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Cocaine and human immunodeficiency virus type 1 gp120 mediate neurotoxicity through overlapping signaling pathways

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Although it has been well documented that drugs of abuse such as cocaine cause enhanced progression of human immunodeficiency virus (HIV)-associated neuropathological disorders, the underlying mechanisms mediating these effects remain poorly understood. The present study demonstrated that exposure of rat primary neurons to both cocaine and gp120 resulted in increased cell toxicity compared to cells treated with either factor alone. The combinatorial toxicity of cocaine and gp120 was accompanied by an increase in both caspase-3 activity and expression of the proapoptotic protein Bax. Furthermore, increased neurotoxicity in the presence of both the agents was associated with a concomitant increase in the production of intracellular reactive oxygen species and loss of mitochondrial membrane potential. Increased neurotoxicity mediated by cocaine and gp120 was ameliorated by NADPH oxidase inhibitor apocynin, thus underscoring the role of oxidative stress in this cooperation. Signaling pathways including c-jun N-teminal kinase (JNK), p38, extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinases (MAPK), and nuclear factor (NF)-κB were also identified to be critical in the neurotoxicity induced by cocaine and gp120. These findings thus underscore the role of oxidative stress, mitochondrial and MAPK signal pathways in cocaine and HIV gp120–mediated neurotoxicity. *Journal of NeuroVirology* (2009) **15,** 164–175.

Keywords: gp120; cocaine; HIV-1–associated neurological disorders; neurotoxicity; primary neurons

Introduction

Mounting evidence suggests that drugs of abuse accelerate the incidence and progression of human immunodeficiency virus (HIV)-1–associated neurological disorders (HANDs) (Aksenov *et al*, 2006; Gurwell *et al*, 2001; Turchan *et al*, 2001). Drug-abusing HIV-1–positive individuals exhibit more severe cognitive impairment compared with the non–drug-abusing HIV-positive counterparts. Epidemiological studies on abused drug users and acquired immunodeficiency syndrome (AIDS) link abuse of cocaine (by different routes), even more than other drugs, to increased incidence of HIV seroprevalence and progression to AIDS (Anthony *et al*, 1991; Baldwin *et al*, 1998; Doherty *et al*, 2000). Cocaine targets the central nervous system (CNS) and is associated with brain dysfunction. Despite the recognized impact of the abuse of psychostimulants on the clinical course of HIV-1–associated brain pathology (Aksenov *et al*, 2006), mechanisms underlying the ability of cocaine to enhance the pathological effects of HIV-1 in the brain remain elusive.

Development of HAND is a consequence of complications associated with HIV-1 infection, culminating from a cascade of virus-host interactions, leading to cytokine and chemokine imbalance, monocytic infiltration, formation of microglial nodules, highly productive infection in the brain

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macrophage/microglia, and neuronal dysfunction and death (Kaul *et al*, 2001; Nath *et al*, 2001). The pathogenesis of HIV encephalopathy revolves around two processes characterized first, by productive replication of the virus in macrophages in the brain, a process that leads to encephalitis, and second, neuronal degeneration that results from byproducts of the infected macrophages, leading to dementia. There is no conclusive evidence of direct infection of neurons by HIV-1, and neuronal death is considered to be a consequence of the toxic effects of viral and cellular toxins that are released from virus-infected and/or activated cells (Dreyer *et al*, 1990; Patel *et al*, 2000). One of the potent and well-characterized HIV-1 toxins implicated in neuronal death is the virus envelope protein gp120, which has been shown to elicit neurotoxicity in rat primary hippocampal neurons (Bansal *et al*, 2000; Meucci and Miller, 1996). Gp120 binding to CXCR4 has been shown to activate proapoptotic signal transduction cascades, including caspase-3 (Biard-Piechaczyk *et al*, 2000; Hesselgesser *et al*, 1998).

Use of cocaine either by snorting, smoking, or by intravenous injection has been known to promote disease progression, including acquisition of secondary opportunistic infections in HIV-1–infected individuals (Fiala *et al*, 1998; Goodkin *et al*, 1998). Mechanism(s) of cocaine-mediated enhancement of HIV disease pathogenesis include its multiple immunomodulatory effects and its ability to cooperate with viral toxins to exacerbate neurotoxicity. For example, cocaine exposure has been shown to aggravate neurotoxic effects of HIV-1 transactivating protein Tat and gp120, thus mediating accelerated neuronal apoptosis (Aksenov *et al*, 2006; Turchan *et al*, 2001). Molecular pathways involved in the combinatorial toxicity of cocaine and Tat on rat dopaminergic neurons include oxidative stress and mitochondrial membrane potential alterations (Aksenov *et al*, 2006). Detailed molecular mechanisms underlying the interaction between cocaine and gp120, however, remain elusive.

In the present study, we hypothesized that cocaine-mediated potentiation of gp120 neurotoxicity will involve converging signaling pathways that will result in amplification of neurotoxic responses. We thus sought to dissect the molecular mechanisms underlying the neurotoxicity induced by cocaine and gp120 with emphasis on c-jun N-teminal kinase (JNK), p38, and extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) pathways. A thorough understanding of how the drugs of abuse and HIV-1 protein(s) cooperate to enhance neurodegeneration can have beneficial implications for development of therapeutic interventions for HIV-infected cocaine abusers.

Results

Cocaine enhanced gp120 toxicity in rat primary neurons

To assess the toxic effects of cocaine and gp120, rat primary neurons were exposed to 1 μM cocaine with or without gp120 (200 ng/ml) for 24 h followed by assessment of cell viability by MTT assay. The concentrations of gp120 and cocaine used in this study were based on previously published reports (Dhillon *et al*, 2007; Peng *et al*, 2008). As shown in Figure 1A, cocaine alone decreased cell viability by 19.9%. Gp120 alone, as expected, decreased cell viability by 14.7%, and in conjunction with cocaine caused statistically significant decrease in cell viability by 36.4%.

The above findings were further confirmed by staining cells with Hoechst 33342, a nuclear dye that stains fragmented nuclei. As shown in Figure 1C, untreated neurons exhibited regular and round-shaped nuclei. In contrast and as expected, condensation and fragmentation of nuclei, characteristic of apoptotic cells, was clearly evident in neurons treated with gp120 or cocaine alone for 24 h. Cotreatment of neurons with gp120 and cocaine caused a dramatic increase in cell death to about 37.6% (Figure 1B and C).

