HIV-1 Tat Induces Neuronal Death via Tumor Necrosis Factor-α and Activation of Non-N-Methyl-D-Aspartate Receptors by a NFκB-Independent Mechanism*

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Human immunodeficiency virus type 1 (HIV-1) infection of the central nervous system may result in neuronal apoptosis in vulnerable brain regions, including cerebral cortex and basal ganglia. The mechanisms for neuronal loss are likely to be multifactorial and indirect, since HIV-1 productively infects brain-resident macrophages and microglia but does not cause cytopathic infection of neurons in the central nervous system. HIV-1 infection of macrophages and microglia leads to production and release of diffusible factors that result in neuronal cell death, including the HIV-1 regulatory protein Tat. We demonstrate in this report that recombinant Tat1–86 and Tat peptides containing the basic region induce neuronal apoptosis in approximately 50% of vulnerable neurons in both rat and human neuronal cultures, and this apoptotic cell death is mediated by release of the pro-inflammatory cytokine tumor necrosis factor α, and by activation of glutamate receptors of the non-N-methyl-D-aspartate subtype. Finally, we show that Tat-induced apoptosis of human neuronal cell cultures occurs in the absence of activation of the transcription factor NFκB. These findings further define cellular pathways activated by Tat, that dysregulate production of tumor necrosis factor α, and lead to activation of glutamate receptors and neuronal death during HIV-1 infection of the central nervous system.

It has been proposed that the mechanisms by which human immunodeficiency virus type 1 (HIV-1) induces widespread neuronal dysfunction and death in the developing and adult central nervous system may involve activation of glutamate receptor subtypes (1, 2). However, the sequence of events that lead to glutamate receptor activation in the setting of HIV-1 infection and low-level chronic inflammation in the central nervous system are complex and poorly understood. Many previous studies on the neuropathogenesis of HIV-1 infection have focused on the neurotoxic effects of the HIV-1 envelope protein, gp120 (3, 4). These studies have revealed that gp120 causes neuronal toxicity in large part via indirect mechanism(s), possibly mediated by intermediary glial cells that in turn produce cellular metabolites that ultimately lead to excitotoxic activation of glutamate receptors. In contrast, the HIV-1 regulatory protein Tat is soluble, secreted, and efficiently taken up by many cell types, including astrocytes and neurons (5, 6). Several studies have implicated both full-length Tat and basic Tat peptides (i.e. Tat amino acid residues 31 to 61 (7)) as mediators of neuronal death. In vitro studies have demonstrated that Tat-induced neuronal cell death occurs via apoptosis (8), and involves activation of a non-NMDA subtype of glutamate receptors (9). In addition, in vivo experiments have revealed that Tat-mediated neurotoxicity can be prevented by pentoxifylline, an agent that blocks the transcription of the pro-inflammatory cytokine tumor necrosis factor α (TNFα) (10).

Previous studies from this laboratory have shown that TNFα-induced neurotoxicity can be prevented in part by blockade of AMPA receptors (11), and that application of TNFα to neuronal cultures results in apoptosis through a NFκB-independent mechanism (12). Hence, we theorized that Tat might up-regulate synthesis and/or release of TNFα in neural cell cultures, which in turn could lead to activation of AMPA receptors and neuronal death. In support of this hypothesis, we now demonstrate that application of Tat to neural cultures induces neuronal apoptosis that can be blocked in part by antibodies to TNFα, and by a non-NMDA receptor antagonist, but not a NMDA or metabotropic glutamate receptor antagonist.

