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Identification of a Human Immunodeficiency Virus Type 1 Tat Epitope That Is Neuroexcitatory and Neurotoxic

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Tat is an 86- to 104-amino-acid viral protein that activates human immunodeficiency virus type 1 expression, modifies several cellular functions, and causes neurotoxicity. Here, we determined the extent to which peptide fragments of human immunodeficiency virus type 1 BRU $Tat_{1–86}$ produced neurotoxicity, increased levels of intracellular calcium ($[Ca^{2+}]_i$), and affected neuronal excitability. $Tat_{31–61}$ but not $Tat_{48–85}$ dose dependently increased cytotoxicity and levels of $[Ca^{2+}]_i$ in cultured human fetal brain cells. Similarly, $Tat_{31–61}$ but not $Tat_{48–85}$ depolarized rat hippocampal CA1 neurons in slices of rat brain. The neurotoxicity and increases in $[Ca^{2+}]_i$ could be significantly inhibited by non-*N*-methyl-D-aspartate excitatory amino acid receptor antagonists. Shorter 15-mer peptides which overlapped by 10 amino acids each and which represented the entire sequence of $Tat_{1–86}$ failed to produce any measurable neurotoxicity. Although it remains to be determined if Tat acts directly on neurons and/or indirectly via glial cells, these findings do suggest that Tat neurotoxicity is conformationally dependent, that the active site resides within the first exon of Tat between residues 31 to 61, and that these effects are mediated at least in part by excitatory amino acid receptors.

It has been estimated that 15,000,000 to 17,000,000 people worldwide are infected with human immunodeficiency virus type 1 (HIV-1) and that one-third of these individuals will develop a dementing illness. HIV-1 infection is now the leading cause of dementia in people less than 60 years of age (15, 23), and the prognosis for HIV-1 dementia is very poor, averaging 6 months from onset to death (10). Clinical features of this complex include motor disabilities as well as behavioral and cognitive changes that range in intensity from memory dysfunction to global dementia (26). Neuropathological findings vary among patients and include microglial nodules, multinucleated giant cells, myelin pallor, astrocytosis, and neuronal loss (25, 32). Most commonly, microglial cells (18, 19, 42) and some astrocytes (29, 31, 35) are infected with HIV-1. A close relationship between neurons and infected glial cells has been shown in vivo (36). As neurons are not infected by HIV-1, it has been hypothesized that HIV-1 viral proteins may produce neuronal dysfunction and/or loss.

One HIV-1 protein so implicated is the *trans*-acting nuclear regulatory protein, Tat. Tat is a nonstructural viral protein composed of 86 to 104 amino acids that is formed from two exons. The first exon contributes to the initial 72 amino acids, and the second exon forms the remaining 14 to 32 amino acids (24). Tat is released extracellularly by infected lymphoid cells (4) and glial cells (34) in vitro. Evidence suggesting that Tat is cytotoxic includes findings that Tat, when injected intracerebroventricularly, is lethal to mice and causes cytotoxicity to neuronal cell lines (30, 40). Although it remains to be determined if Tat acts directly on neurons or indirectly via glial cells, it has been shown that Tat binds specifically to neuronal cell membranes with high affinity, it depolarizes interneurons (30) and the neurotoxic properties of Tat in mammalian neurons are due to activation of excitatory amino acid receptors (22). Some of the same effects, including microglial cell activation, astrocytosis, and neuronal cell loss upon injection into rat striatum, have been reproduced with peptides derived from Tat (13). These pathological features resemble those observed in patients with HIV-1 dementia (25, 32). Similar neuropathological changes have been shown to occur with homologous peptides derived from the Tat protein of another retrovirus, visna virus (13). Thus, HIV-1 Tat may be a causative factor of pathological features associated with HIV dementia.

In this study, we identify an epitope of Tat that is cytotoxic to cultured human fetal neurons and show that mechanisms underlying the Tat-induced neurotoxicity may include Tat activation of excitatory amino acid receptors and increases in intracellular calcium.