Cocaine and gp120–mediated neurotoxicity: role of caspase-3 activation

To corroborate the findings that cocaine and gp120–mediated neurotoxicity involved the apoptotic pathway, we next sought to investigate activation of caspase-3 in lysates of cells treated with gp120 and/or cocaine using the colorimetric assay. As shown in Figure 2A, neurons treated with either gp120 or cocaine demonstrated a significant increase in active caspase-3 compared with untreated control cells ($P < .05$ versus control). Treatment of neurons with a combination of gp120 and cocaine, however, further increased the activation of caspase-3 compared with cells exposed to either agent alone. Caspase-3 activation was further confirmed by immunostaining the cells treated with gp120 and cocaine. As shown in Figure 2B, cells treated with gp120/cocaine for 24 h demonstrated positive staining for activated caspase-3 and simultaneous treatment of neurons with both the agents resulted in a further enhancement of caspase-3 positivity, as quantified in Figure 2C.

Cocaine and gp120–mediated neurotoxicity: role of proapoptotic versus antiapoptotic proteins (Bax/Bcl-xLBcl-xL)

Cell homeostasis is dependent on the fine balance of the pro- and anti apoptotic genes (Bax and Bcl-xL, respectively). Ratio of the expression levels of these

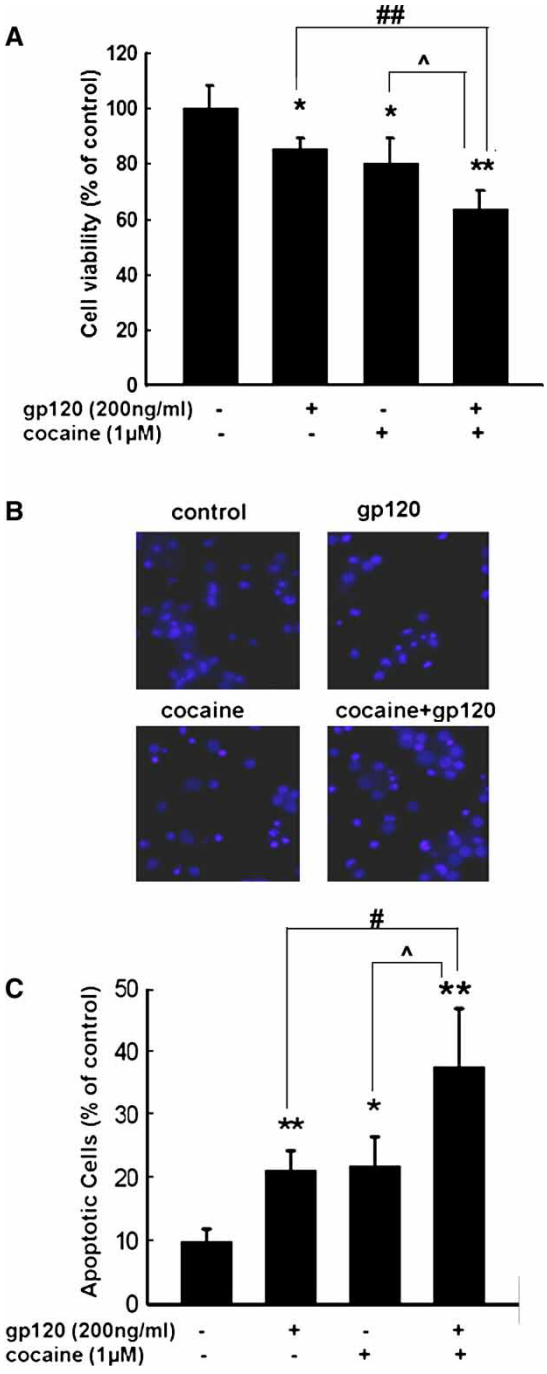

Figure 1 Effects of cocaine and gp120 on cell viability in rat neurons. (**A**) Effects 1 μM cocaine in the presence or absence of gp120 for 24 h on the cell survival of rat neurons using the MTT assay. All the data are presented as mean ± SD of four individual experiments. $^{*}P<.05$;$^{**}P<.01$ versus control group; $\#P<.05$ versus gp120 group; $\hat{}P<.05$ versus cocaine group. (**B**) Rat primary neurons treated with cocaine and/or gp120 for 24 h were stained with Hoechst 33342 by fluorescence microscopy. (**C**) Quantification of Hoechst-positive cells as percentage of control untreated cells. All the data are presented as mean ± SD of four individual experiments. $^{*}P<.05$; $^{**}P<.01$ versus control group; $\#\#P<0.01$ versus gp120 group; $\hat{}P<.05$ versus cocaine group.

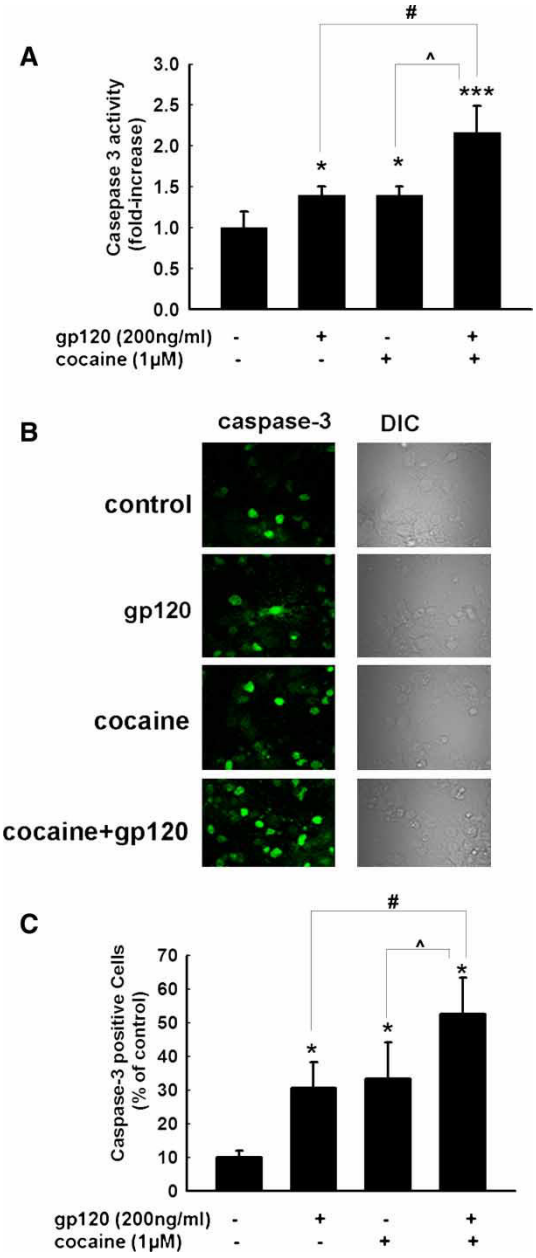

Figure 2 Effects of cocaine and gp120 exposure on caspase-3 activation in rat neurons. (**A**) Primary neurons treated with cocaine and/or gp120 for 24 h were monitored for caspase-3 activity assay in cell lysates. All data in these figures are presented as mean ± SD of four individual experiments. $^{*}P<.05$; $^{***}P<.001$ versus control group; $\#P<.05$ versus gp120 group; $\hat{}P<.05$ versus cocaine group. (**B**) Primary neurons treated with cocaine and/or gp120 for 24 h were monitored for active caspase-3 by immunostaining using anti-cleaved caspase-3 antibody. Immunocytochemical images showing active caspase-3 staining (*left panel*) and differential interference contrast (DIC; *right panel*) were captured using Nikon inverted fluorescence microscope TE02000-E. (**C**) Quantification of caspase-3–positive cells as percentage of control untreated cells using NIH ImageJ software. All the data are presented as mean ± SD of four individual experiments. $^{*}P<.05$ versus control group; $\#P<.05$ versus gp120 group; $\hat{}P<.05$ versus cocaine group.

