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Evaluation of HIV-1 Tat induced neurotoxicity in rat cortical cell culture

Aleida Pérez[1], Albert W Probert[2], Kevin KW Wang[2] and Lamia Sharmeen*[,1]

[1]*Department of Infectious Diseases, Parke-Davis Pharmaceutical Research, Division of Warner and Lambert, 2800 Plymouth Road, Ann Arbor, MI 48105, USA and* [2]*Department of Neuroscience Therapeutics, Parke-Davis Pharmaceutical Research, Division of Warner and Lambert, 2800 Plymouth Road, Ann Arbor, MI 48105, USA*

In a substantial number of cases, Human Immunodeficiency Virus type 1 (HIV-1) infection causes neuronal cell loss and leads to the development of AIDS associated dementia. Several studies have suggested that both host and viral factors contribute to neuronal loss. Here we studied the effect of HIV-1 Tat in primary rat neuronal cells as a model to understand mechanism of neuronal cell death. At nano molar concentration, recombinant Tat induced cell death in primary rat mixed cortical neurons. Tat could also induce uptake of calcium in primary rat cultures. When cells were incubated with NMDA receptor antagonists, MK-801 and D-CPP, cell death and ^{45}Ca uptake were inhibited. Under similar conditions non-NMDA antagonists, NBQX, DNQX and CNQX, and sodium channel antagonist, TTX, did not inhibit Tat induced neuronal cell death. In a similar way HIV associated products from *in vitro* **HIV-1 infected cells induced neuronal cell death which was inhibited by NMDA receptor antagonist. Results presented in this paper suggest that activation of NMDA receptors by HIV-1 Tat is responsible for neuronal cell death in primary rat cortical neurons.** *Journal of NeuroVirology* (2001) **7,** 1–10.

Keywords: neuronal cell death; HIV-1; Tat; NMDA receptors

Introduction

Human Immunodeficiency Virus (HIV-1) infection is associated with neuronal cell death resulting in the development of the neurological disease of AIDS dementia complex. AIDS dementia affects about 30% of the people with AIDS and it is more prevalent in pediatric AIDS. It is characterized by various clinical and neuropathological manifestations that include memory and motor disorder, neuronal loss, and damage to the white matter and astrogliosis (Bloom and Rausch, 1997; Epstein and Gendelman, 1993; Johnson, 1995; Sanders *et al*, 1998; Vitkovic and DaCunha, 1995). In the brain, primarily macrophages and microglia are infected with HIV while restricted infection of astrocytes has been reported (Bloom and Rausch, 1997); He *et al*, 1997; Lavi *et al*, 1998). However, there is no evidence of direct virus infection of neurons.

Several lines of evidence suggest that loss of neurons could be induced by viral and cellular factors that are activated and released upon virus infection. In some cases these effects are transmitted through glutamate receptors with a pivotal role in induction of toxicity observed in AIDS dementia (Adamson *et al*, 1996; Ferrarese *et al*, 1997; Lipton, 1996, 1998). Cellular factors such as tumor necrosis factor alpha (TNF-α) and platelet activating factor (PAF) can prevent glutamate uptake by the astrocytes resulting in activation of glutamate receptors, which can cause neuronal cell toxicity (Nishida *et al*, 1996; Nokta *et al*, 1995; Fine *et al*, 1996). Similarly the HIV-1 envelope protein (gp120) induces neuronal loss through the activation of NMDA receptors (Lipton *et al*, 1991). The effect of gp120 on NMDA receptor is mediated indirectly through arachidonic acid (Dreyer and Lipton, 1997; Maccarrone *et al*, 1998; Ushijina *et al*, 1995).

Tat is a virus encoded non-structural regulatory protein that primarily functions as a transactivator of the HIV promoter. Tat is secreted from infected cells and can be taken up by neighboring

*Correspondence: L Sharmeen
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cells (Frankel *et al*, 1988; Mann and Frankel, 1991). Besides its function on the viral promoter, Tat has been shown to induce chemotaxis, apoptosis and immunosuppression, and cell proliferation in lymphoid cells (Albini *et al*, 1998a,b; Buonaguro *et al*, 1992; Ito *et al*, 1998; Katsikis *et al*, 1997; Lafrenie *et al*, 1996, 1997; Li *et al*, 1995; Scala *et al*, 1994; Westendorp *et al*, 1994). In the brain, Tat is toxic (Magnuson *et al*, 1995; Nath *et al*, 1996; New *et al*, 1997) and induces the expression of the cytokines such as TNF-α, MCP-1 and IL-6 (Chen *et al*, 1997; Conant *et al*, 1998; Sanders *et al*, 1998; Zidovetzki *et al*, 1998). In certain cases, Tat toxicity in human neuronal cells has been suggested to be mediated through the activation of non-NMDA receptors (Magnuson *et al*, 1995; New *et al*, 1998). However Tat from Visna virus, a related lentivirus, has been reported to be toxic in rodent neuronal cells and activates NMDA receptors (Hayman *et al*, 1993; Philippon *et al*, 1994).