EXPERIMENTAL PROCEDURES

Neural Cell Cultures

Primary Human Neuron Cultures—Human fetal brain tissue between gestational ages of 13 to 15 weeks was obtained, with consent, from women undergoing elective termination of pregnancy, under the guidelines of the National Institutes of Health, the University of Rochester Human Subjects Review Board. Adherent blood vessels and meninges were removed and the brain tissue was prepared as described previously, in medium containing N1 components as well as 5% heat-inactivated fetal bovine serum, FSN antibiotic mixture (penicillin at 50 mg/liter, streptomycin at 50 mg/liter, and neomycin at 100 mg/liter), and amphotericin B (Fungizone; 2.5 mg/liter) (8). Cells were plated at a density of 10⁶/ml on 12-mm diameter glass coverslips precoted with polylysine (70,000–150,000 Mw, Sigma), and placed in 24-well culture dishes. Cells were cultured 14 to 28 days at 37 °C in a humidified atmosphere of 5% CO2, 95% air, and the medium was changed every 3 days. Sample cultures were stained for the neuroendocrine-specific protein, PGP 9.5, neuron-specific enolase, microtubule-associated protein-2, synaptophysin, and glial fibrillary astrocyte protein; under these culture conditions neuronal cultures were >70% homogeneous for neurons. The remaining cells were predominantly astrocytes and <5% were microglia-macrophages, as determined by RCA-1 lectin and CD68 staining (13).
SK-N-MC Cell Cultures—Human neuroblastoma SK-N-MC cells were obtained from the American Tissue Culture Collection (14) and cultured in plastic tissue culture dishes (Corning). Cells were grown in a humidified incubator at 37°C with 5% CO₂, fed every 2 days with minimal essential complete medium, and differentiated to a neuronal phenotype, as necessary, by addition of 5 μM retinoic acid (Sigma) for 4–5 days, as described previously (12). Under these conditions the baseline level of apoptosis in neuronal SK-N-MC cells was identical to vehicle-treated cells when measured by TUNEL staining.

Rat Cerebellar Granule Neuronal Cultures—7-Day-old Sprague-Dawley rats were sacrificed and cerebellar brain tissue was harvested according to the guidelines established by the Animal Welfare Act (1987) and NIH policies. Briefly, cerebellum was collected and washed in cold phosphate-buffered saline containing trypsin at 0.25 mg/ml and 0.1% DNase (about 10-ml volume per cerebellum), then minced into 2-mm³ pieces, and triturated with a fire polished pipette, followed by incubation for 20 min at 37°C (15). The tissue was filtered through nylon mesh and the cell suspension was loaded over a two-step Percoll gradient and centrifuged 1,000 g for 30 min. After 1–2 days in culture, 5-fluorodeoxyuridine was added to the culture to 39°C, all as described previously (16).

Albumin and serum-free media components, and shifting the temperature to 39°C, all as described previously (16).

Preparation of HIV-1 Recombinant Tat

Recombinant HIV-1 Tat₁₋₈₆ was expressed and purified as a glutathione S-transferase fusion protein as described previously (8). A thrombin proteolytic digestion procedure was performed to remove the glutathione S-transferase domain from the purified glutathione S-transferase-Tat protein, and Tat preparations were then stored at −70°C until use. Purified Tat₁₋₈₆ was further characterized and quantified by protein assay (Lowry method), SDS-polyacrylamide gel electrophoresis, and by immunoblot analysis, using a polyclonal antibody (Rabbit polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) raised against peptides corresponding to the sequence: CAACGGCAGGGGAATTCCCCTCTCCTT. These antibodies were used to confirm the identity of the recombinant Tat protein (500 nM) or rh-TNFα (40 ng/μg; Genzyme, Cambridge, MA) for 8 h. Cells were then subject to extract preparation and luciferase assay. A representative luciferase assay (Promega, Madison, WI) at about 40 μl/10⁵ cells, and luciferase activity was measured with an LKB Wallace 1250 Luminometer.