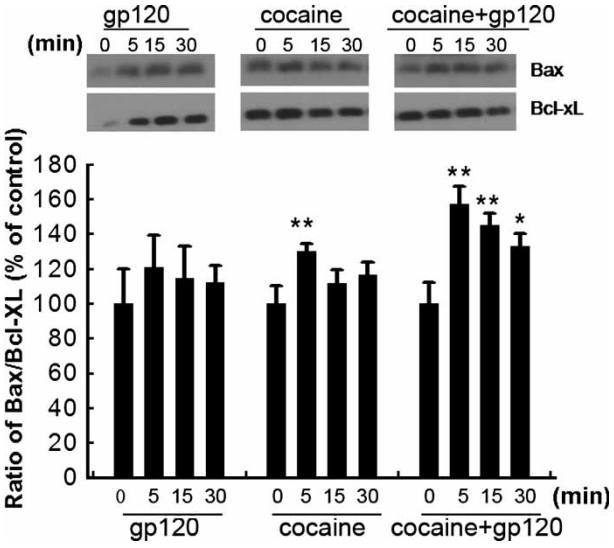

Figure 3 Cocaine enhanced gp120-mediated induction of Bax expression. Rat primary neurons were treated with gp120 and cocaine for varying times (5 to 30 min), followed by cell lysis and detection of proapoptotic (Bax) and antiapoptotic proteins (Bcl-xL) on a Western blot. Cells cotreated with both the agents demonstrated up-regulation of Bax protein, resulting in increased Bax/Bcl-xL ratio. Densitometry scan of the ratio of band intensities of Bax/Bcl-xL from three independent experiments. $*P < .05$; $**P < .01$ versus control group; $\#P < .05$ versus gp120 group; $\hat{}P < .05$ versus cocaine group.

two proteins following cell stimulation determines the cell fate; apoptosis Bax) versus survival (Bcl-xL). We therefore sought to examine the expression of these two proteins in rat primary neurons treated with cocaine and/or gp120 by Western blot analysis. As shown in Figure 3, there was an increase in Bax/Bcl-xL ratio in cells treated with either cocaine or gp120, and this ratio was further enhanced in cells exposed to both cocaine and gp120, thus implicating the amplification of the toxic apoptotic response.

Cocaine and Gp120–mediated neurotoxicity: role of intracellular reactive oxygen species (ROS)

Viral toxins, such as Tat and gp120, are known to mediate neuronal toxicity by increased oxidative stress pathways. We next sought to explore whether cocaine-mediated enhancement of gp120 neurotoxicity involved production of ROS. Briefly, rat primary neurons were treated as described above followed by measurement of intracellular ROS using the DCHF-DA oxidation assay. As shown in Figure 4A and B, treatment of neurons with cocaine or gp120 alone resulted in increased intracellular ROS production compared with untreated cells and this effect was even more augmented in the simultaneous presence of both cocaine and gp120. Because NADPH is a key player in generation of oxidative stress, we next sought to determine whether pretreatment of neurons with NADPH inhibitor apocynin could alleviate cocaine and gp120 elicited toxicity in these cells. As shown in Figure 4C, pretreatment of cells with apocynin followed by exposure of neurons to cocaine and gp120 significantly inhibited neuronal toxicity induced by gp120 and cocaine.

Cocaine and gp120–mediated neurotoxicity: alterations in mitochondrial membrane potential

ROS production plays a critical role in the mitochondrial dysfunction and subsequent cell death. Additionally, changes in mitochondrial membrane potential ($\Delta\psi_m$) have been demonstrated to play a role in HIV protein–mediated neuronal apoptosis (Corasaniti *et al*, 2005). We thus wanted to explore whether cocaine-mediated enhancement of gp120 neurotoxicity also involved alterations in

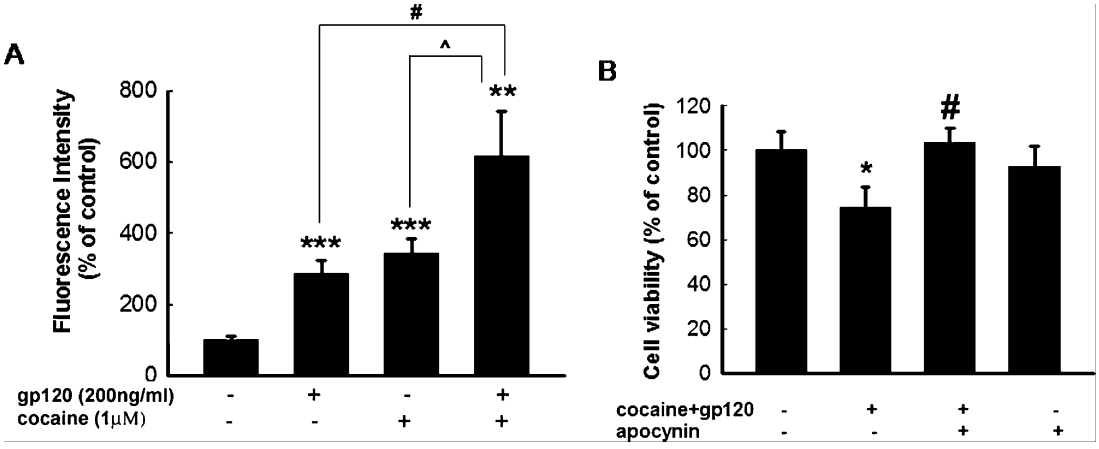

Figure 4 Effects of cocaine and/or gp120 on intracellular ROS production in rat neurons. (**A**) Cells treated with cocaine in the absence or presence of gp120 for 3 h were assessed for production of ROS using DCFH-DA assay. Quantification of ROS fluorescence intensity in different treated groups. The data are presented as mean ± SD of four individual experiments. $**P < .01$; $***P < .001$ versus control group; $\#P < .05$; versus cocaine + gp120 group. (**B**) Inhibition of the NADPH oxidase by apocynin resulted in abrogation of cocaine and gp120 neurotoxicity. The data are presented as mean ± SD of four individual experiments. $*P < .05$ versus control group; $\#P < .05$ versus cocaine + gp120 group.

