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HIV-1 Tat Protein Promotes Neuronal Dysfunction through Disruption of MicroRNAs*

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Over the last decade, small noncoding RNA molecules such as microRNAs (miRNAs) have emerged as critical regulators in the expression and function of eukaryotic genomes. It has been suggested that viral infections and neurological disease outcome may also be shaped by the influence of small RNAs. This has prompted us to suggest that HIV infection alters the endogenous miRNA expression patterns, thereby contributing to neuronal deregulation and AIDS dementia. Therefore, using primary cultures and neuronal cell lines, we examined the impact of a viral protein (HIV-1 Tat) on the expression of miRNAs due to its characteristic features such as release from the infected cells and taken up by noninfected cells. Using microRNA array assay, we demonstrated that Tat deregulates the levels of several miRNAs. Interestingly, miR-34a was among the most highly induced miRNAs in Tat-treated neurons. Tat also decreases the levels of miR-34a target genes such as CREB protein as shown by real time PCR. The effect of Tat was neutralized in the presence of anti-miR-34a. Using *in situ* hybridization assay, we found that the levels of miR-34a increase in Tat transgenic mice when compared with the parental mice. Therefore, we conclude that deregulation of neuronal functions by HIV-1 Tat protein is miRNA-dependent.

Neurodegeneration is characterized by the progressive loss of neurons in the central nervous system. Although the main cause of the cellular dysfunctions in many neurodegenerative diseases is clearly established, there is increasing evidence that neuronal demise results from a combination of genetic and environmental factors that affect key signaling pathways in cell function (1). This view is supported by recent observations that disease-compromised cells in late stage neurodegeneration exhibit profound deregulation of gene expression. MicroRNAs (miRNAs)[3] introduce a novel concept of regulatory control over gene expression, and there is increasing evidence that they may play a profound role in neuronal cell identity as well as multiple aspects of disease pathogenesis (2). In support of this observation, a link between miRNAs and neurodegenerative diseases (*e.g.* Alzheimer, Huntington, and Parkinson) is becoming increasingly evident (3); however, the involvement of miRNAs in developing HIV-1-associated neurocognitive disorders (HAND) remains unclear.

The transactivator regulatory protein (Tat) has been implicated in the pathophysiology of the neurocognitive deficits associated with HIV infection (4). This is the earliest protein to be produced by the proviral DNA in the infected cell. The protein not only drives the regulatory regions of HIV-1 but may also be actively released from infected cells and then interact with the cell surface receptors of other uninfected cells in the brain leading to cellular dysfunction. Tat taken up by uninfected cells (5, 6) can then activate a number of host genes (7–10). The Tat protein is highly potent and has the unique ability to enter cells, including neurons. Importantly, its production is not impacted by the use of antiretroviral drugs once the proviral DNA has been formed. Intracerebral injection of Tat can be lethal to mice within hours of injection (11). In adult animals, Tat affects pre-attentive processes and spatial memory (12). In a Tat transgenic model, there is marked glial cell activation accompanied by neuronal loss (13). Tat causes loss of selective populations of neurons *in vitro* and *in vivo* (14). Regions particularly susceptible to Tat neurotoxicity include the striatum (15), dentate gyrus, and the CA3 region of the hippocampus (16). Furthermore, neuropathological studies from patients with HIV infection show a preferential loss of neurons in the dentate gyrus and striatum (17). Tat is also capable of depolarizing the neuronal cell membrane when applied extracellularly as measured by outside-out membrane patches, thereby providing strong evidence for direct excitation of neurons on the cell surface. Tat induces dramatic increases in levels

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[3] The abbreviations used are: miRNA, microRNA; HAND, HIV-1-associated cognitive disorder; ER, endoplasmic reticulum; CREB, cAMP-response element-binding protein; HN, human neuron; qPCR, quantitative PCR; ISH, *in situ* hybridization; HIVE, HIV encephalitis; IRBIT, inositol 1,4,5-trisphosphate receptor-binding protein; NEFM, neurofilament medium.

of intracellular Ca^{2+} in neurons that is followed by mitochondrial Ca^{2+} uptake, generation of ROS, activation of caspases, apoptosis, and eventually cell loss (18).

In HIV-1-infected cells, it has been shown that miRNAs are conserved factors/processes whose complete suppression is incompatible with cellular viability (19). Hence, virus-encoded RNAi suppressors likely work modestly and in limited settings. Without the ability to fully suppress the RNAi restriction of cells, HIV-1 can further ameliorate the antiviral defense of cells by mutating viral RNA sequences to alter base complementarily with cellular miRNAs. Indeed, there is evidence that selective and evasive nucleotide changes in HIV-1 sequences can be elicited rapidly by siRNA/shRNA-induced RNAi (20). Additionally, HIV-1 can reshape the miRNA expression profile of infected cells (21). One interpretation of this latter finding is that the virus has learned to repress the expression of virus-targeted miRNAs, while enhancing the expression of propitious miRNAs that up-regulate protein factors that benefit HIV-1 replication (22). This notion was supported by the identification of several human miRNAs (*e.g.* miR-28, -125b, -150, -223, and -382) that have the potential to target the 3′UTR of HIV-1 transcripts (23, 24) potentially rendering productive infection into latency (25–27). Using computer-directed analyses, Bennasser *et al.* (28) found that HIV putatively encodes five candidate pre-miRNAs. Several miRNAs were also shown to affect HIV-1 gene expression and replication *in vitro* and in animal models (29). Reciprocally, HIV-1 was shown to affect the levels of several miRNAs to change their expression profile (30). Tat protein was also shown to deregulate expression levels of selected miRNAs, including the neuronal mir-128, in primary cortical neurons (31).

We observed differential expression of multiple miRNAs in primary fetal neurons treated with Tat. Interestingly, some targets of the up-regulated miRNAs have been associated with neurological diseases. For instance, miR-378 has been shown to target the *CYP2E1* gene, a cytochrome p450 isoform whose polymorphism is associated with Parkinson disease (32) and is found tightly associated with dopamine-containing cells in the substantia nigra (33). Accordingly, we observe a 2-fold decrease in the expression of *CYP2E1* in neurons treated with Tat in addition to an increase in miR-1 expression. One interesting target of miR-1 is the *Mef2A* gene. Recently, it was reported that Mef2A induces the expression of miR(379–410) cluster in neurons, and the expression of this cluster is important for dendritogenesis (34). The limited information available regarding the neuropathogenic properties of HIV-1 Tat and its *in vivo* effects gave us the rationale to examine whether Tat contributes to the development of HAND and the mechanisms involved and whether these mechanisms required miRNAs and their target genes.