Here we evaluate the Tat induced cell toxicity using different pharmacological agents in rat neuronal cells. Tat at nanomolar concentrations induces death in rat mixed cortical neurons. Extracellular Tat stimulates a Ca^{+2} flux in the cortical neurons that was inhibited by N-methyl-D-aspartate (NMDA) antagonists, dizocilpine (MK-801) and CPP while non-NMDA antagonists did not have any effect. NMDA antagonists also inhibited cell death, while non-NMDA antagonist or sodium channel blocker (TTX) did not. TNF-α and IL-1β expression was elevated in rat cortical neurons, which suggest that cytokines could be involved indirectly in the Tat induced cell death. These studies strongly suggest that direct activation of NMDA receptors could be responsible for loss of neurons and eventually contribute to development of AIDS dementia.

Results

Assessment of HIV-1 Tat toxicity in rat mixed cortical neurons

We examined toxicity of HIV-1 Tat protein in primary rat cortical neurons. Per cent cell death observed in neuronal culture was calculated by the failure to exclude trypan blue and the leakage of the enzyme lactate dehydrogenase (LDH) in culture medium.

Cells were treated with 1 – 14 μg/ml concentrations of recombinant Tat in culture medium. Following 8 h after addition of Tat, cell death was visible which peaked at 12 h as confirmed by increased LDH release into culture medium. In agreement with others (Magnuson *et al*, 1995; Nath *et al*, 1996; New *et al*, 1997), Tat at concentrations of 0.5 μM or above induced neuronal cell death (Figure 1a). Apoptotic cells demonstrated condensed, brightly stained nuclei indicative of apoptosis (Figure 1c). In a typical culture 20 – 40% apoptotic nuclei were observed which correlated with peak LDH release in culture. Based on morphological examination, most of the cell death occurred in neurons while glial cells remained resistant to Tat induced toxicity. The specificity of purified Tat protein was tested along with a purified HSV-1 exonuclease protein and the neuronal death was only observed with HIV-Tat protein and no such death occurred with either purified HSV-1 exonuclease (Figure 1b). In addition when recombinant Tat was heat treated, the cell culture was still sensitive to Tat induced toxicity demonstrating that endotoxins are not responsible for the observed effect. Under similar conditions trypsin treatment of Tat protein abolished the toxic effect to background level (Figure 1b).

NMDA antagonists inhibit Tat toxicity

To understand the possible mechanisms of Tat induced toxicity, we used a series of pharmacological agents. Cells were incubated with Tat in the presence or absence of N-methyl-D-aspartate (NMDA) anagonists. Figure 2A shows that in the absence of NMDA antagonists Tat induced 63.3% cell death in neuronal culture. However, when cells were incubated with NMDA antagonists, MK801 and D-CPP, Tat toxicity was reversed to that of control untreated cells (30 – 32% cell death respectively). To test whether other glutamate receptors could also mediate the observed toxicity, cells were treated with non-NMDA antagonists. At concentration twice above the reported IC_{50} (Honore *et al*, 1988), no inhibition of Tat induced cell death was observed (Figure 2B). Sodium channel blocker, TTX, has been shown to block toxicity of HIV-1 gp120 (Diop *et al*, 1994), however, TTX did not inhibit Tat mediated cell death (Figure 2B). Therefore, lack of neuroprotection from Na^{+} channel blocker and non-NMDA antagonists suggest that Tat toxicity is mediated through the activation of NMDA receptors.

Effect of Tat on intracellular Ca^{+2} level through NMDA receptors

NMDA receptors appear to participate in excitatory process and neuronal death. One of the characteristics of NMDA induced neuronal cell death is a sustained increase in Ca^{+2}. Earlier intracellular Ca^{+2} dysregulation and neuronal cell death by Tat was reported (Haughey *et al*, 1999; Kruman *et al*, 1998). We tested whether Tat can activate glutamate receptor by measuring uptake of Ca^{+2} by neurons. Rat mixed cortical neurons were incubated with Tat and $^{45}Ca^{+2}$ for 40 min in the presence or absence of NMDA antagonists. Incubation of cells in the presence of NMDA or non-NMDA antagonists did not result in any measurable changes in intracellular calcium. However, when cells were incubated

