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Human immunodeficiency virus type 1 Tat and methamphetamine affect the release and activation of matrix-degrading proteinases

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Human immunodeficiency virus (HIV) dementia (HIVD) is associated with an increase in the number of activated monocytes within the central nervous system (CNS), a pathological feature that may be more remarkable in the setting of superimposed substance abuse. Monocytes may transport HIV to the brain, and, moreover, activated and/or infected monocytes have been shown to release a number of potent neurotoxins. Although the mechanisms responsible for the increase in the CNS ingress of monocytes are multiple, blood-brain barrier (BBB)-degrading matrix metalloproteinases (MMPs) are likely to play an important role. The current study investigates the effects of the HIV-1–encoded protein Tat, and the drug of abuse methamphetamine, on MMP release from brain derived cells. The release of urokinase plasminogen activator (uPA), an activator of MMPs, was also investigated. Mixed human neuron/astrocyte cultures were stimulated with Tat or methamphetamine, and supernatants were analyzed by enzyme-linked immunosorbent assay (ELISA) and/or gelatin substrate zymography. Results showed that Tat and methamphetamine increased the release of MMP-1 from these cultures. Tat also increased supernatant levels of active MMP-2. In addition, both Tat and methamphetamine stimulated the release of the MMP activator uPA, and in a manner that was sensitive to inhibition with pertussis toxin. Together, these results suggest that in HIVD, Tat and methamphetamine may contribute to CNS inflammation by stimulating increased release and/or activation of matrix-degrading proteinases through mechanisms that include Gi/Go-coupled signaling. These results also suggest a potential mechanism for acceleration of HIVD with methamphetamine use. *Journal of NeuroVirology* (2004) **10,** 21–28.

Keywords: blood-brain barrier; methamphetamine; MMP1; MMP-2; Tat; uPA

Introduction

Recent advances in the treatment of human immunodeficiency virus (HIV) have decreased the incidence of symptomatic neurocognitive disease to <10% of acquired immunodeficiency syndrome (AIDS) patients; however, the prevalence of this condition may be increasing (Clifford *et al*, 2002; Sacktor *et al*, 2001). Although the pathogenesis of HIV dementia (HIVD) remains incompletely understood, classical hallmarks include neuronal loss, astrogliosis, and formation of multinucleated giant cells (Spencer and Price, 1992). HIVD is also associated with an increase in the number of activated monocytes within the central nervous system (CNS) (Glass *et al*, 1995), attributed to mechanisms including activation of circulating leukocytes (Pulliam *et al*, 1997) and a compromised blood-brain barrier (BBB) (Power *et al*, 1993). Monocytes may transport HIV to the brain, and, moreover, activated and/or infected leukocytes have been shown to release a number of potent neurotoxins (Epstein and Gendelman, 1993).

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Although the mechanisms of BBB dysfunction in association with drug abuse and HIVD are likely to be multiple, matrix metalloproteinases (MMPs) may play an important role. For example, MMP-2 and -9 degrade collagen IV, a major constituent of this barrier, whereas MMP-1 degrades substrates, including sulfated proteoglycans of the BBB (Nagase and Woessner, 1999; Rosenberg, 2002). Disruption of BBB integrity by MMPs may in turn affect the CNS ingress of leukocytes. In support of this possibility, intraparenchymal injection of select MMPs is associated with leukocyte infiltration of the CNS (Anthony *et al*, 1998; Rosenberg and Navratil, 1997).

MMPs also have effects on matrix proteins of the brain parenchyma, such as laminin, and destruction of parenchymal matrix may therefore also contribute to neuronal dysfunction. Moreover, MMPs have effects on nonmatrix proteins that may be significant (McCawley and Matrisian, 2001). For example, MMPs can activate cytokines, including interleukin (IL)-1β (Schonbeck *et al*, 1998).