EXPERIMENTAL PROCEDURES

Immunohistochemistry—Archival brain samples from HIV-1-infected patients with encephalitis, HIV-1-infected patients with no brain alterations, and normal control brains were obtained from the National NeuroAIDS tissue Consortium at the Manhattan Brain Bank, Mount Sinai School of Medicine, New York. The formalin-fixed and paraffin-embedded tissues, were sectioned at 5 μm thickness and placed on electromagnetically charged glass slides. Sections were deparaffinized in xylene and re-hydrated through descending grades of alcohol up to water. Immunohistochemistry was performed utilizing the avidin-biotin-peroxidase methodology, according to the manufacturer's instructions (Vector Laboratories Inc., Burlingame, CA). Our modified protocol included nonenzymatic antigen retrieval in citrate buffer, pH 6.0, for 30 min at 95 °C in a vacuum oven. After a 20-min cooling period, sections were rinsed with PBS, and endogenous peroxidase was quenched with 3% H_2O_2 in methanol for 30 min. Sections were then rinsed with PBS, and a blocking step was performed with normal horse serum at room temperature in a humidified chamber for 2 h. Primary antibodies were incubated overnight at room temperature and included a mouse monoclonal anti-HIV-1 Tat (1:100 dilution, clone 1102, specific for the N-terminal of Tat, Immunodiagnostics Inc., Woburn, MA) and a mouse monoclonal anti-HIV-1 p24 (1:25 dilution, Clone Kal-1, Novocastra Laboratories, Newcastle, UK). After rinsing with PBS, sections were incubated for 1 h at room temperature with biotinylated anti-rabbit or anti-goat secondary antibodies. The tissue was subsequently incubated with avidin-biotin-peroxidase complexes for 1 h at room temperature according to the manufacturer's instructions (Vectastain Elite ABC Peroxidase kit, Vector Laboratories), and finally, the sections were developed with a diaminobenzidine substrate (Sigma), counterstained with hematoxylin, and coverslipped with Permount (Fisher).

Tat Protein—Recombinant full-length Tat protein prepared from HIV-1 clade B was obtained from the AIDS Reagent Program at National Institutes of Health or purchased from Diatheva s.r.l. (Fano, Italy).

Calcium Measurement—The human neuroblastoma cell line (SH-SY5Y) (35) was grown in DMEM + 10% FBS on MatTek glass bottom plates treated with collagen (MatTek, Ashland, MA). SH-SY5Y was chosen because of its ability to mirror pathways involved in the neurodegenerative process associated with HIV-E (36, 37). The cells (5×10^5) were allowed to adhere and acclimate for 1 day prior to being treated with 10 mM retinoic acid (Sigma) for 4 days to induce differentiation. For calcium measurement and mobilization, the cells were loaded with 5 μM Fluo-4/AM (Invitrogen) for 30 min prior to Tat treatment. Tat was given at 10 ng/ml. Live calcium measurement was made for 2 min prior to Tat treatment and continued for 25 min using Zeiss 510 Meta confocal microscopy system.

MicroRNA Array—Neuronal cell line SY-SY5Y and primary human neurons (HN) (5×10^5) purchased from ScienCell Research Laboratories (Carlsbad, CA) were treated with Tat (10 ng/ml) for 24 h. Total RNA was isolated using TRIzol (Invitrogen). 750 ng of total RNA was used for miRNA array analysis using miRCURY LNA™ microRNA Array, version 11.0 (Exiqon, Woburn, MA). In brief, RNA was labeled using the miRCURY LNA™ miRNA, Hy5 power labeling kit (Exiqon) as per manufacturer's recommendations using Maui SC hybridization chambers (BioMicro Systems, Salt Lake City, UT). Array chips were scanned using Axon GenePix Scanner (Molecular Devices, Downingtown, PA) and Genepix 4000 image capture software (Molecular Devices, Downingtown, PA). Quantile normalization was used using JMP Genomics

(Cary, NC) at Harvard Catalyst-Laboratory for Innovative Translational Technology, and differentially regulated miRNAs were determined. Untreated cells were used as control.

Validation of miRNA by qPCR—miRNA validation was performed using on RNA extracted from primary fetal neurons as described above. First, miRNA cDNA synthesis was performed using Universal cDNA synthesis kit (Exiqon) followed by real time PCR for miR-1, miR-7, and miR-34a using SYBR Green master mix and Universal RT using miRNA specific microRNA LNA™ PCR primer sets (Exiqon).

Western Blot Analysis and Antibodies—Human neuronal SH-SY5Y cells (1×10^6) were treated with 10 pg/ml Tat protein for 24 h. Cells were then harvested and resuspended in lysis buffer as described by the manufacturer (Fermentas, MD). Western blotting (50 μg/sample) was performed on cell lysates 24 h post-transfection as described previously (38). Antibodies against IRBIT, SERP1, NEFM, Drp1, CREB, Bcl-2, STIM1, Orai, SIRT1, Grb-2, and Cox IV were used to detect the level of these proteins.

Genomic Gene Array and Data Analysis Methods—Gene expression profiling was performed using the Affymetrix GeneChip® HG-U133 set (Affymetrix, Santa Clara, CA), composed of two microarrays that represent greater than 33,000 of the best characterized human genes and almost a total of 45,000 probe sets. Labeling, hybridization, and detection of RNA were done at the Genomics Core Facility at Thomas Jefferson University and at Harvard Catalyst-Laboratory for Innovative Translational Technology according to recommendations by Affymetrix. Quality control measures were carried out to ensure that RNA isolated from frozen autopsy material was not degraded or lost. Housekeeping control genes and spike controls were routinely analyzed on the GeneChip® microarrays to confirm the successful labeling of target RNAs. Preliminary data analysis was performed in conjunction with the Thomas Jefferson or Harvard Universities Bioinformatics Program. GeneChip® array results were also correlated with the array design and annotation information using the NetAffx™ Analysis Center. Hierarchical cluster analysis and heat maps were generated using Spotfire DecisionSite 7.2.

p-miR-34a Luciferase Reporter Assay—p-miR-34a-Luc reporter plasmid was purchased from Signosis Inc. (Sunnyvale, CA). The transfection was performed as described previously (7) using SH-SY5Y cells untreated or treated with 10 pg/ml of Tat protein and/or transfected with anti-miR-34a. Luciferase assay was performed 24 h post-transfection.

