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Nrf2-Mediated System x_c^- Activation in Astroglial Cells Is Involved in HIV-1 Tat-Induced Neurotoxicity

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Abstract

HIV-associated neurocognitive disorders (HANDs) affect a large part of HIV-infected patients, despite highly active antiretroviral therapy. HANDs occur in the absence of a direct infection of neurons. Nevertheless, viral proteins (e.g., Tat) are capable to cause neuronal dysfunction via oxidative stress, but the cellular pathways leading to HANDs are not yet fully defined. Here, we investigated the effects of Tat on Nrf2-mediated antioxidant response and system x_c^- expression in U373 human astroglial cells. Moreover, the effect of Tat-producing astrocytes on neuronal cell viability was assessed using SH-SY5Y cells as a culture model. We demonstrated that Tat produced by astrocytes was able to induce Nrf2 activation and system x_c^- expression in astrocytes, thus reducing cell viability of co-cultured neuronal cells. Furthermore, sulfasalazine, a specific system x_c^- inhibitor, was able to reduce extracellular glutamate and to prevent the reduction of neuronal viability, thus demonstrating that the neurotoxic effect was dependent on an increased glutamate release through the transporter. Our findings provide evidence of the involvement of astroglial Nrf2/system x_c^- pathway in the neurotoxicity induced by HIV-1 Tat protein, thereby suggesting how astrocytes may exacerbate neurodegeneration through the conversion of an antioxidant response to excitotoxicity.

Keywords Astrocyte · Glutamate · HANDs · HIV-1 Tat · Nrf2 · System x_c^-

Introduction

HIV-related diseases continue to be a severe public health problem worldwide, with around 40 million people being infected with HIV. Despite highly active antiretroviral therapy, in the late phase of HIV infection, a subset of patients develops neurological complications collectively termed as HIV-associated neurocognitive disorders (HANDs) [1, 2]. Several mechanisms have been proposed to explain why and how low level of viral replication may lead to neurocognitive disorders in the absence of direct infection of neurons [3, 4]. One likely possibility is that glial cell activation and neurotoxicity may arise because of the effects mediated by viral proteins as gp120 and Tat [3, 5]. Note that antiretroviral drugs do not affect Tat availability in infected patients and Tat it is found in the cerebrospinal fluid (CSF) and infiltrating brain macrophages, in treated HIV patients with very low blood viral load [6–8]. Tat can directly interact with neurons after being released by infected macrophages or glia into the extracellular space in the brain [9]. As we previously demonstrated in neuronal SH-SY5Y cells [10], Tat can lead to reduced cell viability eliciting H_2O_2 production by a mechanism involving both polyamine metabolism and N-methyl-D-aspartate (NMDA) receptor activation. However, Tat also induces neuronal loss indirectly at a distant site and, in the meantime, supports the survival of infected astrocytes [11]. Among other HIV-1 proteins, Tat elicits oxidative stress conditions leading to intracellular glutathione decrease in brain endothelial cells and different cell types, including neuronal cells [10, 12]. During oxidative stress conditions, various cell types can upregulate the activity of nuclear factor erythroid 2-related factor 2 (Nrf2), the main orchestrator of the antioxidant response, thus becoming able to counteract intracellular reactive oxygen species (ROS) accumulation and glutathione (GSH) depletion [13]. Astrocytes are more resistant to oxidative insults than isolated neurons; indeed, unlike neurons they are able to strongly upregulate Nrf2-mediated gene expression [14]. Upon modifications in cellular redox state, Nrf2 migrates to the nucleus and sequentially binds to promoter regions,

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known as antioxidant response element (ARE), of many phase II detoxifying and antioxidant genes such as system x_c^- subunit xCT, γ-glutamyl-cysteine ligase (GCL), superoxide dismutase (SOD), glutathione peroxidase (GPX), and heme-oxygenase-1 (HO-1). System x_c^- is an amino acid antiporter that mediates the exchange of extracellular L-cystine and intracellular L-glutamate across the plasma membrane. While the import of L-cystine through this transporter is critical to glutathione production and protection from oxidative stress in astrocytes, the export of glutamate represents a further route of release through which this neurotransmitter may cause excitotoxicity. Thus, system x_c^- has currently been linked to both physiological and pathological processes in the CNS [15]. Although the induction of Nrf2-driven gene expression has been widely indicated as a protective mechanism to counteract the effects of oxidative stress, in many cell types such as astrocytes, the upregulation of xCT elicited by Nrf2 could be a potential source for excitotoxicity due to excessive glutamate release [16]. Despite a lot of experimental and clinical findings indicate astrocytes as the cell population responsible for the bulk of the neuronal death in many neurodegenerative diseases, the cellular pathways leading to such a damage, particularly in HANDs, are not yet clearly defined. Here, we investigated the effect of Tat on Nrf2 activation and system x_c^- expression in human astroglial cells and studied the effects of Tat-expressing astrocytes on neuronal cell viability.

Materials and Methods

Materials

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), 0.25% Trypsin–EDTA solution, gentamicin solution 50 mg/ml, sulfasalazine (SSZ), a specific system x_c^- inhibitor, and MTT assay kit were obtained from Sigma–Aldrich (Milan, Italy). Bradford reagent was obtained from Bio-Rad Italia (Milan, Italy). All chemicals were of analytical or reagent grade and were used without further purification. ARP6017 HIV-1 Tat-B recombinant protein (101 aa), anti-Tat antibody (EVA 3069.1 Mab to HIV-1 Tat Fit Biotech), and pC63.4.1 plasmid were obtained from the Centre for AIDS Reagents, NIBSC HPA UK, supported by the EC FP6/7 Europrise Network of Excellence, the NGIN Consortia, and the Bill and Melinda Gates GHRC-CAVD Project. ARP697 was donated by FIT Biotech, Estonia, Dr. J. Karn.

Construction of Plasmid

The Tat gene encoding 86 amino acids was amplified from the pC63.4.1 plasmid using the following degenerate primers: 5′-GACGAATTCACCATGGARCCRGTRGATCC (forward) and 5′-TTCCKTCGGGCCKGTCGG (reverse). The PCR product was cloned into a pGEM-T Easy vector, finally inserted into the pcDNA3.1 at the EcoRI/KpnI restriction sites. The resulting construct, useful for expression in mammalian cells, was designated pcDNA3.1-Tat, as shown in the supplementary Fig. S1 (Online Resource). The correct orientation and the integrity of inserted Tat cDNA were verified on the purified plasmid by Sanger sequencing, performed by Eurofins GATC Genomics. For the nucleotide sequence, see Table 1. The functional activity of Tat was confirmed using a transactivation assay in IG5/LTR-luciferase cells containing an HIV-1 LTR-luciferase construct [10, 17].

