Apoptotic death of striatal neurons induced by HIV-1 Tat and gp120: differential involvement of caspase-3 and endonuclease G

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Abstract

Human immunodeficiency virus-1 (HIV-1) infection affects the striatum resulting in gliosis and neuronal losses. To determine whether HIV-1 proteins induce striatal neurotoxicity through an apoptotic mechanism, mouse striatal neurons isolated on embryonic day 15 and the effects of HIV-1 Tat_{1-72} and gp120 on survival were assessed *in vitro*. Mitochondrial release of cytochrome c, caspase-3 activation, and neuron survival, as well as an alternative apoptotic pathway involving endonuclease G (endo G), were assessed at 4 h, 24 h, 48 h and/or 72 h using enzyme assays and immunoblotting. Both HIV-1 Tat and gp120 significantly increased caspase-3 activation in a concentration-dependent manner in striatal neurons at 4 h following continuous exposure *in vitro*. Tat_{1-72} and gp120 caused significant neuronal losses at 48 h and/or 72 h. Tat_{1-72} increased cytochrome c release, and caspase-3 and endo G activation at 4 h, 24 h, and/or 72 h. By contrast, gp120 increased caspase-3 activation, but failed to increase cytochrome c or endo G levels in the cytoplasm at 4 h, 24 h, and/or 72 h. The cell permeant caspase inhibitor Z-DEVD-FMK significantly attenuated gp l20-induced, but not Tat_{1-72}-induced, neuronal death suggesting that gp 120 acts in large part through the activation of caspase(s), while Tat_{1-72} induced neurotoxicity was accompanied by activating an alternative pathway involving endo G. Thus, although Tat_{1-72} and gp 120 induced significant neurotoxicity, the nature of the apoptotic events preceding death differed. Collectively, our findings suggest that HIV-1 proteins are intrinsically toxic to striatal neurons and the pathogenesis is mediated through separate actions involving both caspase-3 and endo G.

Keywords

Caspase-3; endonuclease G; cytochrome c; neurotoxicity

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INTRODUCTION

Human immunodeficiency virus-1 (HIV-1) dementia is a neurodegenerative syndrome characterized by cognitive decline, personality change, and motor deficits in humans infected with HIV-1 (McArthur *et al*, 1993; Lipton and Gendelman, 1995). HIV encephalitis (HIVE) often accompanies HIV dementia and is characterized by prominent microglial activation, neuronal losses, dendritic pruning, and decreased density of synapses (Masliah *et al*, 1996; Lipton, 1997). Apoptotic changes are seen with HIVE in both neurons and non-neuronal cells (Ramirez *et al*, 2001; Bonavia *et al*, 2001; Corasaniti *et al*, 2001; Adle-Biassette *et al*, 1995; Gelbard *et al*, 1995; Petito and Roberts, 1995; Shi *et al*, 1996; Kaul *et al*, 2001; Shi *et al*, 1998; Park *et al*, 2001). HIV-1 is neurotoxic by inducing inflammation and through the direct release of toxic viral proteins such as Nef, Vpr, gp120 and Tat (Haughey *et al*, 2001; Brenneman *et al*, 1988; Dreyer *et al*, 1990; Adamson *et al*, 1996; New *et al*, 1997; Kruman *et al*, 1998; Yeung *et al*, 1998; Huang and Bond, 2000; Trillo-Pazos *et al*, 2000; Nath, 2002). The neurotoxicity of gp120 has been demonstrated both in primary human neuronal cultures (Lannuzel *et al*, 1997; Yeung *et al*, 1995) and in transgenic mice (Toggas *et al*, 1994). Gp120 is thought to be intrinsically neurotoxic through an apoptotic mechanism (Barillari *et al*, 1993; Albini *et al*, 1996a,b; Bagetta *et al*, 1996b; Hesselgesser *et al*, 1998; Marzo *et al*, 1998; Meucci *et al*, 1998; Zheng *et al*, 1999) and may also act via inflammatory cytokines (Bagetta *et al*, 1996a, 1999; Lipton, 1997).

HIV-1 Tat protein is not only an intracellular transcriptional activator, but is also secreted by infected cells and is capable of acting as a proto-cytokine. HIV-1 Tat can bind to and activate specific tyrosine kinase receptors including the Flk-1/kinase insert domain (Flk-1/KDR) receptor for vascular endothelial growth factor (VEGF) (Albini *et al*, 1996b) and several classes of integrins (Albini *et al*, 1996a; Barillari *et al*, 1993; Ganju *et al*, 1998; Vogel *et al*, 1993). HIV-1 Tat can trigger apoptosis in PC-12 neuronal cells through the induction of tumor necrosis factor-a release (New *et al*, 1998; Shi *et al*, 1998). Tat is important in the pathogenesis of Kaposi's sarcoma and in the neuroinflammatory changes seen in patients with HIV dementia (Deregibus *et al*, 2002; Kaaya *et al*, 1996).

Apoptosis is often accompanied by the activation of caspases (Creagh and Martin, 2001; Schimmer *et al*, 2001; Stennicke and Salvesen, 2000). Caspase-3 is a member of the CED-3 subfamily of caspases that is activated in several neurodegenerative disorders (Namura *et al*, 1998; Hartmann *et al*, 2000; Su *et al*, 2000). Active caspase-3 has been detected by Western blot in human fetal neural cultures exposed to gpl20 (Zheng *et al*, 1999); however, the specific cell population undergoing caspase activation was not identified. Postmortem studies on the brains of pediatric patients with HIV dementia showed increased procaspase-3 immunoreactivity in neurons (James *et al*, 1999).

The basal ganglia are especially vulnerable to HIV infection (Berger and Nath, 1997; Nath *et al*, 2001) and this may result from unique phenotypic characteristics of striatal neurons compared to other neuron types. For example, striatal neurons are quite susceptible to AMPA-receptor-mediated excitotoxic stimuli, but far less sensitive to NMDA-induced excitotoxicity (Goody *et al*, 2003; Singh *et al*, 2003). The basal ganglia are also a principal target for drug abuse and may be preferentially susceptible to interactions between HIV and

drug abuse (Nath *et al*, 2001; Nath *et al*, 2002; Nath, 2002). For this reason, we examined the mechanisms underlying Tat and gp120 toxicity in striatal neurons and found that Tat_{1-72} and gp120 induced apoptotic cell death in mouse striatal neurons.

Materials and Methods

Materials

Dulbecco's Modified Eagle's Medium (DMEM:F-12), B27, antibiotic-antimycotic (penicillin/streptomycin/amphoterin) were purchased from Gibco/Life Technologies, Grand Island, NY, USA. Purified mouse anti-cytochrome c monoclonal antibody was obtained from BD PharMingen, USA. Insulin, linoleic acid, and protein G beads were purchased from Sigma-Aldrich (St. Louis, MO, USA). Caspase-3 substrates, including Ac-Asp-Glu-Val-Asp- 7-amino-4methylcoumarin (Ac-DEVD- AMC) and a reversible aldehyde inhibitor, Ac-DEVD-CHO (Ac-Asp-Glu-Val-Asp-aldehyde), were obtained either from Bachem Bioscience, Inc. (King of Prussia, PA, USA) or from Molecular Probes, Inc. (Eugene, OR, USA). A cell-permeable pan-caspase inhibitor II (Z-DEVD-FMK) was purchased from Calbiochem-Novabiochem Corporation (San Diego, CA, USA) and used at 30 or 50 μM concentrations. The BCA protein assay kit and reagents were purchased from Pierce (Rockford, IL, USA). Bio-Rad Ready gels (10% and 12%) and Kaleidoscope prestained standards were obtained from Bio-Rad Laboratories (Hercules, CA, USA). An ECL Western blot analysis system containing peroxidase-labeled anti-mouse or anti-rabbit antibodies was purchased from Amersham Pharmacia Biotech (Buckinghamshire, England). Timed, pregnant ICR mice were obtained from Charles River (Charles River, MA, USA).