Validation of miRNA Target Gene Expression—Real time PCR was performed using the following primers (purchased from IDT): 5′-GCCGCGAAGATGGTCGCCAA and 5′-TCGGGGGCATTTCTCGAGGTCT as forward and reverse primers for *SERP1*; 5′-TGGCGCAAGCTCTACTTG and 5′-GGCACTGAAGGCGATGAG as forward and reverse primers for *Orai*; 5′-GTTCTCAGGTGGACCATGTC and 5′-AGTTCTATCAGTGCCAAAGCC as forward and reverse primers for *IRBIT*; 5′-ACTTGACCTCCCTACTGGC and 5′-TCCTCTATCCCGTTGACACC as forward and reverse primers for *Drp-1*; 5′-AAGTGGGAAATGGCTCGTC and 5′-TCTAGTCTCTTCACCCTCCAG as forward and reverse primers for *NEFM*; 5′-GGCAGACAGTTCAAGTCCATG and 5′-CGCTTTGGGAATCAGTTACAC as forward and reverse primers for *CREB*; 5′-TTGTGTCTCCCTTGTCCATG and 5′-GGTCAAATCCCTCTGAGATCC as forward and reverse primers for *STIM1*; and 5′- CAGTGTCATGGTTCCTTTGC and 5′-GTTTCATGATAGCAAGCGGTTC as forward and reverse primers for *SIRT1*. Amplifications were performed on Mastercycler ep realplex Thermocycler (Eppendorf, Hauppauge, NY) using FastStart Universal SYBR Green master mix (Roche Diagnostics). PCR conditions for the amplifications were 95 °C for 5 min followed by 45 cycles of 95 °C for 30 s and 60 °C for 1 min. GAPDH was used as normalizing control using 5′-GCCTTCCGTGTTCCTACC and 5′-CCTCAGTGTAGCCCAAGATG as forward and reverse primers.

Double Labeling Immunofluorescence—Cells in culture were fixed for 3 min in 3% buffered formalin, rinsed with PBS, and blocked with normal horse serum for 1 h. A mouse monoclonal anti-MAP-2 antibody (1:100 dilution, Clone SMI-52, Covance, Berkeley, CA) was incubated for 2 h at room temperature, after which cells were incubated with a fluorescein-tagged anti-mouse antibody for 1 h. Cells were then blocked in normal goat serum and incubated with a rabbit polyclonal anti-CREB antibody (1:200 dilution, Cell Signaling) for hours. Finally, cells were incubated with a rhodamine-tagged anti-rabbit secondary antibody, coverslipped with an aqueous-based mounting media, and visualized with an inverted fluorescent antibody equipped with deconvolution software (Slide Book 5, Intelligent Imaging, CA).

Brain Tissues Prepared from Tat Transgenic Mice—Parental and Tat transgenic mice were described previously, in which *Tat* is under the regulation of the astrocyte-specific glial fibrillary acidic protein promoter and doxycycline, permitting expression mainly in astrocytes cells (13).

In Situ Hybridization (ISH)—miRCURY LNA™ microRNA detection (FFPE), optimization kit 4 (miR-124), and has-miR-34a detection probe (3′-amino-labeled) were purchased from Exiqon. The experiment was performed as suggested by the manufacturer using mice brain tissues (parental and Tat transgenic) or human brain tissues (brain samples from HIV-1-infected patient with encephalitis and normal control brains were obtained from the National NeuroAIDS Tissue Consortium).

Statistics—Statistical analyses were used in all the experiments using Student's *t* tests or unbalanced analysis of variance. Furthermore, each experiment was repeated three times, and five different cell fields were analyzed (each contains 10 cells). The results were considered statistically significant if $p < 0.05$.

RESULTS

Detection of HIV-1 Proteins p24 and Tat in Human Brain Samples—Radiologically, progressive HIV-1 dementia is characterized by cerebral atrophy with diffuse white matter hyperintensity signal change that could be observed in the bilateral peri-ventricular frontal and parieto-occipital regions as displayed in Fig. 1*A* (*right panel*) when compared with the normal control (*left panel*).

Several reports have described the ability of HIV-1-infected macrophages and microglial cells, which eventually release Tat into the extracellular brain parenchyma, where it is then taken up by noninfected cells, including the neurons (5, 39). Based on

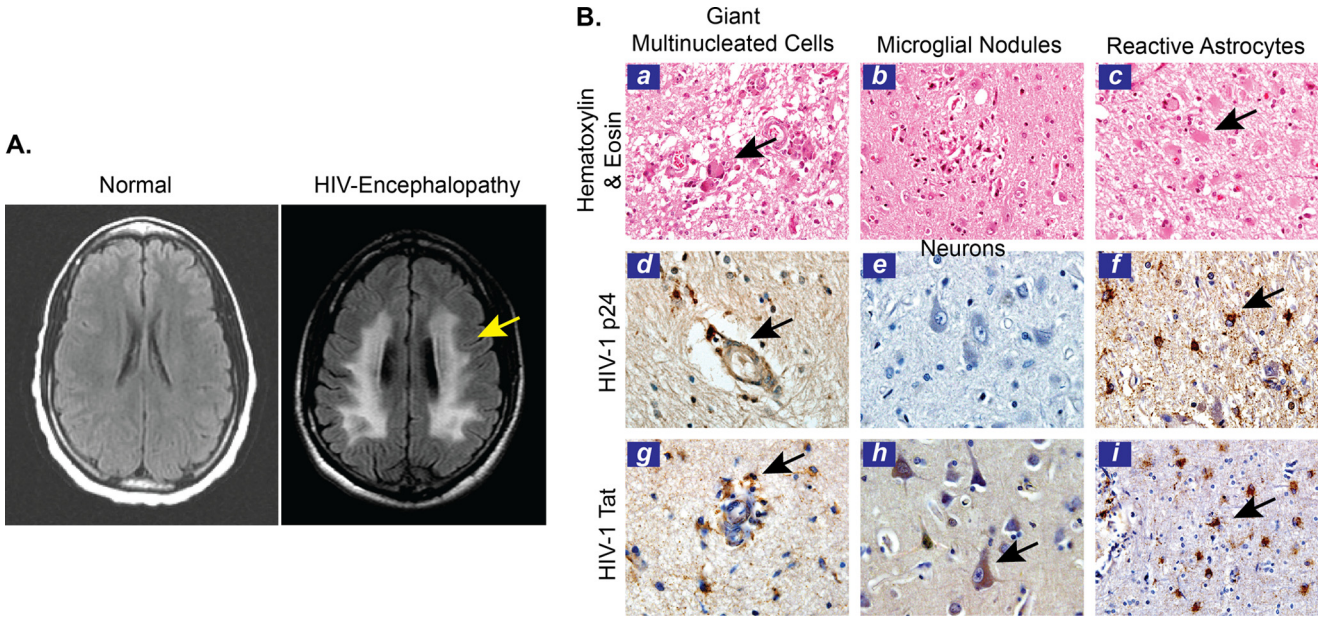

FIGURE 1. **Histopathological and immunohistochemical characterization of HIV encephalitis.** *A,* axial fluid attenuation inversion recovery and magnetic resonance images. Without focal brain parenchymal signal abnormalities in a normal individual (*left panel*) and demonstrating extensive and confluent increased T2 signal in the periventricular, subcortical, and deep white matter consistent with HIV leukoencephalopathy in patient with known HIV-1 infection (*right panel*) are shown. *B,* cases of HIVE were characterized by the presence of perivascular cuffs of inflammatory cells, parenchymal microglial nodules, and reactive astrocytes (*upper panels*, hematoxylin and eosin). The HIV-1 capsid protein p24 was robustly expressed by endothelial and perivascular inflammatory cells as well as astrocytes, indicating active viral infection, but was absent in neurons, cells that are not infected by HIV-1 (*middle panels*). Finally, the transactivator protein Tat was detected in endothelial and perivascular inflammatory cells, as well as astrocytes as expected, but it was also present in the cytoplasm of some and nuclei of other neurons within areas of encephalitis, suggesting that this protein was taken up by uninfected neurons. All panels have original magnification of ×600 except neuronal panels at ×1000.

