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HIV-1 Tat Upregulates Expression of Histone Deacetylase-2 (HDAC2) in Human Neurons: Implication for HIV-Associated Neurocognitive Disorder (HAND)

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Abstract

Histone deacetylases (HDACs) play a pivotal role in epigenetic regulation of transcription and homeostasis of protein acetylation in histones and other proteins involved in chromatin remodeling. Histone hypoacetylation and transcriptional dysfunction have been shown to be associated with a variety of neurodegenerative diseases. More recently, neuron specific overexpression of HDAC2 has been shown to modulate synaptic plasticity and learning behavior in mice. However, the role of HDAC2 in development of HIV-associated neurocognitive disorders (HAND) is not reported. Herein we report that HIV-1 Tat protein upregulate HDAC2 expression in neuronal cells leading to transcriptional repression of genes involved in synaptic plasticity and neuronal function thereby contributing to the progression of HAND. Our results indicate upregulation of HDAC2 by Tat treatment in dose and time dependant manner by human neuroblastoma SK-N-MC cells and primary human neurons. Further, HDAC2 overexpression was associated with concomitant downregulation in CREB and CaMKIIa genes that are known to regulate neuronal activity. These observed effects were completely blocked by HDAC2 inhibition. These results for the first time suggest the possible role of HDAC2 in development of HAND. Therefore, use of HDAC2 specific inhibitor in combination with HAART may be of therapeutic value in treatment of neurocognitive disorders observed in HIV-1 infected individuals.

Keywords

Histone deacetylases; HIV-associated neurocognitive disorder; Tat protein; Synaptic plasticity; Primary human neurons; SK-N-MC neuroblastoma cell line

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1. INTRODUCTION

HIV-associated neurocognitive disorders (HAND) is characterized by development of cognitive, behavioral and motor abnormalities (Antinori et al., 2007), and occur in approximately 50% of HIV infected individuals (Ellis et al., 2007; McArthur and Brew, 2010). Previous studies report that HIV productively infects and replicates in the macrophages, microglia, and monocytes in the brain (Nath et al., 1996; Ellis et al., 2007). The HIV infected cells and the activated immune cells in the brain releases proinflammatory factors and neurotoxins that results in neuronal death. Further, HIV-1 proteins that include Tat, Vpr, and gp120 are known to cause neuronal apoptosis and thereby exacerbate HIV-associated neuropathogenesis (Kruman et al., 1998; Shi et al., 1998).

Recently, a great deal of research has been focused on elucidating the role of chromatin remodeling and transcription regulation in the development of neurological diseases (Davie and Spencer., 1999; Langley et al., 2005; Hildman et al., 2007). Acetylation and deacetylation of histone proteins mediated by histone acetyltransferase (HATs) and histone deacetylases (HDACs) regulates chromatin structure and thereby modulates gene expression and cell signaling (Sun et al., 2003). Experimental evidence suggests that HDAC inhibitors enhance cognitive ability, correct neurodegenerative impairment and help to re-establish long-term memory (Kazantsev and Thompson, 2008; Thomas, 2009). In murine model, treatment with HDAC inhibitor sodium butyrate has been shown to restore the learning ability and long-term memory even after substantial neuronal loss (Fischer et al., 2007). Another study (Vecsey et al., 2007) reported that HDAC inhibitors enhance both memory and synaptic plasticity via cAMP response element-binding protein (CREB)-CBP (CREB binding protein) dependent transcriptional activation. Identification of HDAC(s) that regulate cognitive functions could allow development of specific therapies. More recently, Guan and coworkers (2009) reported that neuronal levels of HDAC2 is inversely related to synaptic plasticity, memory formation and the induction of dendritic structural changes associated with memory. HDAC2 specifically associates with the regulatory elements of the genes involved in plasticity and memory (Guan et al., 2009; Levenson et al., 2004). Further, studies using pharmacological inhibitors of HDACs and mice with conditional alleles to HDAC1 and HDAC2 confirmed that HDAC2 regulates synapse maturation and regulates neuronal function (Akhtar et al., 2009). Another protein that has been implicated in learning and memory is Ca^{2+} /calmodulin kinase II (CaMKII), which is highly expressed in the postsynaptic density and play an important role in long-term potentiation (LTP). Study supporting this hypothesis (Silva et al., 1992) reported deficits in LTP and long-term depression in mice deficient for CaMKII- α (CaMKIIa) gene.