Viral Proteins

The *tat* gene encoding amino acids 1–72 was amplified from HIVbru obtained from Dr. Richard Gaynor through the AIDS repository at the NIH and inserted into an *E. coli* (PinPoint Xa-2) vector (Promega). Recombinant Tat_{1-72} was prepared as described previously (Ma and Nath, 1997) with minor modifications (Gurwell *et al*, 2001). Recombinant gp120 from HIV-SF2 made in CHO cells was a gift from Chiron Inc. (Emeryville, CA, USA), and purified and handled as previously described (Holden *et al*, 1999; Haughey, 2002).

Striatal Neuronal Cultures

Striatal neuronal cultures from embryonic day 15 (E 15) ICR mice were prepared as described before (Goody *et al*, 2003). Striata were dissected from ICR pups, dissociated, and striatal neurons were grown for 5–7 days *in vitro* (DIV) in serum-free DMEM/F-12 medium supplemented with 2% B-27, 1 μg/ml linoleic acid, 25-μg/ml insulin, and 1% antibiotic/ antimycotic. Striatal neurons were seeded at identical densities (4×10^5 cells/cm^2) and grown on poly-D-lysine (0.1 mg/ml) coated Costar-24 well-plates in an incubator maintained at 35–36 °C in 5% CO_2 95% air and high humidity. The cultures described in the present study are enriched in medium spiny neurons, which comprise about 85–95% of neurons in the mouse striatum, and contain about 3–8% astroglia and about 0.4% monocytes/microglia (Hauser, unpublished), but which differ from an alternative mixed neuronal-glial striatal culture system previously characterized by us (Gurwell *et al*, 2001).

Neuronal Viability

To assess neuronal losses following continuous Tat_{1-72} (100 nM) or gp120 (500 pM) exposure, time-lapse, repeated measures analyses of digital photomicrographs of individual neurons were performed as previously reported (Hauser *et al*, 1999; Goody *et al*, 2003). Briefly, neuronal viability was assessed by repeatedly photographing the same neurons at 24-h intervals. Glass coverslips were lightly scored with a diamond marker, before placing into 12-well plates, to aid in finding the same field of neurons (neurons were plated on the opposite side from the score). This method of sampling is systematic, but arbitrary, since all neurons within a region predetermined before the onset of the experiment are assessed. The procedure also selects against background cell death that occurs in many primary neural culture systems, since the small proportion of dying cells are identified before the onset of treatment and are excluded from further study *a priori* without introducing experimenter bias. Neurons were photographed digitally using a Spot 2 camera (Diagnostic Instruments, Sterling Heights, MI) and Nikon Diaphot inverted microscope with phase contrast optics and a 20× objective. About 50–75 neurons were arbitrarily sampled per culture. At least 4–6 cultures, each consisting of cells isolated and maintained from separate mice were assessed per experimental group. Dying neurons were identified by the fragmentation and destruction of the cell body and neurites, as well as the loss of nuclear structure and/or shrinkage (pyknosis) (Fig. 4); detailed criteria for neuronal losses have been previously described (Hauser *et al*, 1999; Goody *et al.* 2003).

The neuronal identity of the dying cells was further confirmed by exposing cells to 0.5 μg/ml ethidium monoazide (EMA) in Dulbecco's PBS for 30 min, permanently binding the EMA to the dying cells through photoaffinity labeling, and co-detecting EMA-positive cells with neuron-specific antigenic markers as previously described (Gurwell *et al*, 2001). Briefly, EMA is excluded from living cells, but intercalates to the DNA of dying cells, where it can be permanently linked via photoaffinity by exposure to a 45 W fluorescent light (15 cm distance) for 30 minutes at room temperature. Cells were then fixed with Zamboni's fixative containing 3% paraformaldehyde. Cell cultures were characterized by immunoreactivity to the neuronal markers, i.e., neuronal nuclear protein (NeuN) (mouse monoclonal anti-NeuN IgG, Chemicon, Temecula, CA, USA; at 1:500 dilution) and PGP 9.5 (rabbit polyclonal anti-PGP 9.5 IgG, Chemicon; 1:1800 dilution).

Caspase-3 Activity

Caspase-3 activity was measured as previously described (Rigamonti *et al*, 2000). Briefly, embryonic mouse striatal neurons grown for 5–7 DIV in serum-free DMEM/F12 medium were treated with vehicle-treated media alone, Tat_{1-72} (100 nM), or gp120 (500 pM). Cells were harvested at 4 h, 24 h, or 72 h after the treatments in ice-cold harvesting buffer (25 mM HEPES, pH 7.5, 5 mM EDTA, 1 mM EGTA, 5 mM magnesium chloride, 10 mM sucrose, 5 mM dithiothreitol, 1% 3-[-(3-chloramidopropyl) dimethylammonio]-l-propanesulfonic acid (CHAPS), 10 μg/ml pepstatin, 10 μg/ml leupeptin and 1 mM PMSF). After freezing and thawing 3 times, the cell lysates were centrifuged for 10 min at 5000 rpm, and the supernatants were centrifuged at 10,000 g for 60 min. The cell lysates thus obtained were stored at −80 °C. Lysates were incubated at 37 °C in a buffer containing 25 mM HEPES, pH 7.5, 10% sucrose, 0.1% CHAPS, and 1 mM dithiothreitol supplemented with 50 μM Ac-

DEVD-7- amino-4- methylcoumarin (AMC) in 96-well Costar plates. As a negative control, DEVD-CHO, a caspase-3 specific inhibitor, was added to the cell lysates 30 min before incubation with caspase-3 substrate, Ac-DEVD-AMC. The increase in fluorescence after the cleavage of the fluorogenic AMC moiety was monitored in a Cytofluor 4000 fluorimeter (Perspective Biosystems, Framingham, MA, USA) using 360 nm excitation and 460 nm emission wavelengths. Caspase-3 activity was expressed as fluorescence units per milligram of total cytosolic protein.

Immunoprecipitation, Immunoblot Analysis, and Cytochrome c Detection

Release of cytochrome c from mitochondria was measured as previously described (Yang *et al*, 1997). After incubation with vehicle-treated medium alone, 100 nM Tat_{1-72}, or 500 pM gp120, striatal neurons were harvested with ice-cold buffer A containing 20 mM Hepes-KOH, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, and 250 mM sucrose. Cell homogenates were centrifuged first at 750 g for 10 min at 4 °C, and the resulting supernatant was centrifuged at 12,000 g for 30 min at 4 °C. The supernatant (cytosolic) fraction and the mitochondrial pellet fraction were used for the detection of cytochrome c. Equal amounts of cytosolic and mitochondrial protein from control and treated cultures were immunoprecipitated with 1 μg of purified mouse anti-cytochrome c monoclonal antibody (clone.6H2.B4 mouse IgG from BD PharMingen) for 3 h at 4 °C with constant rocking. Ten μl of a 50% slurry of protein G-agarose beads in PBS was added, and the tubes were incubated overnight at 4 °C. The beads were pelleted, washed three times with PBS-1% Triton X-100, and suspended in 30 μL of Laemmli sample buffer (Laemmli, 1970). After boiling for 5 min and centrifuging briefly, equal amounts of immunoprecipitated proteins (20 μg protein/lane) in the supernatant were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) on 12% polyacrylamide gels under reducing conditions (4% ß-mercaptoethanol). The proteins were electrophoretically transferred to a polyvinylidenedifluoride (PVDF) membrane. After blocking in TTBS buffer (10 mM Tris/HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween 20) containing 5% skimmed milk powder, the membranes were incubated with primary monoclonal antibodies against cytochrome c (7H8.2C12 mouse IgG, PharMingen, 1:333 dilution) at room temp for 3 h. Finally, proteins were incubated 1 hr at room temperature with a peroxidase-conjugated goat anti-mouse antibody. The blots were developed on Hyperfilm ECL film using Amersham ECL™ reagent (Amersham Pharmacia Biotech, Piscataway, NJ, USA) according to manufacturers' instructions and detected using a phosphoimager (Molecular Dynamics Storm, Amersham Biosciences, Piscataway, NJ, USA) or cooled CCD camera (Kodak Image Station 440 CF, Rochester, NY, USA). The optical density of the band in each lane was expressed relative to the vehicle-treated control in the same blot.

