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Neuronal survival and resistance to HIV-1 Tat toxicity in the primary culture of rat fetal neurons

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Abstract

In this study we report that primary cultures of rat fetal neurons contain subpopulations of cells that may be sensitive or resistant to HIV-1 Tat neurotoxicity. We demonstrate that rapid binding/uptake of Tat 1–86 for 2 hours was sufficient to trigger caspase activation and neurodegeneration in rat fetal midbrain cell cultures. The uptake of Tat was followed by an increase in MCP1 (CCL2) immunoreactivity. Approximately 70% of neurons were able to survive transient or continuous (7 days) Tat exposure. The surviving neurons did not contain bound/internalized Tat, but were able to interact with Tat after medium replacement. These neurons were resistant to Tat toxicity. In neurons that resisted the toxic effects of continuous and repeated Tat treatment, levels of NR2A subunit of the NMDA receptor complex were significantly lower than in controls. We suggest that the subunit composition of NMDAR complexes may be important for the sensitivity of neurons to Tat toxicity.

INTRODUCTION

Neuropathology associated with HIV infection represents a serious medical problem. With the advent of potent antiretroviral therapy, the percentage of AIDS patients with neurological abnormalities is increasing. Despite the small numbers of HIV-infected cells in the brain, HIV-associated neuronal cell damage is widespread. Thus, it has been suggested that neurodegenerative changes in the HIV-infected brain are related to neurotoxicity of viral proteins released from non-neuronal brain cells which harbor the virus. Numerous studies report the presence of neurotoxic HIV-1 proteins in the brain of HIV patients and the capability of viral proteins, such as Tat, gp120, and Vpr to induce neuronal degeneration *in vivo* and *in vitro* (Nath et al, 2002; Wallace, 2006; Hult et al, 2008).

HIV-1 Tat is an 86–101 amino acid-long transactivating nuclear regulatory protein, and is essential for viral replication. Along with other HIV-1 neurotoxic proteins, the presence of Tat in postmortem brain tissue samples obtained from patients with HIV-associated brain pathology has been reported (Hudson et al, 2000). Tat is released extracellularly from unruptured, HIV-infected cells. Tat exits from cells via a leaderless secretory pathway in the absence of permeability changes (Ensoli et al, 1993; Tardieu et al, 1992; Chang et al, 1997) and therefore can interact with uninfected cells in the brain. Tat can bind to several classes of cell surface receptors including heparan sulfate proteoglycan (HSPG) (Liu et al., 2000; Bugatti et al., 2007), a low-density lipoprotein receptor-related protein (LRP), cell adhesion receptors of the integrin family (Urbini et al., 2005), cytokine receptors CCR2, CCR3 and CXCR4

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(Albini et al., 1998; Xiao et al., 2000). Binding and internalization of Tat by neurons is HSPG/LRP-dependent (Liu et al., 2000). Extracellular Tat bound to cell membrane receptor sites is internalized by different types of brain cells and rapidly transported to the nucleus. Tat-mediated changes in gene expression in different types of CNS cells may be a source of indirect neurotoxic effects of Tat in the brain. Tat is known to induce cytokine dysregulation and glial cell activation. Internalized recombinant Tat is able to alter gene expression in neurons (Kolson et al., 1994). However, the relative role of the uptake or membrane interactions in the mechanism of Tat neurotoxicity is not understood. Although the neurotoxic properties of HIV-1 Tat are generally acknowledged, it remains unclear whether continuous production and release of Tat occurs in the brain of HIV patients. Detectable quantities of Tat and other neurotoxic HIV proteins may appear in the brain at certain stages of the HIV neuropathogenesis (Nath et al., 1999; Hudson et al., 2000; Del Valle et al., 2000). Nevertheless, chronic low-level Tat production in the rodent brain may cause neuronal damage, inflammation and behavioral abnormalities (Bruce-Keller et al., 2003). It was demonstrated that even a transient exposure to Tat can cause a prolonged increase in production and release of cytokines by astrocytes and macrophages (Nath et al., 1999).

Direct interactions of Tat with neurons can activate mechanisms of neuronal degeneration. Nanomolar concentrations of Tat cause oxidative stress-associated apoptosis in neurons (Kruman et al., 1998; Aksenov et al., 2006). Changes in mitochondrial potential and ROS production can be observed in cell cultures soon after the beginning of Tat treatment and precede Tat-induced cell death (Aksenov et al., 2006). Tat-mediated apoptosis is believed to be NMDAR-dependent (Self et al., 2004). At the postsynaptic level different neuronal membrane proteins and receptors for neurotransmitters other than glutamate may be involved in complex mechanism of Tat neurotoxicity (Eugenin et al., 2007, Silvers et al., 2007). Tat is known to presynaptically affect the function of dopamine transporter protein, inhibiting specific ligand binding and dopamine uptake (Aksenova et al., 2006; Wallace et al., 2006). Thus, neurotoxic Tat is able to produce alterations in various neurotransmission systems. Analysis of the pattern of HIV-related neuropathology suggests that dopaminergic regions of the brain are particularly vulnerable. Concurrent changes that result from Tat effects on molecular components of dopamine and glutamate transmission may be an important part of the pathogenesis of HIV-associated brain pathology. Results of our Tat toxicity studies in cell culture models suggest that cultured neurons may have different sensitivities to toxic effects of HIV-1 Tat (Aksenov et al., 2006). Very little is known about possible molecular characteristics which may contribute either to specific neuronal sensitivity or resistance against Tat-induced degeneration. This study was designed to analyze the status of key biochemical features linked to the mechanism of Tat neurotoxicity in neurons surviving continuous, transient and repeated exposure to HIV-1 Tat *in vitro*.

MATERIALS AND METHODS

Neuronal Cell Culture

Neuronal cultures were prepared from 18-day-old Sprague-Dawley rat fetuses. Rat midbrains were dissected and incubated for 15 min in a solution of 2 mg/mL trypsin in Ca^{2+} - and Mg^{2+} -free Hanks' balanced salt solution (HBSS) buffered with 10 mM HEPES (Invitrogen, Carlsbad, CA). The tissue was then exposed for 2 min to soybean trypsin inhibitor (1 mg/mL in HBSS) and rinsed three times in HBSS. Cells were dissociated by trituration and distributed to poly-L-lysine-coated culture plates (Costar, Cambridge, MA). Initial plating densities were approximately 160–180 cells/mm². At the time of plating each well contained DMEM/F12 medium (Invitrogen) supplemented with 100 mL/L fetal bovine serum (Sigma Chemicals, St. Louis, MO). After a 24-hr period, the DMEM/F12 medium was replaced with 2% v/v B-27 Neurobasal medium supplemented with 2 mM GlutaMAX and 0.5% w/v D-(+) glucose

(Invitrogen). Two-thirds of the Neurobasal medium was replaced with freshly prepared medium of the same composition once a week. Cultures were used for experiments after 12 days in culture and were >95% neuronal as observed by anti-MAP-2 immunostaining. The reminding (approximately 5%) cells were astrocytes as determined by anti-GFAP/Hoechst staining.

