biotechnolgy, Santa Cruz, CA, USA) that was detected using a fluorescein isothiocyanate (FITC)-labeled secondary antibody (Jackson Laboratories, Bar Harbor, ME, USA).

To assess the effect of Tat on proliferation and neurogenesis, immunocytochemistry was performed on adhered neurosphere/monolayer cultures at days 1, 3, and 5 of neuronal differentiation. Cells fixed with 4% paraformaldehyde were blocked and permeablized for 1 hour with 4% bovine serum albumin (BSA; Sigma-Aldrich) in PBS containing 0.5% Triton X-100. The cells were incubated with anti-mouse Ki67 (Chemicon, Temecula, CA, USA), cyclin D1, or doublecortin (DCX) (Abcam, Cambrige, UK) for 3 h and anti-nestin (Chemicon) or anti-SOX-2 (Chemicon) antibodies and rabbit polyclonal anti-β-III tubulin/Tuj-1 antibody (Covance, Princeton, NJ, USA) for 1 h at room temperature. FITC- or Texas Red–conjugated secondary antibodies and DAPI for nuclear staining were used for detection of primary antibody by immunofluorescence.

TUNEL assay

To assess the effect of HIV-1 Tat on hNPCs and human neuron viability, terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling (TUNEL) assay was performed using the *In Situ* Cell Death Detection Kit, TMR red (Roche, Mannheim, Germany), as per manufacturer's protocol. Human NPCs/neurons obtained after differentiation from hNPCs undergoing apoptosis were identified with the presence of TUNEL-positive cells in images captured from at least five random fields using a Zeiss Axioplan microscope (Carl Zeiss, Heidenheim, Germany) with charge-coupled device camera. For TUNEL assay, a minimum of 100 NPCs/neurons were measured from three to five different fields per experimental group. TUNEL-positive nuclei and total DAPI-positive nuclei were counted, and apoptosis was represented as percentage of TUNEL-positive cells/DAPI.

RNA isolation, RT-PCR, and gene expression profile with oligo DNA microarray

Total RNA was isolated using Trizol (Invitrogen) after 5 days of neuronal differentiation from control and Tat-treated cells following the manufacturer's protocol. To validate the specific neurogenic genes that were modulated by HIV-1 Tat, we performed reverse transcription using the One-Step RT-PCR Kit (Qiagen, Germany) per the manufacturer's protocol. Briefly, reverse transcription was done at 50°C for 30 min, initial PCR activation step at 95°C for 15 min, three-step cycling that consists of denaturation at 94°C for 30 s, annealing at 55°C to 58°C for 30 s, and extension at 72°C for 1 min for 25 cycles, with final extension at 72°C for 10 min.

The expression pattern of genes was normalized with glutaraldehyde-3-phosphate dehydrogenase (GAPDH). The list of primers used and specific conditions are listed in Table 1.

For oligo DNA array, 4 μg of total RNA from each sample was labeled using the True Labeling-AMP Kit (SA Biosciences, Frederick, MD, USA)