MATERIALS AND METHODS

Cultures of human fetal neurons. Brain specimens from fetuses at gestational ages of from 12 to 15 weeks were obtained, with consent, from women undergoing elective termination of pregnancy. All aspects of these studies received approval from the University of Manitoba's Committee for Protection of Human Subjects. Blood vessels and meninges were removed and brain tissue was washed in Opti-MEM (GIBCO) and mechanically dissociated by repeated trituration through a 20-gauge needle. The cells were centrifuged at $270 \times g$ for 10 min and resuspended in Opti-MEM with 5% heat-inactivated fetal bovine serum, 0.2% N2 supplement (GIBCO), and 1% antibiotic solution (10^4 U of penicillin G per ml, 10 mg of streptomycin per ml, and 25 μg of amphotericin B per ml in 0.9% NaCl). Cells (10^5 cells per well) were plated in 96-well microtiter plates and maintained in culture for a minimum of 4 weeks before experimental use. Sample cells were immunostained for the neuronal marker, microtubule-associated protein 2, and only wells in which >70% of the cells were neurons were used for experiments. The remaining cells were predominantly astrocytes, as was determined by staining for glial fibrillary acidic protein, with rare microglia (<1%), which stained for EBM-11 (6).

Neurotoxicity assay. Cell death in neuronal cultures treated with Tat peptides in the absence of fetal bovine serum was determined by trypan blue exclusion 3 h after the addition of the peptides. Neuronal cell counts were determined from five fields chosen randomly. Each field was photographed and coded and then counted by an investigator blinded to its experimental identity. At least 200 cells were counted in each field. Individual experiments were conducted in triplicate wells. The mean percentages of dead cells ± the standard errors of the means

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were calculated from these data, and statistical analyses were performed by one-way analysis of variance, with post hoc analysis by Dunnett's test.

Tat peptides. $Tat_{31–61}$, $Tat_{31–71}$, and $Tat_{48–85}$ were obtained as gifts from the AIDS Reagent Program of the Medical Research Council of the United Kingdom and added to neuronal cultures at concentrations ranging from 7 to 17 μM. Tat peptides (15-mers), each overlapping by 10 amino acids and completely spanning the 86-amino-acid sequence of Tat from HIV_{BRU}, were synthesized on a peptide synthesizer (Applied Biosystems) and purified by reverse-phase high-pressure liquid chromatography. Stock solutions of these peptides were prepared in 0.9% (wt/vol) NaCl. Neuronal cultures were treated with these peptides at 100 μM, and effects on cytotoxicity were determined in triplicate wells as described above. Neurotoxicity experiments with the 15-mer overlapping peptides spanning the region from positions 33 to 72 and the longer peptides spanning positions 31 to 61, 31 to 71, and 48 to 85 were repeated at least three times.

To determine the specificity of $Tat_{31–61}$ neurotoxicity, $Tat_{31–61}$ was incubated with 1:100 dilutions of rabbit anti-Tat serum or normal rabbit serum bound to protein A-coated agarose beads (Pharmacia) for 90 min at room temperature, and this was followed by centrifugation. The supernatants were tested for neurotoxicity. $Tat_{31–61}$ (17 μM) solution was also treated with 0.05% trypsin (Life Tech Inc.) for 30 min at 37°C, and following the addition of trypsin inhibitor (Sigma) (final concentration, 0.1%) for 30 min at 37°C, the effects on neurotoxicity were determined.