Experimental Treatment of Cultures

Recombinant Tat 1–86 (Diatheva, Italy) was added to cell culture medium. Groups of sister cultures growing in 96-well, 24-well plates or in separate 35 mm culture dishes were exposed to 50 nM Tat.

For neurotoxicity experiments cell cultures were treated with Tat and incubated for 2, 24, 48, 96 or 168 hours prior to analyses depending on the experiment design. To study the effect of repeated Tat exposure, 50nM Tat was added to the cultured cells and incubated 48 hours to produce the significant decrease in cell viability. After 48 hours of treatment, medium was removed, cells were washed twice, and the medium was replaced with fresh medium of the same composition with freshly prepared 50nM Tat. Depending on experimental condition, the second incubation was carried out for either 2, 48 or 96 hours.

To study the effects of Tat-conditioned medium (TCM) on neuronal cell cultures, 50 nM of freshly prepared Tat was added to the cell cultures and incubated for 48–96 hours. Following the incubation, TCM was collected. Originally treated cells were immediately supplemented with fresh medium and either used for analysis or returned back to the incubator for the recovery (for up to 48 hours depending on the protocol). Samples of TCM were tested for Tat 1–86 immunoreactivity. To test the toxicity of TCM, samples of Tat-containing conditioned medium were added to another group of sister cultures where the growth medium was removed. To account for the consequences of the medium replacement shock on cell viability, control sister cultures were also treated with conditioned medium taken from another cells with Tat omitted. To ensure that possible toxic effects of TCM were linked to Tat presence in it, samples of Tat-containing and control (no Tat) conditioned medium were passed through 10 kD-cutoff molecular filters before the addition to a new group of cell cultures. The absence of Tat in the filtrates was confirmed by immunoblotting.

The repeated Tat exposure was carried out as described above where initial Tat-containing medium was removed from the corresponding plate wells, cells were rinsed twice with freshly prepared medium, supplemented with another portion of freshly prepared Tat and incubated again according to the experiment design.

Cell viability test

Neuronal survival was determined using a Live/Dead viability/cytotoxicity kit from Molecular Probes (Eugene, OR) in rat fetal midbrain cell cultures prepared in 96-well plates. In accordance with the manufacturer's protocol, neurons were exposed to cell-permeant calcein AM (2 μ M), which is hydrolyzed by intracellular esterases, and to ethidium homodimer-1 (4 μ M), which binds to nucleic acids. The cleavage product of calcein AM produces a green fluorescence (F_{530nm}) when exposed to 494-nm light and is used to identify live cells. Bound ethidium homodimer-1 produces a red fluorescence (F_{645nm}) when exposed to 528-nm light, allowing the identification of dead cells. Fluorescence was measured using a Bio-Tek Synergy HT microplate reader (Bio-Tek Instruments, Inc., Winooski, VT). Each individual F_{530nm} and F_{645nm} value on a plate were corrected for background fluorescence (readings obtained from cell cultures (wells) that were not exposed to calcein AM and ethidium bromide) by the microplate reader KC4 software package (Bio-Tek Instruments, Inc., Winooski, VT). For each individual cell culture (well) on a plate ratios between corrected green and red fluorescence

($F_{530\text{nm}}/F_{645\text{nm}}$, Live/Dead ratios) were calculated. All individual relative numbers of live and dead cells were expressed in terms of percentages of average maximum Live/Dead ratio determined for the set of non-treated control cell cultures (8–16 wells) from the same plate: $[F_{530\text{nm}}/F_{645\text{nm}}]_{\text{well } n} / [F_{530\text{nm}}/F_{645\text{nm}}]_{\text{average max}} \times 100\%$.

ELISA

Changes of the Tat concentration in cell culture medium during continuous incubation were determined using ELISA. Total amount of 5 pmol Tat 1–86 was added to the wells of 96-well plates containing 100 μl of cell culture medium (50 nM final concentration) and midbrain cell cultures prepared as described above. In order to control for changes in the Tat concentration caused by non-specific absorption to the well surface and possible degradation of protein during prolonged incubation, 100 μl aliquotes of medium containing 50 nM Tat 1–86 were added to the wells, which did not contain midbrain cell cultures. Samples of Tat-containing cell culture medium were collected from 96-well culture plates at different time points starting from 1 min and up to 7 days of incubation and stored at -20°C . The collected samples were used for direct ELISA detection of Tat 1–86. Each well of Costar 96-well ELISA plates (Corning Inc, PA) was coated overnight at 4°C using 100 μl of 20 mM carbonate coating buffer, pH 9.6. Cell culture medium samples were diluted 1:10 with D-PBS and 3 μl of each sample were added to the plate wells in triplicates. Control samples of the cell culture medium without Tat were included in each plate. Different dilutions of the stock solution of recombinant Tat 1–86 were used to produce the standard curve ranging from 250 pg/ml to 10 ng/ml with each standard dilution added in triplicates. After overnight incubation at 4°C , plates were rinsed 5 times with PBST (0.05% Tween 20 in PBS, pH=7.4) and blocked with 1% BSA in PBS for 2 hours at room temperature. After blocking, plates were washed again as described above and primary anti-Tat antibodies (Diateva, Italy) diluted 1:1000 in 0.1% BSA-PBST were added to each well except of blanks and no-primary antibody control wells. Plates were kept overnight at 4°C . When the incubation with primary antibodies was completed, plates were again washed 5 times with PBST and secondary antibodies (goat anti-rabbit alkaline phosphatase conjugated, Sigma) diluted 1: 1000 in 0.1% BSA-PBST were added to each well except of blank and no-secondary antibody control wells. After 2 hours of incubation, secondary antibody solution was removed, plates were washed 5 times with PBST and 100 μl of BluePhos phosphatase substrate mixture (KPL Research, Gaithersburg, MD) was added to the plate wells. After 10–20 min of incubation, the absorbance at 650 nm was determined using a Bio-Tek Synergy HT microplate reader.