Cell Extracts and Immunoblotting

Whole cell extracts were prepared from differentiated SK-N-MC cells (5 × 10⁵) by in situ lysis using ELB buffer (50 mls HEPES, pH 7.0, 250 mM NaCl, 20 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethanesulfonyl fluoride) supplemented with various phosphatase inhibitors. Cell lysates containing equal amounts of total protein (15–25 μg) were immediately supplemented with 1% SDS, heated for 5 min at 100°C, fractionated by reducing the media to 50% Dulbecco’s modified Eagle’s medium, 50% Hams F-12 (DF) containing 1% (w/v) bovine serum albumin and serum-free media components, and shifting the temperature to 39°C, all as described previously (16).

Protein Mobility Shift Assays

Electrophoretic mobility shift assays (EMSA) were performed on nuclear extracts using specific DNA sequences (NF-κB E3 and IκB α) and subjected to agarose gel electrophoresis. Gel retardation analyses were performed using recombinant Tat protein. The DNA fragment containing the NF-κB consensus sequence was labeled with [α-32P]dCTP using a nick translation kit (Promega, Madison, WI). Labeled DNA fragment was added to nuclear extracts from SK-N-MC cells and allowed to react for 30 min at room temperature. The resulting DNA-protein complexes were then run on native 4% polyacrylamide gels as described previously (18). The oligonucleotide probe used in this EMSA had the following sequence: CAACGGCAGGGGAATTCCCCTCTCCTT.

Computerized Morphometry and Statistical Analysis

Digitized images of TUNEL-stained neurons in 15 or more microscopic fields were analyzed for numbers of positively stained neuronal somata (21). Rabbit polyclonal antibodies were used to identify neuronal soma. The mean values, and standard errors of mean values were calculated; significance was determined by one-way ANOVA.

RESULTS

Tat has previously been shown to induce TNFα mRNA and protein production in macrophages and astrocytes (21), and results from this laboratory have shown that TNFα is neurotoxic to terminally differentiated (i.e. neuronal) SK-N-MC neuroblastoma cells, but not to undifferentiated SK-N-MC cells (12). We therefore investigated whether recombinant Tat₁₋₈₆ was toxic to terminally differentiated SK-N-MC cells. Fig. 1A demonstrates that when Tat₁₋₈₆ or Tat₄₆₋₆₀ peptide containing

TUNEL assay were fixed in 4% paraformaldehyde, rinsed with phosphate-buffered saline, then post-fixed with a 100% ethanol:acetic acid solution (2:1), and rinsed again with phosphate-buffered saline. Neurons were pretreated with 2% H₂O₂ to quench endogenous peroxidase, prior to the addition of the terminal deoxynucleotidyl transferase. Anti-digoxigenin peroxidase was then added, and catalytically reacted with 0.05% diaminobenzidine in phosphate-buffered saline. TUNEL-stained neurons were then counted from 15 randomly selected fields. Each field of at least 100 neurons was counted for positively stained neurons versus negatively stained cells.

TNFα ELISA

Human TNFα—A solid phase sandwich ELISA kit (Biosource International, Camarillo, CA) was employed to measure human TNFα in culture media from human fetal neuronal cultures and SK-N-MC cultures. Media samples were diluted until within the range of the standard curve.

Murine TNFα—A solid phase sandwich ELISA (Biosource International, Camarillo, CA) was employed to measure TNFα in rat cerebellar granule cell cultures.
the basic domain is applied to undifferentiated SK-N-MC cells with a glial phenotype (i.e. cells not treated with retinoic acid), no significant cell death is observed. In contrast, when either Tat1–86 or Tat46–60 peptide is applied to differentiated SK-N-MC cells with a neuronal phenotype (i.e. retinoic acid treated cells), 20–25% of neuronal cells are TUNEL-stained, indicative of apoptotic death (Fig. 1B). However, Tat65–80 peptide, which lacks the basic, neurotoxic region, is not neurotoxic to neuronal SK-N-MC cells, in agreement with previously published studies (7).