mitochondrial membrane potential. Neuronal mitochondrial membrane depolarization was assessed following exposure of cells to cocaine and/or gp120 for 18 h, using the JC-1 probe, which is a fluorescent lipophilic cationic dye that accumulates in mitochondria in proportion to the $\triangle\psi_m$ that normally exists across the inner mitochondrial membrane (Cossarizza *et al*, 1993). As shown in Figure 5A and B, there was increased membrane depolarization in the neurons exposed to either gp120 or cocaine alone,

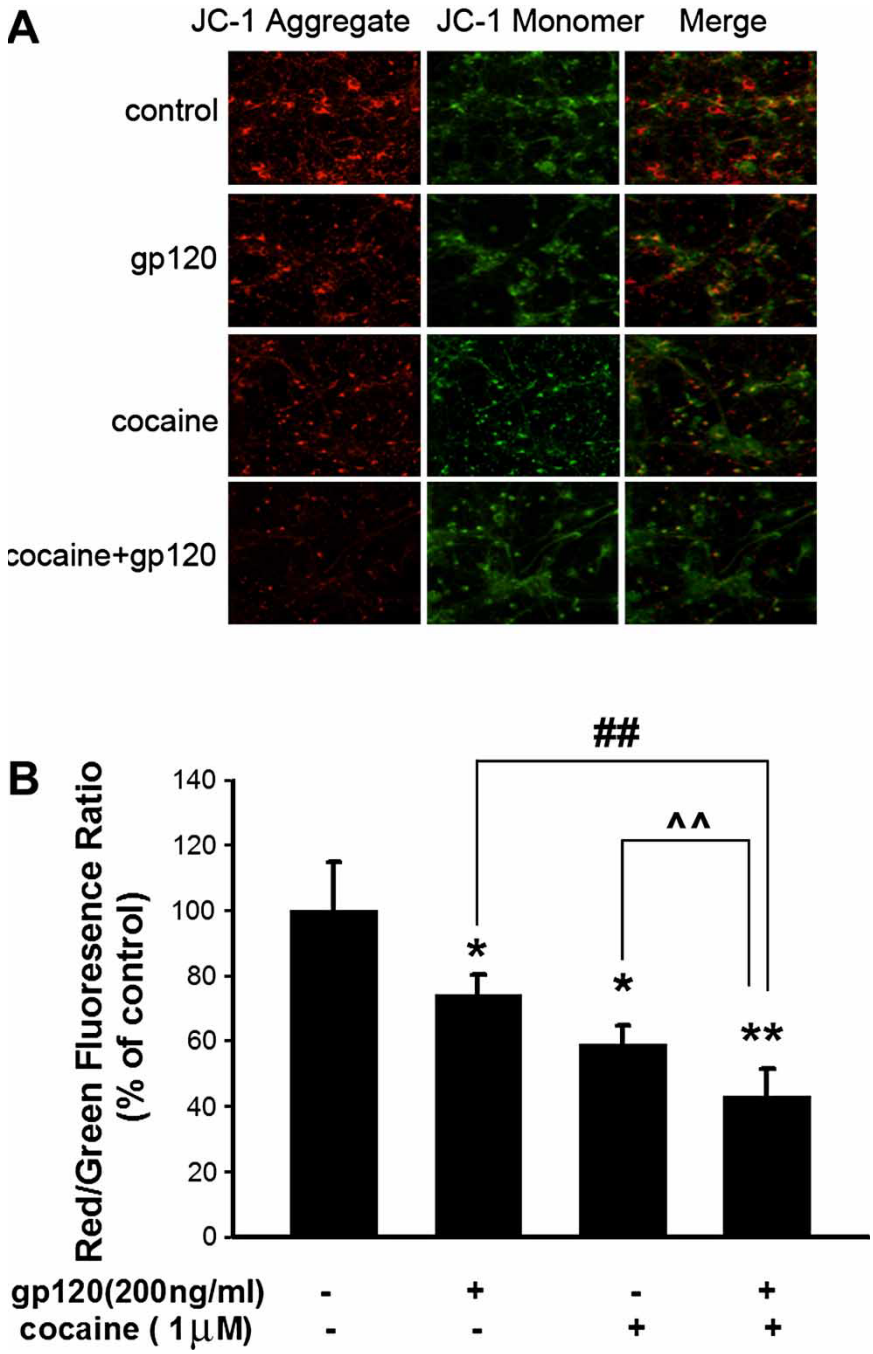

Figure 5 Effects of cocaine and/or gp120 on mitochondria membrane potential in rat neurons. (**A**) Cells treated with cocaine and/or gp120 for 18 h were assayed for mitochondrial membrane potential by staining with JC-1 dye. Cotreatment of cells with cocaine and/or gp120 exposure resulted in reduction of the aggregation of JC-1 dye in the mitochondria (red fluorescence) and decreased ratio of the aggregate (red fluorescence) to monomer JC-1 (green fluorescence) in the cells. (**B**) Quantification of $\triangle\psi$m expressed as a ratio of J-aggregate to JC-1 monomer (red:green) fluorescence intensity using fluorescence plate reader. $^{*}P<.05$; $^{**}P<.01$ versus control group; $\#\#P<0.01$ versus gp120 group; $^{\wedge\wedge}P<0.01$ versus cocaine group.

and this effect was further enhanced in the presence of both cocaine and gp120.

Cocaine and gp120–mediated neurotoxicity: role of MAPK signaling

In order to dissect the signaling pathways mediated by the combinatorial action of gp120 and cocaine, lysates from neurons treated with cocaine and gp120 were run on a Western blot and probed for the activation of signaling proteins involved in the MAPK pathway. There was a time-dependent activation of JNK, p38, and ERK1/2 in the presence of cocaine or gp120 alone. In the presence of both cocaine and gp120, there was time-dependent and a cooperative activation of these kinases, as shown in Figures 6A, 7A, and 8A.