Intracellular calcium recordings. Human fetal neurons were cultured for 4 to 6 weeks as described above in 75-cm^2 T flasks. The flasks were gently tapped manually, and the cells released into the supernatant were replated onto 33-mm-diameter glass coverslips for 7 to 10 days. Intracellular calcium concentrations were measured with fura-2-acetoxymethyl ester (fura-2/AM). Cells were incubated with fura-2/AM for 40 min at 27°C in KREBS buffer containing 111 mM NaCl, 26.2 mM $NaHCO_3$, 1.2 mM NaH_2PO_4, 4.7 mM KCl, 1.2 mM $MgCl_2$, 15 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), 1.8 mM $CaCl_2$, 5 mM glucose, and 1.5 μM bovine serum albumin. Cells were subsequently washed three times with KREBS to remove extracellular fura-2/AM and incubated at 37°C for 5 min. Fura-2/AM-loaded cells were superfused at a rate of 2 ml/min in an open perfusion microincubator at 37°C. Cells were excited at 340 and 380 nm, and emission was recorded at 510 nm with a video-based imaging system (EMPIX, Missassauga, Ontario, Canada). R_{max}/R_{min} ratios were converted to nanomolar $[Ca^{2+}]_i$ according to the method of Grynkiewicz et al. (9).

$Tat_{31–61}$ (100 μM) and $Tat_{48–85}$ (700 μM) were dissolved in KREBS solution and loaded into glass micropipettes and administered by pressure injection (three 15-ms pulses at 8 lb/in^2). The cells nearest the micropipette were monitored for 20 min. Superfusion of cells was stopped during the application of the peptides and was continued after levels of $[Ca^{2+}]_i$ returned to baseline.

Preparation of brain slices. Young Sprague-Dawley rats (14 to 21 days old) were anesthetized with halothane and decapitated. Brains were placed in gassed (95% O_2, 5% CO_2) normal artificial cerebrospinal fluid (118 mM NaCl, 3.0 mM KCl, 1.0 mM NaH_2PO_4, 0.81 mM $MgSO_4$, 2.5 mM $CaCl_2$, 10 mM glucose, and 24 mM $NaHCO_3$) at 4°C for 1 to 2 min and blocked by hand. Slices (200 to 250 μm thick) were prepared and incubated at 28 to 30°C in gassed artificial cerebral spinal fluid for 1 to 4 h. Single slices were then transferred to a continuously perfused (2 to 3 ml/min at 30°C), glass-bottomed recording chamber (Warner Instruments) and held in place by a nylon grid. Neurons were visualized (Hoffman modulation optics) and impaled with sharp glass microelectrodes containing 2.0 M potassium acetate and 1% biocytin (resistance, 100 to 150 MΩ).

Intracellular recording from brain slices. Tat peptides were applied extracellularly to neurons by pressure ejection (General Valve), with pressures of 1 to 20 lb/in^2 and ejection times of 5 to 5,000 ms being used. Initially, five short applications were made at 1 Hz, and doses were increased by increasing the pulse duration; a 5-s continuous application represented a maximum dose. The minimum time for complete evacuation of the pressure electrodes containing 2 μl of solution was 20 s. The maximum dose of $Tat_{31–61}$ and $Tat_{48–85}$ was 670 μM, each applied at 1 to 20 lb/in^2 for 5 s.

To determine the morphology of recorded neurons, biocytin was included in each recording microelectrode, with impalements of 20 min or longer resulting in neurons being filled adequately for histological examination. Slices were fixed overnight at 4°C in 4% paraformaldehyde and were transferred to 10% sucrose for 48 h. Frozen sections (50 μm thick) were exposed to streptavidin CY3 for 3 to 5 h in phosphate-buffered saline (pH 7.4) and viewed with a microscope equipped for epifluorescence microscopy with a rhodamine filter cube to visualize the label CY3. All of the recorded neurons were CA1 hippocampal neurons.

RESULTS

Neurotoxicity of Tat peptides to human fetal neurons. $Tat_{31–61}$ was toxic to human fetal neurons. Maximal toxicity was seen at 0.5 to 2 h as determined by trypan blue exclusion, with a drop in the number of trypan blue-staining cells at 24 h (Fig. 1) because of either rupturing or dislodging of the injured cells. $Tat_{31–61}$ produced significant cytotoxicity; cell loss expressed as mean percentage of total neurons counted ± the standard error of the mean was 7.7% ± 1.0% (Fig. 2). $Tat_{31–71}$ produced 75% less neurotoxicity than did $Tat_{31–61}$, and this level of toxicity was not statistically significant. The neuronal loss of 0.5% ± 0.1% following application of $Tat_{48–85}$ was indistinguishable from control values of 0.5% ± 0.1%. None of the 16 peptides—each 15 amino acids in length, overlapping by 10 amino acids, and spanning the entire molecule of Tat—produced significant neurotoxicity even when used at concentrations of 100 μM, a concentration 100-fold higher than what was used for full-length $Tat_{1–86}$ (Table 1). Toxicity of $Tat_{31–61}$ that was heat treated at 60°C for 30 min (7.7% ± 2.5%) was not statistically different from that of untreated $Tat_{31–61}$ (7.7% ± 1.0%) (Fig. 2).