these observations, we performed immunohistochemical experiments in cases of HIV encephalitis (HIVE) to determine the presence and cellular location of HIV-1 proteins. All cases of HIVE were characterized according to established standards (40) and presented perivascular cuff of inflammatory cells, including giant multinucleated cells (Fig. 1*B*, *panel a*), parenchymal microglial nodules (*panel b*), and reactive astrocytes (*panel c*). The HIV-1 capsid protein p24 was found expressed in the cytoplasm of endothelial cells and perivascular inflammatory cuffs (Fig. 1*B*, *panel d*), a well as reactive astrocytes (*panel f*), but it was completely negative in neurons (Fig. 1*B*, *panel e*), including neurons surrounded by robustly positive astrocytes (*panel f*). Finally, the transactivator protein Tat was detected in endothelial and perivascular inflammatory cells (Fig. 1*B*, *panel g*) and reactive astrocytes (*panel i*) as expected, but it was also present in neurons within areas of encephalitis (Fig. 1*B*, *panel h*). Studies involving the sera and cerebrospinal fluid samples from asymptomatic and AIDS patients also revealed the presence of extracellular Tat correlating with the degree of p24 antigenemia (data not shown).

Tat Induces Ca^{2+} Accumulation inside Cells—Next, we examined whether Tat has the ability to deregulate the neurons. For this purpose, we assessed the effect of extracellular Tat on calcium secretion status. To that end, human neuronal cells, SH-SY5Y (1×10^5) grown in serum-free media, were treated with 10 pg/ml of recombinant Tat protein for 25 min. The cells were incubated with Fluo-4/AM (41), and intracellular calcium (Fig. 2, *green*) was measured every 3 s using confocal microscopy. Interestingly, addition of Tat led to a dramatic increase of intracellular calcium $[Ca^{2+}]_i$ within seconds (Fig. 2) and generated a series of intracellular events that eventually drove the cells to death when compared with the control untreated cells. Our data regarding the ability of Tat to disturb calcium homeostasis corroborate with previous studies (42, 43).

Identification of miRNAs and Their Target Genes Regulated by Tat in HN and SH-SY5Y Cells—Involvement of miRNAs in neuronal degeneration and the ability of Tat to be taken up by neurons gave us the rationale to examine the status of miRNAs and their target genes in Tat-treated neurons. Neuronal cell line SH-SY5Y and primary human cultures of neurons were treated with 10 pg/ml Tat protein for 24 h after which total RNA was isolated and processed for miRNAs array assay. As shown in Fig. 3*A*, several miRNAs were differentially expressed in the presence of Tat protein suggesting that Tat affects the miRNA levels in these cells. We also observed the induction of numerous miRNAs, by Tat, with little or no information on their functions. Most striking is the miRNAs in the chromosome 19 clusters where we observed 14 miRNAs within the cluster being up-regulated. It would be interesting to know the nature of factors that would induce miRNAs expressions from this particular cluster as well as understanding their functions in the context of neuronal function. Similar results were obtained with primary human cultures of neurons (HN) (data not shown).

The levels of the most up-regulated miRNAs were then validated by qPCR in HN and SH-SY5Y cells treated with 10 pg/ml of recombinant Tat protein for 24 h. As shown in Fig. 3*B*, up-regulation of these miRNAs was at least 10-fold in Tat-treated HN cells compared with the control (set at 1). Interestingly, induction of these miRNAs in SH-SY5Y cells followed the same

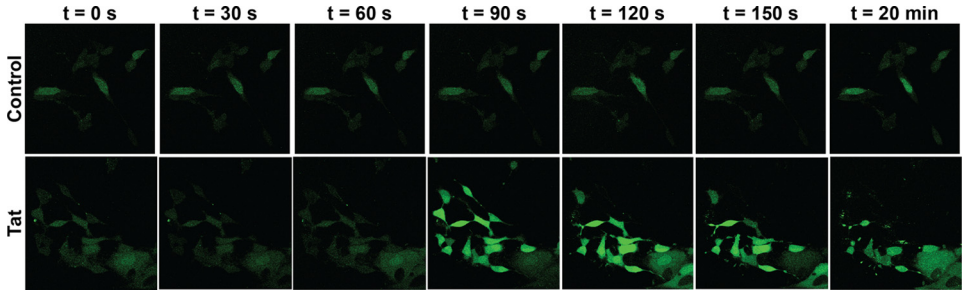

FIGURE 2. **Calcium levels in Tat-treated neurons.** Cells were incubated with Fluo-4/AM (Molecular Probes), and intracellular calcium (*green*) was measured every 3 s using confocal microscopy as described previously (41). The figure represents a montage of images collected from cells at $t = 0$ and 150 s with 30-s intervals and at $t = 20$ min. All measurements were performed using $n = 100$, and the results are statistically significant using Student's t test ($p < 0.05$).