The specificity of these MAPKs in the enhancement of neurotoxicity was further confirmed by pretreating the cells with pharmacological inhibitors specific for the respective signaling pathways. As shown in Figures 6B, 7B, and 8B, pretreatment of cells with p38 inhibitor (SB203580), JNK inhibitor (SP600125), and MEK1/2 inhibitor (U0126) resulted in amelioration of cocaine and gp120–induced neurotoxicity, further confirming the role of these pathways in the enhanced neurotoxicity induced by gp120 and cocaine. Interestingly, pretreatment of cells with the NADPH inhibitor apocynin also

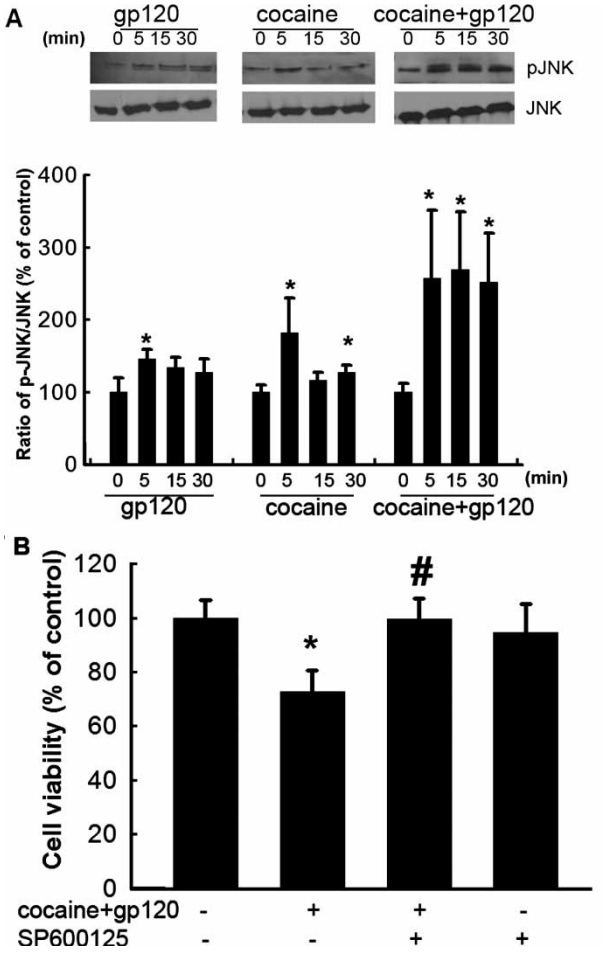

Figure 6 The JNK/MAPK pathway mediated cocaine- and/or gp120-induced neurotoxicity in rat primary neurons. (**A**) Western blot analysis of cytosolic lysates from cocaine and gp120–treated cells for varying times (5 to 30 min) using antibodies specific for the phosphorylated forms of JNK. An antibody against total JNK was used to reprobe the blots for normalization. Densitometry scan of the ratio of band intensities of pJNK/JNK from three different experiments. $*P < .05$ versus control group. (**B**) Inhibition of the JNK pathway by SP600125 resulted in abrogation of cocaine and gp120 neurotoxicity. Values are mean ± SD from three independent experiments. $*P < .05$ versus control group; $\#P < .05$ versus cocaine + gp120 group.

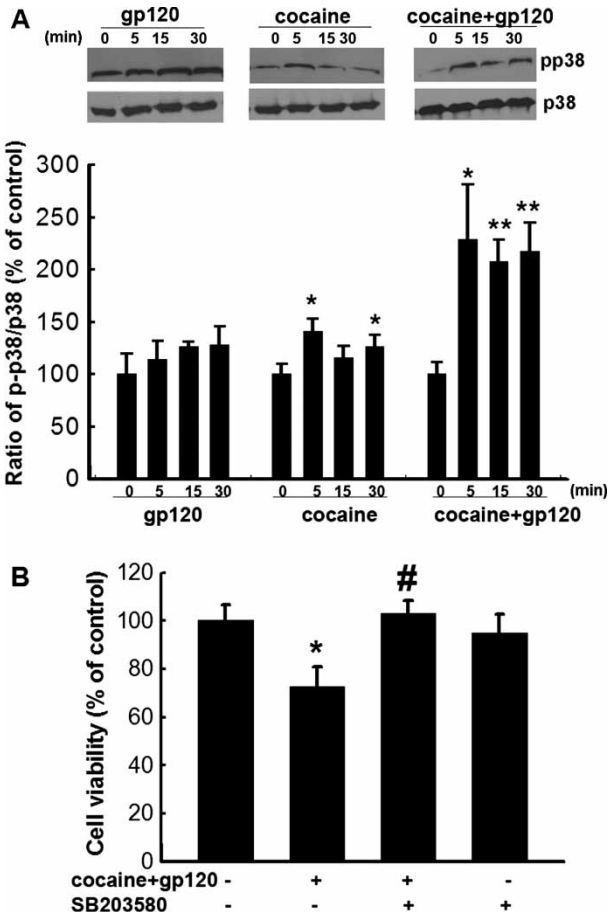

Figure 7 The p38/MAPK pathway mediated cocaine- and/or gp120-mediated neurotoxicity. (**A**) Western blot analysis of cytosolic lysates from cocaine and gp120–treated cells for varying times (5 to 30 min) using antibodies specific for the phosphorylated forms of p38. An antibody against total p38 was used to reprobe the blots for normalization. Densitometric scan of the ratio of band intensities of p38/p38 from three different experiments. $*P < .05$; $**P < .01$ versus control group. (**B**) Inhibition of the pp38/MAPK pathway by SB203580 resulted in abrogation of cocaine and gp120 neurotoxicity. Values are mean ± SD from three independent experiments. $*P < .05$ versus control group; $\#P < .05$ versus cocaine + gp120 group.

resulted in the inhibition of JNK, p38, and ERK activation (data not shown), further corroborating the role of ROS in mediating activation of these respective signaling pathways.

Cocaine and gp120–mediated neurotoxicity: role of NF-κB

Activation of the MAPK cascade has been shown to result in translocation of the downstream transcription factor nuclear factor kappa B (NF-κB) (Kumar *et al*, 1999). It was therefore of interest to next examine whether simultaneous treatment of neurons with cocaine and gp120 could result in enhanced nuclear translocation of NF-κB. Nuclear extracts isolated from rat primary neurons treated with gp120 and cocaine were examined for the translocation of the p65 subunit of NF-κB using Western blot analysis. As shown in Figure 9A, treatment of neurons with both the agents induced time-dependent nuclear translocation of NF-κB p65, an effect that was evident as early as 5 min post treatment and that was sustained for at least 60 min.

Further validation of these findings was done by immunocytochemical analysis. As shown in the Figure 9B, there was a dramatic translocation of NF-κB p65 in the nucleus of cocaine and gp120–treated cells versus the untreated control cells. To further confirm the role of NF-κB in the neurotoxicity induced by gp120 and cocaine, neuronal cultures were pretreated with l-1-tosyllamide-2-phenylethyl chloromethyl ketone (TPCK), an inhibitor of NF-κB,