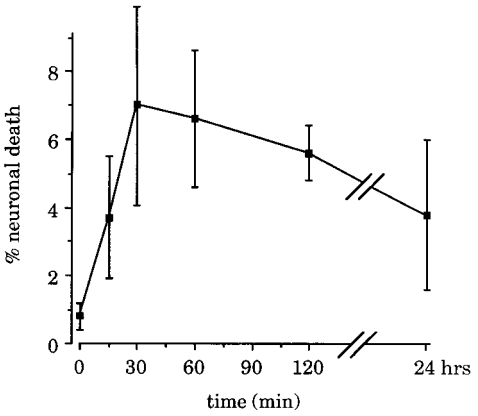

FIG. 1. $Tat_{31–61}$-induced neurotoxicity. Cultures of human fetal neurons were treated with $Tat_{31–61}$ (17 μM), and the amount of neuronal cell death was analyzed by trypan blue exclusion at different time intervals. Maximal neurotoxicity was seen by 0.5 to 2 h. Error bars show standard deviations.

Role of excitatory amino acid receptors in $Tat_{31–61}$-mediated toxicity. Previously, we found that $Tat_{1–86}$-induced neurotoxicity of human fetal neurons was blocked at least in part by

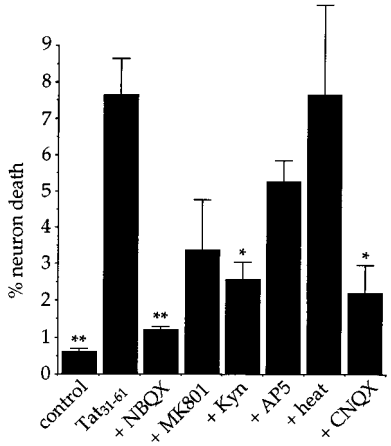

FIG. 2. Excitatory amino acid antagonists attenuate $Tat_{31–61}$ toxicity. Each value represents the mean ± the standard error of the mean. Values marked with an asterisk show significant cell sparing compared with $Tat_{31–61}$ (17 μM) toxicity alone (*, $P < 0.05$; **, $P < 0.01$). Kynurenate (Kyn; 1 mM), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 1 μM), and NBQX (10 μM) significantly decreased $Tat_{31–61}$ toxicity. Some attenuation by MK801 (20 μM) and D,L-2-amino-5-phosphovaleric acid (AP5; 100 μM) was also observed; however, these attenuations were not statistically significant. Heat treatment (60°C for 30 min) of the peptide did not affect its toxic properties.

TABLE 1. Treatment of human fetal neurons with 15-mer Tat peptides[a]

Tat peptide	% Neuron death[b]
1–15	−1.15 ± 0.47 (1)
3–17	−1.20 ± 0.26 (1)
8–22	−0.68 ± 0.38 (1)
13–27	+0.06 ± 0.27 (1)
18–32	−0.04 ± 0.03 (1)
23–37	−0.33 ± 1.20 (1)
28–42	−2.71 ± 0.30 (1)
33–47	+1.80 ± 0.80 (3)
38–52	+0.05 ± 0.15 (3)
43–57	−0.28 ± 0.19 (3)
48–62	−0.34 ± 2.64 (4)
53–67	+0.16 ± 0.88 (5)
58–72	+1.63 ± 2.66 (4)
63–77	−4.30 ± 0.80 (2)
68–82	−1.12 ± 0.37 (1)
72–86	−1.37 ± 0.47 (2)

[a] Sixteen Tat peptides (each 15 amino acids in length, overlapping by 10 amino acids each, and collectively spanning the entire molecule of Tat) from HIV_{BRU} did not produce toxicity.