Immunoblotting

Tat immunoreactivity in cell lysates was determined by Western blotting. Cell lysates prepared from non-treated midbrain cell cultures served as negative controls, samples of Tat 1–86 stock solution were used as positive controls. Cell lysates were prepared from cultures grown in 24-well plates. Tat-treated cell cultures were exposed to 50 nM Tat 1–86 for 15, 30, and 60 min or for 2, 24, 48, 72, and 96 hours before harvesting. At the time point of harvesting growth medium containing Tat 1–86 was removed, cells were washed three times with Dulbecco phosphate-buffered saline, D-PBS, (8 mM Na_2HPO_4 , 1.5 mM KH_2PO_4 , 0.137 M NaCl, and 2.7 mM KCl at pH 7.4) and lysed with CelLyticTM-M mammalian cell lysis buffer (Sigma Chemicals) containing protease inhibitors (protease inhibitors cocktail, Sigma Chemicals). All samples in a group (6 sister culture wells) were pooled together and protein concentration was determined by BCA method (Pierce). Fifteen micrograms of total cell protein were used for immunoblotting analysis of Tat immunoreactivity. Electrophoresis of protein extracts was carried out in the Criterion Cell using precast 12% SDS-PAGE gels (Bio-Rad Laboratories, Inc, Los Angeles, CA). Following the transfer to nitrocellulose membranes, blots were blocked with 3% BSA in TBS. Tat immunoreactivity in samples of growth medium was determined

using dot-blotting. Dot-blots were prepared using Bio-Dot apparatus (Bio-Rad). Following application of samples, blots were blocked with 3% BSA in TBS.

Anti-Tat immunostaining of Western blots and dot-blots was performed using 1:500 working dilution of rabbit polyclonal anti-Tat antibody (Diatheva, Italy). Primary antibodies were diluted with TTBS containing 0.3% BSA. Blots were incubated with primary antibodies overnight at 4°C and then washed 3 times with TTBS on shaking platform. Alkaline phosphatase-conjugated goat anti-rabbit secondary antibodies (Sigma Chemicals, 1:2500 working dilution) were used to detect Tat-immunopositive bands. Blots were incubated with secondary antibodies for 2 hours, washed in TTBS and developed using nitroblue tetrazolium-5-bromo-4-chloro-3-indolyl phosphate (NBT-BCIP) solution (SigmaFast tablets, Sigma Chemicals).

Images of Western blots and dot-blots were captured and analyzed using ChemiDoc™ XRC imaging system with QuantityOne imaging analysis software package (Bio-Rad).

Apoptosis Detection

Following Tat treatment, neuronal apoptosis was analyzed by the Fluorochrome Inhibitor of Caspases (FLICA) Caspase 9 (red fluorescence) or Poly-Caspase (green fluorescence) Apoptosis Detection Kit (Immunochemistry Technologies LLC, Bloomington, MN). The red Caspase 9 FLICA kit contains a sulforhodamine-labeled fluoromethyl ketone peptide inhibitor of caspase 9 (SR-LEHD-). Poly-Caspases Detection Kit contains a carboxyfluorescein-labeled inhibitor of total caspase activity (CR-VAD- fluoromethyl ketone). VAD is an aminoacid sequence targeted by all caspases, while LEHD is targeted only by caspase 9. When covalently coupled to the caspases, the dye is retained within the cell and the fluorescent signal is a direct measure of the number of active caspase enzymes that were present in the cell at the time the reagent was added. FLICA solution was added directly to the cell incubation medium and the plate was returned to the incubator for 1 additional hour. After 1 hour, Hoechst stain was added to the medium for neuronal nuclei counterstaining and incubated for additional 3–5 min at 37°C. Cells were washed twice with wash buffer supplemented with the kit. Red fluorescence of SR-LEHD complex with active caspase 9 was observed immediately by fluorescent microscopy. The results are presented as merged images of red caspase fluorescence and blue Hoechst staining of intact cell nuclei. For the microplate readings of total caspase activity, rat fetal midbrain cell cultures were prepared in black wall 96-well culture plates (Costar). Green fluorescence produced as a result of interaction of CR-VAD- fluoromethyl ketone with active caspases in Tat-treated and control cultures was determined using 488/530 nm –filter set. Sensitivity was automatically adjusted to low signal (no carboxyfluorescein-labeled inhibitor of caspase activity) wells. Blue fluorescent Hoechst neuronal nuclei counterstaining was used to normalize differences in cell density between individual wells.

Immunocytochemistry

For immunocytochemistry experiments Tat-treated cultures and non-treated controls were fixed in acetic alcohol in acetic alcohol (95% ethanol, 5% acetic acid) for 10 min and washed three times (5 min per wash) with Dulbecco phosphate-buffered saline (D-PBS): Na₂HPO₄ (1150 mg/L), KH₂PO₄ (200 mg/L), NaCl (8000 mg/L), KCl (200 mg/L) at pH 7.4. Following the fixation, cultures were blocked with 10% normal horse serum (NHS) in PBS and used for the analysis.

Anti-Tat immunofluorescence—Rabbit polyclonal anti-Tat antibody (Diatheva, Italy) was used to determine Tat 1–86 immunoreactivity. Primary antibodies were diluted 1:500 in 1% NHS/PBS. Cell cultures were incubated with primary antibodies overnight at 4°C. Each set of experiments included cell culture dishes which were left without primary antibody. These

cell cultures served as controls for non-specific binding of secondary antibodies. After the incubation with primary antibodies was complete, plates were washed three times (5 min per wash) with PBS. For the immunofluorescent detection of Tat immunoreactivity goat anti-rabbit IgG conjugated with Alexa 594 dye (Molecular Probes) diluted 1:500 in 1% NHS/PBS was used. Plates were incubated with secondary antibodies for 1 hour at room temperature and then washed 3 times with PBS.

Microscopic images of Tat immunoreactivity in midbrain rat fetal cell cultures were captured using the computer-controlled inverted fluorescent microscope (Nikon Eclipse TE2000-E) under 20X magnification and analyzed using NIS-Elements BR 2.30 imaging software package (Nikon). Individual images of specific Tat immunofluorescence were merged with the subsequent differential interference contrast (DIC) images. Parts of merged immunofluorescent/DIC images were magnified by placing a selection box over the area of interest and saving the selection as a new image with higher resolution.