Chromatin remodeling is also an essential event in HIV-1 life cycle (Margolis, 2010; Richman et al., 2009). HDACs play a critical role during integration and in inducing HIV latency in infected CD4^{+} lymphocytes and cells of the macrophage-monocyte lineage (Archin et al., 2009; Keedy et al., 2009; Ylisastigui et al., 2004). These observations led to the exploration of HDAC inhibitors as a potential adjunct therapeutic approach to induce latent HIV expression in cellular reservoirs. However, no studies have been reported yet on the effect of HIV on HDAC expression in neuronal cells and its role in development of HAND. In the current study, we focused on HDAC2 since it is known to greatly influence synaptic transmission in mature neurons and thus play a critical role in cognitive function. Herein, we tested the hypothesis that HIV-1 neurotoxic protein Tat may modulate expression of HDAC2 by neuronal cells and in turn negatively regulates genes involved in synaptic plasticity or neuronal function, thereby contributing to the progression of HAND.

2. METHODS

2.1. Cell Culture and Reagents

Human neuroblastoma SK-N-MC cells were obtained from ATCC. SK-N-MC cells were grown in Eagle's minimal essential medium (MEM) containing 10% fetal bovine serum (FBS), 5 mM sodium pyruvate, 100 units/ml penicillin, and 100 µg/ml streptomycin at 37°C with 5% CO₂. Primary human neurons (PHNs) were purchased from Sciencell laboratories (Carlsbad, CA) and cultured as per supplier's instructions. Primary neurons are characterized by immunofluorescent method with antibodies to neurofilament, MAP2, and beta-tubulin 3. Also, the cultures are negative for HIV-1, HBV, HCV, mycoplasma, bacteria, yeast and fungi. Trichostatin A was procured from Sigma Aldrich (MO, USA). HDAC2 specific siRNA was purchased from Applied Biosystems, Carlsbad, CA. Antibodies against HDAC2 and CaMKIIa were obtained from Millipore (Billerica, MA) and Sigma Aldrich, respectively.

HIV-1 clade B recombinant Tat protein (86-amino acid) was obtained from NIH AIDS research and reference reagent program (Cat #2222). Tat concentrations used in the current study are in agreement with previously reported (Perry et al., 2010; Mishra *et al*, 2008). Xiao and coworkers (2000) have reported soluble Tat concentration in HIV-infected patient serum to be about 40ng/ml (Xiao et al., 2000). Further, Tat concentration is expected to be much higher in the vicinity of HIV-infected cells than detected in sera from HIV-infected patients (Hayashi et al., 2006). Moreover, recombinant Tat protein is considered to be less potent than Tat released from Tat expressing astrocyte cell line (Li et al., 2008; Nath et al., 2000) suggesting that higher doses of commonly used recombinant Tat protein are needed to adequately mimic the effects of Tat constitutively produced in brain of HIV infected patients (Perry et al., 2010). Thus, the concentration of Tat (10nM) used in the current study is comparable or lower than used previously in similar studies and is biologically relevant.

2.2. HIV-1 Tat treatment of human SK-N-MC neuroblastoma cell line and primary human neurons (PHNs)

SK-N-MC (2×10^6 cells/well) in 6-well plates were serum starved overnight, followed by treatment with HIV-1 Tat protein. For time kinetics experiments, SK-N-MC cells were treated with 10nM (~100ng/ml) Tat protein for different time intervals 4, 8, 12, 24 and 48h. Based on the time kinetics data, dose-response study was done at 24h with 1, 2.5, 5, 10 and 20nM of Tat protein. Primary human neurons (1×10^6 cells/well) in 6-well plates were cultured with 10nM of Tat protein for 24h, followed by gene expression analysis of HDAC2, CaMKIIa and CREB using real time PCR. Heat inactivated Tat protein served as control. HDAC inhibition studies were performed by incubating SK-N-MC cells or primary neurons with Trichostatin A (50nM) for 1h prior to the treatment with Tat protein (10nM) for 24 or 48h. HDAC2 specific siRNA was transfected according to the manufacturer's guidelines (Applied Biosystems).