[b] Values represent the means ± standard errors of the means for the differences between control wells and treated wells. Each experiment was done in triplicate wells. Numbers in parentheses indicate the number of times the peptide was tested.

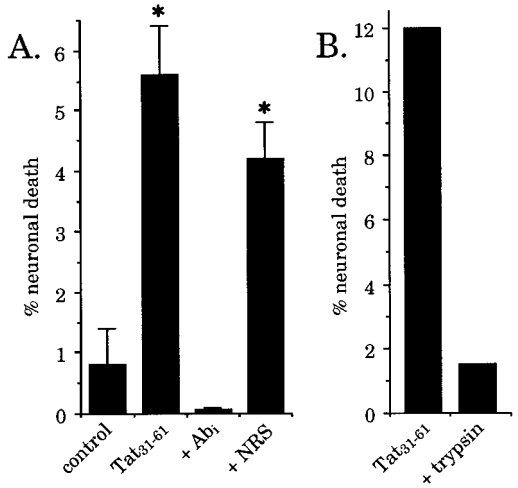

FIG. 3. Specificity of $Tat_{31–61}$ neurotoxicity. (A) This graph represents three individual experiments using triplicate wells. Each value represents the mean ± the standard error of the mean. Values marked with an asterisk show significant cell death compared with that for the control (*, $P < 0.01$). The neurotoxicity of $Tat_{31–61}$ (17 μM) was completely abolished following immunoabsorption with antisera to Tat (Ab_i). Similar treatment with normal rabbit serum (NRS) did not effect the neurotoxicity. (B) Each value represents the mean percentage of cell death above that of the control for a single representative experiment done in triplicate wells. Treatment of $Tat_{31–61}$ (17 μM) with trypsin completely abolished the neurotoxicity.

N-methyl-D-aspartate (NMDA) and non-NMDA excitatory amino acid receptor antagonists (19). To determine the mechanism of the toxicity of $Tat_{31–61}$, the following pharmacological agents were tested: kynurenate (1 mM), a nonselective excitatory amino acid receptor antagonist; D,L-2-amino-5-phosphovaleric acid (100 μM) and dizocilpine (MK801; 20 μM), selective NMDA-receptor antagonists; and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 1 μM) and 2,3-dihydro-6-nitro-7-sulphamoyl-benzo(*F*)quinoxaline (NBQX; 10 μM), selective non-NMDA receptor antagonists. $Tat_{31–61}$-induced neurotoxicity was blocked significantly ($P < 0.05$) by kynurenate, 6-cyano-7-nitroquinoxaline-2,3-dione, and NBQX. MK801 and, to a lesser degree, D,L-2-amino-5-phosphovaleric acid blocked neurotoxicity, although in neither case was the blockade found to be statistically significant (Fig. 2).

Specificity of $Tat_{31–61}$-induced neurotoxicity. The possibility that the neurotoxicity of $Tat_{31–61}$ was due to contaminants was excluded by using only highly purified peptides. Further, the immunoabsorption of $Tat_{31–61}$ with rabbit anti-Tat serum coupled to protein A-conjugated agarose beads (Fig. 3A) and treatment of $Tat_{31–61}$ with trypsin (Fig. 3B) resulted in the loss of Tat neurotoxicity. The neurotoxicity was unaffected by $Tat_{31–61}$ solutions treated with normal rabbit serum coupled to protein A-conjugated agarose beads (Fig. 3A).