Protein Determination

Protein concentrations were determined by the BCA-method using a commercially available kit (Pierce; Rockford, IL, USA).

Statistics

Data were reported as the mean ± SEM of the average values from replicate samples obtained from at least n = 4 separate experiments. Significant overall differences among experimental groups were assessed using ANOVA (Statistica, StatSoft, Inc., Tulsa, OK, USA). If statistically significant differences were noted using ANOVA ($P < 0.05$), multiple group differences were compared *post hoc* using Duncan's test. Paired group differences were assessed using Student's t test. Homogeneity of variances was screened for all data using Levene's test.

RESULTS

We first studied the concentration-dependence of HIV-1 Tat and gp120-induced activation of caspase-3 in striatal neurons at 4 h following continuous exposure *in vitro*. As shown in Figure 1, both HIV-1 Tat and gp120 caused significant increases in caspase-3 activation in a concentration-dependent manner (p<0.0001 versus untreated cultures) at 4 h following continuous exposure in vitro. The optimum concentration of HIV-1 Tat used being 100 nM whereas the optimum concentration of gp120 used being 500 pM for the measurement of neuronal viability and other biochemical studies described in this paper (Fig. 1).

When Tat_{1-72} and gp120 toxicity were assessed by time-lapse photomicrography in primary cultures derived from embryonic mouse striatum, both viral proteins were found to be intrinsically toxic to striatal neurons using time-lapse photomicroscopy (Fig. 2). High rates of death were seen 72 hours after exposure to either Tat_{1-72} (100 nM) (Fig. 2B) or gp120 (500 pM) (Fig. 2D). Although some background neuronal death is normally present in striatal neuron cultures at 7–10 days *in vitro*, increased rates of cytotoxicity were clearly evident following exposure to Tat or gp120 (see Fig. 2A,B). To assess the extent and time-course of viral protein-induced neuronal losses, the effects of Tat_{1-72} and gp120 on neuronal death were quantified before and at 24-h intervals following continuous exposure to viral proteins (Fig. 3). The findings showed that gp120 caused rapid neuronal losses with significant cytotoxicity occurring at 48 h, while Tat_{1-72} caused significant toxicity after 72 h (Fig. 3) (*$P < 0.05$ versus controls treated with medium-vehicle alone).

Because Tat and gp120 toxicity has been associated with activation of caspases and apoptosis in astrocytes and neurons in other brain regions (Kruman *et al*, 1998; Su *et al* 2000; Garden *et al*, 2002; Haughey and Mattson, 2002), we examined the effect of the cell permeant pan-caspase inhibitor Z- DEVD-FMK on rates of Tat and gp120 toxicity in striatal neurons. Tat-induced neuronal death was unaffected in cultures co-incubated with DEVD-FMK (30 μM) (Fig. 2C), while pretreatment with DEVD-FMK protected neurons exposed to 500 pM gp120 (Fig. 2E). The rate of neuronal death in DEVD-FMK treated cultures did not differ significantly from controls treated with medium alone. Incubation with Z-DEVD-FMK (30 μM) significantly attenuated gp120-induced neuronal death (Fig. 3B) ($^{\#}P < 0.01$ versus gp120 treatment alone), but not Tat_{1-72}-induced neuronal death (Fig. 3A). These results suggested that gp120-induced neurotoxicity is mediated by caspase-3, while Tat_{1-72} is neurotoxic through an alternative pathway independent of caspase-3.

To validate the time-lapse studies, striatal neuron death was assessed by co-localized immunofluorescent reactivity for the neuronal nuclear marker (NeuN) in dying cells that cannot exclude EMA at 24 h (Table 1; Fig. 4). The inability to exclude EMA precedes overt pathological changes in neuronal morphology that accompany cell death. Cell cultures were exposed to EMA and assayed at 24 h following Tat or gp120 exposure. Moreover, it was anticipated that the proportion of dying neurons labeled by EMA would be less than that counted by repeated measures, since cells are incubated in EMA for a relatively short duration (30 min) and our experience from time-lapse studies is striatal neurons undergo death quite rapidly (< 24 h). In addition, to similarly assess the role of caspase-3 in Tat and gp120-induced neuronal death, viral protein-toxicity was assessed in the presence or absence of the pan-caspase inhibitor Z-DEVD-FMK (50 μM). The results indicated significant gp120-induced neuronal losses at 24 h that were significantly attenuated by co-treating neurons with Z-DEVD-FMK (Table 1, Fig. 4B,C). In contrast, Tat_{1-72} did not increase the proportion of EMA-positive NeuN-identified neurons at 24 h (Table 1), which agreed with findings that Tat toxicity progresses more slowly than gp120-induced neuronal losses (Fig. 3).

To assess the extent and time-course of viral protein-induced caspase-3 activation, the effects of Tat_{1-72} and gp120 on caspase-3 activation were examined at 4 h, 24 h, and 72 h (Fig. 5). Caspase-3 was significantly elevated following Tat_{1-72} or gp120 exposure at 4 h, 24 h, and 72 h. At 72 h, caspase-3 activity was significantly greater with Tat treatment than gp120 treatment (Fig. 5); however, at this time fewer viable neurons remain following gp120 treatment. Interestingly, despite findings that Z-DEVD-FMK failed to inhibit Tat-induced striatal neuron death (Fig. 4B), Tat nevertheless caused significant increases in caspase-3 activity at 4 h, 24 h, and 72 h (Fig. 5). Lastly, to assure that our assay was specifically assessing caspase-3, Ac-DEVD-CHO, a highly selective (but cell impermeant) caspase-3 inhibitor blocked caspase-3 activity when added to the cell lysates 30 min before incubation with caspase-3 substrate (Ac-DEVD-AMC; data not shown).

A key event in the mitochondrial-mediated pathway for caspase-3 activation is the release of cytochrome c into the cytoplasm (Kluck *et al*, 1997). Increases in cytoplasmic levels of cytochrome c following HIV-1 Tat_{1-72} and gp120 exposure were investigated in striatal neurons at 4 h and 72 h (Fig. 6). As shown in Fig. 6A–C, Tat_{1-72} (100 nM), but not gp120 (500 pM), significantly increased levels of cytochrome c in the cytoplasm (*$P < 0.05$ versus vehicle-treated control cultures; #$P < 0.05$ versus Tat_{1-72}-treated neurons). Tat-induced increases in cytoplasmic levels coincided with declines in mitochondrial levels as expected and β-actin levels in the cytoplasm did not differ among treatments (data not shown). Although gp120 did not increase cytosolic levels of cytochrome c at 4 h or 72 h, this does not exclude the possibility that cytochrome c was involved at other times. The accelerated loss of neurons following gp120 compared to Tat exposure (see Fig. 4), prompts speculation that gp120 might similarly hasten proapoptotic signaling events and induce cytochrome c release prior to 4 h; this notion warrants further investigation.