We further investigated the role of TNFα in the neuronal cell death that occurred after exposure to Tat, by co-incubating neuronal SK-N-MC cells with Tat46–60 peptide, plus a neutralizing monoclonal antibody directed against human TNFα (clone cA2; Ref. 22). Fig. 2A shows that the neuronal cell death that occurs after exposure to Tat46–60 peptide can be markedly reduced with cA2. Similar results were obtained in primary cultures of human fetal neurons when full-length Tat was incubated with cA2 (Fig. 2B), suggesting that both Tat1–86 and Tat46–60 peptide induce neuronal apoptosis in part through synthesis and/or release of TNFα. Because TNFα may be neuroprotective in some rodent neuronal culture systems (23), and because Tat has been demonstrated to have anti-apoptotic effects when applied to serum-deprived rodent PC12 neuronal cells (24), we tested the effects of Tat1–86 on primary cultures of rodent cerebellar granule neurons and immortalized rat dorsal raphe neurons (RN46A). In rodent cerebellar granule neuronal cultures, application of Tat1–86 resulted in apoptosis in 60–70% of primary cerebellar granule neurons, and in approximately 15% of differentiated RN46A neurons (data not shown).

Despite differentiation with retinoic acid, SK-N-MC cultures still contain some cells with glial phenotypes, as do primary human fetal cortical neuronal cultures. Additionally, primary human fetal cortical neuronal cultures contain <5% macrophages (13). Potentially, this population of glial cells and/or macrophages could serve as a source of TNFα, particularly since Chen et al. (21) have demonstrated that Tat1–72 markedly elevated production of TNFα by highly purified cultures of macrophages and astrocytes. We therefore set out to examine whether Tat could induce TNFα production by macrophage and glial cells in a variety of primary neuronal culture systems. Using an ELISA detection method to measure soluble TNFα in culture media, we determined that basal levels of TNFα in a primary human fetal cortical neuronal culture 24 h after the application of vehicle control were below detectable levels of the assay, while treatment with 500 nM Tat1–86 elevated TNFα levels to 176.6 ± 2.3 pg/ml (Fig. 3). In contrast to the full-length protein, the shorter Tat peptide (Tat46–60) containing the basic region elicited a lesser response (TNFα levels to 32.5 ± 0.3 pg/ml), while the non-neurotoxic Tat65–80 had no effect on TNFα production 24 h after addition to neuronal cultures (data not shown). As expected, co-incubation of human neuronal cultures with Tat1–86 or Tat46–60 plus the TNFα neutralizing monoclonal antibody cA2 completely reversed this effect (Fig. 3). Additional experiments were performed to test whether Tat1–86 could elevate TNFα levels in the media of postnatal rodent cerebellar granule neuronal cultures. Here Tat1–86 elevated TNFα only 2-fold, and cA2 was not used to reverse this effect because it does not bind or neutralize rodent TNFα (Ref. 22, data not shown).

We have previously shown that TNFα induces neuronal apoptosis through a mechanism that involves oxidative stress, and is blocked by overexpression of either bel-2 or the caspase inhibitor, crmA, but is independent of NFkB activation (12). NFkB, present in the cytoplasm of most cell types, is normally bound to a member of a family of inhibitor proteins known as IκB. The best characterized member of this family IκB-α, binds specifically to the P50/P65 heterodimer of NFkB in the cytoplasm. When cells are exposed to inducers of NFkB, such as TNFα, the mitogen-activated protein kinase-NIK signaling pathway (25, 26) is activated, which results in stimulation of the multiprotein IκB kinase complex (27–31), followed by serine phosphorylation (Ser32 and Ser36) of IκB-α and subsequent degradation of this protein by the 26 S proteasome complex (32, 33). NFkB is thus released to translocate to the nucleus and activate transcription of target genes. Because Tat1–86 and Tat46–60 peptide induce neuronal apoptosis in part through production of TNFα and activate NFkB in other cell types (21), we wondered whether NFkB activation might play a significant role in mediating Tat-induced neuronal death.
species, media was removed from the cultures and a TNF \(a\) performed to test the effect of Tat 1–86 on the fate of I \(k\)