Anti-NMDAR immunofluorescence—Primary antibodies against NR1 (rabbit monoclonal, Chemicon International, Inc., Temecula, CA), NR2A (rabbit polyclonal, Chemicon International, Inc.), and NR2B (mouse monoclonal, Invitrogen) subunits were used to detect NMDA receptor complex immunoreactivity in Tat-treated and non-treated control rat fetal midbrain cell cultures. Primary antibodies were diluted 1:500 in 1% NHS/PBS. For the detection of anti-NMDAR immunofluorescence, rat fetal midbrain cell cultures were prepared in 24-well plates (Costar). Plates were incubated with primary antibodies overnight at 4°C. Secondary antibodies were goat anti-rabbit IgG (for the anti-NR1 primary) conjugated with Alexa 498 dye (green fluorescence), goat anti-rabbit IgG conjugated with Alexa 594 dye (red fluorescence), and goat anti-mouse IgG conjugated with Alexa 594 dye (Molecular Probes). All secondary antibodies were diluted 1:500 in 1% NHS/PBS. Each plate included no-primary antibody control wells and no-antibody control wells. No-antibody control wells served as blank, low signal wells, for automatic sensitivity adjustment in microplate readings of anti-NMDAR (NR1, NR2A, and NR2B) immunofluorescence. No-primary antibody wells were used for the automatic background correction of immunofluorescent signals. Blue fluorescent Hoechst staining of cell nuclei was used to assess cell densities in all individual wells on the plate. Automatic sensitivity adjustment of Hoechst fluorescence readings was performed using no-Hoechst wells on each plate. Fluorescent signals were measured by Bio-Tek Synergy HT microplate reader using 360/460 nm (blue), 488/530 nm (green), and 594/654 nm (red) filter sets. Anti-NMDAR (NR1, NR2A and NR2B) immunofluorescent signals were normalized to Hoechst fluorescent signals for each individual sister culture (well) on the plate. Each plate included groups of 6 Tat-treated and 6 control sister cultures. Experiments were repeated at least twice to ensure the reproducibility of the results.

Anti-MCP1 immunofluorescence—Rabbit polyclonal anti-MCP1 antibody (Abcam Inc., Cambridge, MA) was used to study MCP1 immunoreactivity in cell cultures. Primary antibodies were diluted 1:500 in 1% NHS/PBS. Cell cultures were incubated with primary antibodies overnight at 4°C. Each plate included no-primary antibody control wells and no-antibody control wells. Secondary antibodies were goat anti-rabbit IgG conjugated with Alexa 498 dye (1:500 working dilution in 1% NHS/PBS). Immunofluorescent signals from anti-MCP1 immunostained wells were analyzed by Bio-Tek Synergy HT microplate reader. Anti-MCP1 immunostained plates included Tat-treated (n=6) and no-treated control (n=6) cell cultures. Anti-MCP1 immunofluorescence was determined using 488/530 nm –filter set. Sensitivity was automatically adjusted to low signal (no-antibody) wells. Individual F₅₃₀ readings were corrected for background fluorescence (readings obtained from no-primary control wells). Blue fluorescent (360/485 nm) Hoechst neuronal nuclei counterstaining was used to normalize differences in cell density between individual wells.

Statistical Analysis

Statistical comparisons were made using ANOVA and planned comparisons were used to determine specific treatment effects. Significant differences were set at $P < 0.05$.

RESULTS

Cell viability changes in rat primary neuronal cell cultures continuously exposed to Tat 1–86

The recombinant Tat (1–86 or 1–72) is known to induce neurotoxicity in rat fetal primary neuronal cell cultures prepared from hippocampus, cortex, or midbrain (Kruman et al, 1998; Bonavia et al, 2001; Aksenova et al, 2006). The decrease in cell viability caused by 48-hour treatment of rat fetal midbrain cell cultures with different doses of Tat 1–86 is shown in Figure 1A. The typical time course of cell viability changes in rat fetal midbrain cell cultures treated with 50 nM dose of Tat 1–86 is shown in Figure 1B. Each time point in the graph represents averaged relative Live/Dead ratios. Individual measurements were carried out in 7–12 sister cultures (wells of the 96-well plate). After 48 hours of treatment, Live/Dead ratios in cultures exposed to Tat 1–86 for 48 hours decreased by 19% ($81 \pm 1.5\%$ of control, $P < 0.05$). After this time point the cell viability in Tat-treated cultures continued to decline. Maximum toxic effect of the continuous exposure of midbrain cell cultures to 50 nM Tat 1–86 was reached after 72 hours of incubation. The exposure of cell cultures to Tat for 96 hours resulted in 29% decrease of neuronal cell viability ($71 \pm 0.7\%$ of control, $P < 0.05$). Cell cultures remained exposed to Tat 1–86 for as long as 7 days. However, no further decline of neuronal viability was observed in Tat-treated cell cultures beyond 96-hour time point. The results of cell viability tests performed at different time points after the start of treatment could be successfully fitted with 4-parameter logistic equation ($R_{\text{sqr}} = 0.998$).

Binding/uptake of Tat 1–86 in rat primary neuronal cell cultures

Figure 2A shows the time course of changes in concentration of Tat 1–86 in the cell culture medium during the continuous exposure of cell cultures for 7 days to 50 nM Tat 1–86. Measurements of Tat concentration in the medium were performed in wells with and without rat fetal midbrain cells. The graph shows that a sharp drop in Tat concentration occurred shortly after the addition of Tat to the wells. After 30 min of incubation, the concentration of Tat in the wells containing midbrain cells decreased from 50 nM to 32.6 ± 0.5 nM. In the wells without cells the concentration of Tat during the same period of time decreased from 50 nM to $43.5 \pm$ nM. We determined that approximately 63 % of the decrease in Tat 1–86 concentration during the first 30 min of incubation was attributed to specific binding/uptake of Tat protein by cultured midbrain cells. Approximately 37% of the decrease were due to non-specific Tat absorption to the well surface.

Each well of the 96-well culture plate contained 100 μ L of growth medium. Therefore, each Tat-treated well at the beginning of the treatment contained total amount of 5 pmol of Tat 1–86. Figure 2B shows that the amount of Tat 1–86 specifically absorbed by midbrain cells increased rapidly within 15 min and stabilized at approximately 1 pmol per well (each well of 96-well plate contained 5000–6000 cells) after 30–60 min of incubation (Figure 2B). Consistently, Tat immunoreactivity (14 kDa polypeptide band) in midbrain cell lysates increased from 15 min to 1 hour incubation time point (Figure 2C).

The decline of Tat concentration that occurred in medium after 1 hour time point was no longer attributed to the specific binding/uptake of the protein by midbrain cells. Western blot analysis demonstrated that the amount of Tat in cell lysates prepared from Tat-treated cell cultures following 2, 24, 48, and 96 hours of exposure to 50 nM Tat 1–86 decreased over time. The 14 kDa Tat band became undetectable in the lysates after 48 hours of incubation (Figure 2 D).

Immunostaining of cell cultures exposed for 2 hours to 50 nM Tat 1–86 with anti-Tat antibodies showed the presence of Tat in nuclei, but in some neurons anti-Tat immunofluorescence was localized in somata, leaving nuclei non-stained (Figure 3). No Tat-immunopositive cells could be observed in midbrain cell cultures treated with 50 nM Tat for 96 hours.

Caspase activity in rat primary neuronal cell cultures continuously exposed to Tat 1–86

Activation of caspase 9 (early caspase) was evident in cell cultures treated with 50 nM Tat 1–86 for 2 hours (Figure 4A). No activated caspase 9 -positive neurons could be found in the non-treated control cultures at this time point (Figure 4B). Results of microplate fluorescent detection of total caspase activity in cell cultures exposed to 50 nM dose of Tat 1–86 for 2, 24, and 48 hours are shown in Figure 3C. Significant increases in total caspase activity was detected in cell cultures exposed to Tat for 2 hours ($127 \pm 11\%$, $P < 0.05$) and 24 hours (145 ± 8.4 , $P < 0.05$). In cell cultures treated with 50 nM Tat for 48 hours or longer, total caspase activity was not different from controls.