Tat Transactivation Assay

To verify the functionality of Tat codified by the construct pcDNA3.1-Tat that has been used for transfection of U373 cells, we performed a transactivation assay based on luciferase activity in 1G5 clonal cell line. 1G5 cells harboring a stable integrated HIV-LTR luciferase reporter system were used to analyze the Tat-mediated transactivation of HIV-LTR. 1G5 cells were transfected with pcDNA3.1-Tat plasmid using Lipofectamine 3000. Then, cells were further incubated for 24 h. Cell lysates were subjected to the luciferase assay, and LTR-dependent luciferase expression was analyzed using a luciferase assay kit as indicated by manufacturer's instructions (Promega Italia Srl, Milan, Italy). Additional data are given in supplementary Fig. S2 (Online Resource).

Cell Cultures, Treatments, and Transfection

U373-MG human glioblastoma astrocytoma cells and SH-SY5Y human neuroblastoma cells were purchased from ATCC (Manassas, VA, USA). Cells were cultured in DMEM supplemented with 10% FBS, 2 mM L-glutamine, and 40 μg/ml gentamicin at 37 °C in a humidified 5% CO_2 incubator. Confluent monolayers of U373 cells were subcultured by conventional trypsinization. For the experiments, 3×10^5 or 2×10^6 cells were seeded in 35- or 100-mm tissue culture dishes, respectively, and grown up to 80% confluence for 18–24 h before treatments. Working solutions of Tat were freshly prepared in culture medium from stock solutions stored at − 80 °C. Where indicated, SH-SY5Y cells were treated with 200 ng/ml HIV-1 Tat recombinant protein in serum-free DMEM. For transfection, 4×10^5 U373 cells were seeded in 60 mm dishes and transfected with Lipofectamine 3000

Table 1 The nucleotide sequence of pCDNA3.1-Tat

ATGGAGCCGGTAGATCCACGCCTCGAACCGTGGAAACA
TCCCGGCTCTCAGCCGAAAACCGCG
TGCACTACCTGCTATTGTAAAAAGTGTTGCTTTCACTG
CCAGGTTTGCTTCACCACTAAAGCCCT
GGGTATCTCCTACGGCCGCAAGAAACGCCGACAACGAC
GCCGTCCGCCTCAGGGCAGCCAGAC
TCACCAAGTTTCTCTGTCCAAACAGCCGACGTCCCAGC
CACGCGGTGACCCGACCGGCCCGACGGAATAG

(Invitrogen) and with 1 μg of pcDNA3.1 or pcDNA3.1-Tat, thus obtaining two stable-transfected cell lines, termed U373-mock and U373-Tat, respectively. After 48 h, the transfected cells were selected adding to the DMEM medium 400 μg/ml of G418 (Geneticin, Sigma). For the maintenance of transfected cells in culture, 200 μg/ml of G418 was used.

MTT Assay

For the experiments to test neuronal viability and glutamate release, transfected-U373 and SH-SY5Y cells were co-cultured in transwell culture system (Falcon® cell culture inserts 0.4 μm pore size). For each sample, 1.5×10^4 neuronal cells were seeded in transwell insert and 3×10^4 astroglial cells were plated in the lower compartment of six-well plate and allowed to grow for 24, 48, 72, and 96 h. Then, at the end of the incubation periods, the viability of SH-SY5Y cells was analyzed by MTT assay.

MTT assay was performed as indicated by manufacturer's instructions on SH-SY5Y cells at the end of each incubation period. Briefly, MTT solution (stock solution of 0.5 mg/ml) was added to the cell culture at the final concentration of 10%. After incubation at 37 °C for 4 h, formazan crystals were dissolved in lysis buffer (4 mM HCl, 0.1% NP40 (*v*/*v*) in isopropanol) and the optical density (O.D.) of each sample was measured using a microplate reader at 570 nm (BioTek ELx800 Absorbance Microplate Reader, Winooski, VT, USA). Cell viability of SH-SY5Y co-cultured with U373-Tat was calculated relative to the viability of neuronal cells co-cultured with U373-mock cells used as control.

Quantitative Real-Time Reverse Transcription–Polymerase Chain Reaction

Total RNA was purified by using TRIzol Reagent (Life technologies Italia-Invitrogen, Monza, Italy) and reverse transcribed into cDNA with GoTaq 2-step RT-qPCR system (Promega Italia Srl, Milan, Italy). cDNA was amplified for the following genes: NAD(P)H:quinone oxidoreductase type 1 (NQO1), catalase (CAT), superoxide dismutase (SOD1), (SOD2), glutathione peroxidase (GPX), system x_c^- (xCT subunit), and glutamate-cysteine ligase (GCLC). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA was examined as the reference cellular transcript. The sequences of primers were reported in Table 2. PCR product quantification was calculated by applying the SYBR-Green method. Reactions were performed in a Rotor gene 6000 machine (Corbett research) using the following program: 45 cycles of 95 °C for 15 s, 60 °C for 60 s, 72 °C for 20 s. GAPDH mRNA amplification products were present at equivalent levels in all cell lysates. The data are calculated relative to the internal housekeeping gene according to the second derivative test (delta–delta Ct (2-ΔΔCT) method).

Table 2 Sequences of primers used for real-time quantitative PCR

Gene	Nucleotide sequence
NQO1	Forward 5′- ATG TAT GAC AAA GGA CCC TTC C -3′
	Reverse 5′- TCC CTT GCA GAG AGT ACA TGG -3′
CAT	Forward 5′- TCA GGT TTC TTT CTT GTT CAG -3′
	Reverse 5′- CTG GTC AGT CTT ATA ATG GAA TT -3′
SOD1	Forward 5′- AGT AAT GGA CGA GTG AAG G -3′
	Reverse 5′- GGA TAG AGG ATT AAA GTG AGG A -3′
SOD2	Forward 5′- AAT GGT GGT GGT CAT ATC A -3′
	Reverse 5′- CCC GTT CCT TAT TGA AAC C -3′
HO-1	Forward 5′- CGG GCC AGC AAC AAA GTG -3′
	Reverse 5′- AGT GTA AGG ACC CAT CGG AGA A -3′
GPX3	Forward 5′- CAT TCG GTC TGG TCA TTC TG -3′
	Reverse 5′- CCT GGT CGG ACA TAC TTG A -3′
System X_c^-	Forward 5′- GGT GGT GTG TTT GCT GTC -3′
	Reverse 5′- GCT GGT AGA GGA GTG TGC -3′
GCLC	Forward 5′- TTG CAA AGG TGG CAA TGC -3′
	Reverse 5′- GAA ACA CAC CTT CCT TCC -3′
GAPDH	Forward 5′- TTG TTG CCA TCA ATG ACC C -3′
	Reverse 5′- CTT CCC GTT CTC AGC CTT G -3′