A.

miRs upregulated in Tat-treated HN and SH-SY5Y (>2 fold)		
miR-1	miR-200a	miR-512-3p
miR-7	miR-302b	miR-515-5p
miR-34a	miR-302c	miR-518a
miR-34b	miR-369-5p	miR-518d
miR-96	miR-371-3p	miR-519b-3p
miR-137	miR-373	miR-519c
miR-141	miR-378	miR-520a
miR-142-3p	miR-383	miR-520b
miR-146a	miR-429	miR-520d
miR-147	miR-449a	miR-520e
miR-148a	miR-496	miR-520h
miR-182	miR-507	miR-592
miR-199a-3p	miR-509-3p	miR-603

miRs downregulated in Tat-treated HN and SH-SY5Y (< 2 fold)		
miR-33b	miR-508-3p	miR-497
miR-135b	miR-517c	miR-770-5p
miR-188-5p	miR-570	miR-E1183
miR-380	miR-579	

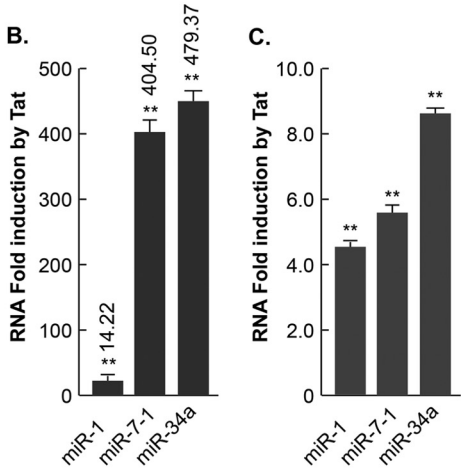

FIGURE 3. **miRNA levels in Tat-treated cells.** *A,* list of the miRNA expressions that are positively or negatively regulated ($2 <$ Tat $<$ 2-fold up) in Tat-treated SH-SY5Y cells and HN cells (data not shown). *B* and *C,* fold changes of three selected miRNAs in Tat-treated HN (*B*) and SH-SY5Y (*C*) cells compared with control (U6 set at 1) obtained by qPCR. The experiment was repeated three times, and the results are statistically significant using Student's t test ($p < 0.05$) as indicated by **; ** was compared with the *Mock* control group. Only 10 pg/ml of rTat were used for treatment.

profile observed in HN cells; however, this induction was between 5- and 10-fold (Fig. 3*C*).

Because Tat is not the only HIV-released viral proteins, we performed miRNA array using RNA isolated from SH-SY5Y cells treated with either Vpr or gp120 proteins (HIV-1_{JR-FL}) or with supernatant collected from HIV-1_{JR-FL}-infected U937 cells. miRs-1, -7, and -34a were among the most up-regulated miRNAs as confirmed by qPCR (data not shown).

Tat Induces Pri-miR-34a in SH-SY5Y Cells—To examine whether Tat induces these miRNAs via induction of transcription, we therefore analyzed the expression of pri-miR-34a using RNA isolated from Tat-treated SH-SY5Y cells. miRNAs are initially transcribed as long primary transcripts called pri-miRNAs that are processed in a series of endonuclease reactions to produce the mature miRNA species. Transcription of pri-miRNAs appears to be regulated by the same mechanisms that control mRNA expression. To that end, qPCR was performed using the following pri-miR-34a primers: forward, 5′-CGTCACCTCTTAGGCTTGGA-3′; and reverse, 5′-CATTGGTGTCGTTGTGCTCT-3′. *β-Actin* primers were used as a control: forward, 5′-TTCCAGCCTTCCTTCCTGG-3′, and reverse, 5′-TTGCGCTCAGGAGGAGCAAT-3′. As shown in Fig. 4*A*, Tat protein induces the pri-miR-34a when compared with the control untreated cells. The highest induction was observed at 48 h post-treatment. These results confirm the ability of Tat protein to deregulate the miR-34a at transcriptional levels.

Specificity of miR-34a Induction by Tat—It is well known that the specificity of miRNA is obtained when a specific miRNA targets its binding site at 3′UTR downstream of a luciferase RNA and represses the expression of luciferase enzyme activity. Therefore, we sought to validate our observations and determine the specificity of induction of miR-34a by Tat. To that end, we transfected SH-SY5Y cells with p-miR-34a-luciferase reporter plasmid as described previously (7). The cells were untreated, treated with 10 pg/ml Tat protein, or transfected with anti-miR-34a prior to the addition of Tat protein. As shown in Fig. 4*B*, rTat decreases the luciferase activity yet failed to do so in the cells transfected with anti-miR34a. These results confirmed the ability of Tat to induce miR-34a and allow it to bind to its binding site at 3′UTR of the luciferase vector.

Identification of Genes Regulated by Tat in Neurons—To further confirm the role of Tat in affecting neuronal deregulation, we also performed gene array assay. Because miRNA regulates mRNA levels of its target genes, increased expression of certain

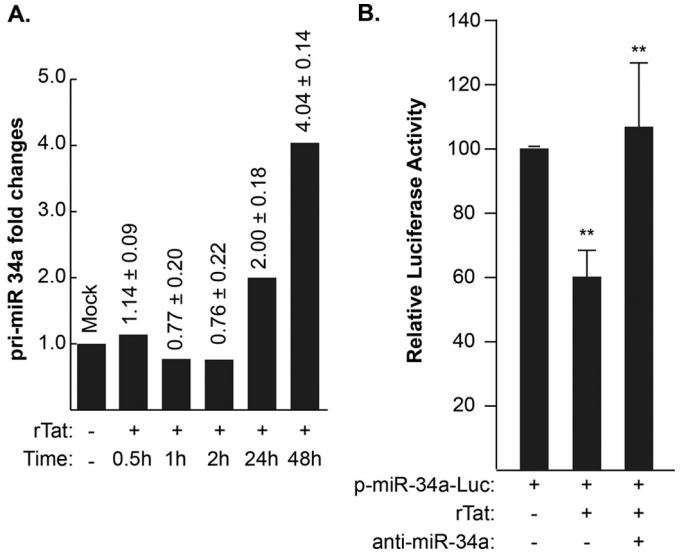

FIGURE 4. **Specificity of miR-34a induction by Tat protein.** *A,* induced pri-miR-34a expression in SH-SY5Y cells. The pri-miR-34a was significantly increased in Tat-treated cells at 48 h as measured by qPCR when compared with the mock. Each sample was performed in three separate experiments. *B, histogram* displays the luciferase activity as measured in SH-SY5Y cells transfected with 10 ng of p-miR-34-Luc reporter plasmid alone or in the presence of anti-miR-34a as indicated. The cells were untreated or treated with 10 pg/ml rTat protein. The experiment was repeated six times, and the results are statistically significant using Student's *t* test ($p < 0.0002$) as indicated by **; ** was compared with the *Mock* control group.

miRNAs is expected to be associated with a decrease in the level of its target gene(s). For gene array assay, mRNAs were prepared from SH-SY5Y cells untreated or treated with 10 pg/ml Tat protein for 24 h. We then investigated the levels of several selected genes involved in neuronal regulation and also known to be miR-1, miR-7, and miR-34a/b targets. Interestingly, gene array assay showed that the levels of the selected genes decreased by at least 2-fold, whereas the levels of their associated miRNAs increased in Tat-treated cells compared with the mock untreated (Table 1). Functions of the genes are listed (Table 1, right column). These results further supporting the hypothesis that Tat is involved in neuronal deregulation. Similar results were obtained with HN cells treated with Tat (data not shown). Note that Table 1 does not include all the regulated genes.