2.3. Quantitative Real Time PCR (qRT-PCR)

Gene expression was quantitated using real time PCR. Upon desired treatment period, cells were harvested, RNA from cell pellets was extracted using RNeasy mini kit (Qiagen, GmbH, Germany) followed by cDNA synthesis using high capacity reverse transcriptase cDNA kit (Applied Biosystems) to perform qRT-PCR using Taqman gene expression assays (Applied Biosystems) for HDAC2 (Hs00231032_m1), CaMKIIa (Hs00392405_m1), CREB (Hs00231713_m1) and GAPDH (Hs99999905_m1). GAPDH served as an internal control. Relative abundance of each mRNA species was assessed using brilliant Q-PCR master mix from Stratagene using Mx3000P instrument which detects and plots the increase in fluorescence versus PCR cycle number to produce a continuous measure of PCR

amplification. Relative mRNA species expression was quantitated and the mean fold change in expression of the target gene was calculated using the comparative C_T method (Transcript Accumulation Index, $TAI=2^{-\Delta\Delta CT}$). All data were controlled for quantity of RNA input by performing measurements on an endogenous reference gene, GAPDH. In addition, results on RNA from treated samples were normalized to results obtained on RNA from the control, untreated sample.

2.4. Western Blot Analysis

To assess protein expression, SK-N-MC cells were treated with HIV-1 Tat protein (10nM), for 12, 24 or 48h. After incubation, cells were harvested and lysed using lysis buffer with a complete cocktail of protease inhibitors (Pierce Chemical Co., Rockford, IL). Total protein content in these samples was determined using Bradford dye reagent (Pierce Chemical Co., Rockford, IL). 10µg total protein was resolved on 10% Tris-HCl gel by polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and were probed with primary antibody against HDAC2 (Millipore, 1:2500), CaMKIIa (Sigma Aldrich, 1:2500), and β -actin (Cell Signaling Technology, 1:1000) followed by secondary goat antirabbit IgG antibody (Cell Signaling Technology, 1:2500). Immunoreactive bands were visualized using a chemiluminescence's system according to the manufacturer's instructions (Pierce Chemical Co., Rockford, IL).

2.5. Flow cytometry analysis

Flow cytometry using EPICS-XL instrument (Beckman, Brea, CA) was used to study the effect of HIV-1 Tat protein on HDAC2 expression in SK-N-MC cells. Approximately, 1×10^6 SK-N-MC cells were treated with Tat (10 nM) for 24h and cells were harvested, trypsinized, washed and fixed and permeabilized with Cytotfix/Cytoperm solution (BD Bioscience). Cells were incubated with primary monoclonal antibody against HDAC2 (Millipore) followed by secondary goat antirabbit IgG conjugated with fluorescein isothiocyanate (FITC) (Millipore). Cells were gated based on unlabeled and secondary control antibody. Stained cells were subject to light scatter analysis and the results are expressed as overlay histogram of mean fluorescence intensity of different treatments.

2.6. Statistical analysis

Experiments were performed at least three times and the values obtained were averaged. All results are expressed as mean $\pm$ s.e.m. Statistical analysis of two groups was performed by Student's t test, while more than two groups were analyzed using one way ANOVA followed by Bonferroni's multiple comparison test. Differences were considered significant at $p \leq 0.05$. Data analysis was performed with the Statistical Program, GraphPad Prism software (La Jolla, CA).

3. RESULTS

3.1. HIV-1 Tat upregulates HDAC-2 expression in SK-N-MC cells

In order to evaluate the role of HDAC2 in progression of HAND, we studied the effect of HIV-1 Tat protein on expression of HDAC2 gene by SK-N-MC cells. Data presented in Fig. 1a demonstrate that Tat significantly increased the expression of HDAC2 gene by SK-N-MC at 12h ($p < 0.01$), 24h ($p < 0.0001$) and 48h ($p < 0.01$), respectively. Based on the time kinetics results, a 24h time point was selected to perform dose-response studies. Results show a significant upregulation in HDAC2 gene expression by SK-N-MC cells at 5nM ($p < 0.02$), 10nM ($p < 0.002$) and 20nM ($p < 0.002$) of Tat treatment compared to control cultures (Fig. 1b).

Further to gene expression studies, we observed a time-dependent increase in HDAC2 protein expression by western blot analysis. SK-N-MC cells were incubated in the presence of 10nM Tat for 12h, 24h and 48h and then analyzed by immunoblotting using specific antibody to quantitate HDAC2 levels. The results indicate significant upregulation of HDAC2 protein levels at 12h ($p<0.01$), 24h ($p<0.0001$) and 48h ($p<0.0001$) of Tat treatment compared to control cultures (Fig. 2a).