Effect of Tat peptides on intracellular calcium. Human fetal brain cells were treated with $Tat_{31–61}$ (100 μM) and $Tat_{48–85}$ (700 μM) and analyzed by video imaging to determine the effect of Tat peptides on intracellular calcium. For $Tat_{31–61}$, peak increases in $[Ca^{2+}]_i$ of 955 ± 280 nM were reached within 0.21 ± 0.06 min ($n = 19$) (Fig. 4). NBQX (10 μM) did not itself affect $[Ca^{2+}]_i$ but almost completely blocked responses to $Tat_{31–61}$; the time to peak was delayed to 1.0 ± 0.14 min, and increases in $[Ca^{2+}]_i$ were significantly ($P < 0.01$) reduced to 69 ± 26 nM ($n = 13$), i.e., 7% of the original response. After NBQX washout, $Tat_{31–61}$-induced peak increases in $[Ca^{2+}]_i$ were 770 ± 256 nM (81% of the original response). These responses to $Tat_{31–61}$ after NBQX washout were not significantly different from values obtained for $Tat_{31–61}$ prior to NBQX treatment ($n = 13$) (Fig. 4). Treatment of neurons with $Tat_{48–85}$ even at a concentration sevenfold greater than that of $Tat_{31–61}$ resulted in a negligible change in $[Ca^{2+}]_i$ of 7 ± 3 nM ($n = 13$).

Electrophysiological properties of Tat peptides. To determine whether $Tat_{31–61}$ or $Tat_{48–85}$ had neuroexcitable properties, we performed intracellular recordings from hippocampal CA1 pyramidal neurons in rat brain slices and applied peptides extracellularly. $Tat_{31–61}$ elicited depolarizations in 6 of 10 neurons tested (Fig. 5). The depolarizations were similar in appearance to those elicited by $Tat_{1–86}$ or kainate (22). In contrast, when $Tat_{48–85}$ was similarly applied to hippocampal

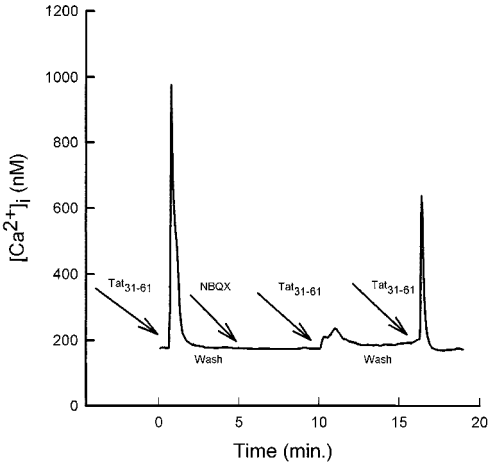

FIG. 4. A representative trace depicting the response of a single neural cell showing blockade of $Tat_{31–61}$ response with NBQX. Baseline $[Ca^{2+}]_i$ was 177 nM. The application of $Tat_{31–61}$ (100 μM) resulted in an increase in $[Ca^{2+}]_i$ to 799 nM. In the presence of NBQX (10 μM), the $Tat_{31–61}$ response was diminished to 59 nM (applied 4 min after the application of NBQX), and following a washout, reapplication of $Tat_{31–61}$ resulted in a $[Ca^{2+}]_i$ of 461 nM.