Neurotoxicity of Tat-containing conditioned medium from neuronal cell cultures continuously exposed to Tat 1–86

To determine if Tat 1–86 remains neurotoxic during continuous incubation, we collected the medium from 50 nM Tat- treated cultures and non-treated controls under sterile conditions 96 hours after the beginning of the experiment and applied the samples of cell-conditioned medium to a new set of cell cultures (Figure 5). At 96-hour time point the Tat concentration in cell-conditioned medium still contained 18.9 ± 0.7 nM Tat 1–86 (determined by ELISA). The 4 day-exposure of cell cultures to 96 hour-conditioned Tat containing medium decreased neuronal cell viability to $67 \pm 1.5\%$ ($P < 0.05$) of controls in which medium was replaced with 96 hour-conditioned medium without Tat. Tat immunoreactivity could be successfully removed from the medium by filtration through 10 kD-cut off molecular filters. After the filtration samples of the Tat-containing 96 hour-conditioned medium did not show detectable Tat immunoreactivity in dot-blotting and did not produce neurotoxicity (Figure 5).

Cell viability changes in rat primary neuronal cell cultures transiently exposed to Tat 1–86

To determine whether or not the continuous presence of Tat in the growth medium was required for neurotoxicity, rat fetal midbrain cell cultures were exposed to 50 nM Tat 1–86 for 2 hours and then re-incubated in the growth medium without Tat for 48 hours. No significant changes of Live/Dead ratios were detected immediately after the end of 2 hour-exposure of cell cultures to Tat. Forty eight hours after the transient exposure to Tat Live/Dead ratios in cell cultures were $78 \pm 4.1\%$ ($P < 0.05$) of control (Figure 6).

Cell viability changes in rat primary neuronal cell cultures following the second Tat exposure

To test the effect of repeated Tat treatment on the viability of surviving neurons, we replaced the Tat-containing medium in rat fetal midbrain cell cultures after 96 hours of incubation with the fresh dose of 50 nM Tat 1–86. In another group of Tat-treated cell cultures medium was replaced with the portion of growth medium not containing Tat. Changes of Live/Dead ratios were monitored in both groups for additional 48 and 96 hours. At the time of medium replacement Live/Dead ratios in Tat-treated cell cultures were $70.8 \pm 2.9\%$ of non-treated control. No additional cell death occurred in these cell cultures 48 or 96 hours after the beginning of the second treatment with Tat (Figure 7).

Tat 1–86 binding/uptake and caspase activity in rat primary neuronal cell cultures repeatedly treated with Tat 1–86

Despite the presence of Tat in the growth medium, no Tat immunoreactivity was detected in cultured neurons surviving 96 hours of continuous exposure to 50 nM Tat 1–86. No cell-

associated Tat immunoreactivity was present in cell cultures at the beginning of the second treatment of surviving neurons with a new 50 nM dose of Tat (data not shown). Two hours after the addition of a fresh portion of Tat-containing medium the presence of cell-bound/internalized Tat became evident again. The Tat immunoreactive band was present again on Western blots of cell lysates and Tat-positive neurons could be observed by immunofluorescent microscopy of cultures treated with a new dose of Tat 1–86. (Figure 8). However, no increase in total caspase activity was observed either 2 hours or 24 hours after the start of the second Tat treatment (data not shown).

MCP1 (CCL2) immunoreactivity in rat fetal midbrain cell cultures continuously exposed to Tat

Neurons positively stained with anti-MCP1 antibodies were present in rat fetal midbrain cell cultures (Figure 9A). We analyzed effects of Tat exposure on anti-MCP1 immunofluorescence in cultures treated with 50 nM Tat 1–86 for 2, 24, 48, and 96 hours. Microplate readings of plates stained for MCP1 immunoreactivity (normalized to Hoechst staining of intact cell nuclei) demonstrated a significant increase in MCP1 levels in Tat-treated cell cultures following 24 and 48 hours of exposure. In cell cultures treated with Tat for 96 hours the surviving neurons exhibit MCP1 immunoreactivity levels not significantly different from non-treated controls (Figure 9B).

NMDA receptor subunits (NR1, NR2A, NR2B) immunoreactivity in rat fetal cultured neurons surviving the continuous exposure to Tat

NR1, NR2A and/or NR2B immunofluorescent signals were measured in control and Tat-treated midbrain cell cultures. A significant decrease of NR2A immunoreactivity (normalized to Hoechst fluorescence) was observed in cultures treated with 50 nM Tat 1–86 for 96 hours (Figure 10).

DISCUSSION

Neurotoxic effects of HIV-1 Tat protein in a variety of cell culture models are well documented (Kruman et al, 1998; Bonavia et al, 2001; Aksenova et al, 2006). Continuous exposure to different doses of recombinant Tat 1–72 or Tat 1–86 for hours or days increases numbers of apoptotic neurons in Tat-treated cell cultures. Specifically, we have reported that in rat hippocampal cell cultures maximum neurotoxic effect of 48-hour treatment with Tat 1–72 could be observed at concentration of 100–150 nM of this recombinant variant of HIV-1 Tat in the growth medium (Aksenov et al, 2006). Consistent with our studies in primary cultures of rat fetal hippocampal neurons, we found that different doses of recombinant full-length Tat 1–86 cause the cell death in primary midbrain cell cultures. Doses of Tat 1–86 that produced maximum effect were in the same concentration range (100–150 nM) as previously reported for Tat 1–72 in hippocampal cell cultures. The full-length open reading frame of HIV-1 Tat is composed of the two exons of the viral *tat* gene and encodes a protein of approximately 101 amino acids. The LAI/Bru strain of HIV-1 encodes an 86-amino acid full-length Tat protein due to a premature stop codon within the second *tat* exon. In the late stage of the infection cycle, a C-terminally truncated Tat, encoded only by the first *tat* exon, is generated when unspliced viral RNAs are exported to the cytoplasm by the viral Rev protein. Both Tat 1–72 and Tat 1–86 can transactivate the HIV promoter and are neurotoxic.

Most of our recent studies of HIV-1 Tat neurotoxicity were carried out in primary cultures of rat fetal neurons (90–95% neuronal, 5–10% astrocytes). Highly purified cultures of embryonic neurons allow for a more precise focus on neuron-specific signaling events and on neuronal proteins.