Preparation of Nuclear and Total Extracts

After treatments at the indicated time points, the cells were mechanically detached with a scraper in cold PBS. Nuclear extracts were prepared by adding buffer A (10 mM Hepes, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.1% NP40, protease inhibitor cocktail) to the cell pellets to separate nuclei from cytosol. After incubation for 10 min on ice and subsequent centrifugation at 12,000 rpm for 10 min at 4 °C, pellets containing nuclear fractions were resuspended in buffer C (20 mM Hepes pH 7.9, 420 mM NaCl, 1.5 mM $MgCl_2$, 25% glycerol, 1 mM EDTA, 1 mM EGTA, 0.5 mM DTT, 0.05% NP40, 1 mM PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin) and incubated on ice for 30 min. A final centrifugation at 14,000 rpm was carried out, and the supernatants were collected, quickly frozen in liquid nitrogen, and stored at − 80 °C. The total protein content of nuclear extracts was determined according to Bradford method [18]. To show the quality of the nuclear separation, both nuclear and cytosolic fractions were blotted for the nuclear marker lamin A and cytoplasmic marker vinculin, respectively. The image of the western blot has been provided as supplementary Fig. S3 (Online Resource).

For total extracts, the cells were mechanically detached with a scraper in cold PBS. Total extracts were prepared by adding buffer TEEN (10 Mm Tris HCl pH 7.4, 1 mM EGTA, 1 mM EDTA, 150 mM NaCl, 1% Triton X-100, protease inhibitor cocktail) to the cell pellets. After incubation for 20 min on ice and subsequent centrifugation at 14,000 rpm

for 20 min at 4 °C, the supernatants were collected, quickly frozen in liquid nitrogen, and stored at − 80 °C. The total protein content was determined according to Bradford method.

Analysis of Nrf2 Activation, System x_c^-, and Tat Expression by Western Blotting

To evaluate Nrf2 activation, equal amounts (40 μg proteins/sample) of nuclear extracts were subjected to electrophoresis in an 8% polyacrylamide gel and transferred to nitrocellulose membranes. Membranes were blocked with 5% non-fat dry milk for 1 h and incubated overnight at 4 °C with a polyclonal anti-Nrf2 antibody (1:1000; Abcam Italy, Prodotti Gianni S.p.A., Milan, Italy) or with a polyclonal anti-lamin A (1:2000, Abcam, Italy). To evaluate system x_c^- and Tat expression, equal amounts (10 μg proteins/sample) of total extracts were subjected to electrophoresis in an 8% polyacrylamide gel and transferred to nitrocellulose membranes. Membranes were blocked with 5% non-fat dry milk for 1 h and incubated overnight at 4 °C with polyclonal anti-system x_c^- antibody (1:5000; OriGene), anti-Tat antibody (1:1000 Fit Biotech), or polyclonal anti-actin (1:1000, Sigma, Italy). Lamin A and actin were used as the reference protein amounts for nuclear and total extracts, respectively. Secondary peroxidase-labeled anti-rabbit IgG antibody (1:10000) was