Table 1 List of primers used for RT-PCR for validation of OligoArray data

Gene	Accession number	Primer sequences	AT	AS
TUJ-1	NM_006086	F: 5′ GGCCAAGTTCTGGGAAGTC 3′ R: 5′ CGTTGTAGTAGACGCTGATCC 3′	56	106
GAPDH	NM_002046	F: 5′ CCACAGTCCATGCCATCACT 3′ R: 5′ GAGCTTGACAAAGTGGTCGT 3′	55	450
PTN	NM_002825	F: 5′ GCTGAAGCAGGGAAGAAAGAG 3′ R: 5′ CTTCAGGGCTGTGTTCAGGT 3′	57	262
CSPG5	NM_006574	F: 5′ GTGGAGAACATAGGGGCCTT 3′ R: 5′ CAATGGTGGAGAGGGAGAAG 3′	55	271
JAG 1	NM_00214	F: 5′ TCGTGCTGCCTTTCAGTTTC 3′ R: 5′ TTTTGTTGCCATTCTGGTCA 3′	55	296
KAL 1	NM_000216	F: 5′ GTTTGGTGCCAGAATCACAAG 3′ R: 5′ ACCCCAGAGCACTCATTGTC 3′	55	256
CHD 6	NM_03221	F: 5′ CTTCCTTCAATAGATGAAAATGAAA 3′ R: 5′- ACCTCCTCCTCCACTGTCCT 3′	55	279
MTSS1	NM_014751	F: 5′ATCTGCTCTCACCAGGATGTGC 3′ R: 5′ GCCACTTTCTTCCATTCTTCCA 3′	60	128
PCDHB2	NM_18936	F: 5′ TGCCAGGGATTTAGACATTGG 3′ R: 5′ GTCCTGCAAGTTTTCTGGGA 3′	55	292
PBX 1	NM_002585	F: 5′ CCATCTCAGCAACCCTTACCC 3′ R: 5′CCATTGAGTGACTGCACGCTC 3′	55	302
NRXN3	NM_004796	F: 5′ GGATGTGTCCTTCCGCTTC 3′ R: 5′ TCTGGTCCTTTGCTGGAGTTAC 3′	57	171
PCDHB5	NM_015669	F: 5′ CCCCCGAATTTTTACAATCA 3′ R: 5′ GAAATCCAATGCCCTTTTCA 3′	55	209

Note. AT = annealing temperature; AS = amplicon size.

and cDNA was synthesized at 42°C for 50 min, followed by 75°C for 5 min, then cooled to 37°C. The total cRNA was synthesized using total cDNA and biotinylated UTP, 10 mM (Perkin Elmer, Waltham, MA, USA). After purification using SuperArray ArrayGrade cRNA Cleanup Kit (SA Biosciences), 4 μg of cRNA was used for hybridization to GE Array containing 263 genes related to human neurogenesis and neural stem cell (SA Biosciences) following the manufacturer's protocol and analyzed using GEArray Expression Analysis Suite 2.0 software (SA Biosciences). Only genes with more than 20% alterations in their expression levels were assessed further.

Human phospho-MAPK array

To determine relative level of phosphorylation of mitogen-activated protein kinases (MAPKs) and other serine/threonine kinases, the Human Phospho-MAPK Array Kit (R&D systems, Minneapolis MN, USA) was used following the manufacturer's protocols. Briefly, cell lysates were prepared from undifferentiated hNPCs as well as hNPCs undergoing neurogenesis in the presence or absence of HIV-1 Tat (100 ng/ml; 24 h) using the lysis buffer supplied in the kit. After blocking the nonspecific sites, 300 μg of lysate was used on each array and incubated with Human Phospho-MAPK Array for overnight at 4°C. Unbound materials were washed and a cocktail of phospho-site–specific biotinylated antibodies were used to detect phosphorylated kinases via streptavidin–horseradish peroxidase (HRP) and chemiluminiscence. Phospho-MAPK array data were developed on x-ray film as well as by the Gel-Doc system and the array image analyzed using Image-J (version 1.38) analysis software. The average signal (pixel density) of the pair of duplicate spots were determined and normalized with the positive control value.

Western blot analysis

Cell lysates were prepared using NP40 lysis buffer containing protease inhibitors cocktail (Roche, Bielefeld, Germany) from hNPCs undergoing neurogenesis for 24 h in the presence or absence of Tat. 30 μg of protein was used for detecting the level of phosho-ERK1/2 with anti-phospo ERK1/2 (Cell Signaling Technology, Beverly, MA, USA) as described previously (Mishra *et al,* 2008). The band intensities were measured using densitometry and normalized with β-tubulin.

Statistical analysis

Experiments for each condition and assays were repeated three to five times from hNPCs that were obtained from three different fetal brain samples. Results from each set of experiment were averaged, counted as $n = 1$ for statistical analysis, and presented as mean ± standard deviation (SD). Statistical significance between groups was calculated either using Student's t test within two groups and with Fisher's analysis of variance (ANOVA) where comparisons were made with more than two groups. All values of $P < .05$ were taken as statistically significant.

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Supplementary material available online

Supplementary Table S1.
Supplementary Figure S1.