Although MMPs are known to be released by activated glia (Gottschall and Yu, 1995), and to be elevated in association with HIVD, the effect of HIV-specific stimuli on MMP release from neural cells has not been well investigated. The HIV *trans*-activating protein Tat, however, is among those factors that may contribute to such release. This protein is required for viral replication and has been reported to regulate activation of cellular transcription factors (Conant *et al*, 1996; Kumar *et al*, 1998), to stimulate the secretion of various chemokines and cytokines (Conant *et al*, 1998b; Mayne *et al*, 2000; Sawaya *et al*, 1998; Schmidtmayerova *et al*, 1996), to up-regulate adhesion molecule expression in endothelial cells, and to contribute to leukocyte chemotaxis (Dhawan *et al*, 1997). Tat also causes neurotoxicity (Bonavia *et al*, 2001; Magnuson *et al*, 1995). Studies have shown that in non-neural cells, Tat can stimulate the release of MMPs (Kumar *et al*, 1999). In addition, Tat neurotoxicity can be prevented by the use of MMP inhibitors (Johnston *et al*, 2001).

Of additional interest with respect to the possibility that Tat influences MMP activity is its potential to increase the release of urokinase plasminogen activator (uPA), a critical activator of MMPs in numerous experimental settings (Carmeliet *et al*, 1997; Mazzieri *et al*, 1997). MMPs can be activated by disruption of the interaction between the active site zinc and a conserved pro-domain cysteine, which may occur with or without proteolytic removal of the pro-domain. Although many stimuli can activate MMPs, including organomercurials and the detergent sodium dodecyl sulfate (SDS), uPA is a relevant physiological activator. It stimulates the formation of plasmin, which then activates numerous pro-MMPs through cleavage (Woessner and Nagase, 2000).

As opposed to Tat, the effects of methamphetamine on MMP and urokinase release have not been previously studied. This compound, however, is known to increase extracellular levels of neurotransmitters, which may in turn affect MMP release (Sundstrom *et al*, 2001), and to increase the DNA binding activity of transcription factors that influence MMP release (Flora *et al*, 2002). In addition, previous studies have shown that cocaine, which may also increase extracellular levels of catecholamines, can increase BBB permeability (Zhang *et al*, 1998).

The current study investigates the possibility that Tat and methamphetamine stimulate the release of MMP-1, MMP-2, and uPA from CNS-derived cells. Cultures containing not only astrocytes but neurons are used in all experiments because methamphetamine's effects would be expected to be most profound in the presence of neuronal cells. Hypothetically, methamphetamine may stimulate the release of catecholamines from neurons, which could then stimulate increased DNA-binding activity of transcription factors critical to the expression of MMPs in both neurons and astrocytes. In addition, because both Tat and catecholamines may stimulate signaling through G protein–coupled receptors (GPCRs), this study examines the ability of pertussis to inhibit select effects of Tat and methamphetamine.

Results

Norepinephrine is associated with increased DNA-binding activity of AP-1 in cultures of human brain-derived cells

Previously, we and others have shown that HIV-1 Tat increased the DNA-binding activity of nuclear factor (NF)-κB and/or activator protein (AP)-1, two transcription factors that may be critical to MMP-1 expression (Bond *et al*, 1999; Conant *et al*, 1996; Lim and Garzino-Demo, 2000; Sato *et al*, 2002). In rat brain, it has also been shown that methamphetamine stimulates increased binding of AP-1 (Flora *et al*, 2002). To test the hypothesis that this increase may be mediated in some part by catecholamines known to be released in response to methamphetamine, we performed electrophoretic mobility shift assays using extracts from unstimulated and norepinephrine stimulated cultures. As shown in Figure 1A, exposure of cells to 50 μM norepinephrine (NE) was associated with an increase in the DNA-binding activity of AP-1. Treatment of cells with 50 μM acetylcholine (Ach) did not produce a similar effect. Subsequent supershift studies demonstrated that this activity was likely to be composed of *fos-jun* heterodimers, which, as opposed to some AP-1 family member combinations, are likely to increase AP-1–mediated transcription. In similar experiments, we also observed that 50 μM dopamine and 50 μM serotonin could increase the DNA binding activity of AP-1 (data not shown). Together, these results suggest that neurotransmitters whose extracellular concentrations may be increased following acute exposure