We additionally investigated the role of endonuclease-G (endo G), a mitochondrion-specific nuclease that translocates into the nucleus during apoptosis (Parrish *et al*, 2001; Li *et al*, 2001). To test whether endo G is released following HIV-1 protein neurotoxicity, we

exposed neuronal cultures to Tat_{1-72} (100 nM) or gp120 (500 pM) for 4 h and 72 h (Fig. 7A–C). Tat_{1-72}, but not gp120, significantly increased the translocation of endo G from mitochondria into the cytoplasm at 4 h following exposure ($*P < 0.05$ versus medium-treated control cultures). The results suggested that Tat can induce apoptosis by activating endo G in addition to caspase activation, while gp120 failed to activate the endo G apoptotic pathway. This difference may explain why Z-DEVD-FMK was able to significantly attenuate gp120 neurotoxicity but unable to allay Tat_{1-72} toxicity in the present study.

DISCUSSION

The work presented here indicates that HIV-1 Tat_{1-72} and gp120 are toxic to striatal neurons, and suggests they trigger neurodegeneration by activating caspase-3 and/or endonuclease G apoptotic pathways. Although the mechanisms underlying HIV-induced cell death in the nervous system are not fully understood, results presented herein suggest that underlying neurotoxic pathways are highly complex and likely to differ for each viral protein. HIV-1 viral products can be released by infected microglia and astroglia (Ensoli et al, 1990; Munis et al, 1992; Robert-Guroff *et al*, 1990; Chang et al, 1997; Shutt and Soil, 1999; Jones et al, 1998; Jones et al, 2000), and can also induce the production and release of proinflammatory cytokines and cellular toxins in neural cells (Yeung *et al*, 1995; Adamson *et al*, 1996; Bagetta *et al*, 1996a; Kaul and Lipton, 1999; Nath *et al*, 1999). Numerous lines of evidence suggest that extracellular proinflammatory cytokines and cellular toxins can induce neuronal apoptosis (Gabuzda et al, 1986; Gendelman *et al*, 1994; Shi *et al*, 1998; New *et al*, 1998; Bagetta *et al*, 1999; Holden *et al*, 1999; Zheng *et al*, 1999; Park *et al*, 2001; Takahashi et al, 1996). Furthermore, several studies suggest that HIV-1 Tat and gp120 interact with neuronally-expressed receptors to activate multiple pro-apoptotic signaling cascades in neurons (Ma and Nath, 1997; Moore *et al*, 1997; New *et al*, 1997; Shi *et al*, 1998; Piller *et al*, 1999; Bonavia *et al*, 2001; Corasaniti *et al*, 2001; Haughey *et al*, 2001).

The present study shows that both Tat_{1-72} and gp120 activated the apoptotic effector caspase-3. This is consistent with reports that HIV proteins, including Tat, Vpr, gp41 and Nef, can initiate apoptotic cascades in other neural cell types (Adamson *et al*, 1996; Yang *et al*, 1997; Kruman *et al*, 1998; Bartz and Emerman, 1999; Liu *et al*, 2000; Trillo-Pazos *et al*, 2000; Parrish *et al*, 2001; Li *et al*, 2001). The protective role of caspase inhibitors in experimental models of HIVE supports the involvement of caspases in the pathogenesis of neuroAIDS (Garden *et al*, 2002). The pan caspase inhibitor Z-DEVD-FMK (Z-VAD-FMK) is an important tool to assess the role of caspases in cell death, while the substrate used to detect activity (Ac-DEVD-AMC) is selective for caspase-3 (Lei *et al*, 2002). Interestingly, although both Tat_{1-72} and gp120 caused increases in caspase-3 activity followed by neuronal cell death, only gp120 neurotoxicity was significantly blocked by caspase inhibition suggesting that Tat_{1-72} can induce neuronal death independent of caspase activation.

The release of cytochrome c from mitochondria into the cytosol can be a key event in the apoptotic process (Springer *et al*, 1999). Our results indicate that exposure of mouse striatal neurons with Tat alone significantly increased cytochrome c levels in the cytoplasm following 4 h or 72 h exposure, while gp120 failed to induce significant increases in cytochrome c in the cytoplasm.

Endonuclease G is primarily synthesized in a propeptide form in mammalian cells and its import into mitochondria is mediated by an amino-terminal mitochondrion-targeting sequence (Ruiz-Carillo and Renaud, 1987; Cote and Ruiz-Carillo, 1993). Upon entry into the mitochondria, the signal peptide is cleaved-off and the mature endo G can be released from mitochondria following an appropriate apoptotic signal (Li *et al*, 2001). We report here that exposure of mouse striatal neurons with HIV-1 Tat$_{1-72}$ significantly increased the translocation of endo G from mitochondria into the cytoplasm and this may be sufficient to induce death despite the presence of caspase inhibition. Thus, endo G levels in brains of HIV infected patients may be an indirect indicator of the presence of Tat. In contrast, gp120 did not induce endo G translocation from mitochondria at 4 h or 72 h suggesting an alternative apoptotic pathway different from Tat. Studies in progress are more directly addressing the role of endo G in Tat neurotoxicity.

Collectively, these findings suggest the involvement of at least two independent pathways, which execute neuronal apoptosis in neuro-AIDS. The existence of multiple apoptotic pathways may underlie fundamental differences in Tat versus gp120 mediated neurotoxicity in the striatum. Understanding the differences in HIV protein-mediated neurotoxicity is critical to the development of new therapeutic approaches for HIV dementia.