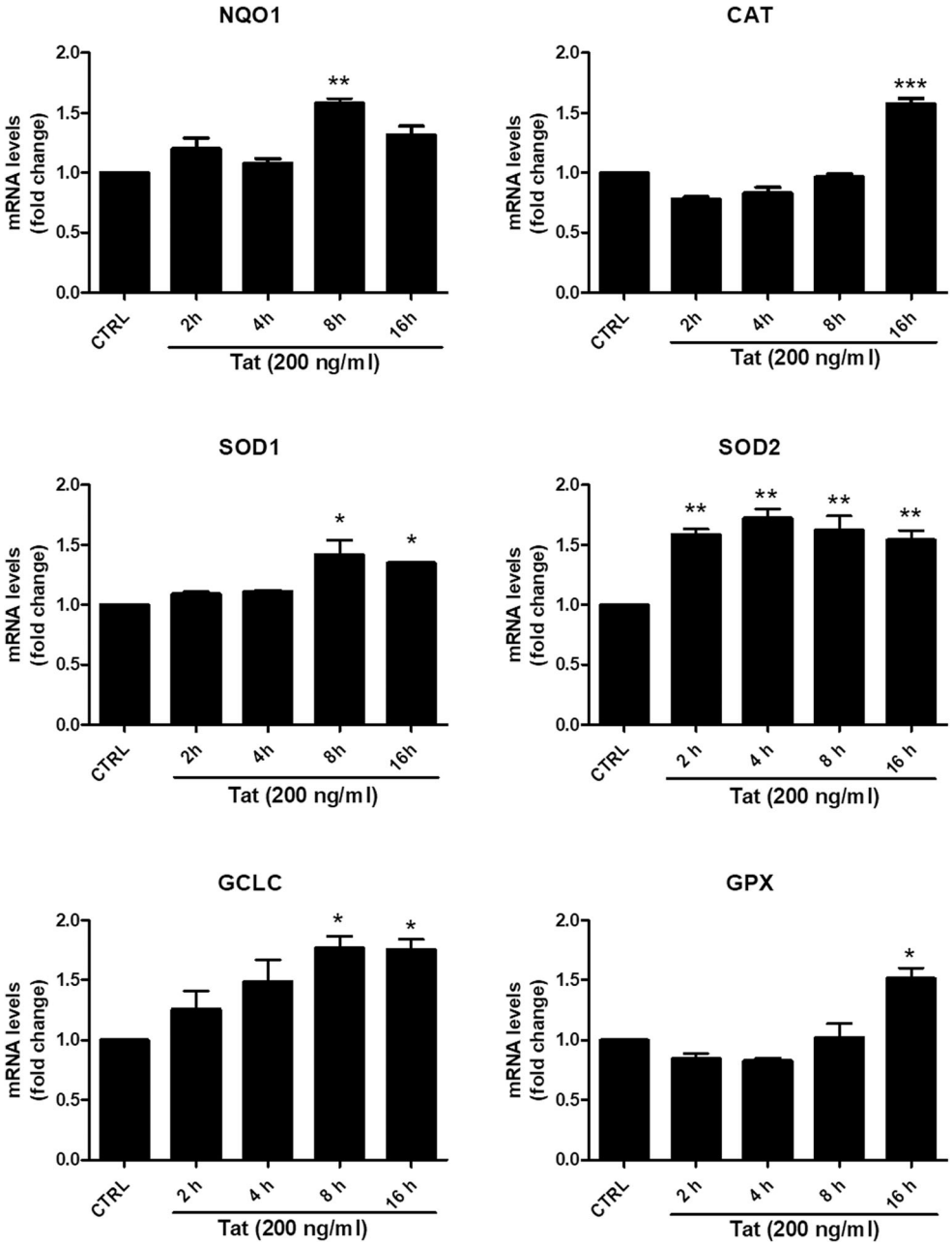

Fig. 1 Effects of Tat on ARE-driven gene expression in U373 cells. U373 were treated with Tat (200 ng/ml) for 2, 4, 8, and 16 h. After incubation at 37 °C, the cells were homogenized and total RNA has been purified to assess mRNA levels of several genes (NQO1, CAT, SOD1, SOD2, GPX, and GCLC) by RT-qPCR. Data are calculated relative to the internal housekeeping gene (GAPDH) and are the means ± SEM from three separate experiments, each performed in duplicate. One-way ANOVA, followed by Bonferroni's test, was used to determine significant differences. ***$p \le 0.001$ vs CTRL; **$p \le 0.01$ vs CTRL; *$p \le 0.05$ vs CTRL

from Bio-Rad Italia (Milan, Italy). Immunoreactive bands were detected using ECL Western blotting detection reagents (GE Healthcare, Milan, Italy) and captured by Chemi DocTM XRS 2015 (Bio-Rad Laboratories, Hercules, CA, USA). Densitometric analysis was performed using Image Lab software (Version 5.2.1; © Bio-Rad Laboratories). The optical density of each band was measured and normalized with respect to the relative loading control band (lamin A for Nrf2 and actin for system x_c^-).

Measurements of Glutamate Concentration in Cell Supernatants

To evaluate glutamate release in supernatants of co-cultures, Glutamate Assay (Biovision) was performed as indicated by manufacturer's instructions. Briefly, 20 μl of each sample supernatant was collected in a 96-well plate and assay buffer was added up to 50 μl final volume. Then 100 μl of the reaction mix was added to each well, the plate was incubated for 30 min at 37 °C, protected from light, and optical density (OD) was measured at 450 nm in a microplate reader. Glutamate concentration of each sample was calculated using glutamate standard curve (0, 1.3, 6.5, 13, 26, 40, 53, 67 μM).

Statistical Analysis

All data are expressed as the mean ± standard error of the mean (SEM) of *n* observations. Statistical analysis was performed by one-way ANOVA and subsequently by Bonferroni post-test. Differences are considered statistically significant at $p \leq 0.05$.

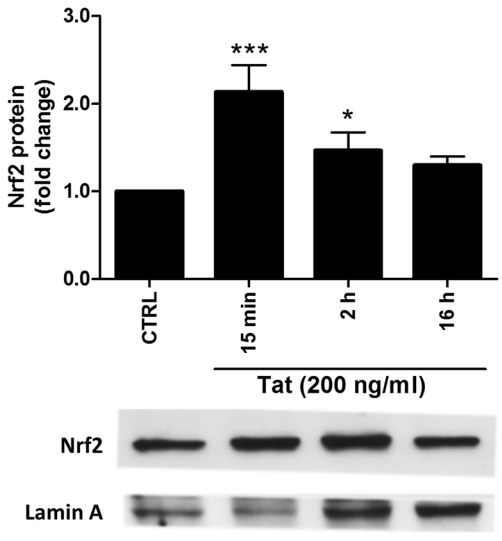

Fig. 2 Effects of Tat on Nrf2 nuclear translocation in U373 cells. Cells were treated with Tat (200 ng/ml) for the indicated time points. The histogram shows the densitometric analysis of the western blots for each sample. Values are calculated relative to the nuclear lamin A content and are the means ± SEM from three separate experiments, each performed in duplicate. One-way ANOVA, followed by Bonferroni's test, was used to determine significant differences. ***$p \leq 0.001$ vs CTRL; *$p \leq 0.05$ vs CTRL

Results and Discussion

Free radical generation and oxidative stress play pivotal roles in many neurodegenerative diseases, including HANDs [19]. Several studies indicate that HIV-1 viral proteins, such as gp120 and Tat, are able to increase nitro-oxidative stress in the brain [20, 21]. Previously, we reported that HIV-1 Tat can induce the production of ROS (e.g., H_2O_2) and neuronal cell

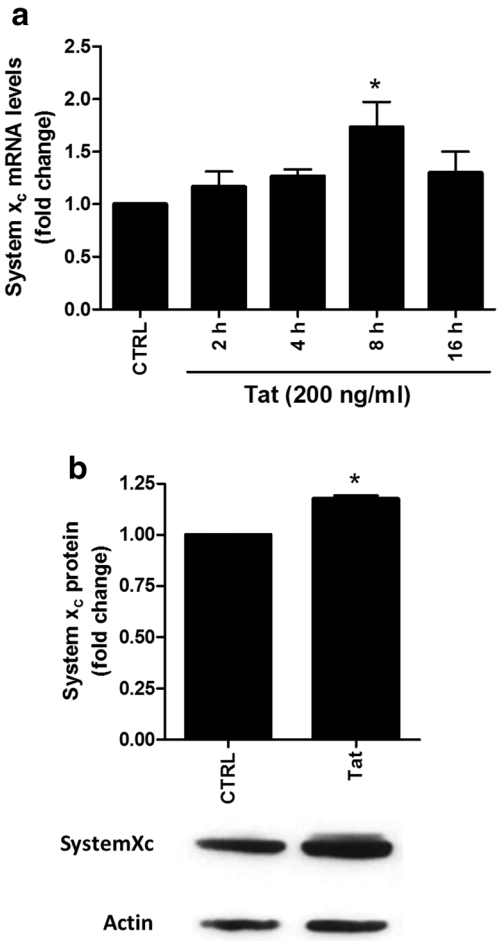

Fig. 3 Effects of Tat on system x_c^- gene (**a**) and protein (**b**) expression in U373 cells. **a** U373 were treated with Tat (200 ng/ml) for 2, 4, 8, and 16 h. After incubation at 37 °C, the cells were homogenized and total RNA has been purified to assess mRNA levels of system x_c^- gene by RT-qPCR. Data are calculated relative to the internal housekeeping gene (GAPDH) and are expressed as the mean fold change compared with control. Each value represents the mean ± SEM of three independent experiments. One-way ANOVA, followed by Bonferroni's test, was used to determine significant differences. *$p \leq 0.05$ vs CTRL. **b** U373 were treated with Tat (200 ng/ml) for 24 h. Data are calculated relative to the internal housekeeping protein actin and are expressed as the mean fold change compared with control. Each value represents the mean ± SEM of three independent experiments. One-way ANOVA, followed by Bonferroni's test, was used to determine significant differences. *$p \leq 0.001$ vs CTRL