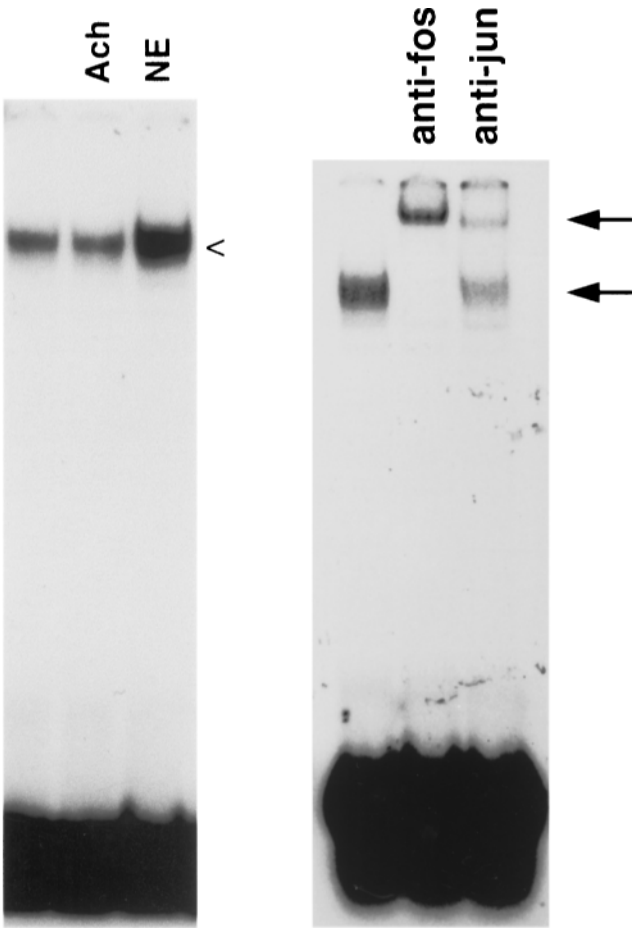

Figure 1 Treatment with norepinephrine is associated with increased DNA-binding activity of AP-1 in cultures of human brain-derived cells. Nuclear extracts from unstimulated, acetylcholine stimulated (Ach), and norepinephrine stimulated (NE) cultures were incubated with a radiolabeled DNA-binding sequence for AP-1 and analyzed by electrophoresis (**A**). Free probe can be appreciated at the bottom of the image, whereas probe that migrated more slowly due to the presence of bound AP-1 protein can be observed closer to the top (*arrowhead*). From this image, it can be appreciated that NE was associated with an increase in the DNA binding activity of AP-1. In (**B**), extracts from NE treated cells were incubated with both the DNA probe and an antibody to an AP-1 family member (c-*fos* or -*jun*). The upper arrow indicates a band likely to represent a complex of probe, AP-1, and antibody in that its migration is retarded even with respect to that of AP-1 and probe alone (*lower arrow*).

to methamphetamine, and sometimes chronic as well (Wang *et al*, 2000), can increase the DNA-binding activity of transcription factors important to MMP-1 expression.

Tat and methamphetamine stimulate the release of MMP-1 from brain cells

Human neuron/astrocyte cultures were incubated with Tat (100 nM) or methamphetamine (500 μM) for 24 h as indicated, after which supernatants were harvested and analyzed by enzyme-linked immunosorbent assay (ELISA) for MMP-1. As seen in Figure 2, both Tat and methamphetamine significantly increased the release of MMP-1 into the supernatants, $P = .04$ and $P = .05$, respectively.