Tat1–86, Tat46–60, or Tat65–80. 24 h after addition of either vehicle or Tat plates for 2–3 weeks prior to treatment with vehicle or recombinant

Cultures of primary human fetal cortical neurons were grown in 24-well

detected with an I \(k\) antibody cA2 (10 \(g/ml, Centocor Inc.) for 24 h. Note that SK-N-MC cells were from a different stock and passage than SK-N-MC cells used in Fig. 1, thus the relative amount of Tat-induced neurotoxicity is different from the results presented in Fig. 1B. Addition of cA2 by itself to either SK-N-MC or primary human neuronal cultures was non-toxic (data not shown). Cultures were then fixed, TUNEL stained, and analyzed for TUNEL-positive neuronal cells as described under “Experimental Procedures.” *, \(p < 0.01\) from vehicle control. Data shown here are from a single, representative experiment replicated three times.

**FIG. 3.** Tat1–86 elevates TNF \(a\) in primary neuronal cultures. Cultures of primary human fetal cortical neurons were grown in 24-well plates for 2–3 weeks prior to treatment with vehicle or recombinant Tat1–86, Tat46–60, or Tat65–80. 24 h after addition of either vehicle or Tat species, media was removed from the cultures and a TNF \(a\) ELISA was performed in 96-well plates as described under “Experimental Procedures.” *, \(p < 0.01\) from vehicle control. Data shown here are from a single, representative experiment replicated three times.

To investigate whether Tat1–86 could transduce a signal for activation of NF\(\kappa\)B in neuronal SK-N-MC cells, studies were performed to test the effect of Tat1–86 on the fate of I \(k\). In untreated SK-N-MC cells, a single 37-kDa form of I \(k\) was detected with an I \(k\)B-\(\alpha\)-specific antiserum (Fig. 4A). Incubation of the cells with Tat1–86 (500 nM, at the LD\(_{50}\) for neuronal apoptosis; see Ref. 8) for 2–6 h did not induce any detectable change in steady state levels of I \(k\)B-\(\alpha\) (Fig. 4A; compare the intensity of the I\(\kappa\)B-\(\alpha\) band to that of the upper, nonspecific band, which provides a control for total protein content in these extracts). I\(\kappa\)B-\(\alpha\) levels remained unaltered even when de novo synthesis of I\(\kappa\)B-\(\alpha\) was inhibited through the addition of a protein synthesis inhibitor, cycloheximide. In order to confirm that neuronal SK-N-MC cells are responsive to other activators of NF\(\kappa\)B, we incubated the cells with human recombinant TNF \(a\) (40 ng/ml) for various time periods. As expected, this led to the rapid degradation of I\(\kappa\)B-\(\alpha\), which was prevented by preincubation of the cells for 15 min with 50 \(\mu\)M of proteasome inhibitor MG132 (Fig. 4A). Parallel studies showed that TNF \(a\) potently activated NF\(\kappa\)B-driven luciferase activity in transiently transfected neuronal SK-N-MC cells (12). These results suggest that the mitogen-activated protein kinase-NIK-I\(\kappa\)B kinase signal transduction pathway is fully functional in neurodiplated SK-N-MC cells. Additional experiments were performed to confirm that Tat1–86 did not activate NF\(\kappa\)B in neurally differentiated SK-N-MC cells. First, immunoblot experiments were conducted using an I\(\kappa\)B-\(\beta\)-specific antiserum; no change in I\(\kappa\)B-\(\beta\) levels was detected in Tat1–86-treated cells (data not shown). Second, EMSA studies were performed. Constitutive nuclear expression of NF\(\kappa\)B was detected in unstimulated, undifferentiated SK-N-MC cells (data not shown). NF\(\kappa\)B levels remained unaltered after exposure of cells to retinoic acid, and were similarly unchanged upon exposure to Tat1–86 (Fig. 4B). Interestingly, Tat1–86 treatment in the presence of an anti-TNF \(a\) neutralizing antibody (cA2) had no influence on activation of NF\(\kappa\)B, indicating that the low levels of TNF \(a\) generated due to Tat1–86 treatment of neurally differentiated SK-N-MC cells are not sufficient to trigger the activation of NF\(\kappa\)B.

