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Preferential sensitivity of human dopaminergic neurons to gp120-induced oxidative damage

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The dopamine (DA)-rich midbrain is known to be a key target of human immunodeficiency virus (HIV)-1. Studies of simian immunodeficiency virus (SIV)-induced neuropathogenesis recently established that there is a major disruption within the nigrostriatal dopaminergic system characterized by marked depletion of dopaminergic neurons, microglial cell activation, and reactive astrocytes. Using a human mesencephalic neuronal/glial culture model, which contains dopaminergic neurons, microglia, and astrocytes, experiments were performed to characterize the damage to dopaminergic neurons induced by HIV-1 gp120. Functional impairment was assessed by DA uptake, and neurotoxicity was measured by apoptosis and oxidative damage. Through the use of this mesencephalic neuronal/glial culture model, we were able to identify the relative sensitivity of dopaminergic neurons to gp120-induced damage, manifested as reduced function (decreased DA uptake), morphological changes, and reduced viability. We also showed that gp120-induced oxidative damage is involved in this neuropathogenic process. *Journal of NeuroVirology* (2009) **15**, 401–410.

Keywords: dopamine; gp120; neurons; oxidative stress

Introduction

Human immunodeficiency virus (HIV)-associated neurocognitive disorder (HAND), which includes asymptomatic neurocognitive impairment, mild neurocognitive disorder (MND), and the more severe HIV-associated dementia (HAD), continues to be an important clinical manifestation of HIV-1 infection in the age of antiretroviral therapy. Although the rates of HAD have declined due to antiretroviral therapy, it still remains the most common form of dementia in adults under age 40 and remains an independent risk factor for mortality despite antiretroviral therapy (Boisse *et al*, 2008). Clinically, HAD presents as subcortical dementia, with predominant basal ganglia involvement, leading to Parkinsonism, psychomotor slowing, as well as cognitive and behavioral impairment (Koutsilieri *et al*, 2002b).

Research over the past two decades has revealed that a network of factors, including the virotoxin gp120 and mediators released from activated glial cells, are involved in the neuropathogenesis of HIV (D'Aversa *et al*, 2005; Gendelman, 2005; Ghorpade *et al*, 2005; Gonzalez-Scarano and Martin-Garcia, 2005; Kaul and Lipton, 2006; Kaul *et al*, 2005; Mattson *et al*, 2005). The HIV-1 envelope protein gp120 enables HIV-1 entry into cells via its interaction with CD4 and the chemokine receptors CCR5 and CXCR4 and is one of the toxic viral proteins released by HIV-1–infected cells (D'Aversa *et al*, 2005). gp120 has a direct toxic effect on neurons (Corasaniti *et al*, 2000; Kaul and Lipton, 1999; Meucci *et al*, 1998), and by interacting with microglia/macrophages, facilitates the release of other neurotoxic mediators (Bezzi *et al*, 2001; Brenneman *et al*, 1988; Garden *et al*, 2002; Yi *et al*, 2004). Studies in our laboratory have shown that gp120-mediated toxicity is enhanced

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by substances of abuse such as morphine (Hu *et al*, 2005) and cocaine (Hu *et al*, 2006). More recent work has demonstrated that the nigrostriatal dopaminergic system is a critical brain region for the neuronal dysfunction and death seen in HAD (Aylward *et al*, 1993; Ferris *et al*, 2009; Gelman *et al*, 2006; Itoh *et al*, 2000; Koutsilieri *et al*, 2002a; Koutsilieri *et al*, 2002b; Nath *et al*, 2000; Paul *et al*, 2005; Sardar *et al*, 1996; Silvers *et al*, 2006; Wang *et al*, 2004). Dopamine (DA) is the main neurotransmitter in the nigrostriatal dopaminergic pathway and depletion of DA in this mesencephalic area underlies the clinical manifestations of Parkinson's disease (PD); symptoms of PD have been well characterized in HAD (Koutsilieri *et al*, 2002b; Sardar *et al*, 1996). Recent studies of simian immunodeficiency virus (SIV)-induced neuropathogenesis established that there is a major disruption within the nigrostriatal dopaminergic system characterized by marked depletion of dopaminergic neurons, microglial cell activation, and reactive astrocytes (Czub *et al*, 2004; Scheller *et al*, 2005); these same histopathological abnormalities mirror those observed in HAD. The presence of gp120 expression in the brains of patients with HAD has been demonstrated, particularly in the area of the basal ganglia (Jones *et al*, 2000). One noteworthy feature of the substantia nigra that may help explain the susceptibility of this brain region to HIV-1–related damage is that this area contains a high concentration of microglial cells (Lawson *et al*, 1990), which are the only brain cell type that can support productive HIV-1 infection and are a rich source of neurotoxic reactive oxygen species (ROS) and cytokines/chemokines (Chao *et al*, 1995; Cosenza *et al*, 2002; Koenig *et al*, 1986; Wiley *et al*, 1986).