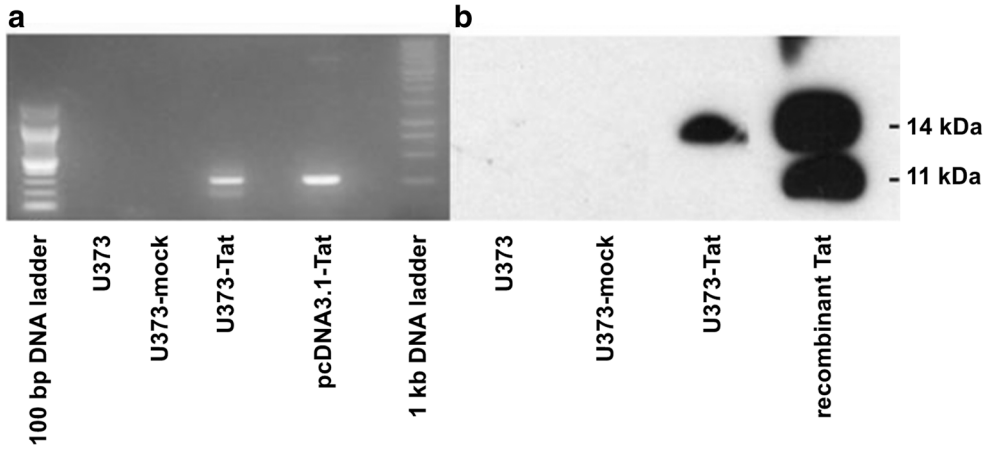

Fig. 4 Expression of Tat in stably transfected U373-Tat cells. **a** Untransfected (U373), pcDNA3.1-transfected (U373-mock), and pcDNA3.1-Tat-transfected (U373-Tat) cells were homogenized and total RNA has been purified to assess mRNA levels of Tat by RT-PCR. The PCR product of the GAPDH gene was taken as the reference cellular transcript. **b** Cells were mechanically harvested, and the total extracts were prepared as specified in the Materials and Methods section to assess Tat protein levels by western blot analysis with anti-Tat. pcDNA3.1-Tat vector and recombinant Tat (ARP697 HIV-1 Tat-B) were used as technical control to verify the specificity of PCR reaction and western blotting, respectively

death by a mechanism involving both polyamine metabolism and NMDA receptor activation [10]. In several cell types including astrocytes, ROS can trigger a protective antioxidant cell response through the transcriptional induction of phase II detoxifying and antioxidant genes (i.e., ARE genes) [13].

Here, we found that in human U373 astroglial cells the treatment with recombinant Tat (101 aa; 200 ng/ml) for 2, 4, 8, and 16 h was able to increase the mRNA expression of antioxidant enzymes, such as NQO1, CAT, SOD1, SOD2, GCLC, and GPX. As shown in Fig. 1, we observed an increase of mRNA expression of all the genes analyzed. The maximum was reached between 2 and 8 h for SOD1, SOD2, and GCLC whereas CAT and GPX expression peaked at 16 h after Tat treatment. Gene upregulation was maintained until 24 h post-treatment for all the genes analyzed (data not shown).

Given that ARE genes are mainly regulated by Nrf2, we investigated whether Tat could activate this transcription factor in astroglial cells. In particular, U373 cells were treated with Tat (200 ng/ml) for 15 min, 2 h, and 16 h, and the levels of Nrf2 were measured in nuclear extracts by western blot analysis. As shown in Fig. 2, Tat induced a 2.5-fold increase of the nuclear Nrf2 levels already at 15 min post-treatment, this time frame being compatible with the transcriptional induction of antioxidant ARE genes. Since activation of the astrocytic Nrf2 pathway represents a principal regulator of the large array of Nrf2-dependent antioxidant genes, these findings would be in harmony with the idea that astrocytes play a key role in providing antioxidant support to nearby neurons. Indeed, it is thought that post-mitotic neurons survive and are functional for many decades despite their relatively low intrinsic antioxidant defenses because of the strong antioxidant support they receive from surrounding glial cells, particularly astrocytes [14, 22–24].

Nrf2 can regulate also the expression of system x_c^- that along with other genes is involved in GSH homeostasis. Therefore, we explored whether Tat may induce the

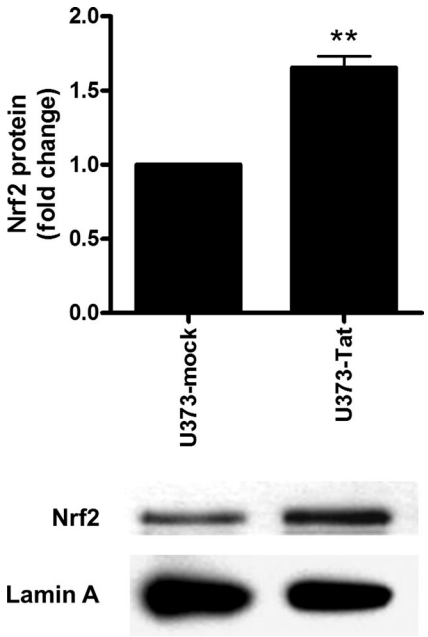

Fig. 5 Effects of endogenously produced Tat on Nrf2 nuclear translocation in U373-Tat cells. pcDNA3.1-transfected (U373-mock) and pcDNA3.1-Tat-transfected (U373-Tat) cells were mechanically harvested, and the nuclear extracts were prepared as specified in the Materials and Methods section to assess Nrf2 levels by western blot analysis with anti-Nrf2 and anti-lamin A. The histogram shows the densitometric analysis of the western blots for each sample. Values are calculated relative to the nuclear lamin A content and are the means ± SEM from three separate experiments, each performed in duplicate. One-way ANOVA, followed by Bonferroni's test, was used to determine significant differences. $^{**}p \leq 0.001$ vs U373-mock

upregulation of this antiporter in cells treated for 2, 4, 8, and 16 h. As shown in Fig. 3, we found that Tat (200 ng/ml) induced a significant increase of xCT mRNA levels at 8 h post-treatment (panel A). We also detected an increase of system x_c^- protein levels (20%) at 24 h post-treatment in whole cell extracts as shown in the panel B of Fig. 3. Note that the effect of Tat protein added in culture medium could be decreasing over time.