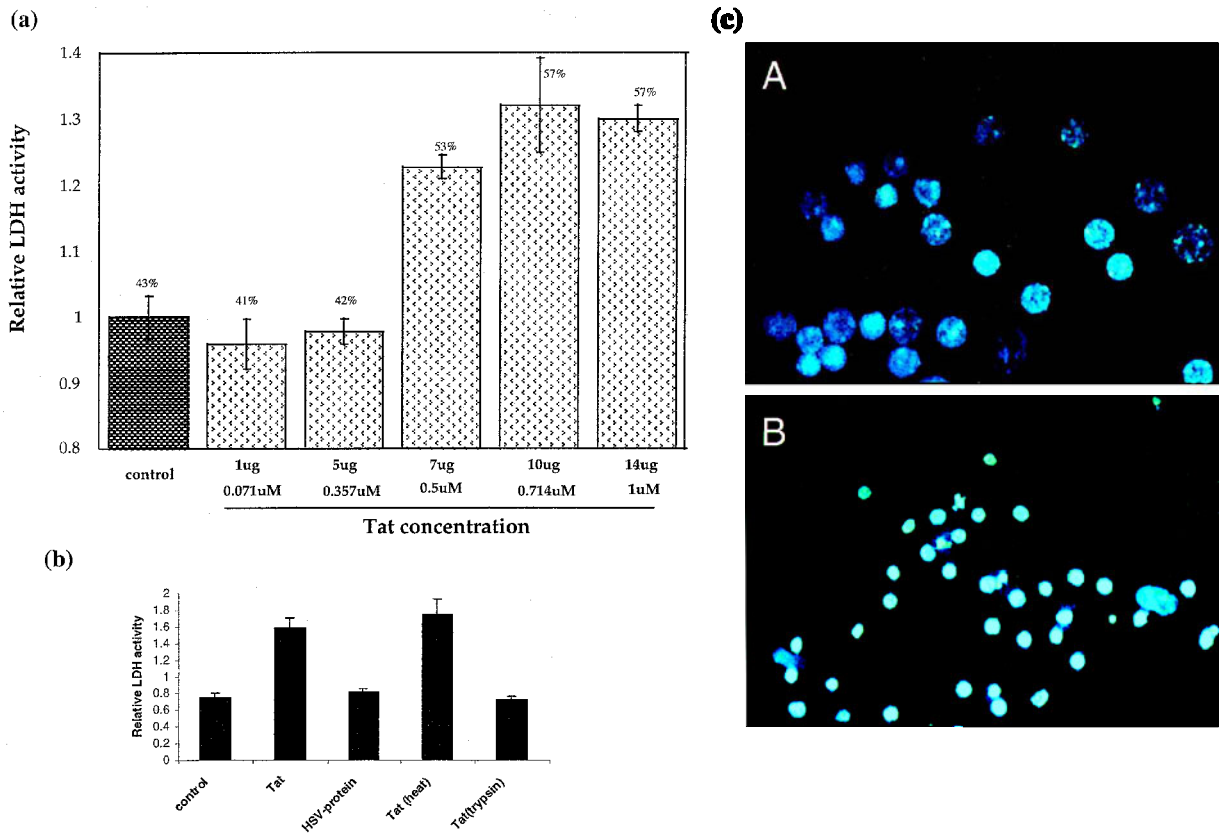

Figure 1 (**a**) Effect of Tat on rat mixed cortical neurons. Rat mixed cortical neurons were incubated with various concentrations of Tat and cells incubated at 37°C for 12 h. Cell death was measured by lactose dehydrogenase (LDH) release in the media. Results are plotted as relative LDH activity to that of the control wells. Results are shown as the mean ± s.d. (n=8). Percent cell death are shown. (**b**) Specificity of Tat protein on toxicity in rat cortical cultures. (**c**) Hoechst 33258 staining of neuronal culture without Tat (A) and with Tat (B). In (**b**) Hoechst 33258 staining revealed many condensed brightly stained nuclei.

with Tat, an increase in $^{45}Ca^{+2}$ uptake by neurons was observed (Figure 3). Only addition of NDMA antagonists, MK801 and DCPP inhibited the Tat induced calcium flux. The non-NMDA antagonists did not have any effect. This result demonstrates that NMDA receptors were activated by Tat resulting in release of intracellular calcium.

Tat and expression of cytokines

In lymphoid and neuronal cells, Tat can induce expression of various cytokines (Buonaguro *et al*, 1992; Conant *et al*, 1998; Zidovetzki *et al*, 1998). We assessed Tat mediated induction of cytokines by either RT – PCR or RNase protection assay. By RT – PCR, modest increase (1.5 – 2.5-fold) in rat TNF-α RNA was detected at 3 and 24 h after incubation with Tat. In addition to TNF-α, induction of IL-1β, IL-1α and IL-6 were detected as early as 3 and 24 h following addition of Tat in rat neuronal cell culture (Figure 4B). Since TNF-α has been shown to cause neuronal death (Fine *et al*, 1996; New *et al*, 1998; Nokta *et al*, 1995; Selmaj and Raine, 1988; Shi *et al*, 1998), we examined if TNF-α alone could cause cell death in rat neuronal cortical culture. Rat recombinant TNF-α (0.01 – 0.1 μg/ml) was added to same cell culture, but no cell death was detected (data not shown).