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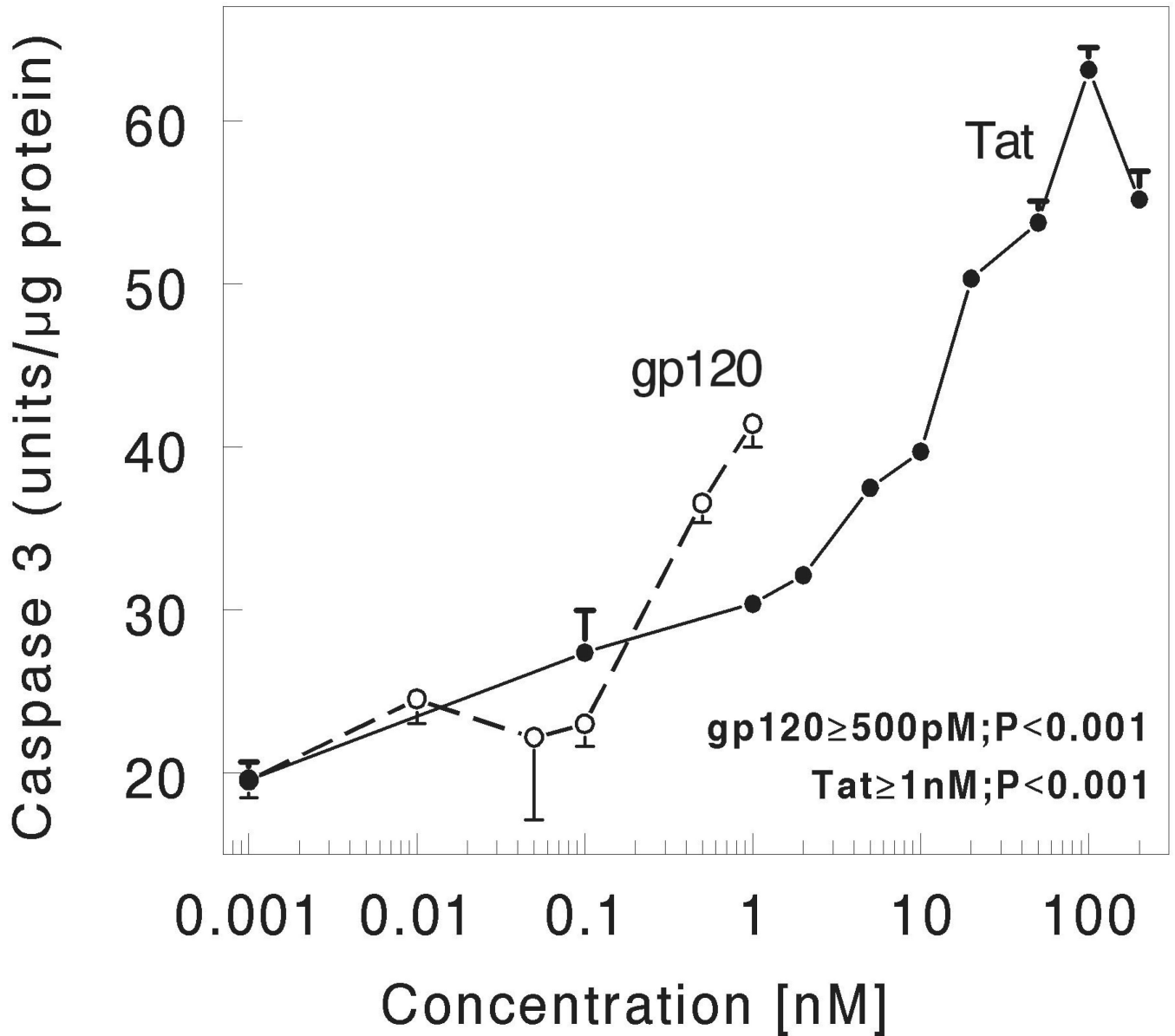
Figure 1.
HIV-1 Tat and gp120 caused concentration-dependent increases in caspase-3 activation in striatal neurons at 4 h following continuous exposure *in vitro*. Striatal neurons were grown for 7 days in culture and incubated with varied concentrations of HIV-1 Tat (closed circles with solid line) and gp120 (open circles with dotted lines) for 4 h. Caspase-3 activity was measured as described in the Methods section. Results are the mean ± the S.E.M from n=4 experiments.
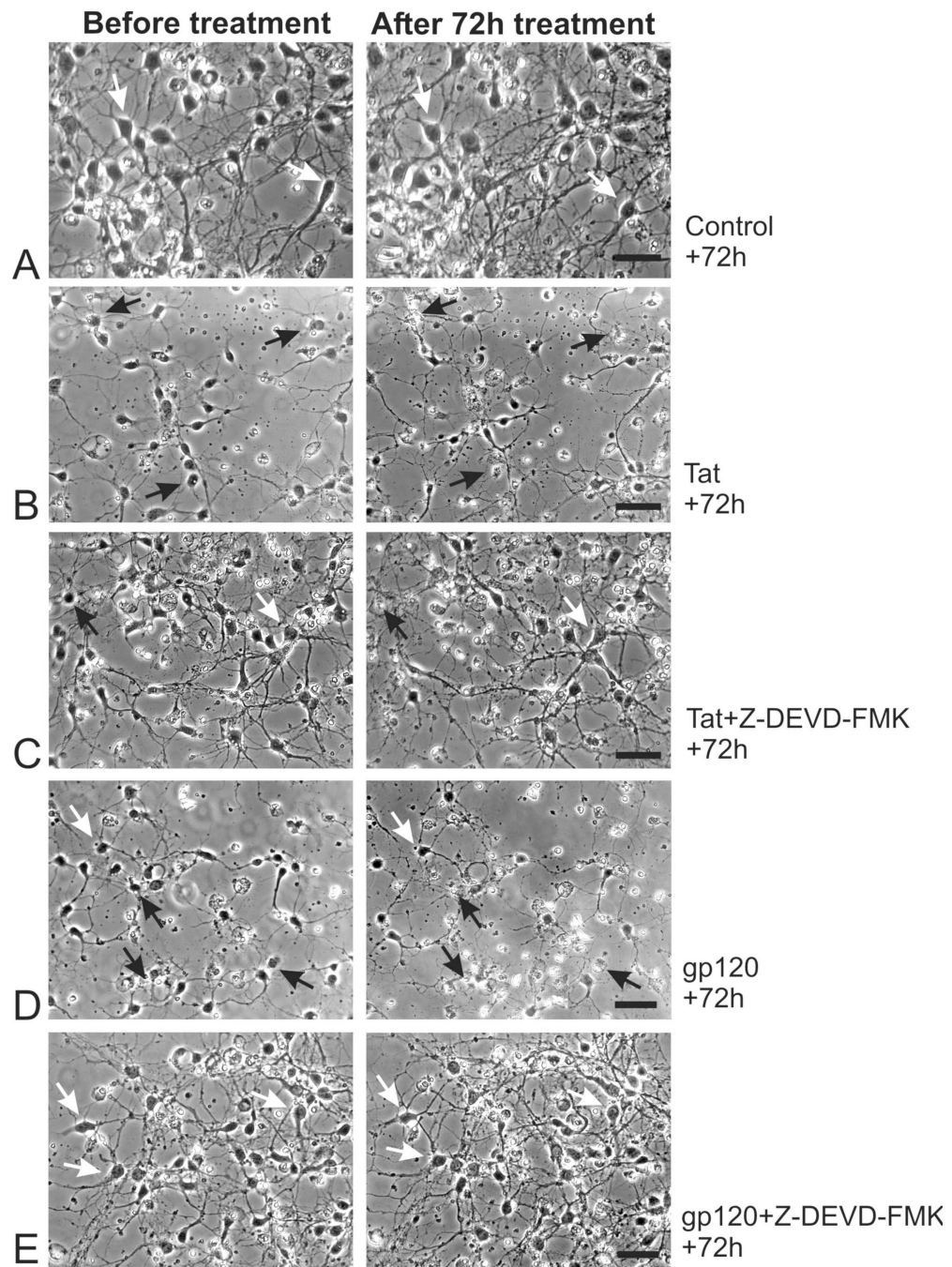
Before treatment   **After 72h treatment**

Figure 2.
Time-lapse digital photomicrographs showing the effects of HIV-1 Tat_{1-72} or gp120 on striatal neuron survival prior to (Before treatment; left column) and at 72 h following exposure (right column) in *vitro* (A–F). The effects of Tat or gp120 were additionally assessed in the presence or absence of Z-DEVD-FMK (30 μM) (DEVD) a caspase inhibitor applied 4 hours prior to viral protein exposure. Tat_{1-72} (100 nM) (B) and gp120 (500 pM) (D) were neurotoxic compared to treatment with media alone (A). However, only gp120 (E),