Although HIV-1 Tat is clearly neurotoxic in vivo and in vitro (King et al, 2006), information about the time course of the development of Tat-mediated neurodegeneration is very limited. The majority of experiments that we report in this study were done in rat fetal midbrain cell cultures. Therefore, we present the data that describe the progress of cell viability changes induced by recombinant Tat variant 1–86 in this particular experimental model. However, the similar time course of the cell death can be observed in rat fetal hippocampal and/or cortical neuronal cell cultures treated with Tat 1–86 under the same experimental conditions (unpublished observations). Our observations of the development of Tat-induced decline in cell viability are consistent with previous studies that used similar conditions of treatment to investigate the development of apoptosis in rat primary neuronal cell cultures exposed to Tat (Bonavia et al, 2001; Perry et al, 2005). Neuronal cell death becomes evident in rat fetal neuronal cell cultures following more than 24 hours of the exposure to toxic dose of HIV-1 Tat protein. However, direct interactions of Tat with cultured neurons, which includes binding of Tat to neuronal cell membrane, the uptake of the protein and translocation of it into the nuclei, require a very brief exposure time (Chandra et al., 2005). Our results consistently confirm that the absorption of Tat by rat fetal neurons occurs within minutes after the addition of Tat to the culture medium. In our previous studies we reported increased free radical production and mitochondrial dysfunction following 2 hour exposure of rat hippocampal cell cultures to 50 nM Tat 1–72 (Aksenov et al., 2006).

As we report in the current study, the activation of early caspase 9 becomes evident as soon as 2 hours after the beginning of Tat treatment. The time period during which Tat 1–86 immunoreactivity is detectable in cell lysates overlaps with the increase in early and total caspase activities. The detection of activated caspases was performed directly in alive cells. The immunodetection of cell-bound Tat immunoreactivity required the fixation of cell cultures or preparation of cell lysates. Therefore, we were unable to investigate the co-localization of bound/internalized Tat 1–86 with activated caspases in neurons in our experiments. Our observations are in agreement with the suggestion that direct interactions of Tat induce oxidative stress-dependent apoptosis in neurons (Kruman et al, 1998, Perry et al, 2005; Aksenov et al, 2006).

Images of Tat immunoreactivity in cultured rat fetal midbrain neurons treated with 50 nM Tat for 2 hours showed that at the time when the level of cell-bound Tat was maximal, the culture contained populations of Tat-positive and Tat-negative neurons. This indicates that rat fetal neurons in culture may differ in their ability to interact with extracellular Tat.

Our study demonstrates that even a brief exposure of cultured neurons to Tat is sufficient to trigger neuronal apoptosis. This finding underscores the key role of rapid direct interactions of Tat with cultured neurons in the development of Tat neurotoxicity. Previously, the sustained increase of cytokine and chemokine expression was reported in astrocytes transiently exposed to Tat (Nath et al, 1999). However, the “hit-and-run” neurotoxic effects of Tat in primary neuronal cell cultures have not been previously documented. Our results are supportive to the suggestion that transient encounters of brain cells with neurotoxic HIV-1 proteins, such as Tat, can initiate the process of cerebral dysfunction (Nath et al, 1999). The continuous presence of neurotoxic Tat in the brain may not be required for the development of HIV-associated neurodegeneration.

We determined that the period of elevated caspase activity correlated well with cell viability changes in cultures continuously exposed to Tat. The return of total caspase activity to control levels after 48 hours of treatment was followed by the stabilization of neuronal viability in Tat-treated cell cultures. About 70% of the cell population was able to survive prolonged exposure to toxic doses of Tat. During the period of continuous incubation, the concentration of extracellular Tat in cell culture medium decreased as a result of a combination of initial non-

specific absorption, binding/uptake of the protein by cultured midbrain cells, and chemical degradation.

Tat 1–86 remaining in cell-conditioned medium after the 96 hour of incubation induced the cell death in cultures that were not previously exposed to Tat. Thus, the survival of a significant part of cell population in cultures continuously treated with Tat could not be explained by the loss of the toxic properties of Tat-containing medium. We suggest that rat fetal neuronal cell cultures initially contain a subpopulation of neurons (approximately 30% of total cell population in primary cultures prepared from the rat fetal midbrain), in which direct and rapid interactions with Tat trigger caspase-dependent apoptotic cascades.

We observed that the continuous exposure of primary cultures of rat fetal neurons to HIV-1 Tat was associated with the decrease of Tat immunoreactivity in cell lysates and disappearance of Tat-immunopositive cells from cultures. It was suggested that incubation with toxic Tat resulted in the selective loss of neurons able to bind/uptake Tat from the medium. However, we found that the ability of surviving neurons to internalize Tat 1–86 could be restored by the replacement of old Tat-containing medium with a new dose of Tat 1–86. As it follows from our results, the exposure to Tat may activate adaptive mechanisms that stimulate the removal of cell-bound Tat protein and limit its re-absorption from the medium.

Due to the lack of fixed conformation, Tat is able to interact with a variety of intracellular and extracellular molecules (Shojania and O'Neil, 2006). Therefore, Tat was reported to produce various changes in neuronal structure and function. Some of the documented effects of Tat (activation of caspases, for instance) are undoubtedly linked to its mechanism of toxicity. Some may reflect adaptive cell responses to Tat insults.

Many studies suggested that binding to chemokine receptors plays an important role in Tat interactions with mammalian cells. Tat mimics chemokine features and is able to displace binding of β -chemokines from the chemokine receptors CCR2 and CCR3, but not CCR1, CCR4, and CCR5 (Albini et al, 1998). The physiological ligand of CCR2, monocyte chemoattractant protein-1, MCP1 (CCL2), which is expressed and secreted by different brain cell types, can compete with Tat for binding to CCR2 receptors. Neurons in the rat brain express CCR2 (Kalkonde et al., 2007; Huerta et al., 2004) and MCP1 (CCL2) (Banisadr et al, 2005). Recently MCP1 (CCL2) was shown to be protective against Tat neurotoxicity in neuronal cell cultures (Eugenin et al., 2007). Tat was shown to enhance the expression of MCP1 (CCL2) in astrocytes and microglia (D'Aversa et al, 2005; El-Hage et al, 2006). Increased MCP1 (CCL2) production and secretion may help to inhibit Tat interactions with CCR2 and attenuate the following changes in CCR2-dependent signaling. However, the effects of Tat on MCP1(CCL2) expression in neurons have not been explored. Therefore, we investigated MCP1(CCL2) expression in rat fetal midbrain cell cultures during the continuous Tat treatment. In our experiments we detected increased MCP1(CCL2) immunoreactivity in neuronal cell cultures exposed to Tat 1–86. The increase in MCP1(CCL2) levels correlated with the decrease in Tat immunoreactivity in cell extracts, which makes it possible to suggest that MCP1 (CCL2) expression may be related to changes in levels of cell-bound Tat immunoreactivity levels. Changes in MCP1 may reflect compensatory neuronal response to interactions of Tat with CCR2 receptors. Maximum increase in MCP1 immunoreactivity levels coincided with the end of the period of increased total caspase activity in Tat-treated cell cultures. In this study we did not investigate whether or not the exposure to Tat caused the decline in relative numbers of CCR2-expressing neurons. In the surviving neurons after 96 hours of treatment, when Tat 1–86 immunoreactivity became undetectable in the cell lysates, MCP1(CCL2) was not different from controls. CCR2 is not the only type of neuronal membrane receptor protein that participates in binding and internalization of Tat. The observed changes in MCP1(CCL2) expression could not fully explain why neurons continuously exposed to Tat could not re-