**FIG. 2.** A neutralizing antibody to TNF \(a\) blocks Tat-mediated neurotoxicity. Panel A, cultures of SK-N-MC cells were grown in 24-well plates for 2 days, then treated with media containing 5 \(\mu\)M retinoic acid for 4 days; and Panel B, cultures of primary human fetal cortical neurons were grown in 24-well plates for 2–3 weeks prior to treatment with vehicle, Tat46–60 recombinant Tat1–86, or Tat + the neutralizing monoclonal antibody cA2 (10 \(\mu\)g/ml, Centocor Inc.) for 24 h. Note that SK-N-MC cells were from a different stock and passage than SK-N-MC cells used in Fig. 1, thus the relative amount of Tat-induced neurotoxicity is different from the results presented in Fig. 1B. Addition of cA2 by itself to either SK-N-MC or primary human neuronal cultures was non-toxic (data not shown). Cultures were then fixed, TUNEL stained, and analyzed for TUNEL-positive neuronal cells as described under “Experimental Procedures.” *, \(p < 0.01\) from vehicle control. Data shown here are from a single, representative experiment replicated three times.

These biochemical analyses of NF\(\kappa\)B activation were also verified by direct functional analysis of the transactivation ability of NF\(\kappa\)B, with a \(\kappa\)B-driven luciferase reporter (\(p\kappa\)B-\(\kappa\)onaLUC, Refs. 12 and 19). Briefly, this reporter plasmid was introduced into differentiated SK-N-MC cells by transient transfection and cells were then treated with neurotoxic
Tat Neurotoxicity Occurs by TNFα and AMPA Receptor Activation

Using primary human fetal cortical neuronal cultures, we have previously demonstrated that TNFα activates non-NMDA (i.e. AMPA) receptors, resulting in neuronal death (11). We therefore performed analogous experiments, using Tat1–86. Neurons were treated with full-length Tat in the presence of a non-NMDA receptor antagonist, to establish whether Tat-mediated neurotoxicity resulted from non-NMDA receptor activation (as would be predicted for a TNFα mediated effect). In agreement with our previous results (11), co-incubation of Tat1–86 with the AMPA receptor antagonist, 6-cyano-7-nitroquinoxaline-2,3-dione resulted in an approximately 50% reduction in neuronal apoptosis, using primary cultures of either human fetal cortical neurons (Fig. 5A) or rat cerebellar granule neurons (Fig. 5B). In both culture systems, the NMDA receptor channel antagonist dizocilpine (MK-801) was ineffective in ameliorating Tat1–86-mediated neurotoxicity (Fig. 5, A and B). Additionally, the metabotropic glutamate receptor antagonist 2-amino-3-phosphonopropionic acid (AP-3) was ineffective in antagonizing Tat1–86-mediated neurotoxicity in rodent cerebellar granule neuronal cultures (Fig. 5B).

**DISCUSSION**

HIV-1 Tat has been shown to cause apoptosis of human peripheral blood mononuclear cells, and a T cell line (34–37), in addition to neuronal cells. In earlier work (8), we had speculated that Tat-induced neuronal apoptosis was due in part to TNFα-mediated signaling and glutamate receptor activation. In the present report, we have experimentally examined this hypothesis, and we have shown that intact HIV-1 Tat, or a basic Tat peptide, is able to induce neuronal apoptosis through a mechanism that involves extracellular release of TNFα, and activation of neuronal non-NMDA receptors. As previously noted, Tat-induced neurotoxicity is confined to vulnerable neurons, and astroglia are spared (8).