but not Tat (C), induced neuronal losses were significantly attenuated by DEVD. Black arrows represent dying neurons, white arrows show viable neurons; scale bars = 25 μm.
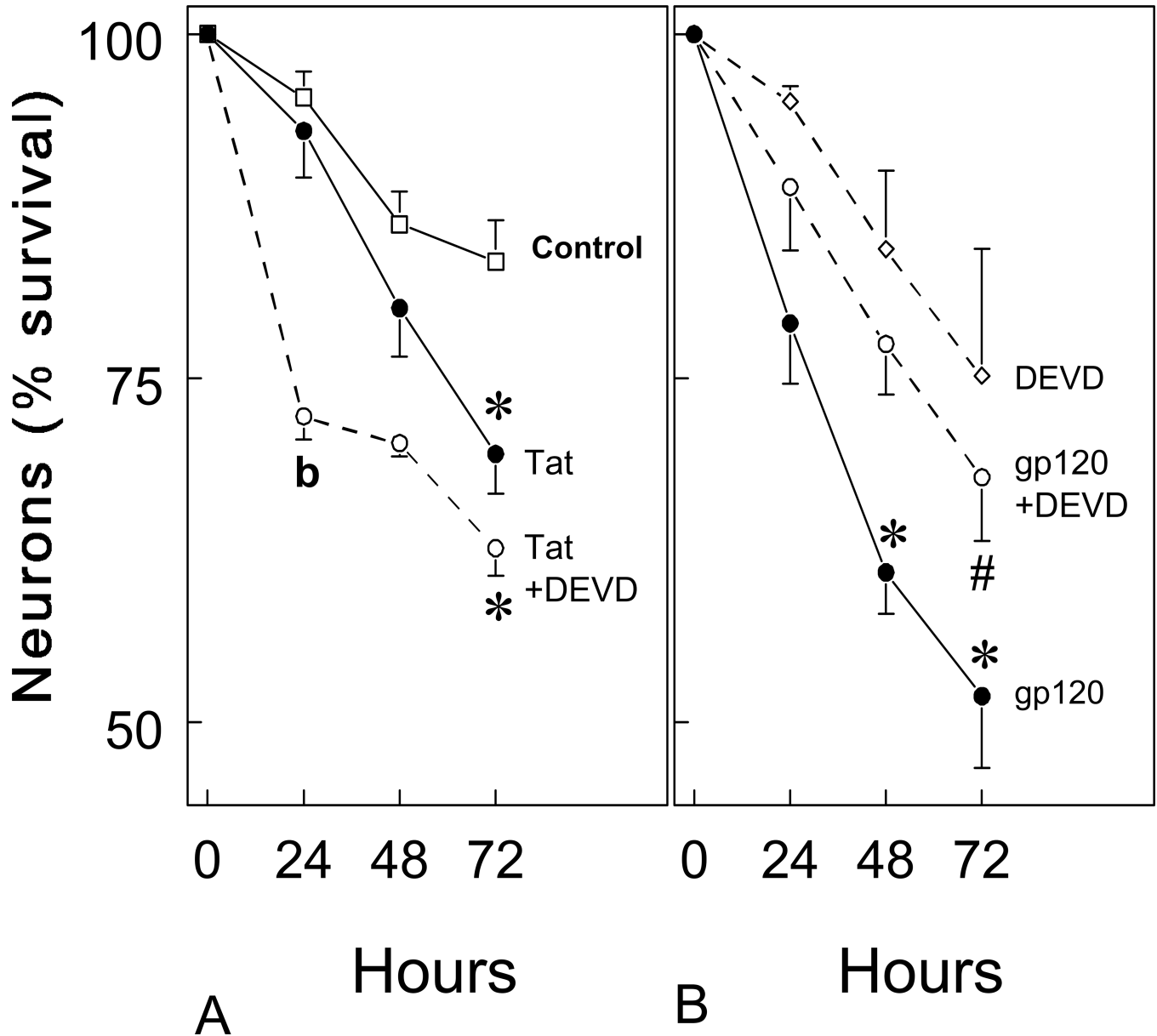
Figure 3.

HIV-1 Tat_{1-72} and gp120 reduced striatal neuron survival at 24, 48, and 72 hours *in vitro*. Both Tat (100 nM) (A) and gp120 (500 pM) (B) significantly increased the proportion of dying neurons at 48 and/or 72 h (*$P < 0.05$ versus vehicle-treated controls). The cytotoxic effects of gp120, but not Tat_{1-72}, were significantly attenuated by the caspase inhibitor Z-DEVD-FMK (30 µM; [#]$P < 0.01$ versus gp120 treatment alone). Interestingly, Z-DEVD-FMK exposure appeared to enhance Tat toxicity at 24 h ([b]$P < 0.05$ versus Tat-treated or vehicle-treated cultures). About 50–75 neurons were arbitrarily sampled per culture. At least 4–6 separate cultures, each consisting of cells isolated and maintained from separate mice were assessed per experimental group. Rates of neuronal death in DEVD-FMK treated cultures did not differ significantly from vehicle-treated controls; DEVD = Z-DEVD-FMK.
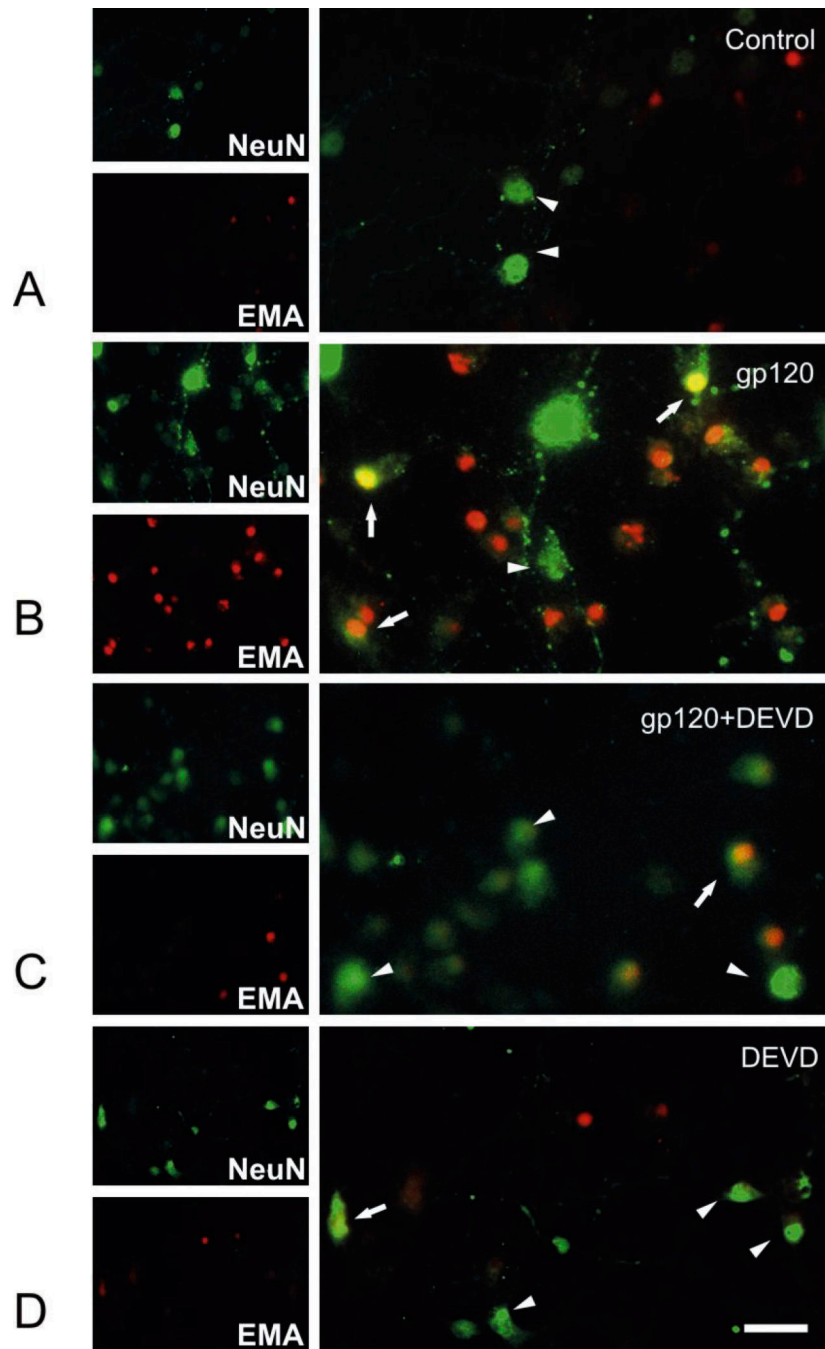
Figure 4.
Fluorescent photomicrographs showing the co-localization neuronal nuclear (NeuN) marker and ethidium monoazide (EMA) in striatal neuron cultures at 24 h following exposure to medium alone (A), gp120 (500 pM) with or without Z-DEVD-FMK (DEVD) (50 μM) (B,C), or DEVD alone (50 μM) (D). Cell cultures were incubated with DEVD for 4 h prior to exposure to gp120. gp120 treatment increased the proportion of dying neurons and the toxicity was prevented by co-administering DEVD (see Table 1). Viable NeuN immunofluorescence neurons (arrowheads) appear green and exclude EMA (red