NMDA antagonists can block HIV infection mediated neuronal toxicity

Results presented above demonstrate that Tat can induce cell death in mixed cortical neurons through the activation of NMDA receptors. Although Tat protein in nanomolar concentration induced cell death, it is not clear if such concentration could exist in brain. It is quite possible that such concentration could reach in localized area where active virus replication occurs. To test this possibility we chose *in vitro* HIV infected cell culture and asked if it could induce similar neuronal cell death that could be blocked by NMDA receptor antagonists. HeLa-CD4 cells were infected with HIV-1_{IIIB} (a T-tropic strain) at multiplicity of infection of 1. After 3 days, virus particles from cell free supernatant was removed by centrifugation on a 20% sucrose cushion. The clarified cell free supernatant was added to neuronal culture. An increase in cell death ($P<0.05$) was detected but under same

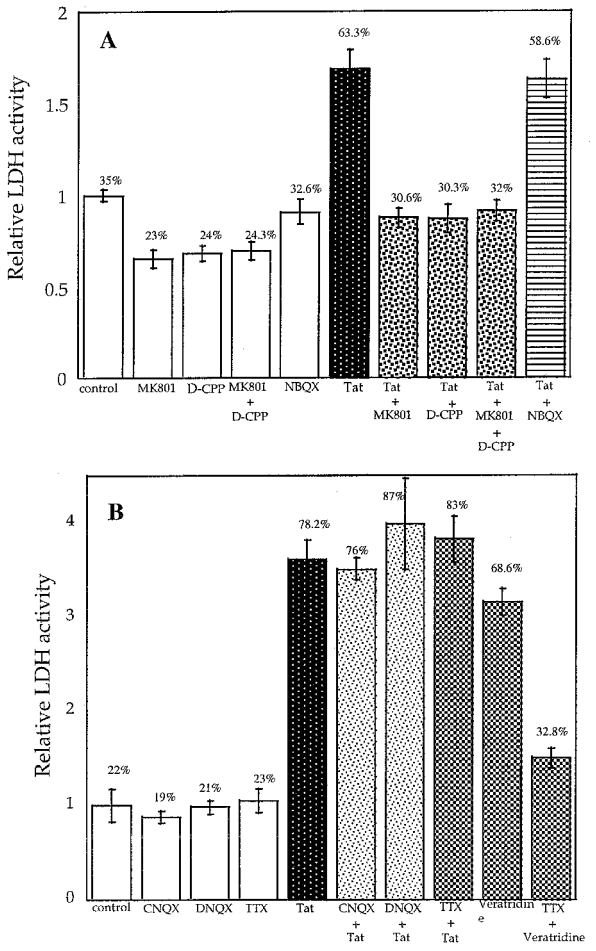

Figure 2 NMDA antagonists inhibit Tat induced cell death in rat mixed cortical neurons. Cells were pre-incubated with NMDA antagonists (**A**) or non-NMDA antagonists and sodium channel blocker (**B**) for 60 min at 37°C. Tat was added at a concentration of 0.7 μM and cells were incubated at 37°C for an additional 12 h. Cell death was measured by LDH release in the media. Results are plotted as relative LDH activity to that of the control. Results are shown are the mean ± s.d. (n=8). Per cent cell death are shown.

condition uninfected cell free supernatant did not cause similar level of cell death (Figure 5). Incubation with NMDA antagonist, D-CPP, decreased LDH activity to that of background level. The toxicity observed with infected-cell free supernatant is not due to secretion of cellular factors, since removal of virus and/or viral products failed to induce cell death. The possibility that the neurotoxicity of infected culture media was due to a nonspecific contaminant was excluded by immunoprecipitation. Since Tat and gp-120 were detected in supernatant (data not shown), each of the product was removed from culture medium by immunoabsorption. First, immunoabsorption of Tat with mouse anti-Tat antiserum coupled to protein G-conjugated agarose beads (Figure 5) reversed infected culture induced neurotoxicity to background level. In addition to Tat antibody, immunoabsorption of gp-120 with rabbit anti-gp120 coupled to protein A-conjugated agarose beads (Figure 5) also reversed the neurotoxicity. The specific contribution of secreted gp-120 induced neurotoxicity from HIV-infected culture is currently under investigation with pharmacological agents in our laboratory. Interestingly, infected media induced neurotoxicity could be reversed with NMDA antagonists as shown for recombinant Tat induced neurotoxicity. Therefore these results suggest that virus protein products including Tat in infected culture may induce cell death in rat mixed cortical neurons through the activation of NMDA receptors.

Discussion

Human immunodeficiency virus (HIV-1) invades the central nervous system resulting in the development of AIDS dementia. In this study, we show that rat mixed cortical neurons could be used as a model to elucidate the mechanism of HIV-Tat induced neuronal death. The observed effects of Tat induced toxicity on human brain neurons is in agreement with what has been previously reported (Nath *et al*, 1996; New *et al*, 1997; Sabatier *et al*, 1991). We have extended these studies to identify receptors that are involved in this process by using receptor specific antagonists, MK-801 and D-CPP to block Tat induced neurotoxicity. Tat induced calcium flux was also inhibited by NMDA antagonists which supports involvement of NMDA receptors.