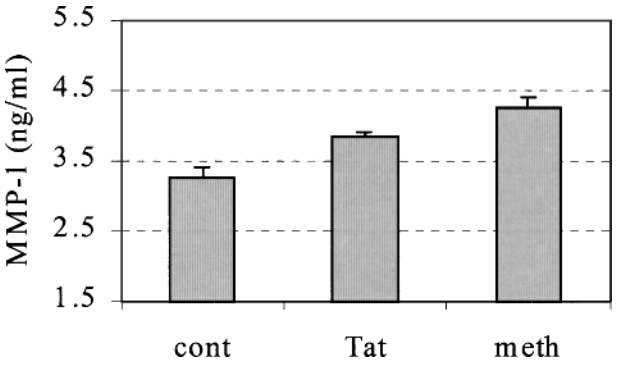

Figure 2 Tat- and methamphetamine-stimulated release of MMP-1. Mixed neuron/astrocyte cultures were serum starved for 24 h followed by treatment with Tat (100 nM) or methamphetamine (500 μM). Supernatants were harvested after 24 h and assayed by ELISA for MMP-1. The difference between unstimulated and Tat stimulated MMP-1 was significant at $P = .04$, and that between unstimulated and methamphetamine stimulated MMP-1 was significant at $P = .05$.

Tat stimulates the release of MMP-2 from brain cells

Mixed cultures were treated with Tat at 100 nM and 1 μM as indicated, and supernatants collected after 24 h were run on a gelatin zymogram. As seen in Figure 3A, active MMP-2 (*arrowhead*) at 65-kDa was only apparent in the cells treated with Tat. Consistent with some constitutive expression by astrocytes, pro-MMP-2, which runs at 72 kDa, can be appreciated in supernatants from both unstimulated and Tat-stimulated cultures. A higher molecular weight band was also observed in the Tat-labeled lane that was absent from the control lane. Based on molecular weight (92 kDa), this band is likely to represent pro-MMP-9. Figure 3B illustrates that supernatant levels of active MMP-2 are increased following Tat treatment, and that this effect was dose dependent. In this figure, two activated forms of MMP-2 can be appreciated. Although it appears from both Figure 3A and B that the total amount of pro-plus active MMP-2 in supernatants is increased in association with Tat, it also appears as though Tat treatment may have up-regulated the production of stimuli which activate MMP-2 by proteolysis.

Methamphetamine was also tested for its ability to effect an increase in supernatant levels of pro- or active MMP-2, but as determined by zymography, levels were not detectably increased by 24 h (data not shown).

Tat and methamphetamine stimulate the release of uPA from brain cells

Mixed neuron/astrocyte cultures were treated with serum-free medium (SFM) alone or SFM containing

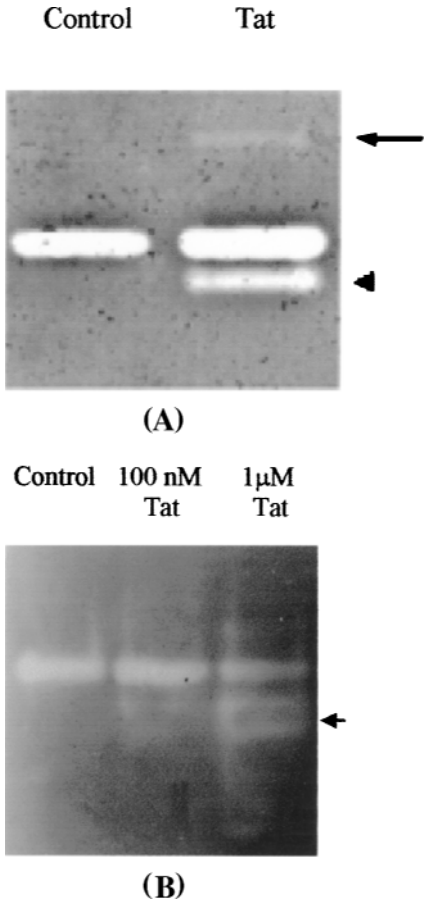

Figure 3 Tat is associated with increased supernatant levels of active MMP-2. (**A**) Neuron/astrocyte cultures were stimulated with Tat (100 nM) and after 24 h, supernatants were harvested assessed by gelatin substrate zymography. Active MMP-2 is observed in Tat lane (*arrowhead*). The predominant band seen in both lanes represents pro-MMP-2, which is apparent on zymograms due to the presence of detergent in the renaturation buffer. A higher molecular weight band, which, based on molecular weight, represents pro-MMP-9, can also be appreciated in Tat-stimulated supernatants. (**B**) The experiment was repeated with different doses of Tat (100 nM and 1 μM) to show that the increase in active MMP-2 is dose dependent.