fluorescence) from their nuclei. Non-viable neurons (arrows) fail to exclude EMA (red) and their nuclei appear yellow. Tat (100 nM) was also assayed but showed no effect at 24 h (see Table 1). The large photomicrographs (right-side) are composite images of NeuN reactivity (upper left insets) and EMA (lower left insets); scale bar = 20 μm.
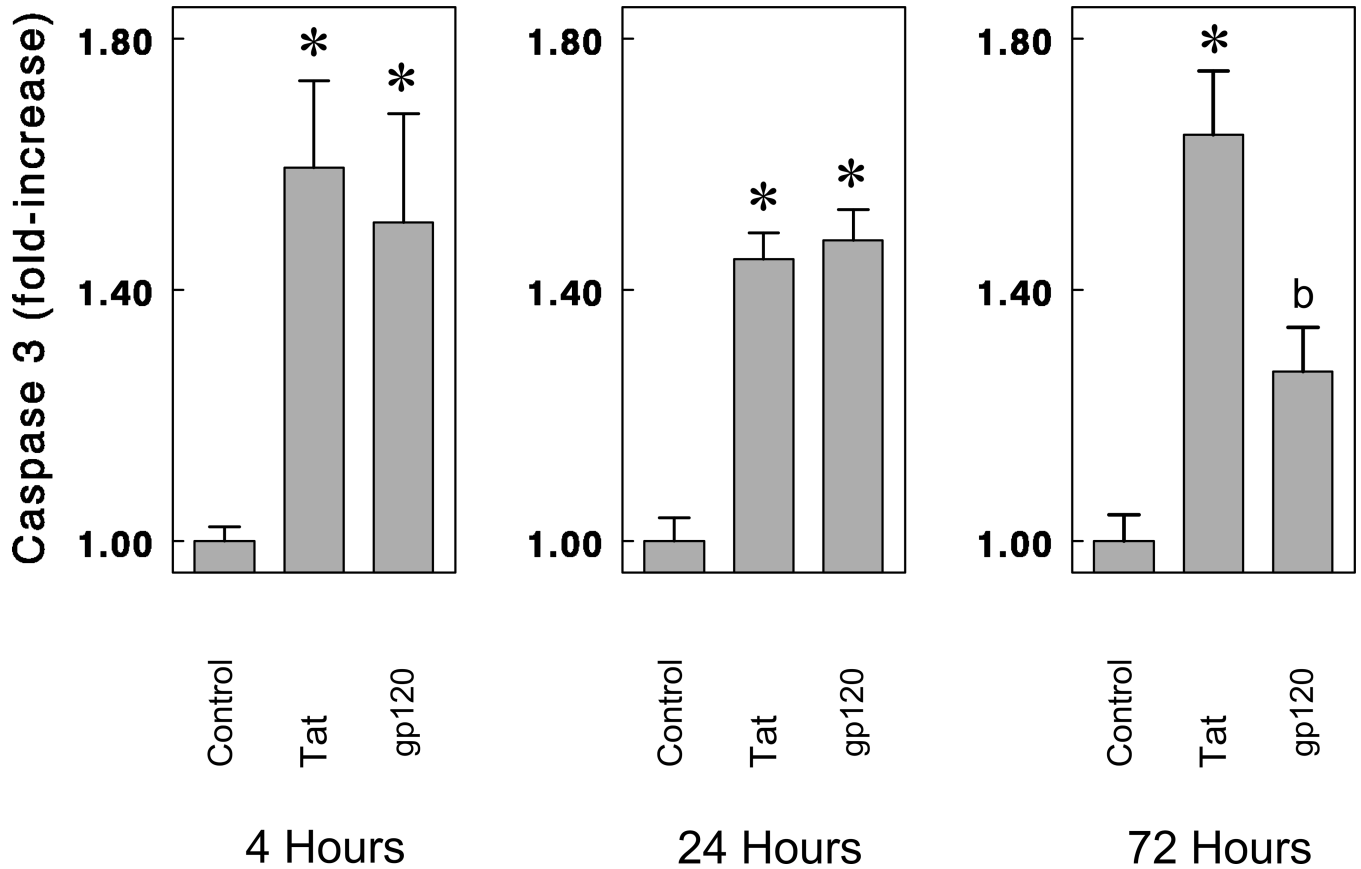
Figure 5.
Effects of Tat$_{1-72}$ and gp120 on caspase-3 activation in striatal neurons at 4 h, 24 h, and 72 h following continuous exposure *in vitro*. Both Tat (100 nM) and gp120 (500 pM) significantly increased caspase-3 activity at 4 h, 24 h, and 72 h. Caspase-3 activity was expressed as fluorescence units per μg of cytosolic protein and averaged from triplicate determinations each from at least n = 4 separate experiments. Mean caspase-3 activity in vehicle-treated (control) cultures was 69.71 units/μg protein (*P < 0.015 versus vehicle-treated cultures; ANOVA, *post hoc* Duncan's test; [b]P< 0.05 versus vehicle-treated or Tat-treated cultures; ANOVA, *post hoc* Duncan's test).
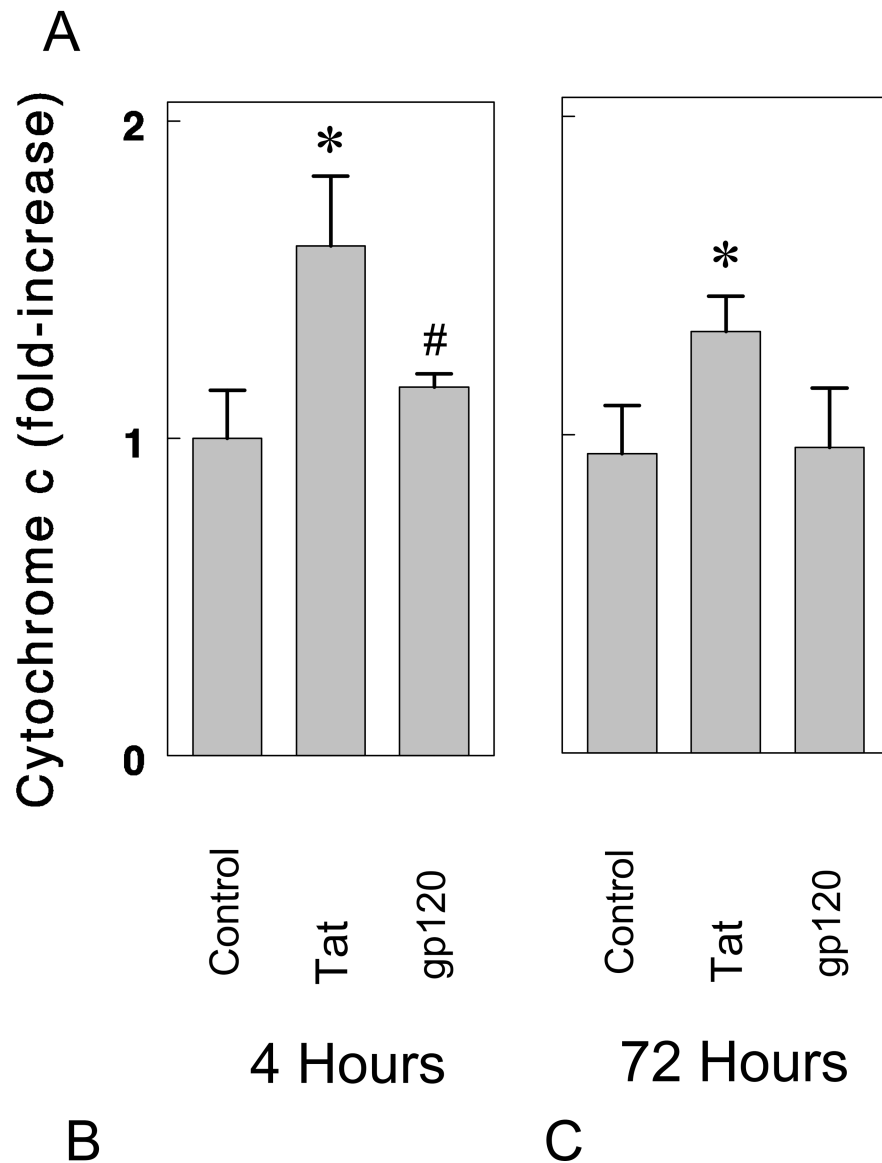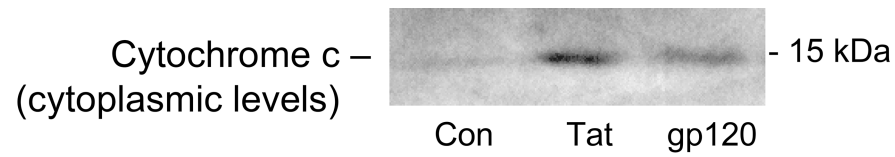
Figure 6.
HIV-1 Tat$_{1-72}$ increases cytoplasmic levels of cytochrome c in striatal neurons *in vitro*. Tat$_{1-72}$ (100 nM), but not gp120 (500 pM), significantly increased levels of cytochrome c in the cytoplasm at 4 (A & B) and 72 hours (C) (*$P < 0.05$ versus vehicle-treated control (Con) cultures; #$P < 0.05$ versus Tat-treated neurons; n = 4 experiments).
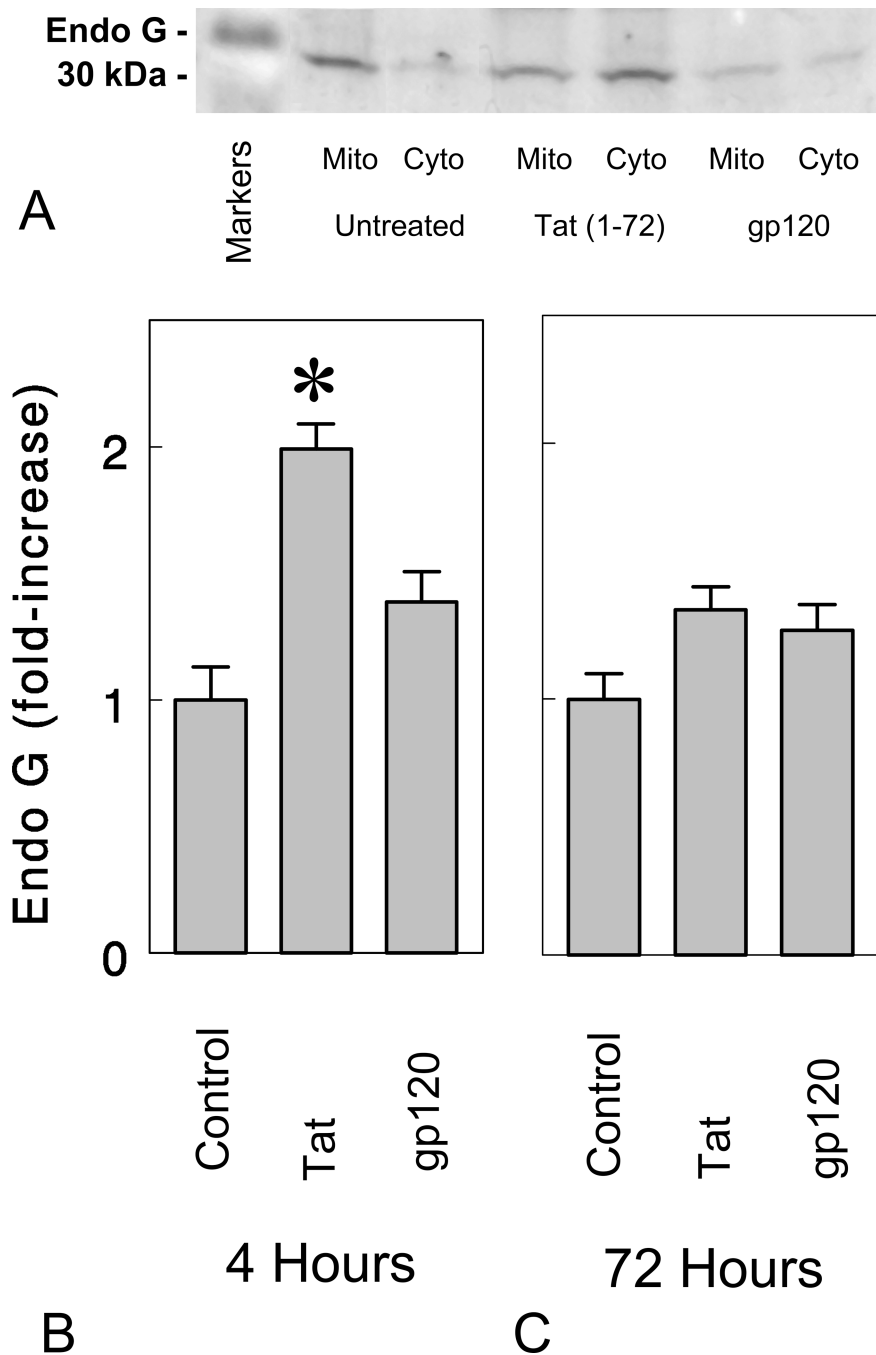
Figure 7.
Effects of Tat and gp120 on endonuclease G (endo G) in the cytoplasm at 4 and 72 h following continuous treatment. HIV-1 Tat_{1-72} (100 nM) increased cytoplasmic levels of in striatal neurons at 4 hours *in vitro* (*$P < 0.05$ versus vehicle-treated control cultures), while gp120 (500 pM) failed to increase endo G levels in the cytoplasm (Cyto) at 4 h or 72 h. Although cytoplasmic changes in endo G are shown (B,C), mitochondrial (Mito) levels were