Validation of Target Genes Expression by qPCR—Next, we sought to validate our data using SH-SY5 cells (5×10^5) that we treated with 10 pg/ml Tat protein for 24 h. Total RNA was prepared from one set (set 1) and processed for quantitative PCR, and protein extracts were prepared from the second set (set 2) and used for Western blot analysis.

The status of nine target genes (*IRBIT, SERP1, NEFM, Drp1, CREB, Bcl-2, STIM1, Orai,* and *SIRT1*) that are known to be targets of miRs-1, -7, and -34a was examined. These genes are known to be involved in neuronal regulation. For example, it has been shown that dephosphorylation and inhibition of the IRBIT released with inositol 1,4,5-trisphosphate leads to Ca^{2+} release from the ER and causes aberrant neuronal regulation (49), whereas the stress-associated endoplasmic reticulum protein 1 (SERP1) has been shown to stabilize ER membrane proteins during stress (50) and protects unfolded target proteins against degradation during ER stress. Similarly, maintaining the proper axon diameter size by NEFM is essential for the conduction of nerve impulses (51). Furthermore, distribution of the mitochondria inside the axon is essential to control ATP levels and cell-to-cell communication. The NEFM polypeptide (52) protein and the Dynamin-related protein (Drp-1) (53) are the main proteins involved in these events. Furthermore, ER stress activates CREB through TOX3, and CREB is a target gene of miR-34a and is involved in neurite growth and synaptic plasticity (54, 55). Phosphorylated CREB has been shown to protect the neuron by regulating the Bcl2 and brain-derived neurotrophic factor (a gene involved in synaptic plasticity) promoters (56, 57). Moreover, STIM1 functions as a calcium sensor in the ER (58, 59). Upon activation of the inositol 1,4,5-trisphosphate receptor, the Ca^{2+} concentration in the ER decreases, which is sensed by STIM1 and leads to the activation of the "store-operated" Orai Ca^{2+} ion channels in the plasma membrane, via protein-protein interaction (60). Finally, it has been shown that activation of miR-34a inhibits SIRT1, a gene that regulates synaptic plasticity and cellular senescence. Activation of SIRT1 deacetylates and stabilizes p53 and thus prevents cell death (46).

As shown in Fig. 5, the RNA and protein levels of these genes decreased, as measured by qPCR (*A*) and Western blot (*B*), respectively, in Tat-treated cells when compared with the controls untreated. These results suggest that Tat affects ER stress, ER Ca^{2+} release, mitochondrial distribution, and synaptic plasticity leading to neuronal deregulation.

To further validate our observations, we performed an immunohistochemistry assay using brain tissues prepared from control/parental or Tat transgenic mice. As shown in Fig. 5*C*, Tat reduces the levels of CREB protein (rhodamine) and promotes its translocation to the nucleus of the cells when compared with the control. MAP-2 (fluorescein) was used to demonstrate the neuronal nature of the cells and DAPI (Fig. 5*C*, *blue*) was used to highlight the nuclei.

Effect of Tat Is miRNA-dependent—Induction of the specific miRNAs by Tat and inhibition of their target genes prompted us to examine the status of the target genes in the absence of their associated miRNA. SH-SY5Y cells were transfected with anti-miR-34a for 24 h and then treated with 10 pg/ml Tat protein. Total RNA was isolated, and the levels of selected genes that are regulated by miR-34a were examined by qPCR. As shown in Fig. 6*A*, Tat decreased the mRNA levels of these genes (*red*) in the presence of miR-34a but not in its absence (*yellow*). Interestingly, the RNA levels of these genes did increase in cells transfected with anti-miR-34a (Fig. 6*A*, *green*), which corroborate with previous data regarding the ability of anti-miR34a to rescue the cells from apoptosis and to up-regulate miR-34a target genes (62). These results placed miR-34a as a major player involved in neuronal deregulation once induced by Tat.

To further confirm this hypothesis, we performed ISH assay to examine the status of miR-34a *in vivo* in the brain of Tat transgenic mice. ISH of brain tissues prepared from control or Tat transgenic mice showed that Tat increases the levels of miR-34a *in vivo* when compared with the control parental mice (Fig. 6*B, arrows*).

It is well know that only neurons in the proximity of infected astrocytes or microglia are lost. Therefore, we hypothesize that

TABLE 1

Tat regulates the level of genes involved in neuronal regulation

Table represents some of the cellular genes that are differentially expressed (suppressed) in Tat-treated neurons and involved in neuronal regulation and their associated miRNAs.

miRs	Genes (Ref.)	Genes names	Functions
miR-1	*SERP-1* (44)	Stress-associated endoplasmic reticulum protein 1	Stabilizes ER membrane during stress
miR-7	*NEFM* (45)	Neurofilament medium polypeptide	Nerve functions and maintains axonal diameter
	Drp-1	Dynamin-related protein	Mitochondrial distribution inside the axon and division
miR-34a/b	*CACNB1*	Voltage-dependent L-type Ca^{2+} channel subunit $\beta 1$	Controls Ca^{2+} release
	SIRT-1 (46)	Sirtuin	Synaptic plasticity and neuron protection
	STIM1 (47)	Stromal interaction molecule 1	Controls Ca^{2+} influx
	CREB (48)	cAMP-response element-binding	Transcription factor
	IRBIT	Inositol 1,4,5-trisphosphate receptor-binding protein released with inositol 1,4,5-trisphosphate	Control Ca^{2+} release from the ER