In addition to immunoblot analysis for protein quantification, flow cytometry analysis using specific antibodies was performed to quantitate HDAC2 protein expression by SK-N-MC treated with Tat protein. SK-N-MC cells were incubated in the presence of 10nM Tat concentration for 24h and then analyzed by flow cytometry. The results are expressed as the percentage of HDAC2 positive cells. The exposure of SK-N-MC cells to Tat protein produced significantly higher levels of HDAC2 ($p<0.003$) compared to control cultures (Fig. 2b).

3.2. Trichostatin A inhibits Tat induced upregulation of HDAC2

Trichostatin A (TSA) is a nonselective HDAC inhibitor that targets both class I and II HDAC enzymes (Thomas, 2009). We investigated whether TSA could inhibit Tat induced overexpression of HDAC2. SK-N-MC cells were pretreated with TSA (50nM) for 1h followed by culturing with 10nM of Tat for 24h. Results presented in Fig. 3a shows a significant increase in HDAC2 gene expression ($p<0.0001$) on Tat treatment, whereas cultures pretreated with TSA showed complete abrogation of HDAC2 gene upregulation by Tat treatment. These results were further confirmed by western blot and flow cytometry to analyze the HDAC2 protein levels. Data presented in Fig. 3b and Fig. 3c show that TSA completely inhibited the increase in HDAC2 protein levels in SK-N-MC cells on treatment with Tat. SK-N-MC cells treated with TSA alone had no significant difference in the expression of HDAC2 gene compared to control cultures.

3.3. Tat downregulates synaptic plasticity-related genes

HDAC2 is known to be enriched at the promoters of genes that regulate memory and synaptic plasticity (Guan et al., 2009). Further, previous study (Vecsey et al., 2007) reports that HDAC inhibitors enhance memory and synaptic plasticity through CREB-CBP dependent transcriptional activation. We evaluated the effect of Tat on expression of CREB and CaMKIIa by SK-N-MC cells. Fig. 4a shows that Tat (10nM) significantly downregulated CREB ($p<0.05$) and CaMKIIa ($p<0.05$) gene expression by SK-N-MC cells at 24h compared to untreated control culture, whereas SK-N-MC cells pretreated with TSA (50nM) showed complete reversal of Tat mediated downregulation of CaMKIIa and CREB genes. These results were further confirmed by analyzing CaMKIIa protein levels using western blot. Data presented in Fig. 4b indicates that Tat treated SK-N-MC cells showed a significant downregulation of CaMKIIa protein levels at 24h ($p<0.05$) and 48h ($p<0.05$), respectively. However, this downregulation of CaMKIIa was completely reversed in SK-N-MC cells that were treated with TSA (50nM) prior to treatment with Tat.

3.4. HDAC2 specific siRNA inhibits Tat mediated effects on neuroblastoma cells

In order to demonstrate the specificity of HDAC2 in Tat mediated effects on SK-N-MC cells, we undertook the following experiment using HDAC2 specific siRNA to see whether it could block the effect of HIV-1 Tat protein. The HDAC2 knock down was validated by investigating the effect of HDAC2 specific siRNA on the HDAC2 gene expression in siRNA-transfected SK-N-MC cells (Fig. 5a). The results obtained show that HDAC2-specific siRNA transfection (30nM) inhibited the gene expression of HDAC2 (lane 4) compared to untransfected control (lane 1) or scrambled siRNA-transfected cultures (lane 3). Further, HDAC2 siRNA transfected cultures treated with Tat (10nM) produced

significantly lower level of HDAC2 gene expression (lane 5) compared to untransfected SK-N-MC cells treated with Tat (lane 2, $p<0.0001$). These HDAC2 knock down was further confirmed by analyzing the protein levels using western blot. Results (Fig. 5b) indicate that HDAC2 siRNA transfected SK-N-MC cells inhibited the HDAC2 protein expression (lane 3) compared to untransfected control (lane 1). Further, Tat treated SK-N-MC cells showed a significant upregulation of HDAC2 protein levels ($p<0.001$) compared to untransfected control, however this upregulation was inhibited in HDAC2 siRNA transfected cells (lane 4, $P<0.0001$).