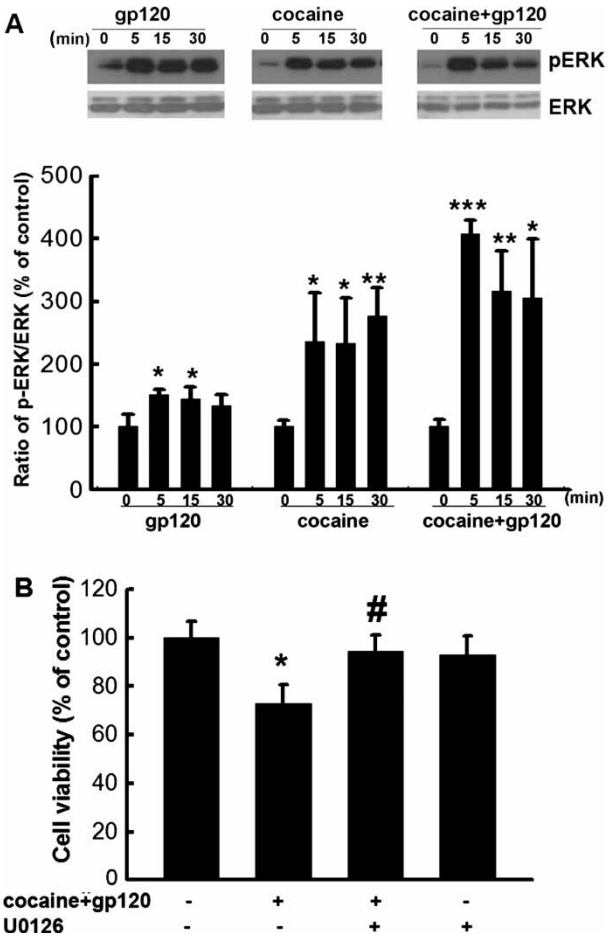

Figure 8 The ERK/MAPK pathway mediated cocaine- and/or gp120-induced neurotoxicity. (**A**) Western Blot analysis of cytosolic lysates from cocaine and gp120–treated cells for varying times (5 to 30 min) using antibodies specific for the phosphorylated forms of ERK. An antibody against total ERK was used to reprobe the blots for normalization. Densitometry scan of the ratio of band intensities of pERK/ERK from three independent experiments. $*P<.05$; $**P<.01$; $***P<.001$ versus control group. (**B**) Inhibition of the ERK pathway by U0126 resulted in abrogation of cocaine and gp120 neurotoxicity. Values are mean ± SD from three independent experiments. $*P<.05$ versus control group; $\#P<.05$ versus cocaine + gp120 group.

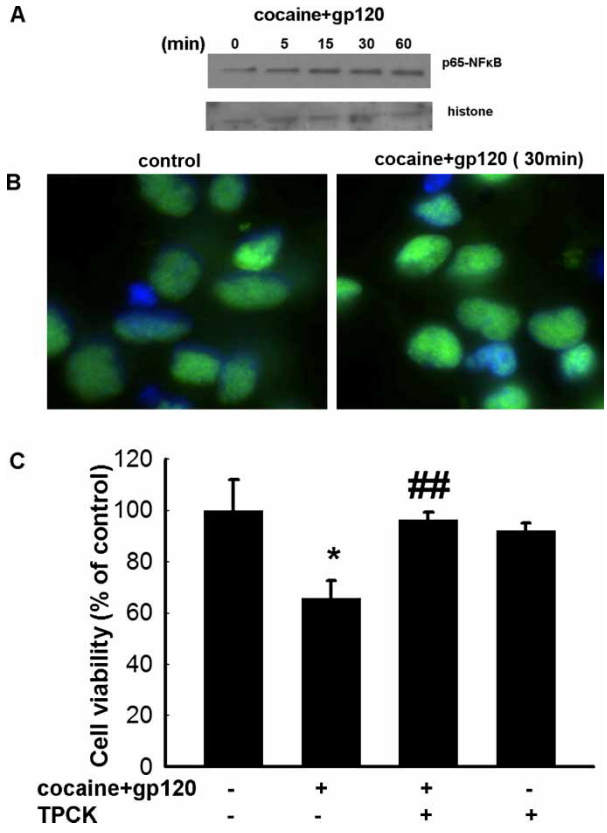

Figure 9 NF-κB plays a role in the cocaine and gp120–induced neurotoxicity. (**A**) Western Blot analysis of nuclear extracts from cocaine and gp120–treated cells for varying times (5 to 60 min) using an antibody specific for the p65 subunit of NF-κB. (**B**) Rat primary neurons grown on coverslips were treated with cocaine and gp120 for 30 min and stained with an anti-NF-κB p65 antibody, followed by treatment with an Alexa Flour 488–conjugated secondary antibody. Slides were mounted in Slow Fade antifade reagent (with DAPI, blue nuclear stain) and images were captured by confocal microscopy (magnification × 250). After 30 min, nuclear translocation of NF-κB was clearly evident in the treated cells, as show by the green (NF-κB) and blue (DAPI) stains overlapping. (**C**) Inhibition of the NF-κB using the specific inhibitor TPCK resulted in abrogation of cocaine and gp120 neurotoxicity. Values are mean ± SD from three independent experiments. $*P<.05$ versus control group; $\#\#P<.01$ versus cocaine + gp120 group.

followed by treatment of cells with both the agents. Neurotoxicity induced by cocaine and gp120 was significantly abrogated in the presence of the NF-κB p65 inhibitor TPCK (Figure 9C), thus confirming the involvement of NF-κB activation in mediating cocaine and gp120 neurotoxicity.

Discussion

Although there is ample evidence suggesting that drugs of abuse such as cocaine can cooperate with HIV-1 gp120 to induce neurotoxicity (Turchan *et al*, 2001), the mechanisms involved in this process remain elusive. The present study demonstrated that cocaine in cooperation with gp120 exacerbated toxicity in rat neuronal cultures via the apoptotic pathway involving intracellular ROS production, mitochondrial membrane potential loss, and activation of the MAPK and NF-κB signaling pathways.

Neuronal apoptosis, a consequence of neuronal dysfunction, is one of the features of HIV-associated dementia that can be induced by a variety of cellular and viral factors, including viral proteins Tat and gp120 (Bansal *et al*, 2000). Additionally, drugs of abuse can also cooperate with the viral proteins to further exacerbate neurotoxicity. In concordance with the previously reported findings by Turhcan *et al* (2001), we also demonstrated amplification of gp120 toxicity in rat neuronal cell cultures cotreated with cocaine. Furthermore, in line with findings by Nassogne *et al* (1997) demonstrating that cocaine induces apoptosis in cortical neurons of fetal mice, we also observed toxicity of rat neurons with cocaine treatment.

Apoptosis or programmed cell death is a consequence of concerted activation of proteolytic cascade involving a family of proteases such as caspase-3. To dissect the apoptotic pathway involved in cocaine and gp120–mediated neurotoxicity, activation of caspase-3 was monitored in treated cells using both the colorimetric and immunostaining assays. Indeed there was an increased activation of capase-3 in cells treated with both the agents compared with cells treated with either agent alone. Our results are consistent with the previously published report in embryonic locus coeruleus neurons demonstrating that cocaine-induced activation of caspase-3 plays a critical role in signaling pathways leading to apoptosis (Dey and Snow, 2007). These findings were further confirmed by assessing the relative levels of the anti- (Bcl-xLBcl-xL) and pro- (Bax) apoptotic gene products. Treatment of neurons with both gp120 and cocaine resulted in increased apoptosis as evidenced by increased pro-apoptotic to anti-apoptotic ratio (Bax/Bcl-xL), further validating the combined neurotoxicity induced by gp120 and cocaine.