The pro-apoptotic effect of a basic Tat peptide on SK-N-MC cells differentiated to a neuronal phenotype (Fig. 1, A and B) was strongly reminiscent of the effect of TNFα on these same cells (12). This led us to consider the possibility that Tat-mediated neurotoxicity may be due, at least in part, to TNFα. We were able to confirm this using a neutralizing monoclonal antibody (cA2) directed against TNFα, both in neuronal SK-N-MC cell cultures and in primary human fetal neuronal cultures (Fig. 2, A and B). This finding is consistent with results from earlier experiments, in which the in vivo neurotoxicity of basic Tat peptides in murine brain could be partially blocked by co-administration of a specific inhibitor of TNFα (10).

Chen et al. (21) have recently demonstrated that Tat1–72 up-regulates production of TNFα at the transcriptional and translational level in human macrophages and fetal astrocytes. In their study, using highly purified cultures of macrophages, 100 nM Tat1–72 increased production of TNFα protein to 15 ng/ml 4 h after exposure, with levels of TNFα remaining at 10 ng/ml 24 h after exposure. In contrast, using highly purified cultures of fetal astrocytes, 1 μM Tat1–72 increased production of TNFα to 1 ng/ml transiently 1 h after exposure, with a return to near-baseline levels by 4 h. We performed a TNFα ELISA 24 h after application of full-length Tat (Tat1–86) and have detected TNFα levels of approximately 0.18 ng/ml. We have previously shown that TNFα levels in this range are neurotoxic.
to human fetal cortical neurons (11). Furthermore, if one considers the fact that macrophages and microglia comprise ≤5% of primary human cortical neuronal cultures grown in defined (N1) medium (13), the present levels of TNFα (0.18 ng/ml) would be consistent with the production of approximately 3.5 ng/ml TNFα by a pure population of human macrophages; this would be quite consistent with the results of Chen and co-workers (21). The findings that Tat46–60 produces 20% of TNFα levels elicited by Tat1–86 in these neuronal culture systems, yet Tat46–60 is able to induce neuronal apoptosis with equal efficacy as Tat1–86, and is blocked to the same degree by cA2 (Fig. 1B), remain to be reconciled. Possible explanations include different rates of cellular internalization and different time courses of TNFα production for Tat46–60 compared with Tat1–86. Additionally, basal values of murine TNFα in rodent cerebellar granule neuron cultures were approximately 70–80 pg/ml, and doubled to approximately 140–160 pg/ml with Tat-induced stimulation 24 h later (data not shown). This may reflect the lower concentration of macrophages and microglia in rodent cerebellar granule neuron cultures or species-specific differences in Tat-induced production of murine TNFα.

Chen et al. (21) have also demonstrated that Tat1–72-mediated production of TNFα by monocytoid cells and astroglia occurs via activation of nuclear factor κB (NFκB). In contrast, we demonstrate here that Tat1–86 and Tat46–60-induced neuronal apoptosis is independent of NFκB activation (Fig. 4, A and B). If Tat-mediated neurotoxicity is due in part to production of TNFα, with subsequent pro-apoptotic signaling, this is in good agreement with our previously published studies demonstrating that TNFα-mediated neuronal apoptosis is also independent of NFκB activation (12). Further support for this notion has been provided by the work of Hu and co-workers (38), who have shown that TNFα-mediated induction of apoptosis and activation of NFκB occur via distinct signal transduction pathways. Alternatively, the constitutive level of NFκB activation may be high enough in basal (i.e., control) neuronal SK-N-MC cells to mediate Tat-induced neurotoxicity (Fig. 4). However, the very low level of apoptosis in untreated neuronal SK-N-MC cultures mitigates against the idea of a threshold elevation for NFκB. Furthermore, in the SK-N-MC model system, it appears that Tat-induced production of TNFα is also independent of NFκB activation; it is unknown whether this is also true for primary human and rodent neuronal cultures. One possibility may be that Tat-induced production of TNFα in neuronal SK-N-MC cells occurs via a post-transcriptional mechanism which is known to occur in lipopolysaccharide-stimulated mononuclear cells (39–41).