Additionally, to evaluate whether Tat-induced effects on synaptic plasticity associated genes are mediated through HDAC2, we investigated the effect of siRNA directed against HDAC2 for its potential effect to reverse Tat-induced downregulation of CaMKIIa and CREB genes. The data presented in Fig. 5c show that SK-N-MC cells treated with Tat (10nM) significantly downregulated CaMKIIa (lane 3, $p<0.05$) and CREB (lane 4, $p<0.001$) gene expression compared to untransfected control culture (lanes 1 and 2, respectively). However, SK-N-MC cells transfected with HDAC2 specific siRNA and treated with Tat completely reversed Tat-induced downregulation of CaMKIIa (lane 5, $P<0.05$) and CREB (lane 6, $P<0.05$) gene expression compared to untransfected Tat-treated culture (lanes 3 and 4, respectively).

3.5. Tat modulates HDAC2, CaMKIIa and CREB gene expression by PHNs

To confirm the observations made with SK-N-MC neuroblastoma cell line, additional experiments were conducted with primary human neurons (PHNs). PHNs were cultured with 10nM Tat and gene expression was analyzed by qRT-PCR as described above for SK-N-MC cells. As seen in Fig. 6, Tat treatment significantly increased the expression of HDAC2 ($p<0.0001$) by PHNs, whereas the expression of CaMKIIa ($p<0.01$) and CREB ($p<0.01$) was significantly downregulated. Transfection of PHNs with HDCA2 siRNA reversed Tat mediated upregulation of HDAC2 ($p<0.0001$), and downregulation of CaMKIIa ($p<0.001$) and CREB ($p<0.01$) genes. Also, pre-treatment of PHNs with TSA blocked the Tat mediated effects on HDAC2 ($p<0.001$), CaMKIIa ($p<0.001$) and CREB ($p<0.001$) genes. This further supports our premise that HDAC2 may play an important role in development of HAND by downregulating the expression of genes that regulate memory and synaptic plasticity.

4. DISCUSSION

During the last decade, advances in HAART has remarkably improved the immune outcome and survival rates of HIV-1 infected individuals, however the associated neurological abnormalities remain among the most common clinical disorders observed in HIV infected patients (Ellis et al., 2007; Woods et al., 2009; McArthur and Brew, 2010). HIV-1 induced neuronal damage mainly results from the inflammatory products (Persidsky and Gendelman, 2003) and neurotoxic substances released by the infected cells as well as activated uninfected cells (Nath et al., 1996; Kruman et al., 1998; Shi et al., 1998; Ellis et al., 2007). HAND is characterized by neuroinflammation, including astrogliosis, multinucleated giant cells, and neuronal apoptosis that is linked to progressive virus infection and immune deterioration (Kanmogne et al., 2007; Toborek et al., 2005; Ikezu, 2009;). HAND comprises a hierarchy of progressively more severe patterns of CNS involvement that range from asymptomatic neurocognitive impairment (ANI), to minor neurocognitive disorder (MND), to the most severe HIV-associated dementia (HAD) (McArthur and Brew, 2010).

In recent years, the role of chromatin remodeling and transcriptional dysfunctions has emerged as an important regulatory element in the development of neurological disorders. Histone hypoacetylation and transcriptional dysfunction has been implicated in a large

number of neurodegenerative disorders (Butler and Bates, 2006; Kazantsev and Thompson, 2008; Thomas, 2009). Much of the research has focused on the interplay between HATs and HDACs that modulate the net balance of histone acetylation levels. In addition to histone modification, other non-histone proteins also are substrates for HATs and HDACs, namely tubulin, transcription factors such as CREB, NF- κ B, p53, and others (Chuang et al., 2009). Accumulating evidences suggest that HDAC inhibitors enhance cognitive ability, correct neurodegenerative impairment and help to re-establish long-term memory.