pertussis toxin (PTX, 100 ng/ml) for 1 h, and then stimulated with fresh SFM or SFM containing 100 nM Tat and/or 500 μM metamphetamine. Supernatants were harvested 24 h later and assayed for uPA by ELISA. Data, which represent the mean + SE of three experiments, are shown in Figure 4 and demonstrate that both Tat and methamphetamine caused an increase in supernatant levels of uPA. In addition, PTX inhibited the ability of stimuli to increase supernatant levels of uPA ($P = .04$ Tat versus Tat + PTX). PTX was not toxic in these experiments as determined by increased release of lactate dehydrogenase (LDH).

Of interest, although methamphetamine increased supernatant levels of uPA, this drug was not associated with an increase in supernatant levels of active MMP-2 (see above). Methamphetamine's effect on uPA could be delayed with respect to that of Tat so that differences in MMP-2 activity cannot be detected by 24 h. Alternatively, Tat, but not methamphetamine, may up-regulate the production of another protein required for either MMP-2 or uPA-stimulated MMP-2 activation.

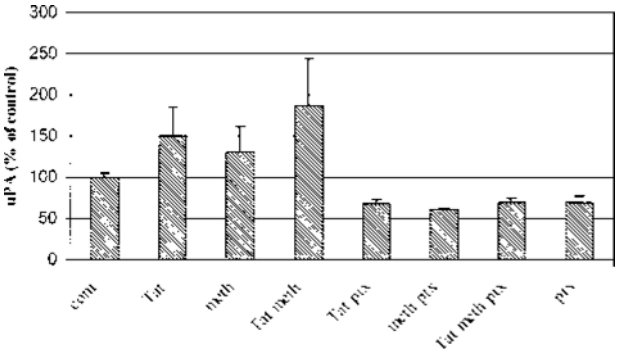

Figure 4 Tat- and Methamphetamine-stimulated release of uPA. Cells were preincubated in serum-free medium, with or without pertussis toxin (100 ng/ml, 1 h), followed by addition of 100 nM Tat, 500 μM methamphetamine, or both as indicated. Supernatants were harvested 24 h later and their uPA levels measured by ELISA. Pertussis toxin reduced the effect of Tat on the cells when compared to Tat alone ($P = .04$).

Discussion

In the present study, we have shown that HIV Tat and methamphetamine can stimulate increased release and/or activation of MMP-1 and MMP-2 from neural cells. We have also shown that these compounds increase release of the MMP activator uPA. The latter converts plasminogen to active plasmin, which itself contributes to cellular matrix degradation and also stimulates the activation of MMPs including MMP-1 and -2 (Festuccia *et al*, 1998; Woessner and Nagase, 2000). It should be remembered that mixed cultures were used in these experiments. This allows for potentially important cell-cell interactions and may better mimic the *in vivo* environment, but does not allow for conclusions regarding the potential behavior of cultures containing a single cell type.

In a number of studies, Tat has been shown to interact with GPCRs, including CXCR4 (Xiao *et al*, 2000) and CCR2 (Albini *et al*, 1998). In addition, methamphetamine stimulates the release of neurotransmitters, which may in turn activate GPCR signaling. The pertussis toxin sensitivity of the uPA release described herein is therefore not without precedent.