Although HIV could be detected in the central nervous system (CNS) early in the course of virus infection, symptoms of dementia do not appear until later stages of the disease. The neurotoxicity induced by HIV infection is not unique since it is observed with other members of the lentivirus family. Visna virus encoded Tat is neurotoxic in primary rodent cells (Hayman *et al*, 1993; Philippon *et al*, 1994; Strijbos *et al*, 1995). The mechanism of visna virus encoded Tat toxicity was indirect where Tat induced expression of nitric oxide synthase (NOS) (Hayman *et al*, 1993) or cytokines (Philippon *et al*, 1994) resulted in over-activation of the NMDA receptors. In a similar system, direct activation of non-NMDA receptors could not be ruled out (Strijbos *et al*, 1995). Recently, it has been shown that Tat can activate non-NMDA receptors through tumor necrosis factor alpha (TNF-α) in human mixed cortical neuron (New *et al*, 1998). However, in our system, Tat was found to activate almost exclusively the NMDA receptor. Non-NMDA antagonists, NBQX, CNQX, DNQX did not block Tat induced neurotoxicity. The sodium channel blocker, TTX blocks Na^{+2} channel and thus release of glutamate at the presynaptic terminals. The lack of protection by TTX against Tat induced neurotoxi-

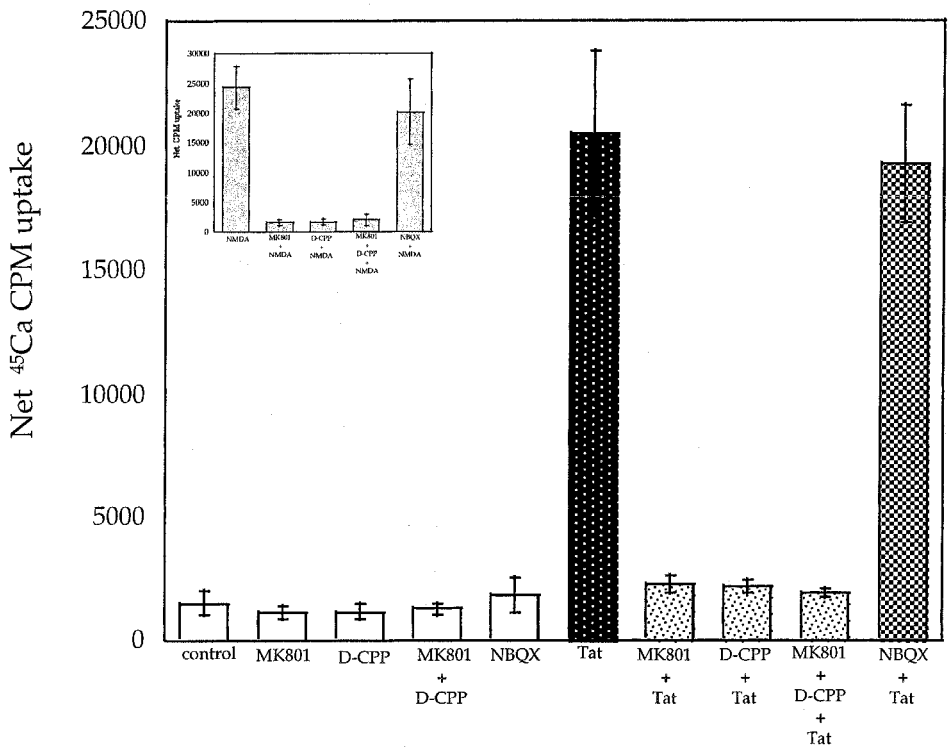

Figure 3 Tat elicits a Ca^{+2} uptake in cortical neurons. Cells were pre-incubated with NMDA antagonists for 60 min at 37°C followed by Tat (0.7 μM) and ^{45}Ca (1 μCi/ml) for 40 min as described in Materials and methods. Results are plotted as net CPM shown as the mean ± s.d. (n=4).

city suggest that Tat acts at the 'post-synaptic' receptor levels (NMDA, Non-NMDA receptors, Figure 2A,B). It appears that results supporting either NMDA or non-NMDA receptor could reflect differences in neuronal culture model, which might represent different extent of apoptotic and necrotic cell death. The culture used in these experiments have 47.5% neurons, 47.5% astroglia, but less than ~5% microglia (data not shown). Direct comparison of NMDA and non-NMDA pathways in a model system will be very useful for mechanistic understanding of neuronal death. It is well established that AMPA, kainate receptors (Non-NMDA receptors) are present in this culture and both AMPA and Kainate can kill neurons (Hajimohammadreza *et al*, 1995). Pharmacological agents are often used as an alternative tool to distinguish between biological pathways that may overlap. It is quite possible that in all neuronal toxicity, activation of NMDA receptors plays a key role in the overall scheme of cell death. We do not know whether Tat directly interacts with NMDA receptor or not. Since a modest (2 – 3-fold) increase in TNF-α RNA was observed (Figure 4) following addition of Tat in rat mixed cortical neurons, an indirect mechanism of Tat through activation of cytokines and thus its contribution to neurotoxicity needs further investigation. The recombinant rat TNF-α did not induce cell death in this system, therefore these results do not support a role of TNF-α on Tat mediated NMDA activation. It is possible that the regulation or the mechanism of action of TNF-α is distinct between human and rat cortical neurons. Alternatively, TNF-α may be induced in small percentage of glial cells in mixed cortical cultures which is not sufficient to induce neuronal death. We also detected activation of IL-6, IL-1α and IL-1β (Figure 4B), in Tat treated neuronal culture. It is known that AIDS induced dementia is associated with a late stage of the disease when severe inflammatory reactions could be followed by infiltration of infected macrophages. High level of viremia may lead to increased level of viral factors like Tat which may induce proinflammatory cytokines and chemokines in the brain. Since inflammatory cytokines contribute to neuronal loss, the indirect effect through inflammatory cytokines and its synergistic contribution to neuronal death, needs careful examination in an appropriate model.