A growing body of evidence also supports the role of "oxidative stress" in HIV-1 neuropathogenesis (Aksenov *et al*, 2003, 2006; Minghetti *et al*, 2004; Mollace *et al*, 2001; Pocernich *et al*, 2005; Sheng *et al*, 2000; Turchan-Cholewo *et al*, 2006; Wallace *et al*, 2006). Brain sections obtained from patients with acquired immunodeficiency syndrome (AIDS) dementia show intense immunostaining for nitrotyrosine, indicating that reaction between nitric oxide (NO) and has led to peroxynitrite formation, resulting in oxidative damage (Boven *et al*, 1999). Furthermore, it has been reported that levels of the lipid peroxidation product HNE (4-hydroxy-2-nonenal) are increased in brain tissues of patients with HAD (Haughey *et al*, 2004). Among the cell types in the brain parenchyma, microglia are most capable of generating large quantities of the free radical (Chao *et al*, 1995), which is rapidly metabolized to the highly cytotoxic ROS hydrogen peroxide and hydroxyl radical. Unlike microglia from certain rodent species, human microglia do not express inducible nitric oxide synthase (iNOS) (Lee *et al*, 1993; Peterson *et al*, 1994; Rock *et al*, 2005), but when activated, they release abundant amounts of interleukin (IL)-1β, which in turn induces iNOS and production of the reactive nitrogen species (RNS) NO by human astrocytes (Hu *et al*, 1999). ROS, RNS, and peroxynitrite are regarded as the key mediators of oxidative damage in HAD.

Based upon findings that the mesencephalon, and specifically dopaminergic neurons, are important to HIV neuropathogenesis, as well as the growing appreciation that oxidative stress plays an important role in HIV neuropathogenesis, experiments were performed using a human mesencephalic neuronal/-glial culture model to characterize the damage to dopaminergic neurons induced by gp120 by assessing functional impairment as measured by DA uptake, and to investigate neurotoxicity by assessing apoptosis and oxidative damage.

Results

Although the dopaminergic mesencephalic region is recognized as a key target area in HIV-1 neuropathogenesis, studies of the effects of gp120 specifically on human dopaminergic neurons have not been reported. Thus, we have developed an *in vitro* human mesencephalic neuronal/glial culture model (Figure 1A, B) that contains approximately 55% neurons, 40% astrocytes, and 5% microglia (Figure 1C). Additionally, double-staining using anti-NeuN (neuron-specific nuclear protein) or anti-MAP-2 (microtubule-associated protein-2) in combination with anti-GABA (γ-aminobutyric acid), anti-ChAT (choline acetyltransferase), or anti-TH (tyrosine hydroxylase) antibodies showed that the neurons are composed of 30% to 35% GABA-immunereactive (IR) neurons (GABAergic), 10% to 15% ChAT-IR neurons (cholinergic), and 5% to 10% TH-IR neurons (DAergic) relative to the neuronal cell population. Experiments performed in our laboratory have determined that the human mesencephalic neuronal/glial cultures do not contain noradrenergic cells by using the noradrenergic-specific marker, dopamine β-hydroxylase (DBH) (data not shown), a finding similar to other mesencephalon models (Bennett *et al*, 1995). The relative amount of TH-IR neurons is comparable to what has been reported previously in human (2% to 5%) (Dong *et al*, 1993; Silani *et al*, 1994) and in rodent (1% to 2%) (Liu *et al*, 2000; Yang *et al*, 2007) mesencephalic neuronal/glial cultures.