Previous studies with HIV have focused on critical role of HDAC in inducing HIV latency (Richman et al., 2009). HDAC complexes are recruited to the highly conserved initiator region of the HIV promoter, which ultimately represses HIV gene and virus production thus promoting quiescence of HIV within the resting CD4 T cells (Ylisastigui et al., 2004). However, no studies have been reported on the effects of HIV-1 proteins on HDAC expression in neuronal cells and its role in progression of HAND. In the current study, we especially focused on HDAC2 expression in neuronal cells in response to HIV-1 neurotoxic protein Tat. HDAC2 plays an important role in embryonic development, affects cytokine signaling and is often over expressed in solid tumors (Kramer et al., 2009). Further, HDAC2 greatly influence synaptic transmission in mature neurons and thereby is critical factor in cognitive function. Our studies showed that Tat significantly upregulated HDAC2 expression in a time-and dose-dependent manner by SK-N-MC cells (Fig. 1). These observations were further confirmed at protein levels using flow cytometry and western blot analysis (Fig. 2). Guan and coworkers (2009) have shown that overexpression of HDAC2 in mice resulted in decrease in dendritic spine and synapse densities, which is linked to impaired synaptic plasticity and memory ability. Another study using pharmacological inhibitors of HDACs and mice with conditional alleles to HDAC1 and HDAC2 confirmed that HDAC2 regulates synapse maturation (Akhtar et al., 2009). Anderson and coworkers (2003) have reported dysfunction of synaptic transmission and plasticity changes in murine model of human HIV-1 encephalitis (HIVE). Studies have also documented a significant decline in working memory in HIV positive subjects compared to HIV negative controls (Stout et al., 1995). In another study, neuroimaging revealed hippocampal dysfunction in HIV positive women and this was associated with deficits in verbal episodic memory, working memory and visual memory (Maki et al., 2009). Further, HIV patient with severe HAD reports substantial disruption of post-synaptic elements, which are characterized by sinuous, shortened and vacuolated dendrites that lead to reduction in synaptic density (Masliah et al., 1992).

Earlier studies (Vecsey et al., 2007) with HDAC inhibitors have reported to enhance memory and synaptic plasticity through CREB-CBP dependent pathway. Interestingly, HIV-1 Tat is also known to interact with at least five different HATs in HIV-1 infected cells, which includes CBP/p300, p/CAF, GCN5, Tip 60, and TAF_{II}250 (Easley et al., 2010). CBP and p300 are transcriptional activators and they function by interaction with transcription factor CREB, as well as HATs such as p/CAF. We further evaluated the expression of CREB and CaMKIIa by neuronal cells in response to Tat treatment. Our results showed that HDAC2 upregulation in neuronal cells was associated with concomitant down regulation in CREB and CaMKIIa genes that regulate synaptic plasticity. It is well demonstrated that CREB-CBP pathway regulates the expression of CREB target genes that are known to be involved in long-term memory and LTP by acetylating histones at the regulatory regions of key CREB target genes. In our study, Tat mediated downregulation of CREB gene is in concurrence with earlier report (Zauli et al., 2001) where HIV-1 Tat decreased CREB expression in PC12 neuronal cell line. Further, downregulation of CREB pathway has been reported to decrease EGR1 (Early growth response factor 1) expression (Guan et al., 2009). EGR1 is a target gene for CREB and a key molecule involved in hippocampal-associated memory and learning and it is reported to be significantly downregulated in hippocampal

neurons of the SIV-infected macaques (Gersten et al., 2009). Another protein, CaMKIIa merits discussion, since previous knockout studies in mice clearly demonstrate that CaMKIIa is essential for synaptic plasticity and learning (Silva et al., 1992; Lisman et al., 2002). Further, Gupta and coworkers (2010) have reported that CaMKII expression and activation is significantly downregulated in rat hippocampal neuronal cultures treated with culture supernatant harvested from HIV-1 infected human monocyte-derived macrophages. In the same study, the authors observed that CaMKII activation was decreased in hippocampus and frontal cortex of SIV-infected macaques compared to uninfected animals (Gupta et al., 2010). Interestingly, clinical studies in patients with HAD have also demonstrated a significant decrease in CaMKII subunits in human brain cortex (Gelman et al., 2004).

Additionally, we found that treatment of SK-N-MC cells with TSA or HDAC2 specific siRNA resulted in significant decrease in HDAC2 gene expression and reversed the inhibitory effect of Tat on CREB and CaMKIIa expression (Fig. 3–5). Previous studies have shown that injection of TSA reduces the HDAC2 gene expression in caudate putamen and nucleus accumbens of rats self-administering cocaine (Host et al., 2009). Similarly, Li and coworkers (2008) have reported significantly lower expression of HDAC2 transcripts in *in vivo* recovered blastocysts treated with TSA (Li et al., 2008). Currently, we are investigating the mechanism of TSA mediated inhibition of HDAC2 upregulation induced by Tat.