uptake it from the medium. However, our study indicates that MCP1 may be one of the factors involved in the mechanism of inhibition of direct Tat toxicity to neurons. Further studies are needed to investigate other mechanisms that may cause the decrease of absorption of extracellular Tat and stimulate clearance of internalized Tat from the cells. For instance, increased expression of extracellular proteinases able to degrade Tat bound to the cell membrane may contribute to the reversible inhibition of Tat binding observed in our experiments.

While the inhibition of Tat uptake by the surviving Tat-treated neurons could be reversed by the replacement of the medium, this subpopulation of neurons was completely resistant to toxic effects of a new dose of Tat. Thus, the ability of these neurons to resist the Tat treatment should be linked to certain biochemical features that preclude Tat-induced neuronal apoptosis without a detectable inhibition of Tat binding.

Since NMDA receptors are known to play the key role in the mechanism of Tat-mediated apoptosis (Chandra et al., 2005; King et al, 2006), we examined the expression of NMDA receptors in neurons that survived prolonged Tat exposure. NMDA receptor complexes are composed of two NR1 subunits and at least one type of NR2 subunits with predominantly NR2A or NR2B subunits in adult rat hippocampus (Wenzel et al., 1997). Results of anti-NR1 immunostaining demonstrated that the exposure of midbrain cell cultures to Tat did not lead to the selective loss of NR1-expressing neurons. Thus, the insensitivity of neuronal survivors to repeated Tat treatment was not due to the decreased expression of NMDARs. However, these results did not exclude the possibility that Tat-resistant neurons may have NMDAR complexes with different subunit composition. HIV-1 Tat affects NMDAR-mediated calcium channeling either by altering the function of polyamine-sensitive site presumably located on NR2B (Prendergast et al, 2002; Self et al, 2004) subunit of NMDAR or by releasing the Zn^{+2} -mediated inhibition of NR1/NR2A NMDA receptor subtype (Chandra et al., 2005). The relative contribution of NR2A and NR2B-containing NMDA receptor subtypes to Tat neurotoxicity has not been investigated. However, several studies suggested that the NMDAR subunit composition may be important for the sensitivity of neurons to NMDA-mediated excitotoxicity (Zou and Baudry, 2006; Brewer et al, 2007). We found that the resistance to Tat was associated with the decreased level of NR2A immunoreactivity in the surviving rat fetal midbrain neurons. The observed changes could result from Tat-mediated down-regulation of NR2A expression and/or from the death of neurons expressing NR2A-containing NMDARs. Along with the absence of statistically significant changes in NR1 and NR2B subunits, the decreased expression of NR2A indicates that in Tat-resistant rat midbrain neurons the composition of NMDARs may be shifted toward the relative prevalence of 2NR1/2NR2B over 2NR1/2NR2A or 2NR1/NR2A/NR2B subunit complexes. As it was demonstrated by Brewer and co-authors (Brewer et al, 2007), the enhanced excitotoxic neuronal vulnerability of aging hippocampal neurons is associated with an increase in NR1, NR2A, but not NR2B subunit expression. We suggest that changes in the subunit composition of NMDAR complexes due to the decreased NR2A expression may, at least in part, contribute to the ability of the surviving neurons to resist Tat-induced apoptotic death.

In our studies of HIV-1 Tat toxicity we employ primary neuronal cultures isolated from brain regions that are known to be particularly vulnerable to HIV-associated neuropathology: midbrain, hippocampus, and cortex. All these regions contain neurons that express components of glutamate and dopamine neurotransmission systems. Neurons in primary midbrain, hippocampal, and cortical cell cultures are sensitive to Tat-mediated injury (Bonavia et al, 2001; Aksenov et al, 2006; Aksenova et al, 2006). Dysfunctions of dopamine transmission have long been identified as critical determinants of subcortical dementias, such as Parkinson's disease and HIV-associated dementia (HAD). Dopamine and glutamate have been shown to extensively interact to regulate different physiological functions. There is increasing evidence

that concurrent alterations of dopamine and glutamate function may play an important role in the pathogenesis of these diseases (Missale et al, 2006; Silvers et al, 2007).

The changes in NMDAR subunit composition, which result from the exposure of neurons to HIV-1 Tat, may be coupled with changes in expression and functioning of components of dopamine neurotransmission system. Neurons in rat fetal midbrain cell cultures express dopaminergic markers (tyrosine hydroxylase, fully-functional dopamine transporter, dopamine receptors) and NMDA receptors. Therefore, the primary culture of rat fetal midbrain neurons is a good model to study interactions between the dopamine D1 and the glutamate NMDA receptors in the mechanism of Tat toxicity. We previously reported that treatment of rat fetal midbrain cell cultures with Tat for 48 hours results in the decline of dopamine transporter- (Aksenova et al, 2006) and D1- specific ligand binding (Silvers et al, 2007). In striatal postsynaptic densities (PSD), the dopamine D1 receptor is a part of oligomeric complex with the NMDA receptor (Fiorentini et al, 2006). Activation of D1 receptors can selectively change NR2B phosphorylation and trafficking, which may result in relative increase of NMDAR complexes that include either one or two NR2B subunits (Hallet et al, 2006). Dopamine D1 receptor activation potentiates NMDA transmission and is required for NMDA-mediated long-term potentiation (Fiorentini and Missale, 2004). Rapid interaction with extracellular Tat inhibits neuronal dopamine uptake in vitro (Wallace et al, 2005). The selective antagonist of D1 receptors have been shown to attenuate Tat-induced death of rat fetal midbrain neurons (Silvers et al, 2007). Collectively, our studies suggest that interactions between the dopamine D1 and the glutamate NMDA receptors may be relevant to the mechanism of Tat neurotoxicity. Further studies are needed to clarify the importance of the composition of oligomeric NMDA receptor complexes and dopamine-glutamate interactions for neuronal sensitivity to neurotoxic effects of HIV-1 Tat.

The investigation of biochemical features of neuronal cells that survive Tat-mediated toxic insults may provide valuable information for the development of protective therapeutic strategies for patients suffering from HIV-associated neuronal dysfunction.