Previous studies of Tat-induced neurotoxicity in human fetal neuronal cultures have demonstrated that blockade of non-NMDA receptors results in complete amelioration of this neurotoxicity in a relatively small percentage (~10%) of the total number of neurons (9). However, these studies differ from the present work in at least two important methodological details. In particular, Magnuson and colleagues (9) quantitated cell death by trypan blue exclusion at 3 h after application of Tat and glutamate receptor antagonists. In the present study, TUNEL staining was used to examine apoptosis, at a 24-h time point. The trypan blue exclusion method is not a specific indicator of apoptosis, and would be expected to provide an indication of the number of neurons undergoing necrosis; apoptotic cells would still be expected to exclude this vital dye, particularly at an early time point in the programmed cell death pathway (42). Consistent with this, unpublished studies from our laboratory demonstrate that application of basic Tat peptides results in neuronal apoptosis, not necrosis, using the TUNEL–trypan blue double-stain method of Perry et al. (43).

The extent of Tat-induced neuronal apoptosis, and the efficacy with which the AMPA/kainate receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione inhibited this effect (Fig. 5), is similar to results from our published studies, in which we examined the effect of exogenous TNFα on primary human neurons, in the presence and absence of 6-cyano-7-nitroquinoxaline-2,3-dione (11). One interpretation of these findings is that, in approximately half the neurons that die following exposure to Tat, production of soluble TNFα is followed by activation of AMPA and/or kainate receptors, and subsequently by apoptotic death of vulnerable neurons. This occurs in the absence of NFκB activation. This pro-apoptotic pathway is presumably distinct from the very rapid Tat-mediated depolarization of rat hippocampal CA1 neurons, that occurs over a period of seconds, and which can be blocked by non-NMDA

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**Fig. 5.** Tat-mediated neurotoxicity is blocked in part by AMPA receptor antagonism. Panel A, cultures of primary human fetal cortical neurons were grown in 24-well plates for 2–3 weeks; and Panel B, cultures of rodent cerebellar granule neurons were grown for 7 days prior to treatment with either vehicle or recombinant Tat1–86 in the presence and absence of the NMDA receptor channel antagonist MK-801 (10 μM), the AMPA/kainate receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (10 μM), or the metabotropic glutamate receptor antagonist AP-3 (2 μM). Cultures were then fixed, TUNEL stained, and analyzed for TUNEL-positive neuronal cells as described under “Experimental Procedures.” *, p < 0.01 from vehicle control. Data shown here are from a single, representative experiment replicated three times.

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2 D. R. New, unpublished data.

3 D. R. New, S. B. Majirwar, L. G. Epstein, S. Dewhurst, and H. A. Gelbard, unpublished data.
Tat Neurotoxicity Occurs by TNFα and AMPA Receptor Activation

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4 D. R. New and H. A. Gelbard, unpublished data.

receptor antagonists (9).

Furthermore, an additional pro-apoptotic pathway that is independent of AMPA receptor activation, but is responsive to neurotoxic species of Tat and TNFα, exists in neuronal SK-N-MC cells, which lack functional AMPA receptors.4

The results reported here may offer an explanation for the observed 50% reduction in expression of the GluR-A subunit of AMPA receptors in brains of individuals with HIV-1 infection, at both the mRNA and protein level (44). Thus, Tat-mediated release of TNFα may induce apoptosis in vulnerable neurons expressing AMPA receptors within the central nervous system of persons with AIDS. Future studies will be necessary to characterize further the molecular mechanisms of Tat-mediated neurotoxicity, to better understand how Tat or TNFα can activate non-NMDA receptors, and to examine whether Tat exerts its effects in whole or in part through the induction of oxidative stress (45).

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