Several mechanisms have been proposed to explain HIV induced degeneration of the CNS. The viral protein HIV gp120, induces neurotoxicity through the activation of NMDA receptors (Corasaniti *et al*, 1996; Fontana *et al*, 1997; Lipton *et al*, 1991; Raber *et al*, 1996; Toggas *et al*, 1996). The mechanism proposed for gp120 mediated activation

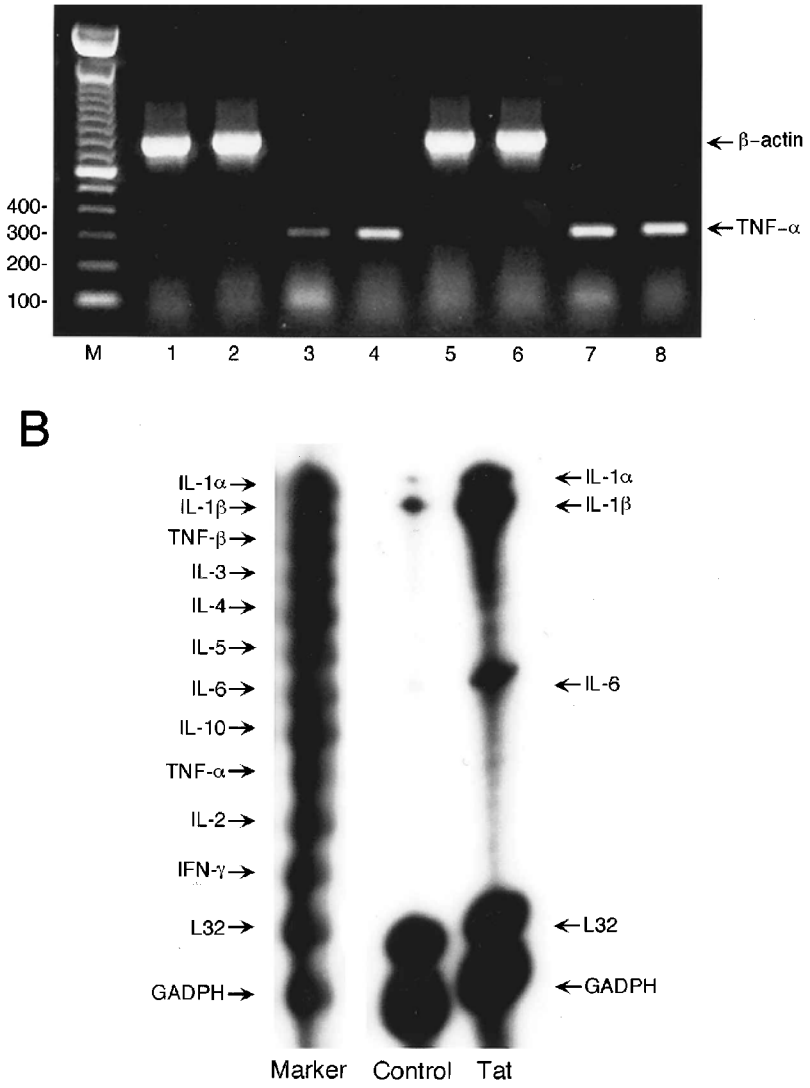

Figure 4 Tat induces the expression of cytokines. (**A**) Total RNA was extracted from rat mixed cortical neurons untreated (lanes 1 – 4) or treated with Tat (0.7 μM) (lanes 5 – 8) for 3 h (lanes 5, 7) or 24 h (lanes 6, 8). Untreated controls for 3 h (lanes 1, 3) or 24 h (lanes 2, 4). RT – PCR of rat TNF-α and internal control β-actin was performed as described in Materials and methods. PCR products were resolved in a 1.5% agarose gel and stained with ethidium bromide. M represents 100-bp molecular size markers. (**B**) RPA assay for detection of rat cytokines with rCK-1 probe as described in Materials and methods. Marker lanes represent different sizes of protected RNA bands. Control untreated and Tat treated cultures are indicated.