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Figure 1.

The dose-response and the time course of decreased neuronal cell viability in primary rat fetal midbrain cell culture exposed to HIV-1 Tat 1-86. A. The graph shows the decrease in Live/Dead ratios produced by different doses of recombinant Tat 1-86 after 48 hours of treatment. The relative (compared to non-treated controls) cell viability decrease was determined by the formula: $(1 - [F_{530nm}/F_{645nm}]_{well\ n} / [F_{530nm}/F_{645nm}]_{average\ max}) \times 100\%$. Data presented as mean values, n of sister cultures analyzed = 6–15 per each Tat 1-86 concentration. B. The graph represents relative (compared to non-treated controls) changes in Live/Dead ratios following the addition of 50 nM Tat 1-86. Data presented as mean values, n of sister cultures analyzed = 7–12 per each time point. The curve represents the best fit equation for the set of

mean values: a 4 parameter logistic curve. The best fit function was selected using SigmaPlot 8.0 Regression analysis.

Figure 2.

Binding/uptake of Tat 1–86 by cultured rat fetal midbrain neurons during the continuous exposure of cell cultures to 50 nM dose of Tat. A. The graph shows changes in Tat 1–86 concentration (measured using anti-Tat ELISA) in the medium during continuous incubation with rat fetal midbrain cell cultures. The control curve shows results obtained from the wells containing only 50 nM Tat in the medium, but no rat fetal midbrain cells. Data presented as mean values $\pm$ SEM ($n=3$ per each time point). B. The graph shows amounts of Tat 1–86 per well specifically absorbed by midbrain cells during the first 2 hours of treatment. Data presented as mean values $\pm$ SEM ($n=3$ per each time point). Panel C shows the increase of bound/internalized Tat in cell lysates within 15–60 min after the addition of 50 nM Tat to the cell culture medium. Panel D shows the decrease of bound/internalized Tat in cell lysates during 2–96 hours of continuous exposure to 50 nM Tat 1–86.

Figure 3.

The representative image of Tat immunoreactivity in rat fetal midbrain cell cultures exposed to 50 nM Tat 1–86 for 2 hours. Red arrowheads point at Tat-immunopositive cells, white arrowheads point at cells that do not contain Tat. Colored boxes mark areas of the selection, which are shown as new magnified images with greater resolution.

Figure 4.

Caspase activity in rat fetal midbrain cell cultures continuously exposed to 50 nM Tat 1–86. Microscopic images of the caspase 9 activity (red fluorescence) in rat fetal midbrain cell cultures treated for 2 hr with Tat (Panel A) and in non-treated control (Panel B). The graph in Panel C shows the time course of total caspase activity in rat fetal midbrain cell cultures continuously exposed to 50 nM Tat 1–86. The graph represents relative (compared to non-treated controls) changes in CR-VAD- fluoromethyl ketone green fluorescence (normalized to Hoechst fluorescent signal) following the addition of 50 nM Tat 1–86. Data presented as mean values $\pm$ SEM, n of sister cultures analyzed = 7–12 per each time point. *- P<0.05.

Figure 5.

Tat-containing growth medium remains neurotoxic after prolonged incubation with rat fetal midbrain neurons. Live/Dead ratios were determined 4 days (96 hours) after the replacement of the medium in test groups of non-treated cell cultures (n=12) with 96 hour-conditioned growth medium that contained (TCM 96) or did not contain (CM 96) Tat 1–86. The results were compared with control cultures in which the medium was not replaced. The second graph in the panel illustrates decreased toxicity of the conditioned medium from Tat-treated cell cultures after the removal of Tat by ultrafiltration. Graphs show relative (% versus control) changes in cell viability. Data presented as mean values $\pm$ SEM. * - $P < 0.05$. Dot-blot on the

right side of each graph show results of testing of conditioned medium samples for Tat immunoreactivity.

Figure 6.

The cell viability decrease in rat fetal midbrain cell cultures transiently exposed to 50 nM Tat 1–86. The graph represents relative (compared to non-treated controls) Live/Dead ratios determined 48 hours after the replacement of the medium in cultures pretreated with Tat for 2 hours and cultures that were not exposed to Tat (control group). Data presented as mean values $\pm$ SEM, n of sister cultures analyzed = 7–12 per each group. * - $P < 0.05$

Figure 7.

Cell viability changes in rat primary neuronal cell cultures following the second Tat exposure. The graph shows the results of the repeated treatment of cultures that survived 96 hours of continuous exposure to Tat with a fresh portion of Tat-containing medium of the same concentration. The graph represents relative (compared to non-treated controls) Live/Dead ratios determined in cell cultures treated with 50 nM Tat for 96 hours and in surviving cell cultures that were re-exposed to 50 nM Tat 1–86 for either extra 48 or 96 hours. Data presented as mean values $\pm$ SEM, n of sister cultures analyzed = 7–12 per each group.

Figure 8.

Tat 1–86 binding/uptake in rat primary neuronal cell cultures repeatedly treated with Tat 1–86. The image of Western blot illustrates the restoration of Tat bound/internalized immunoreactivity levels in cell lysates 2 hours after the replacement of 96 hour-old Tat-containing medium with a new 50 nM dose of Tat 1–86. Lane 1- lysate prepared from cell culture treated for 96 hours with 50 nM Tat 1–86. Lane 2- lysate prepared from the same culture in which old Tat-containing medium was replaced with new portion of 50 nM Tat (2 hour exposure). The image shows re-appearance of Tat-positive cells in the cultures pre-incubated with Tat for 96 hours. Image of Tat immunofluorescence was taken 2 hours after the addition of a new 50 nM Tat 1–86 dose.

Figure 9. Changes in MCP1 (CCL2) immunoreactivity in rat fetal midbrain cell cultures exposed to Tat. Microscopic image illustrates the presence of MCP1-immunopositive cells in rat fetal midbrain cell culture. The graph represents relative (compared to non-treated control) changes in MCP1 (CCL2) immunofluorescence normalized to Hoechst (cell density) fluorescent staining during the continuous exposure of cultures to 50 nM Tat 1–86. Data presented as mean values $\pm$ SEM, n of sister cultures analyzed = 6 per each group. *- P < 0.05.

Figure 10.

NMDA receptor subunits (NR1, NR2A, NR2B) immunoreactivity in rat fetal cultured neurons surviving the continuous exposure to Tat. The graph represents NR1, NR2A, NR2B immunofluorescence (A_{528}/A_{460}) normalized to Hoechst (cell density) fluorescent staining in control non-treated cell cultures and in cultures treated with 50 nM Tat 1–86 for 96 hours. Data presented as mean values $\pm$ SEM, n of sister cultures analyzed = 7–12 per each group. * - $P < 0.05$.