In other cell types, both uPA and MMP expression may be influenced by activation of the transcription factors AP-1 (Catterall *et al*, 2001; Hui *et al*, 1998; Sato *et al*, 2002) and NF-κB (Barchowsky *et al*, 2000; Bond *et al*, 1999). Both Tat and neurotransmitters such as dopamine, whose extracellular levels may

be increased by methamphetamine, are known to affect the DNA-binding activity of AP-1 and/or NF-κB (Conant *et al*, 1996; Ishihara *et al*, 1998; Lim and Garzino-Demo, 2000; Luo *et al*, 1999; Ott *et al*, 1998). Though we did not link MMP release to increased MMP expression and/or to alterations in transcription factor binding, our results are consistent with the known ability of Tat and catecholamines to affect both AP-1 and NF-κB. With respect to human brain-derived cells in particular, this ability was herein confirmed for norepinephrine and AP-1.

MMPs have been well studied for their ability to disrupt BBB basement membrane integrity. BBB disruption may in turn facilitate CNS damage mediated by increased infiltration of leukocytes and/or serum-derived toxins. MMPs may, however, also degrade interneuronal proteins, such as sulfated proteoglycans and laminins, that support survival.

Previously, we and others have shown that MMPs may be elevated in association with CNS inflammation/degeneration (Conant *et al*, 1999; Lindberg *et al*, 2001; Lorenzl *et al*, 2003; Shapiro *et al*, 2003), and that these enzymes can be toxic to select populations of neurons *in vitro* (Gu *et al*, 2002; Johnston *et al*, 2001; Vos *et al*, 2000). Although such toxicity may follow from extracellular matrix (ECM) destruction as has been suggested (Vos *et al*, 2000), other mechanisms may be involved.

One ECM-independent mechanism by which MMPs/uPA activated MMPs involves the cleavage of nonmatrix proteins to generate potential cell surface receptor signaling ligands (McCawley and Matrisian, 2001). For example, MMP-1 can cleave insulin-like growth factor–binding proteins and may therefore affect insulin-like growth factor signaling (Fowlkes *et al*, 1994). MMP-7 can cleave cell surface Fas ligand, which may be beneficial or detrimental to cell survival, depending on the system studied (Powell *et al*, 1999). MMP-9 may be involved in the activation of IL-1β (Schonbeck *et al*, 1998) and can potentiate IL-8 activity (Van den Steen *et al*, 2000). Proteolytic processing by MMP-2 can generate endothelin fragments, which can signal cell surface receptors to alter cell adhesion molecule expression (Fernandez-Patron *et al*, 1999). In addition, numerous MMPs can cleave SDF-1α (stromal derived factor) and may thereby generate fragments with altered signaling properties (McQuibban *et al*, 2001).

Another mechanism by which uPA and MMPs may affect cell survival might involve direct effects on cell surface receptors. In recent years, a number of MMPs have been associated with specific cell surface proteins. For example, MMP-9 binds to CD44 (Yu and Stamenkovic, 1999), MMP-2 to $\alpha_v\beta_3$ (Brooks *et al*, 1996), and pro-MMP-1 to both $\alpha_2\beta_1$and $\alpha_1\beta_1$ (Dumin *et al*, 2001; Stricker *et al*, 2001). uPA has likewise been shown to associate with a particular cell surface receptor (Yebra *et al*, 1999). Such binding interactions may serve to localize enzymatic activity, disrupt cell matrix interactions so that cell motility may be facilitated, serve to mediate internalization and degradation of the bound protease, and/or stimulate signaling through the bound receptor (Conant *et al*, 2002; Yebra *et al*, 1999).

Taken together, the data presented in this study suggest that in HIVD, Tat and methamphetamine might increase the release of both uPA and select MMPs from cells of the brain parenchyma. Given the broad range of potentially detrimental effects that may result from excess proteinase levels in the CNS, these results may be of importance to understanding the pathogenesis of the CNS damage, which often occurs with HIV infection and may be exacerbated by superimposed drug abuse (Nath *et al*, 2001).

Materials and methods

Tat, methamphetamine, and neurotransmitters
Recombinant Tat_{1-72} HIV_{BRU} was made as previously described (Ma and Nath, 1997). Methamphetamine was obtained from the National Institute on Drug Abuse (Rockville, MD, USA). Neurotransmitters were obtained commercially from Research Biochemicals International.