of NMDA receptor was indirect in which gp120 from infected macrophages produced arachidonic acid, which in turn inhibited the uptake of glutamate by astrocytes (Maccarrone *et al*, 1998; Ushijina *et al*, 1995). Additionally, gp120 stimulated the secretion of the NMDA agonist cysteine (Lipton, 1996, 1998). It is important to differentiate between effects of gp-120 and Tat, so that their contribution to neuronal degeneration is properly understood. One of the strategies employed in studies to understand the biological effect is using relevant biological models (Wang *et al*, 1999). For neuronal cells, although the primary cultures are often a complex system, results from these studies could be a useful guide in selection of appropriate models for future investigation. HIV-infected macrophages in brain may lead to exposure of localized high level of envelope proteins. Alternatively, improperly processed HIV on budding virus from infected macrophages could affect neighboring neuronal cells and induce cell death. It may not be unreasonable to expect that in late stage of infected individuals, Tat is also produced in the vicinity of neuronal cells. Therefore, Tat could be

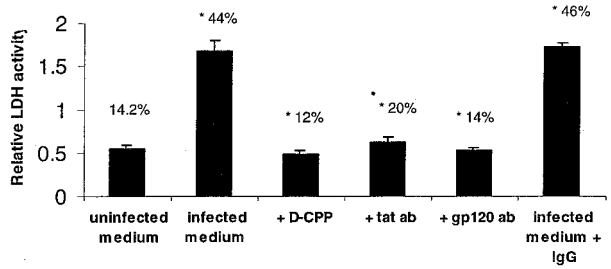

Figure 5 NMDA antagonists block HIV toxicity. Rat mixed cortical neurons were incubated with supernatant from uninfected (control), infected cells for 12 h in the presence or absence of D-CPP and following removal of Tat and gp120 specific products by immunoprecipitation. Cell death was measured by LDH release and trypan blue exclusion in the media. Results are plotted as relative LDH activity to that of the control. Results are shown as the mean ± s.d. (n=8). Per cent death are shown. Asterisk indicates statistical significance $P<0.05$ for HIV-infected culture and $P<0.01$ for the rest of the experiments.

released in milieu of neighboring cells. The *in vitro* infected culture (Figure 5) system demonstrates that both gp120 and Tat are viable candidates for such effect and may be used to understand the details of the pro inflammatory pathways that specifically contribute to neuronal loss. Using pharmacological agents, we have demonstrated that the effect of Tat is mediated through NMDA receptor.

The activation of NMDA receptors by HIV and viral products seems to be a common pathway for the degeneration of neuronal cells in AIDS patients (Epstein and Gendelman, 1993; Lipton, 1996, 1998). It is important to note that modulation of receptors may depend upon type of cell death produced by respective injurious stimuli. Interestingly, the activation pathway of NMDA receptor can be linked to other neurodegenerative diseases such as cerebral ischemia. Direct interaction of viral proteins such as Tat with NMDA receptors could provide a target for the development of therapeutics for the treatment of AIDS dementia.

Materials and methods

Cells, virus and reagents

NMDA antagonists dizocilpine (MK-801) and D-CPP, non-NMDA antagonists 1,2,3,4-Tetrahydro-6-nitro-2, 3-dioxo-benzo [f] quinoxaline-7-sulfonamide (NBQX), 6-Cyano-7-nitroquinoxaline-2, 3-dione (CNQX), 6,7-Dinitroquinoxaline-2, 3-dione (DNQX) were purchased from RBI Research Biochemical International (MA, USA) and sodium channel blocker, tetrodotoxin (TTX) was from Sigma (St. Louis, USA). All chemicals were stored as recommended by the manufacturer. HIV-1_{IIIb} (ABI) was used for all virus infection. HeLa-CD4-LTR-lacZ (Kimpton and Emerman, 1992) was obtained from AIDS repository and maintained in Dulbecco's Modified Eagles Media (DMEM, Gibco-BRL)+10% fetal bovine serum (FBS; Gibco-BRL) and 200 μg/ml of Gentamicin (G418, Gibco-BRL).

The synthetic codon optimized tat gene encoding amino acids 1 – 86 (HIB-1_{Bru}) was expressed as a His-tag fusion protein in *Escherichia coli* DH5αF′ IQ (Life Technologies) using a pEt-3 (Novagen) expression vector. The removal of His-tag and purification of Tat was previously described (Frankel and Pabo, 1988; Kirsch *et al*, 1996). The purified Tat was 90% pure following a single step of column purification based on estimation from immunostained band. This protein was then purified as a single band by HPLC 0 – 60% acetonitrile gradient in water with 0.1% Trifluorocaetic acid (TFA). Batch of 3 ml fractions were collected, lyophilized immediately and argon overlay was applied to each vial. Endotoxin contamination of Tat (at a concentration of 100 ng/ml) was determined to be <1.0 pg/ml in Limulus amebocyte lysate assay (BioWhittaker, Walkersville, MD, USA). The biological activity of the recombinant protein was tested in HeLa-LTR-Lacz transfectants. Addition of 300 – 400 pmoles of recombinant Tat activated Lacz expression from LTR promoter by 15 – 20-fold. Tat monoclonal antibody was purchased from BabCO (Berkley, CA, USA) and gp-120 monoclonal antibody was purchased from ABI, Inc.