Oxidative stress has been implicated in the pathogenesis of various neurodegenerative diseases and is critical for manifestation of apoptotic responses (Suh *et al*, 2007). In the present study cotreatment of rat primary neurons with both cocaine and gp120 enhanced ROS production as compared to neurons treated with either agent alone. ROS can be generated from several sources such as NADPH oxidase, a superoxide-generating enzyme long recognized to be a key player of microglial activation (Gao *et al*, 2003; Qin *et al*, 2004; Zhang *et al*, 2004). Recent studies have also identified a critical role for NADPH oxidase in neurons, where it may be involved in the normal physiological redox signaling response (Serrano *et al*, 2003; Tejada-Simon *et al*, 2005; Vallet *et al*, 2005). Activation of NADPH oxidase in neurons can contribute to cell death under stress stimuli. In the present study, NADPH inhibitor apocynin significantly abrogated neurotoxicity induced by cocaine and gp120, thus underscoring the role of NADPH-ROS pathway in neuronal toxicity.

Work by various groups has suggested the mitochondria as a link between the initial apoptotic signal and the end point biochemical reactions leading to cell death (Kluck *et al*, 1997; Yang *et al*, 1997). Response to death stimuli, mitochondrial membranes are permeabilized, ultimately resulting in activation of caspases (Kroemer, 1999). In the present study, cotreatment of primary neurons with both cocaine and gp120 resulted in a significant decrease in mitochondrial membrane potential compared with cells treated with either agent alone. These findings are consistent with a previous report indicating that cocaine potentiated Tat-induced alterations in mitochondrial membrane potential (Aksenov *et al*, 2006).

Having determined the role of oxidative stress pathway in the combined neurotoxic response mediated by cocaine and gp120, we next sought dissect the downstream signaling pathways critical for this effect. MAPK signaling pathways including JNK, p38, and ERK modulate diverse cellular events, such as cell death and survival (Jiao *et al*, 2007). For example, it has been well documented that activation of JNK and p38 plays a role in cell death (Xia *et al*, 1995), whereas ERK signaling is critical for neuronal survival (Jia *et al*, 2007). In the present study, treatment of neurons with gp120 alone led to a rapid and transient phosphorylation of JNK and p38 and cotreatment of neurons with both cocaine and gp120 resulted in further activation of these key signaling mediators. These findings were consistent with the previous report that cocaine induced activation of JNK and p38 in fetal locus coeruleus neurons (Dey and Snow, 2007).

The extracellular signal–regulated kinases (ERK1 and ERK2, abbreviated ERK1/2) are prosurvival factors in the (MAPK family and contribute to the regulation of proliferation and differentiation (Johnson and Lapadat, 2002; Wada and Penninger, 2004). A recent study by Subramaniam and colleagues (Subramaniam *et al*, 2004), however, suggests that

ERK1/2 also has a key proapoptotic role in neuronal apoptosis. In our study also we observed that similar to the JNK/p38 pathway, there was a time-dependent activation of the ERK/MAPK pathway in the presence of both cocaine and gp120, an effect that was inhibited by the ERK/MAPK inhibitor U0126. Additionally, inhibition of the JNK, p38, and ERK/MAPK activation in neurons pretreated with the NADPH inhibitor further lends credence to the findings suggesting oxidative stress-mediated regulation of ERK phosphorylation (Kishida *et al*, 2005).

Downstream of the MAPK pathway is the transcription factor NF-κB, which translocates into the nucleus upon activation to initiate transcription of the various target genes (Dent *et al*, 2003). Our findings clearly demonstrated enhanced nuclear translocation of NF-κB in cells cotreated with cocaine and gp120. These findings are consistent with the previous reports implicating the role of NF-κB in the long-term adaptation of nucleus accumbens neurons to cocaine (Ang *et al*, 2001). Further corroboration of these findings has also been reported in PC12 cells, wherein cocaine exposure resulted in significant induction of the pivotal transcription factors such as c-fos, c-jun, SP-1, and NF-κB (Imam *et al*, 2005). Modulation of signaling pathways by cocaine and gp120 has also been convincingly demonstrated in various other published studies (Hargrave *et al*, 2003; Lee *et al*, 2001).

Taken together, these findings suggest that cocaine or gp120 alone are able to elicit similar signaling transduction pathways in neurons, involving oxidative stress, mitochondrial membrane potential loss, activation of JNK, p38, and ERK/MAPK pathways, and resulting downstream activation of NF-κB (Figure 10). Simultaneous treatment of neurons with both the agents leads to an even further activation response analogous to a ''perfect storm'' scenario in the cells. These responses lead to enhanced neurotoxicity and have serious implications in the exacerbation of HIV-associated CNS disease progression in cocaine-abusing HIV-1–infected patients. Defining the molecular pathways in response to drugs of abuse and viral toxins can help pave the way for designing therapeutic strategies aimed at treatment of HAND in drug-abusing population.

Materials and methods

Materials

Viral gp120 (IIIB strain) was obtained from the AIDS Research and Reference Reagent Program of National Institutes of Health. The specific MAPK/ERK kinase (MEK) 1/2 inhibitor U0126, p38 inhibitor SB203580, and JNK inhibitor SP600125 were purchased from Calbiochem (San Diego, CA). Nuclear factor (NF)-κB inhibitor l-1-tosyllamide-2-phenylethyl chloromethyl ketone (TPCK) was purchased from Sigma Chemicals (St. Louis, MO).

Animals and primary neuron culture

All animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Kansas Medical Center. For isolation of neurons, cortices and striatum were dissected from brains of embryonic day 18–19 Sprague-Dawley rats (Charles River Laboratories). Briefly, dissociated cells were seeded and maintained in Dulbecco's modified Eagle's medium/F12 supplemented with 10% fetal bovine serum and 1% antibiotics for 24 h, following which the cultures were supplemented with serum-free neurobasal medium containing B27 (50:1), 2 mM glutamax, and 1% antibiotic. After 7 days of culturing, the initial plating cultures consisted of 97%