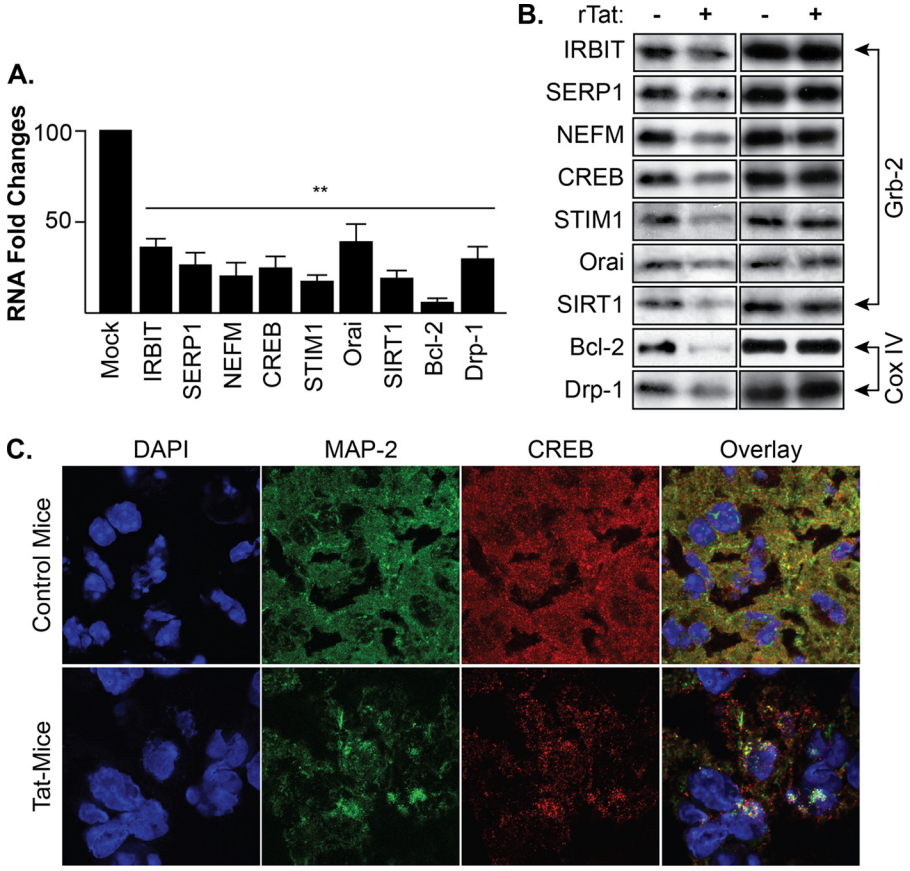

FIGURE 5. **miRNAs and their target genes levels in Tat-treated cells.** *A* and *B,* qPCR and Western blot analysis point to the ability of Tat to decrease the RNA and proteins levels of the indicated genes. Anti-IRBIT, -SERP1, -NEFM, -Drp1, -CREB, -Bcl-2, -STIM1, -Orai, -SIRT1, -Grb-2, or -Cox IV antibodies were used as indicated. Fold changes are displayed as *histograms*. The experiment (qPCR) was repeated three times, and the results are analyzed by Student's *t* test statistical significance level; $p < 0.05$ is indicated by ** compared with the mock control group (one-way analysis of variance test). *C,* distribution of CREB protein (*red*) in the brain of Tat transgenic mice compared with the control parental mice. MAP-2 (*green*) and DAPI (*blue*) staining were used as a control.

if induction of miR-34a is specific, its levels should remain normal in unaffected neurons. To confirm this hypothesis, we examined the levels of miR-34a in the brain tissue of an HIV-1-infected patient with encephalitis where the affected neurons are lost. Interestingly, the level of miR-34a did not change or modestly decreased in the examined tissue when compared with the control tissue as detected by ISH (Fig. 6*C, dark spots*), which confirms our hypothesis and the specificity of miR-34a induction. Interestingly, induction of miR-34a in the brain tissue of Tat transgenic mice was significantly high and comparable with the data obtained by qPCR using SH-SY5Y (compare *B* in Figs. 3 and 6). However, this was not the case when using human brain tissues. We believe that the presence of additional factors such as viral proteins (*e.g.* Vpr, gp120; and Nef) and/or toxins (*e.g.* TNF-α) neutralize the effect of Tat and keep the induction of miR-34a under control (Fig. 6*C*).

Validation of Data in HN Cells—Finally, we validated our data in primary human cultures of neurons (HN). The cells were treated with 10 pg/ml of Tat protein for 3 h. RNA was isolated and used for qPCR assay. The mock (untreated cells) and GAPDH were set at 1. As shown in Table 2, the numbers represent the fold decrease of the selected genes following treatment of HN cells with Tat protein, which corroborate with our data obtained with neuronal cell line, SH-SY5Y. These results also prove that SH-SY5Y cell line is a good model to be used in future studies.

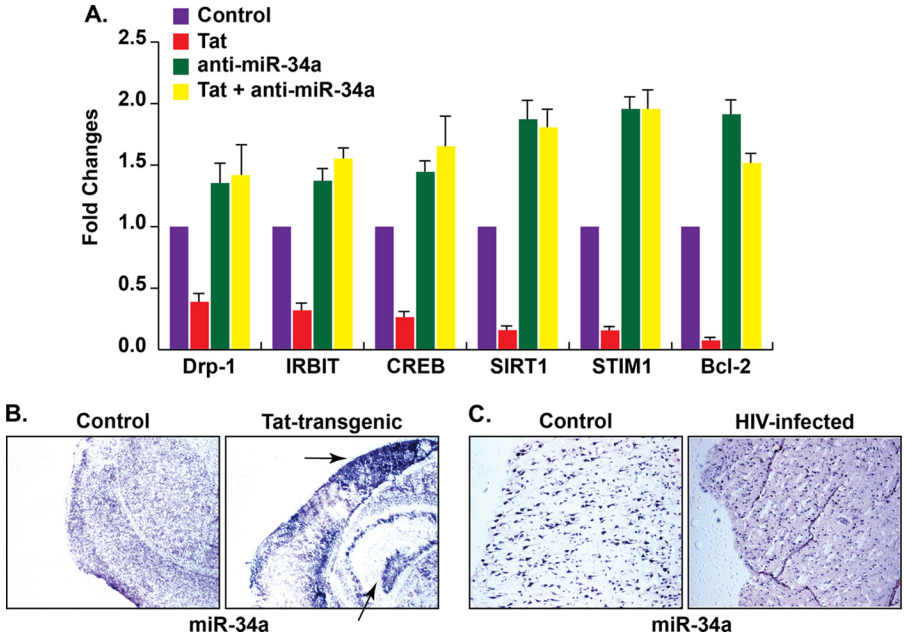

FIGURE 6. **Tat failed to affect selected genes in the presence of anti-miR-34a.** *A,* qPCR results point to the ability of Tat to decrease the RNA levels of the selected genes in the absence of anti-miR-34a (*red*) but not in its presence (*yellow*) as indicated. Fold changes are displayed as *histograms*. The experiment was repeated three times, and the results were analyzed by Student's *t* test statistical significance level, $p < 0.05$. *B* and *C,* distribution of miR-34a in the brain of Tat transgenic mice (*arrow*) compared with the control mice (*B*) or in human brain tissue of an HIVE patient (*C*). miR-34a localizes more to the cortical region in the mice.

TABLE 2

Effect of Tat on selected genes in HN cells

qPCR results point to the ability of Tat to decrease the RNA levels of several selected genes in HN cells as indicated. The numbers represent the fold decrease of the selected genes. The experiment was repeated three times, and the results were analyzed by Student's *t* test statistical significance level, $p < 0.05$.