In order to demonstrate the biological relevance of these studies, we performed additional experiments using PHNs. We found that, like the neuroblastoma cells, Tat induced the expression of HDAC2 gene and concomitantly inhibited both the expression of CREB and CaMKIIa by PHNs (Fig. 6). Further, the Tat mediated effects were reversed on treatment of PHNs with TSA or HDAC2 specific siRNA.

To summarize our discussion, we propose the model highlighted in Fig. 7. This model encompasses that Tat induced upregulation of HDAC2 leads to transcriptional repression and ultimately neurocognitive impairment. However, inhibition of HDAC2 leads to transcriptional activation and improves neurocognition. To conclude, our results demonstrate for the first time that HIV-1 Tat modulates expression of HDAC2 by neuroblastoma SK-N-MC cell line and PHNs. These studies support our hypothesis that HDAC2 overexpression in neurons may play an important role in progression of HAND by downregulating memory and synaptic plasticity associated genes. However, future investigation of chromatin remodeling and transcriptional dysfunction may help to elucidate the exact molecular mechanism involved in the development of HAND. Further, this work suggests that use of HDAC2 inhibitor might be of therapeutic significance in the treatment of HAND.

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Figure 1. HIV-1 Tat protein upregulates HDAC2 gene expression in SK-N-MC cells

a, SK-N-MC cells (2×10^6) were treated with HIV-1 Tat protein (10nM) for 4, 8, 12, 24 and 48h. RNA was extracted and reverse transcribed followed by quantitative real time PCR for HDAC2 gene. Relative expression of mRNA species was calculated using the comparative C_T method. Data are expressed as mean $\pm$ s.e.m of three independent experiments performed in replicates. Statistical significance was determined using ANOVA (* $P < 0.01$ and *** $P < 0.0001$).

b, SK-N-MC cells (2×10^6) were treated with HIV-1 Tat protein (1, 2.5, 5.0, 10 and 20nM) for 24h. RNA was extracted and reverse transcribed followed by quantitative real time PCR for HDAC2 gene. Heat-inactivated (HI) Tat protein served as control. Relative expression of mRNA species was calculated using the comparative C_T method. Data are mean $\pm$ s.e.m of three independent experiments performed in replicates. Statistical significance was calculated by ANOVA (* $P < 0.02$ and ** $P < 0.002$).

Figure 2. HIV-1 Tat protein upregulates HDAC2 protein levels in SK-N-MC cells
a, SK-N-MC cells (2×10^6) were treated with 10nM Tat protein for 12, 24 and 48h. The HDAC2 protein level in all the samples was quantitated by western blot analysis. Data presented shows a representative blot indicating modulation of HDAC2 protein expression and a bar graph representing the mean $\pm$ s.e.m of % densitometric values of HDAC2 protein levels (% of control) of 3 separate experiments performed. Statistical significance was determined using ANOVA (*P<0.01 and ***P<0.0001).
b, SK-N-MC cells (1×10^6) were cultured with 10nM of Tat protein for 48h. The number of HDAC2 expressing cells was quantitated using FACS analysis. Stained cells were subjected to light scatter analysis. Upper panel is a scatter plot showing the gated cell population, whereas the lower panel is a representative overlay histogram showing the cell count vs log fluorescence. The bar graph represents the % HDAC2 positive cells $\pm$ s.e.m of three separate experiments. Statistical analysis was determined using Student's t test (**P<0.003).

Figure 3. TSA inhibits Tat induced overexpression of HDAC2 in SK-N-MC cells

a, SK-N-MC cells were cultured with or without Tat (10nM) and TSA (50nM) for 24h. RNA was extracted and amplified by qRT-PCR with GAPDH and HDAC2-specific primers. Relative expression of mRNA species was calculated using the comparative C_T method. Data are mean $\pm$ s.e.m of three independent experiments performed in replicates. Statistical analysis of performed using ANOVA based on comparison with the untreated control (***) $P < 0.0001$).