Really, in the CNS, Tat is continuously released in the extracellular space by astrocytes that represent the main reservoir of the virus, as they are susceptible of latent infection by HIV [9, 11]. Therefore, we analyzed both Nrf2 activation and ARE-driven gene expression in astroglial cells transfected to express and secrete Tat protein, in this way simulating a condition occurring in seropositive patients, such as the infection of astrocytes. To this aim, we generated a stably U373 cell line transfected with pcDNA3.1-Tat (U373-Tat) that constitutively produces high levels of Tat, as shown in Fig. 4 (cDNA in panel a and protein in panel b). In U373-Tat cells, we found a significant 2-fold increase of Nrf2 activation (see Fig. 5) and a 2.5-fold increase of the expression of Nrf2-dependent genes (Fig. 6), particularly those involved in GSH homeostasis, such as GCLC (panel a), and those involved in ROS scavenging such as CAT (panel b). As control cells, we used U373

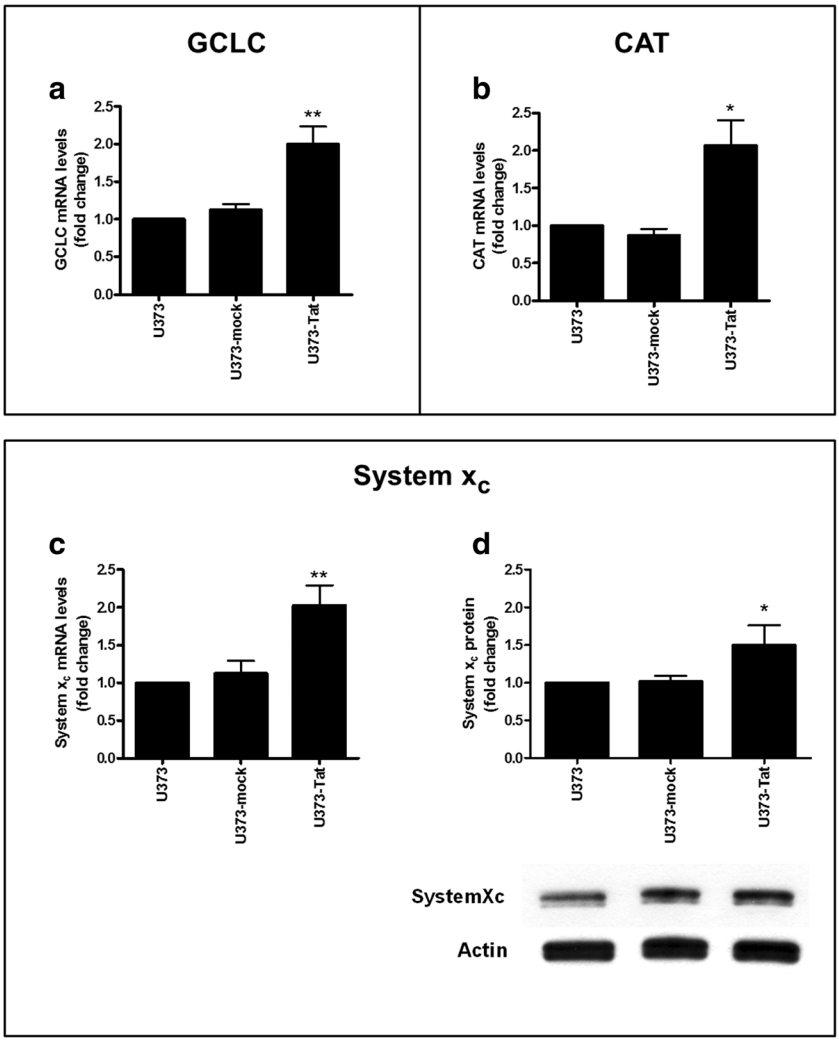

Fig. 6 Effects of endogenously produced Tat on mRNA expression of ARE genes in U373-Tat cells (**a**–**c**). Un-transfected (U373), pcDNA3.1-transfected (U373-mock), and pcDNA3.1-Tat-transfected (U373-Tat) cells were homogenized and total RNA has been purified to assess mRNA levels of GCLC (**a**), CAT (**b**), and system x_c^- (**c**) by RT-qPCR. Data are calculated relative to the internal housekeeping gene (GAPDH) and are expressed as the mean fold change compared with control. Each value represents the mean ± SEM of three independent experiments. One-way ANOVA, followed by Bonferroni's test, was used to determine significant differences. *$p \leq 0.05$ and **$p \leq 0.01$ between U373-Tat and U373-mock. **d** Effects of endogenously produced Tat on system x_c^- protein expression in U373-Tat cells (**d**). Cells were mechanically harvested, and the total extracts were prepared as specified in the Materials and Methods section to assess system x_c^- protein levels by western blot analysis with anti-system x_c^- and anti-actin. The histogram shows the densitometric analysis of the western blots for each sample. Values are calculated relative to the actin content and are the means ± SEM from three separate experiments, each performed in duplicate. One-way ANOVA, followed by Bonferroni's test, was used to determine significant differences. *$p \leq 0.05$ between U373-Tat and U373-mock

transfected with the empty vector pcDNA3.1 (U373-mock). As expected, we observed a significant upregulation of also system x_c^-, at both mRNA and protein levels, in U373-Tat when compared to control cells (Fig. 6, panels c and d, respectively). In particular, xCT mRNA levels were 2.3-fold higher. Together, these findings indicate that, in astroglial cells, endogenously produced Tat can induce an effective antioxidant response and suggest how infected astrocytes can counteract oxidative stress induced by HIV infection.

However, the upregulation of system x_c^- can increase extracellular glutamate release and potentially cause excitotoxicity [15, 16]. Thus, we wondered whether Tat-producing astroglial cells release higher levels of glutamate in the extracellular space in comparison to control cells. To this aim, we measured the glutamate levels in the supernatant of U373-Tat co-cultured with SH-SY5Y neuronal cells in comparison with U373-mock/SH-SY5Y co-cultures. As expected, Tat-producing cells released twice the amount of glutamate respect to control cells. The higher levels of glutamate were reached after 48 h of co-culture and were kept unchanged up to 72 h of co-culture (the last time point analyzed) (Fig. 7, panel a). To demonstrate that glutamate was produced