Fetal rat cerebrocortical mixed cultures

Primary rat cell isolation was carried out in compliance with the NIH guide for animal care and use of laboratory animal and the Parke-Davis animal use committee. Cerebrocortical cells were harvested from fetal rats (Sprague Dawley) on their 18th day of gestation and cultured with DMEM/F12 (Gibco-BRL) media containing heat inactivated 10% horse and 6% fetal bovine serum (Gibco-BRL) in 24- or 12-well poly-L-lysine-coated plates as previously described (Probert *et al*, 1997). Non neuronal cell division was halted at 3 days into the culture with 35 μg/ml of uridine (Sigma, St. Louis, USA) and 15 μg/ml of 5-fluoro-2-deoxyuridine (Sigma, St. Louis, USA). Cells were cultured for 12 – 14 days at 37°C until they were ready for experiments.

Tat toxicity in rat cerebrocortical neurons

Cells in 12- or 24-well plates at day 14 post-isolation were washed once with serum free DMEM. Recombinant Tat was added at concentrations ranging from 0.001 to 1 μM in serum free media for 12 – 16 h at 37°C. For NMDA and non-NMDA antagonist, cells were pre-incubated with the pharmacological agents for 60 min at 37°C and during Tat treatment. To measure cell death, lactose dehydrogenase (LDH) activity was tested. LDH activity was measured by using CytoTox 96 (Promega) following manufacturer's instructions using 25 μl of cell media. LDH activity was presented as normalized activity to that of the control. In parallel with cytotoxic assay, we monitored cell death by

exclusion of trypan blue and the percentage of cell death was calculated. Apoptosis was monitored by staining with Hoechst 33258 which reveals condensed brightly stained nuclei under fluorescent microscope. To test specificity of Tat neurotoxicity, heat treatment of Tat at 60°C for 30 min and 10 μM of Tat solution with 0.1% trypsin (Life Tech Inc.) for 30 min at 37°C were carried out prior to addition of Tat to cell culture.

$^{45}Ca^{+2}$ accumulation studies

This assay was carried out according to the method of Birrell *et al* (1993). Briefly cells were washed three times with Mg^{+2} free HBSS containing 2.5 mM Ca^{+2} and incubated with MK-801 (1 μM) or D-CPP (100 μM) for 60 min at 37°C. Recombinant HIV-1 Tat was added at a concentration of 0.7 μM in conjunction with ^{45}Ca (ICN; 1 μCi/ml) for 40 min at 37°C. Cells were washed three times with DMEM (Gibco-BRL) and lysed in 250 μl of deionized water overnight at room temperature. ^{45}Ca β-emissions in the intracellular contents were counted by scintillation spectroscopy.

RT–PCR and RNase protection assays

Cells were incubated with Tat in serum free media for 3 and 24 h at 37°C. Total RNA was isolated with TRIzol Reagent (Gibco-BRL) following manufacturer's instructions. Total RNA was treated with DNase I (Gibco-BRL) prior to RT–PCR or RNAse protection (RPA) assays. For RT–PCR, primers for the amplification of rat TNF-α and β-actin were purchased from Clontech. RT–PCR reaction was performed with superscript one-step RT–PCR (Gibco) with cDNA synthesis cycle at 50°C for 30 min and 94°C for 2 min. cDNA amplification of 35 cycles at 94°C for 30 s, 60°C for 1 min and 72°C for 1 min followed by an incubation at 72°C for 10 min. For RPA assay, rCK1 probe was purchased from PharMingen. Probes were labeled with ^{32}P-dUTP and T7 transcription kit (PharMingen, CA, USA) as described by the manufacturer's instructions. RNase protection assay was performed with a RiboQuant RPA kit (PharMingen, CA, USA) as directed by the manufacturer.

Virus products and toxicity of rat mixed cortical neurons

HeLa-CD4 cells were infected with HIV-1_{IIIb} at multiplicity of infection of 0.1 at 37°C for 5 days. At day 5, cellular debris were removed from infected cell culture by low speed centrifugation. To rat mixed cortical neurons 0.5 ml of infected cell free supernatant was added in the presence or absence of NMDA inhibitors and incubated for 12 h at 37°C. Virus or virus related products were removed from HeLa-HIV infected cell free supernatant on 20% sucrose cushion by centrifugation at 20 000 ×g for 2 h at 4°C. Cleared cell free supernatant was added to rat mixed cortical neurons as described above. To measure HIV toxicity, LDH activity was measured as described above. To determine specificity of Tat neurotoxicity in comparison to gp-120 specificity, clarified media from infected cell culture was incubated with 1 : 100 dilution of mouse monoclonal anti-Tat antibody or mouse IgG_1/k isotype bound to protein G-coated agarose beads (Pharmacia) for 120 min at room temperature, followed by centrifugation. The immunodepleted supernatant was tested for neurotoxicity. Similar procedure was carried out for immunodepletion of gp120 from infected clarified supernatant with a rat monoclonal gp120 antibody.

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