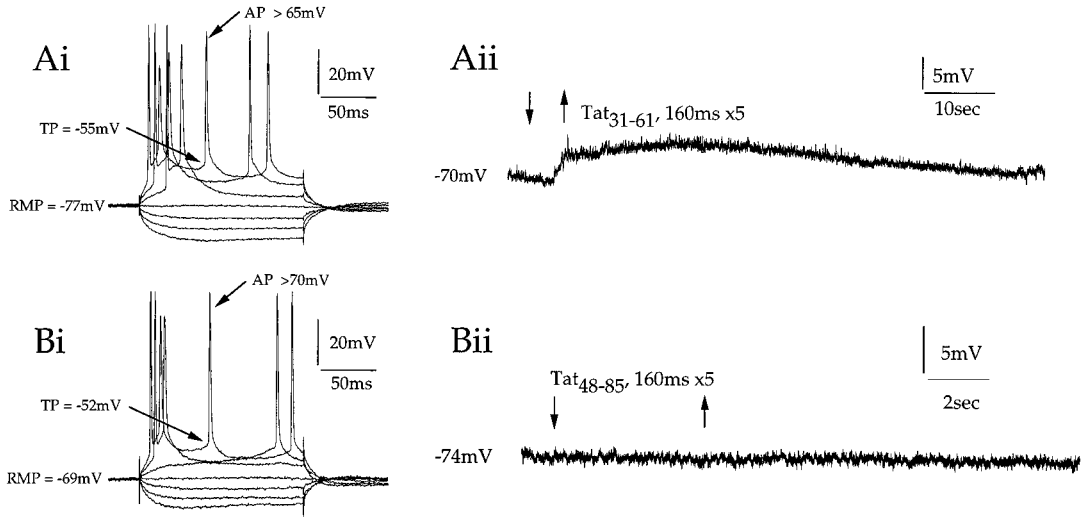

FIG. 5. Electrophysiologic properties of Tat_{31-61}. This figure shows representative intracellular recordings of two rat hippocampal neurons during intracellular current injection (−0.6 to 0.6 nA in 0.2-nA steps) and exposure to Tat_{31-61} and Tat_{48-85} applied extracellularly by pressure injection. (Ai and Bi) These hippocampal CA1 pyramidal neurons have normal current-voltage responses, resting membrane potentials (RMP), membrane input resistances, thresholds for action potential generation (TP), and action potentials (AP). (Aii) The neuron demonstrated in Fig. 4Ai shows a change in membrane potential (depolarization) when exposed to Tat_{31-61}. (Bii) The neuron demonstrated in Fig. 4Bi showed no change in the membrane potential when exposed to Tat_{48-85}.

neurons, no alterations in the resting membrane potentials were seen in any of the five neurons examined.

DISCUSSION

HIV-1 proteins including Tat, gp120, Nef, and Rev have been shown to be cytotoxic and have been implicated in the pathogenesis of HIV-1 dementia (8, 20, 21, 41). Previously, we showed that full-length Tat (Tat_{1-86}) caused neuronal cell death and increased neuronal excitability through interactions with NMDA- and non-NMDA-type excitatory amino acid receptors (22). Further, we showed that Tat_{1-86} significantly increased levels of intracellular calcium in mixed cultures of human fetal brain (11, 22). In this paper, we report that in terms of neurotoxicity, neuronal depolarization, and calcium mobilization, an active region of Tat resides in the amino acid sequence from positions 31 to 61.

HIV Tat protein influences a large number of viral and host functions. For some of these, the functional regions have been defined. The basic region which contains an arginine-rich region (positions 49 to 57) has been reported to be important for nuclear localization of Tat (16), cell attachment (38, 39), and cytotoxicity (14, 30). The cysteine-rich region (positions 22 to 37) was found to be responsible for the metal binding properties of Tat (5, 16), and Tat (positions 1 to 58) was shown to be required for the inhibition of antigen-induced lymphocyte proliferation (37). The RGD (arginine-glycine-aspartic acid) sequence in the second exon of Tat (positions 72 to 74) is a well-known integrin receptor recognition sequence (1) and has been shown to be important in mediating adhesion and cell aggregation (2, 17). Our finding here that the distinct, conformationally dependent region of Tat within the first exon contained within positions 31 to 61 causes excitation of neurons, increases in intracellular calcium, and neurotoxicity suggests that Tat has a potentially important role in mediating central nervous system effects in HIV-1-infected individuals.

It appears clear that neuroexcitatory and -toxic properties of full-length Tat as well as Tat_{31-61} are mediated predominantly through interactions with non-NMDA and, to a lesser degree, NMDA excitatory amino acid receptors. Possible explanations for this include specific interactions of Tat with the non-NMDA receptors or a differential expression of non-NMDA receptors in fetal neurons. It has been shown that non-NMDA receptors are expressed earlier and are more abundant than NMDA receptors in fetal brains (28). Thus, if Tat were to cause a release of a glutamate-like substance from glial cells that activates both types of excitatory amino acid receptors, it would appear as though the toxicity is mainly mediated via non-NMDA receptors. Further studies are needed to determine if Tat acts directly on excitatory amino acid receptors or indirectly via action on glial cells.