Cell culture
Brain specimens were obtained from human fetuses of 12 to 14 weeks' gestational age with consent from women undergoing elective termination of pregnancy and approval by the Johns Hopkins University Institutional Review Board. Neuronal cultures were prepared as described previously (Magnuson *et al*, 1995). Briefly, the meninges and blood vessels were removed and the specimens washed in Opti-MEM (Invitrogen) prior to mechanical dissociation by repeated trituration through a 20-gauge needle. Cells were then pelleted at 270 $\times$ *g* for 10 min and subsequently suspended in Opti-MEM with 5% heat-inactivated fetal bovine serum, 0.2% N2 supplement (Invitrogen), and 1% antibiotic solution (penicillin G 10^3 units/ml, streptomycin 10 μg/ml, and amphotericin B 25 μg/ml) and plated in tissue culture flasks. The cells were maintained in culture for at least 1 month before neurons were released through gentle shaking and then plated onto flat bottomed plates, in which they were maintained for 5 days before experiments were conducted. The purity of cultures was established by immunostaining for MAP-2 (Dako, Carpinteria, CA).

Nuclear extracts
Nuclear extracts were prepared 30 min following cell stimulation according to the method of Andrews and Faller (1991). Cells are lysed in a hypotonic buffer, and the nuclei are subsequently extracted with a high-salt buffer. Centrifugations of less than 30 s are carried out in a room temperature microfuge and between steps cells are placed on ice. In addition, extracts can be prepared from limiting numbers of cells

(5×10^5), which further facilitates the near simultaneous preparation of extracts from multiple cell culture samples.

Nucleic acid probes
The AP-1 consensus sequence oligonucleotide was obtained from Promega and end-labeled with ^{32}P using T4 polynucleotide kinase. The sequence for the AP-1 probe was as follows: 5′-CGC TTG ATG AGT CAG CCG GAA-3′.

Electrophoretic mobility shift assays
Electrophoretic mobility shift assays were also performed as previously described (Conant *et al*, 1996). Binding reactions contained 5 μg of nuclear proteins and 1 ng of ^{32}P-labeled probe. Antibodies for supershift were obtained from Santa Cruz Biotechnology, sc-44, (*jun*) and Dr. Michael Iadarola (c-*fos*), and used as previously described (Conant *et al*, 1998a).

Zymography
SDS–polyacrylamide gel electrophoresis (PAGE) gelatin zymography was used to detect both pro and active MMP-2 in cell culture supernatants obtained from unstimulated control cells and Tat-stimulated cells. Samples were electrophoresed on a BioRad polyacrylamide gel containing gelatin (10%) as a substrate. The gel was renatured in 2.5% Triton X-100, then incubated overnight at 37°C in development buffer (50 mM Tris, pH 7.5, 5 mM $CaCl_2$). On the next day, the gel was stained with Coomassie Blue and after destaining, the presence of MMP-2 was observed as clear bands on a blue background. The molecular weights of these bands were inferred by comparison to prestained standards (Biorad).

ELISA
The amount of uPA and MMP-1 released into the supernatant harvested from unstimulated control cells and Tat- and/or methamphetamine-stimulated cells was measured using commercially available ELISA kits (Dako and Amersham respectively) according to the manufacturer's instructions. The MMP-1 ELISA detects both pro- and active MMP-1 and has a sensitivity of 1.7 ng/ml. The sensitivity of the uPA ELISA kit is 25 pg/ml.

Toxicity assays
Potential cytotoxicity by pertussis was evaluated by measurement of lactic acid dehydrogenase in cell culture supernatants using a commercially available kit (Tox-7, Sigma).

Statistical analysis
Data analysis was carried out using the Microsoft Excel 2000 software package. Mean comparisons were done using Student's t test. Statistical significance was set at $P \leq .05$. Results shown in bar graphs were expressed as mean ± SEM relative to untreated controls.

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