also assessed and varied inversely to cytoplasmic levels in control and Tat-treated neurons as expected (A). The results show the mean ± SEM of n = 4 experiments.

TABLE 1

Effects of Tat_{1-72} or gp120 and caspase inhibition on striatal neuron viability at 24 h following viral protein exposure. gp120 treatment caused significant neuronal losses at 24 h that could be significantly attenuated by pretreating cultures with the caspase inhibitor, Z-DEVD-FMK (DEVD)[a]

Treatment	Non-viable neurons (%)[b]
Vehicle-treated controls	0.84 ± .37
DEVD (50 μM)	0.67 ± .41
HIV-1 Tat_{1-72} (100nM)	1.00 ± .38
HIV-1 Tat_{1-72} (100 nM) + DEVD (50 μM)	1.17 ± .24
HIV-1 gp120 (500 pM)	1.71 ±.31[*]
HIV-1 gp120 (500 pM) + DEVD (50 μM)	0.60 ±.33

[a]Striatal neurons were continuously exposed to HIV-1 proteins and/or the soluble caspase inhibitor Z-DEVD-FMK (DEVD) and assayed at 24 h *in vitro*.

[b]Neuronal viability was assessed by determining the proportion of neuronal nuclear (NeuN) immunoreactive neurons that failed to exclude ethidium monoazide (EMA) following 30 min incubation in EMA (see Fig. 3); percentage non-viable neurons = [(EMA and NeuN-positive neurons)/(total NeuN-positive neurons) * 100].

[*]$P < 0.05$ versus vehicle-treated control or gp120-treated cultures.