At present, the cause-and-effect relationships for neuronal depolarization, toxicity, and build-up of intracellular calcium remain uncertain. It is known that excessively high levels of intracellular calcium ultimately lead to cell death (7). Indeed, in some of our first experiments, we found that microinjection of high concentrations of Tat_{31-61} led to dramatic increases in the levels of intracellular calcium and cell demise (11). Given that excitation of NMDA and non-NMDA receptors results in increased levels of intracellular calcium, that sufficiently high levels can lead to cell death (7), and that the time course of neuronal depolarization by Tat precedes the Tat-induced increases in intracellular calcium, it is possible that the calcium response is secondary to increased neuronal excitability. If so, then extracellular calcium may enter the cell through calcium-permeable excitatory amino acid receptor-coupled channels or alternatively through depolarization-induced openings of voltage-sensitive calcium channels (7). Results of our video imaging of calcium in brain cells treated with Tat or Tat_{31-61} suggest that calcium levels adjacent to the plasma membrane are highest initially as well as during periods of maximal increases in intracellular calcium, findings which are consistent with the Tat-induced influx of extracellular calcium (12). However, these data do not exclude the possibility that calcium released from intracellular pools contributes to the Tat-induced rises in intracellular calcium.

The tertiary structure of the Tat molecule, as governed by its length, appears to be important in mediating neurotoxicity. Tat peptides 15 amino acids in length do not produce significant

increases in cell death. A 31-mer Tat peptide ($Tat_{31–61}$) produced significant levels of neurotoxicity. At a 10- to 20-fold-higher concentration of the peptide compared with that in our previous study with full-length Tat, the amount of toxicity was small; however, the pharmacological properties of the neurotoxicity were similar to those of full-length Tat (22). Increasing the length of the Tat peptide to 41-mers ($Tat_{31–71}$) reduced the degree of neurotoxicity. Further, heat treatment of $Tat_{31–61}$ did not change its toxicity. However, we and others have previously shown that similar heat treatment of $Tat_{1–86}$ resulted in the loss of toxicity (22, 40), suggesting that $Tat_{31–61}$ has a stable tertiary configuration. $Tat_{31–61}$ includes the core region (positions 32 to 47) and the basic region (positions 48 to 57); both regions are probably essential for neurotoxicity. These core and basic domains of Tat are highly conserved and have exposed hydrophobic and helical regions available for membrane interaction (3, 33) which may mediate neurotoxicity. Sabatier et al. (30) reported neurotoxicity with the basic region alone. In their studies, death of mice following intracerebroventricular injections was used as a measure of toxicity, but pathological confirmation of neurotoxicity was not included. We, however, did not observe any toxicity with two 15-mer peptides ($Tat_{43–57}$ and $Tat_{48–62}$) and another 38-mer peptide ($Tat_{48–85}$), all of which contained the basic region of Tat. These results are consistent with those of Weeks et al. (40), who were also unable to demonstrate any toxicity with the basic peptide $Tat_{49–58}$ in neuronal cell lines. The basic region, however, does play a role in cell surface binding (40).

Circulating or intracerebral levels of Tat have not yet been determined. Localization of Tat by immunohistochemistry in the brain has been difficult because of the cross-reactivity of anti-Tat antibodies with normal brain antigens (27). Our studies and those of others show that micromolar concentrations of Tat are required to produce neurotoxicity (22, 27, 30). Although it might be unlikely for such high levels to be present in the circulation, it is conceivable that these levels may be achieved in close vicinity to HIV-infected cells. Alternatively, Tat may act synergistically with other neuroexcitatory and/or toxic molecules released from HIV-infected cells, since Tat has been shown to be released extracellularly from HIV-infected cells in vitro and neurons are frequently observed in close proximity to HIV-infected glial cells (29, 36). Clearly, further studies are needed to determine the biological relevance of Tat neurotoxicity.

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