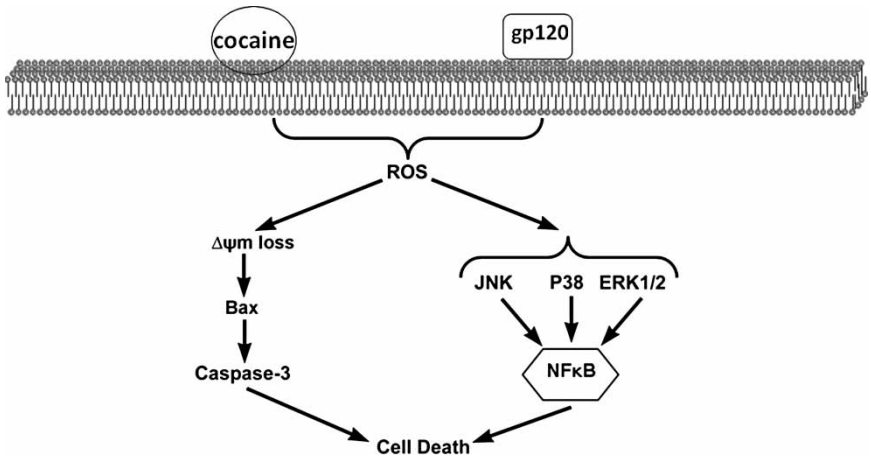

Figure 10 Schematic illustration of signaling pathways involved in the neurotoxicity mediated by cocaine and gp120 in rat primary neurons. Treatment of primary neurons with cocaine and gp120 results in increased intracellular ROS production, mitochondrial membrane potential loss, increased proapoptotic protein (Bax) expression, and increased caspase-3 activation. Other signaling pathways activated by both gp120 and cocaine involve the JNK, p38, and ERK/MAPK pathways converging in the activation of NF-κB and ultimately culminating into neuronal death.

neuron-specific nuclear protein–immunoreactive neurons as described earlier (Yao *et al*, 2005).

MTT assay
Cell viability was measured by 3-(4,5-dmethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. Briefly, cells were collected and seeded in 96-well plates at a density of 10^5 cells/cm^2. Different seeding densities were optimized at the beginning of the experiments. Rat primary neurons were exposed to fresh medium containing various concentrations of cocaine (0.5, 1, 10 μM) with or without 200 ng/ml gp120. After incubation for up to 24 h, 20 μl MTT tetrazolium salt dissolved in Hank's balanced salt solution at a final concentration of 5 mg/ml was added to each well and incubated in the CO_2 incubator for 4 h. Finally, the medium was aspirated from each well and 200 μl of dimethyl sulfoxide was added to dissolve the formazan crystals and the absorbance of each well was obtained using a Dynatech MR5000 plate counter at test and reference wavelengths of 570 and 630 nm, respectively.

Hoechst staining
Rat primary neurons were treated with gp120 and/or cocaine and fixed followed by staining with Hoechst 33324 (Sigma). The morphological features of apoptosis (cell shrinkage, chromatin condensation, and fragmentation) were monitored by fluorescence microscopy (Olympus BX 60; Tokyo, Japan). At least 400 cells from 12 randomly selected fields per dish were counted, and each treatment was performed in triplicate.

Caspase-3 activity assay
Activity of caspase 3 was analyzed using the Caspase 3 Colorimetric Assay Kit Systems (R&D Systems, Minneapolis, MN) following the manufacturer's instructions. Briefly, rat primary neurons were plated at a density of 2×10^6 cells per well in 6-well plates. Following treatment, cells were collected and lysed with 50 μl lysis buffer for 10 min on ice. The lysate was centrifuged at $200 \times g$ for 5 min and was incubated with 50 μl of 2 × reaction buffer containing 0.5 μl dithiothreitol (DTT) and 5 μl of the caspase-3 colorimetric substrate, DEVD-pNA. Two hour post incubation at 37°C, caspase-3 protease activity was measured in a spectrophotometer at a wavelength of 405 nm. Absorbance was normalized to the protein concentration of each lysate, which was determined using the BCA Protein Assay Reagent (Pierce, Rockford, IL). The changes in caspase-3 activity in treated cells were presented relative to the values obtained from the untreated samples. Each experiment consisted of three replicates and was repeated at least three times.

Immunocytochemistry
Cells were fixed in 4% paraformaldehyde, followed by blocking with phosphate-buffered saline (PBS) containing 10% bovine serum albumin. After blocking, cells were incubated at 4°C overnight with the anti-cleaved caspapse-3 antibody (Cell signaling, Danvers, MA). Following washes, cells were then incubated with the secondary goat anti-rabbit Alexa Fluor 488–conjugated antibody (1:500). For negative controls, cells were treated as described above, except that the primary antibody treatment was omitted.

Reactive oxygen species (ROS) assay
Intracellular production of ROS was measured by 2',7'-dichlorfluorescein diacetate (DCFH-DA) oxidation. Primary neurons were treated with gp120 and/or cocaine for 3 h and then incubated with DCFH-DA (Sigma) at 20 μM for 30 min. After incubation, cells were washed with PBS and the fluorescence was visualized immediately at wavelengths of 485 nm for excitation and 530 nm for emission by a Nikon Optical TE2000-S inverted fluorescence microscope. Total green fluorescence intensities in every well were quantitated using NIH Image J software.

Analysis of mitochondrial membrane depolarization
The change in mitochondrial membrane potential in the neurons was monitored using the mitochondrial membrane potential detection kit (Cell Technology, Mountain View, CA) according to the manufacturer's instructions. Briefly, rat primary cultured cortex neurons cultured in either 24-well plate (1×10^5 cells per well) or 96-well plate (3×10^4 cells per well) were treated with gp120 and/or cocaine followed by treatment with 1 × JC-1 reagent diluted in serum-free culture medium for 20 min at 37°C in 5% CO_2. Thereafter, cells were rinsed once in 1 × rinsing buffer provided in the kit. Uptake of the dye was then assessed using the Nikon inverted fluorescence microscope TE2000-E (Nikon, Tokyo, JAPAN). Fluorescence was also measured using the FL600 fluorescent plate reader (Bio-Tek Instruments, Winooski, VT) at the excitation wavelengths of 485 nm and 535 nm.

Western blotting
Treated cells were lysed using the Mammalian Cell Lysis kit (Sigma) and the NE-PER Nuclear and Cytoplasmic Extraction kit (Pierce). Equal amounts of the corresponding proteins were electrophoresed in a sodium dodecyl sulfate–polyacrylamide gel (12%) in reducing conditions followed by transfer to PVDF membranes. Blots were blocked with 5% nonfat dry milk in phosphate-buffered saline and probed with antibodies recognizing the phosphorylated forms of JNK, p38, ERK1/2, Akt (Cell Signaling; 1:200), NF-κB p65 (Cell Signaling; 1:1000), and Bax, Bcl-xL antibodies (Sigma; 1:4000). The secondary antibodies were alkaline phosphatase–conjugated to goat anti mouse/rabbit immunoglobulin G (IgG)

(1:5000). Signals were detected by chemiluminescence (Pierce Chemical).

Statistical analysis

Data were expressed as mean ± SD. Significance of differences between control and samples treated with various drugs was determined by one-way analysis of variance (ANOVA) followed by post hoc least significant difference (LSD) test. Values of $P < .05$ were considered to be statistically significant.

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