Gene	HN cells
IRBIT	0.436 ± 0.044
SERP1	0.322 ± 0.042
NEFM	0.234 ± 0.019
CREB	0.379 ± 0.043
STIM1	0.251 ± 0.035
Orai	0.611 ± 0.053
SIRT1	0.339 ± 0.048
Bcl-2	0.103 ± 0.006
Drp-1	0.447 ± 0.061

DISCUSSION

In this study, we demonstrated the ability of HIV-1 Tat to cause neuronal deregulation through a miRNAs-dependent pathway. First, we identify the miRNAs and their target genes (with implications in neuronal physiology) affected by the presence of Tat in human neurons (primary cultures and cell line), and later we validated the levels of these miRNAs and genes by qPCR, Western blot, and immunohistochemical and *in situ* hybridization assays.

Tat has been shown to causes neuronal death through a pathway that implicates TNFα and NF-κB (63) or through its interaction with GSK-3β (64). Furthermore, Tat was shown to deregulate neuronal calcium homeostasis, a phenomenon that also leads to neuronal death (42, 65, 66). Tat was also shown to promote cell death through mitochondrial hyperpolarization (18, 43). In addition to the mentioned studies, Tat was shown to up-regulate the levels of p53 and p73, a phenomenon that also leads to neuronal death (7). Conversely, Tat was shown to protect the cells from apoptosis through induction of neuronal differentiation and survival by NGF pathway (67).

Similarly, small RNA in general and miRNAs in particular have been described to be involved in neuronal deregulation and in the development of neurodegenerative diseases. In this regard, miRNAs have been shown to be involved in psychiatric disorders (68) such as schizophrenia and bipolar disorders (69). Furthermore, the link between miRNAs and neurodegenerative diseases such as Alzheimer, Huntington, and Parkinson diseases is becoming increasingly evident (2, 70). miRNAs were also shown to be involved in the pathogenesis of neuroblastoma and astrocytoma (71, 72). Strikingly, miR-34a is among the miRNAs involved in these events. Identified by Welch *et al.* (73), miR-34a was shown to be involved in cell cycle progression, cellular senescence, and apoptosis; however, it is unknown what all its targets are in mediating such functions. Among known miR-34a targets, *p53* and *SIRT1* genes were the most studied and shown to be involved in apoptosis or cell survival (59). miR-34a was recently shown to behave like a tumor suppressor in brain tumors and glioma stem cells (74). In addition to its involvement in tumors and neurodegenerative diseases, miR-34a was also shown to play a role in psychiatric problems. In this regard, miR-34a was described to be one of the miRNAs involved in mood disorders (68), schizophrenia, and bipolar disorders (69). miR-34a was also shown to be functionally linked to Bcl2 down-regulation by preventing the phosphorylation of CREB even though no miR-34a seeds exist in these genes (55), which corroborate our data. Finally, Tat mediated down-regulation of CREB has been shown to accompany the prevention of CREB phosphorylation through the PI3K pathway (75).

Interestingly, although we demonstrated that the level of miR-34a increases in Tat-treated neurons and in neurons

treated with supernatant prepared from HIV-1-infected U-937 cells, this level decreases in peripheral blood mononuclear cells isolated from HIV-1-infected patients (26). The difference is mainly due to the fact that peripheral blood mononuclear cells, and not neurons, can be infected with HIV-1. Therefore, we established the relation between the microRNAs (mainly miR-34a), Tat, and neuronal regulation as demonstrated by Tat-dependent induction of miR-34a as shown in Fig. 4 and indirectly the inhibition of miR-34a target genes by Tat. Such down-regulation of miR-34a target genes would likely lead to physiological changes in neurons that in turn would cause neuronal deregulation, neuronal loss, and eventually the development of HAND. We also demonstrated that such a phenomenon could be prevented in the presence of anti-miR-34a. These results further confirm our hypothesis regarding the involvement of miRNAs in neurodegenerative diseases, and further elucidation of Tat-induced gene regulation may provide novel explanation regarding the development of HAND.

Alternatively, our results raised a challenging question regarding the ability of HIV-1 proteins (*e.g.* Tat) to play a role in neuronal deregulation in the highly active antiretroviral therapy (HAART) era. The answer to this highly challenging question and the pathways involved in the development of HAND, besides the one studied here, remain unclear. Nevertheless, there are compelling neuropathological data showing that the HAND disease process occurs with the ongoing presence of virus, and despite the therapy, HAND remains very prevalent (76–78). However, in a recent study, it was described that HIV-1 infects multipotent hematopoietic stem and progenitor cells. These cells allow the virus to hide and to be reactivated and re-infect additional cells even in the highly active antiretroviral therapy (HAART) era (79). Although the reasons for the reactivation of latent viruses are unclear, it was described that deregulation of miRNAs could lead to latent HIV reactivation (78). As a result, it is expected that viral proteins released by latent reactivated viruses continue to play a role along with miRNAs in the development of HAND.

In this regard, an important question remains to be answered. How does Tat deregulate the miRNAs and what is/are the mechanism(s) involved? The answer to this question remains unclear. However, it is well described that HIV-1 benefits from deregulating the miRNAs of the host cell (26). This theory is supported by the fact that miRNA-processing enzymes Drosha and Dicer are silenced to reduce generation in the cell of mature miRNAs (Ref. 26 and references within). This phenomenon can lead to a robust HIV-1 replication and preventing some miRNAs (*e.g.* miR-150 or miR-223) from silencing, reducing, or even delaying viral replication (23). This observation corroborates with the results published by Houzet *et al.* (26) where they showed the down-regulation of these two miRNAs in T-cells. Furthermore, viruses such HIV-1 may use an alternative mechanism involving miRNAs to better replicate. For example, the HIV-1 RNA structure TAR has been reported to be processed by Dicer to release miRNAs that could be involved in chromatin remodeling (Ref. 61 and references within). Because neurons do not support viral replication, the above-described mechanisms cannot explain the pathway used by Tat to deregulate the miRNAs, and therefore additional studies are required to decipher these mechanisms even though our data shown in Fig. 4*A* support the idea that Tat induces transcription of these miRNAs and that induction of miR34a by Tat is specific as shown in Fig. 4*B*.

In summary, although we demonstrate that Tat is able to affect neuronal dysfunction by altering miRNA expressions, the pathway used by Tat needs to be further evaluated. It may be that the increased expression of miR-34a may simply be the result of Ca^{2+} mobilization because this mobilization mediates numerous signaling pathways. To clarify this hypothesis, we examined the target genes involved in calcium homeostasis and deregulated in neurons in the presence of Tat (data not shown). Gene array data point to the ability of Tat to affect the expression levels (positively or negatively) of several genes involved in calcium homeostasis, further demonstrating the role Tat plays in altering homeostasis/mobilization of calcium in the cell. These results led us to suggest the presence of a tight link between Tat calcium release and the development of HAND. However, considering that several studies have shown how Tat can also affect signal transduction pathways, elucidating the exact pathway that soluble Tat hijacks in neurons may prove useful in understanding HAND and may provide novel therapeutic targets.

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