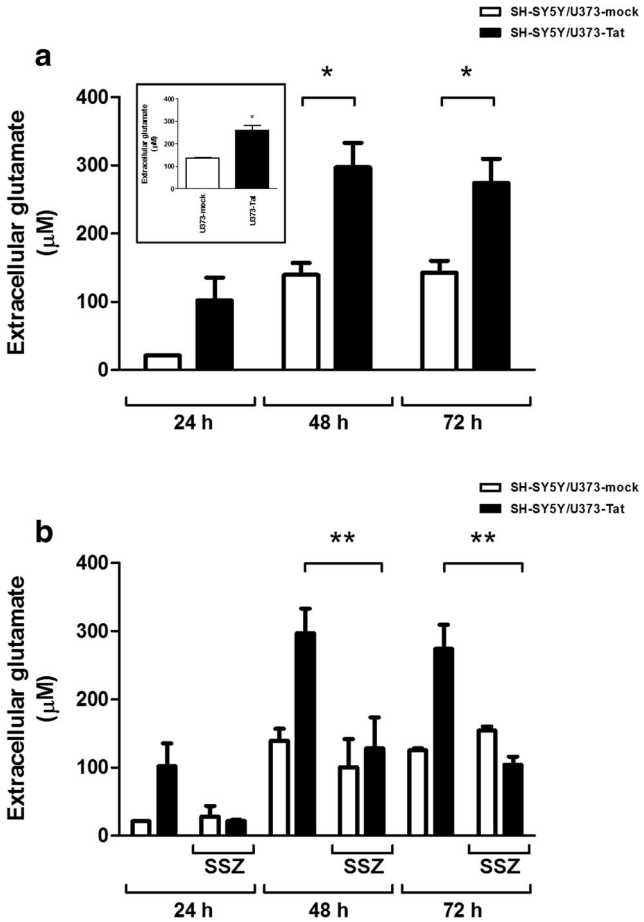

Fig. 7 Effects of endogenously produced Tat and system x_c^- activity on extracellular glutamate release in SH-SY5Y/U373-Tat co-cultures. **a** SH-SY5Y cells were co-cultured with U373 cells stably transfected with pcDNA3.1 (U373-mock) or with pcDNA3.1-Tat (U373-Tat) for 24, 48, and 72 h. The Glutamate Assay was performed as specified in the Materials and Methods section. The histogram shows the extracellular glutamate release (μM). Values are calculated relative to a glutamate standard curve (μM). Results are the means ± SEM from three separate experiments, each performed in duplicate. One-way ANOVA, followed by Bonferroni's test, was used to determine significant differences. $*p \leq 0.05$ vs SH-SY5Y/U373-Mock (white). The inset shows glutamate release in stably transfected mono-cultures, U373-mock or U373-Tat, for 48 h. $*p \leq 0.05$ vs U373-mock. **b** Co-cultures were treated for 24, 48, and 72 h alone or with SSZ and Glutamate Assay was performed. The histogram shows the extracellular glutamate release (μM). Values are calculated relative to a glutamate standard curve (μM). Results are the means ± SEM from three separate experiments, each performed in duplicate. One-way ANOVA, followed by Bonferroni's test, was used to determine significant differences. $**p \leq 0.01$ between SH-SY5Y/U373-Tat and SH-SY5Y/U373-Tat + SSZ

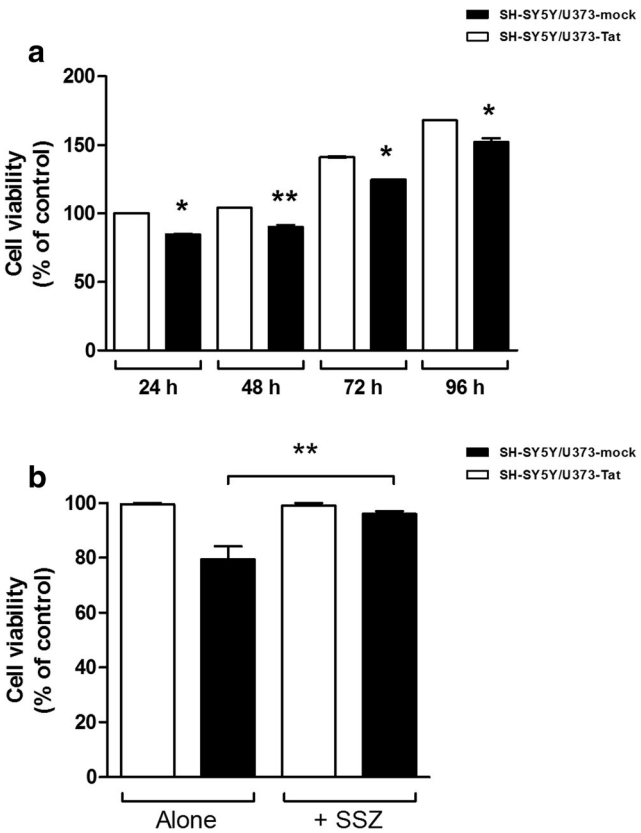

Fig. 8 Effects of endogenously produced Tat and system x_c^- activity on SH-SY5Y viability co-cultured with U373-Tat cells. **a** SH-SY5Y cells were co-cultured with U373 cells stably transfected with pcDNA3.1 (U373-mock) or with pcDNA3.1-Tat (U373-Tat) for 24, 48, 72, and 96 h. **b** Co-cultures were treated for 48 h alone or in the presence of SSZ. MTT cell viability assay was performed as specified in the Materials and Methods section. The histogram shows the percentage of control. Values are calculated relative to the viability of neuronal cells co-cultured with U373-mock cells and are the means ± SEM from three separate experiments, each performed in duplicate. One-way ANOVA, followed by Bonferroni's test, was used to determine significant differences. **a** $*p \leq 0.05$ and $**p \leq 0.01$ vs SH-SY5Y/U373-mock. **b** $**p \leq 0.01$ between SH-SY5Y/U373-Tat alone and SH-SY5Y/U373-Tat + SSZ

by astroglial cells, we measured the glutamate levels in the supernatant of transfected U373 mono-cultured for 48 h. As shown in the inset of Fig. 7a, Tat-producing cells (U373-Tat) released about twice the amount of glutamate respect to control cells (U373-mock). It is noteworthy that our findings are consistent with previous data indicating increased glutamate levels in CSF of patients with HIV dementia [25], thus providing an explanation about the involved mechanisms at molecular and cellular level. In our opinion, this represents an important point and confers a physiological relevance to our study.

Finally, to verify whether Tat-elicited glutamate release occurred through system x_c^- activation, we analyzed the levels of the amino acid in the supernatants of co-cultures treated without or with sulfasalazine (SSZ), a specific system x_c^- inhibitor. As shown in Fig. 7 (panel b), we found that SSZ treatment prevented Tat-induced glutamate release, maintaining its levels comparable to the control at all the time points analyzed. These findings clearly indicate that Tat released by astroglial cells induces an increased glutamate release by eliciting system x_c^- upregulation.

Afterwards, the viability of neuronal cells under these experimental conditions has been investigated. As shown in Fig. 8a, the viability of neuronal cells co-cultured with U373-Tat for 48 h was significantly decreased with respect to neuronal cells co-cultured with U373-mock. In particular, we observed 20% less viability in SH-SY5Y cells co-cultured with astrocyte-producing Tat. To verify whether the reduced neuronal viability, induced by Tat, was effectively due to the increased export of glutamate through system x_c^-, we performed an MTT assay in co-cultures treated for 48 h alone or in the presence of SSZ. As shown in Fig. 8b, SSZ was able to prevent neuronal toxicity in U373-Tat/SH-SY5Y co-cultures, restoring neuronal viability at the control level. These findings demonstrate for the first time that Tat-induced neurotoxicity is mediated by an increase of glutamate release due to system x_c^- upregulation in astroglial cells. Moreover, our study suggests that latently infected astrocytes, as occur in the CNS of HIV-infected patients, may mediate neurodegeneration through this pathway.