b, SK-N-MC cells were treated with or without 10nM Tat protein and TSA for 24h. The HDAC2 protein level in all the samples was quantitated by western blot analysis. Data presented shows a representative blot indicating modulation of HDAC2 protein expression and a bar graph representing the mean $\pm$ s.e.m of % densitometric values of HDAC2 protein levels (% of control) of 3 separate experiments performed. Statistical significance was determined using ANOVA (**) $P < 0.001$).

c, SK-N-MC cells (1×10^6) were cultured with or without 10nM of Tat and TSA protein for 48h. The number of HDAC2 expressing cells was quantitated using FACS analysis. Upper panel is a scatter plot showing the gated cell population, whereas the lower panel is a representative overlay histogram showing the cell count vs log fluorescence. The bar graph represents the % HDAC2 positive cells $\pm$ s.e.m of three separate experiments. Statistical analysis was performed using ANOVA based on comparison with the untreated control (**) $P < 0.001$).

(a)

(b)

Figure 4. Tat downregulates CaMKIIa and CREB expression in SK-N-MC cells

a, SK-N-MC cells (2×10^6) were treated with Tat protein (10nM) $\pm$ TSA (50nM) for 24h. RNA was extracted and reverse transcribed followed by quantitative real time PCR for CamKIIa and CREB genes. Relative expression of mRNA species was calculated using the comparative C_T method based on comparison with normalized untreated control (TAI=1). Data are expressed as mean $\pm$ s.e.m of three independent experiments performed in replicates. Statistical significance was determined using ANOVA (* $P < 0.05$).

b, SK-N-MC cells (2×10^6) were treated with 10nM Tat protein $\pm$ TSA (50nM) for 24 and 48h. The CaMKIIa protein level in all the samples was quantitated by western blot analysis. Data presented shows a representative blot indicating modulation of CaMKIIa protein expression and a bar graph representing the mean $\pm$ s.e.m of % densitometric values of CaMKIIa protein levels (% of control) of 3 separate experiments performed. Statistical significance was determined using ANOVA based on comparison with the untreated control (* $P < 0.05$).

Figure 5. HDAC2 knock down using siRNA reverses Tat mediated effects on CaMKIIa and CREB

a, SK-N-MC cells were transfected with HDAC2-specific siRNA (30nM) according to manufacturer's protocol (Applied Biosystems). Our results indicate silencing of HDAC2 gene at 24h (~60% inhibition of HDAC2 gene expression) post-transfection. Data represent mean $\pm$ s.e.m of three separate experiments and statistical analysis was done using one way ANOVA (** $P < 0.0001$).

b, SK-N-MC cells were transfected with HDAC2-specific siRNA and then cultured with Tat (10nM) for 24h and HDAC2 protein expression was analyzed using western blot. Data presented shows a representative blot indicating the knock down of HDAC2 and a bar graph representing the mean $\pm$ s.e.m of % densitometric values of HDAC2 protein levels (% of control) of 3 separate experiments performed. Statistical significance was determined using ANOVA (** $P < 0.001$ and *** $P < 0.0001$).

c, SK-N-MC cells were transfected with HDAC2-specific siRNA and then cultured with Tat (10nM) for 24h and the CaMKIIa and CREB specific mRNA expression was quantitated using qRT-PCR. Relative expression of mRNA species was calculated using the comparative C_T method based on comparison with normalized untreated control (TAI=1). Data is expressed as mean $\pm$ s.e.m of three independent experiments. Statistical analysis was done using one way ANOVA followed by Bonferroni's multiple comparison test (* $P < 0.05$ and ** $P < 0.001$). The symbol (#) denotes statistically significant differences between the Tat treated samples and the untreated control.

Figure 6. Tat induces HDAC2 and inhibits CaMKIIa and CREB gene expression by PHNs
 PHNs (1×10^6) were cultured with or without Tat for 24h. RNA was extracted; reverse transcribed, and amplified using gene specific primer for HDAC2, CaMKIIa and CREB. In a separate experiment the reversal of Tat induced effects by TSA and HDAC2-specific siRNA was evaluated. Data is expressed as mean $\pm$ s.e.m of three experiments and the statistical analysis was performed using one way ANOVA followed by Bonferroni's multiple comparison test (* $P < 0.01$, ** $P < 0.001$ and *** $P < 0.0001$). The symbol (#) denotes significant differences between the Tat treated samples and the untreated control.

Figure 7.
Schematic representation showing the modulatory effect of Tat on HDAC2