Note that glutamate release via system x_c^- from both astrocytes and microglia has been shown to enhance excitotoxicity of cortical neurons [26–30]. Very recently, it has been found

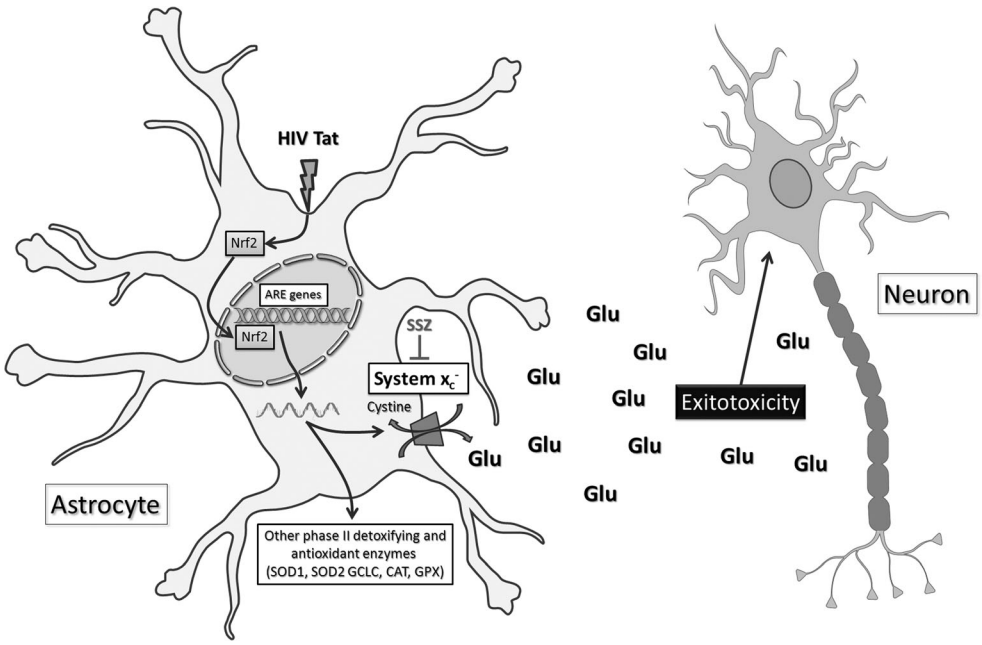

Fig. 9 Proposed model for Tat effects on Nrf2 translocation, ARE genes upregulation, system x_c^- activation, and glutamate release during HANDs. In astrocytes, Tat triggers an antioxidant response through the transcriptional induction of Nrf2-dependent ARE genes (e.g., SOD1, SOD2 GCLC, CAT, GPX, and system x_c^-), thus enabling these cells to sustain latent HIV infection. While the import of L-cystine through the transporter system x_c^- is critical to protection from oxidative stress (i.e., glutathione production), the export of glutamate represents a route of release through which this neurotransmitter may cause neuronal death. We propose that, also in HANDs, astrocytes can exacerbate neurodegeneration through the conversion of oxidative stress to excitotoxicity via system x_c^-.We have used two experimental approaches to mimic different exposure conditions: "acute" using a single treatment with Tat (ranging from 15 min to 16 h) and "chronic" using Tat-transfection, respectively. To analyze the process simulating a "chronic" condition, the experiments on glutamate release and neuronal viability have been performed using co-cultures of neuronal cells with stably transfected-astrocytes producing Tat for 24 to 96 h. For more details, see text. Abbreviations: ARE, antioxidant response element; CAT, catalase; GCLC, γ-glutamyl-cysteine ligase; Glu, glutamate; GPX, glutathione peroxidase; NQO1, NAD(P)H:quinone oxidoreductase type 1; Nrf2, nuclear factor erythroid 2-related factor 2; SOD, superoxide dismutase; SSZ, sulfasalazine

that system x_c^- contributes to increase glutamate excitotoxicity in the neocortex of a mouse model (Dach-SMOX) displaying a constant and chronic oxidative stress [31]. Several data reported that during neuroinflammation activated microglia and astrocytes release and maintain high level of extracellular glutamate [30]. Moreover, it has been recently observed that system x_c^- could mediate methamphetamine-induced neurotoxicity by eliciting oxidative stress, microgliosis, and glutamate-related toxicity [32].

Early evidence reporting that system x_c^- could be a source of excitotoxic glutamate derives from a study on microglia that, because of a sustained need for oxidative protection, expresses high levels of the transporter [33]. More recently, Gupta et al. reported that Tat elicits microglial glutamate release via system x_c^- induction, thus suggesting that Tat-induced glutamate release might contribute in part to neurologic dysfunctions associated with HIV infection [34]. In astrocytes, it has been reported that the upregulation of system x_c^- by interleukin-1β was able to enhance hypoxic neuronal injury [26]. In fact, neurons co-cultured with astrocytes were found to be more susceptible than neurons alone to hypoxic cell death after treatment of cultures with IL-1β, an effect that was mediated by increased efflux of glutamate through system x_c^- [26]. Previously, we reported that the expression of IL-1β as well as of nitric oxide synthase was elicited in astrocytes by HIV gp120 [21]. These findings indicate how inflammatory pathways and nitrosative stress may converge to an intersection point, represented by system x_c^- activation, and suggest a likely explanation for the mechanism involved in excitotoxicity induced by HIV infection.

In conclusion, Tat can induce in astrocytes both Nrf2 activation and the upregulation of ARE-driven genes, including system x_c^-. The latter causes a reduced cell viability of co-cultured neuronal cells (Fig. 9). It should also be noted that upon stimulation with recombinant Tat, human neuronal cells are able to activate a slight Nrf2-mediated antioxidant response, as we previously reported [35]. Given the involvement of astrocytes in CNS pathology, it is not surprising that the ability to exacerbate neurodegeneration through the conversion of oxidative stress to excitotoxicity via system x_c^- has been linked to a variety of disorders, including Alzheimer's disease [36], Parkinson's disease, AIDS [37], multiple sclerosis [38], and ALS [39]. The present study shed light on Nrf2/system x_c^- pathway and may help to better understand the role of astrocytes as the cell population responsible for the bulk of the neuronal death in HANDs.

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