In our first experiments, a more profound loss of DAergic neurons was noted when compared to GABAergic and cholinergic neurons, demonstrating their relative sensitivity to the detrimental effects of gp120 compared to other neurons of the mesencephalon (Figure 2A). To further characterize the neuronal impairment induced by gp120, we chose to investigate functional impairment of these cells by assessing DA, GABA, and choline uptake. In support of the notion that gp120 impairs DAergic neurons preferentially, we found that exposure to

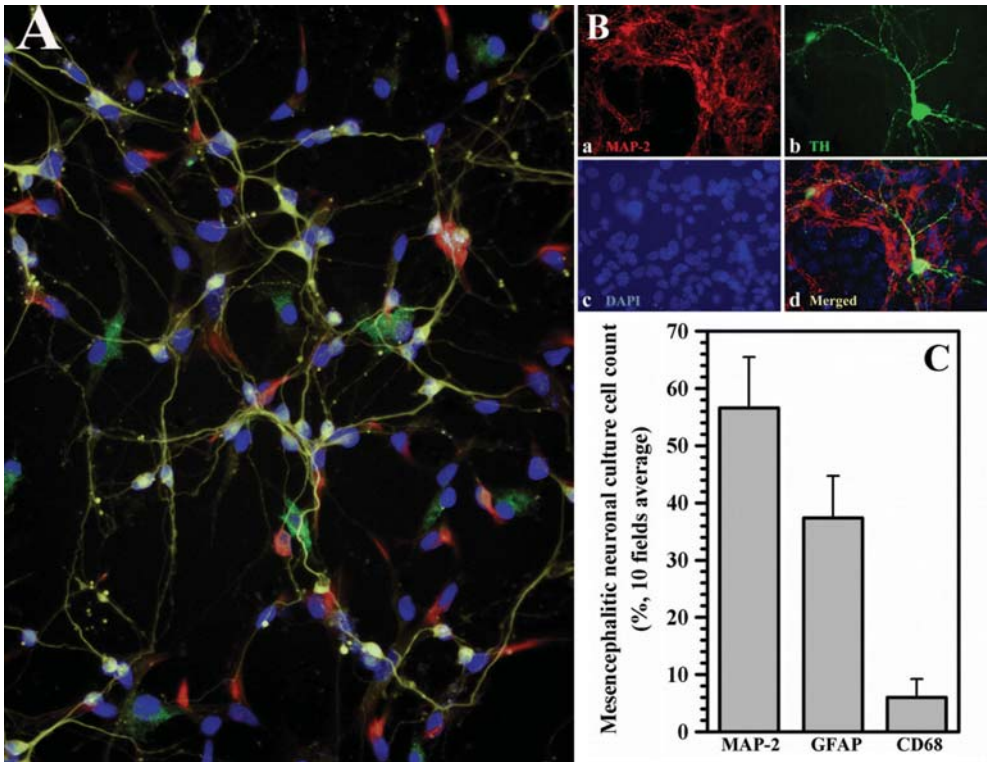

Figure 1 Composition of human mesencephalic neuronal/glial cultures. Mesencephalic neuronal/glial cultures grown for 2 weeks were (**A**) immunostained with mouse anti-human CD68 (microglial marker, green; labeled with anti-mouse NL493) and rabbit anti-GFAP (astrocyte marker, red; labeled with anti-rabbit NL557), followed with biotinylated mouse anti-MAP-2 (neuronal marker, yellow; labeled with streptavidin NL637) and DAPI as nuclei counter stain (blue) (20×). Individual photomicrograph was acquired using Zeiss Axioscope (Jena, Germany) and the merged image was processed by Adobe Photoshop. (**B**) Mesencephalic neuronal/glial cultures grown for 2 weeks and stained for MAP-2 (red, **a**), tyrosine hydroxylase (TH, dopaminergic neuronal marker, green, **b**), and DAPI (blue, **c**), followed by a merged image (**d**) (20×). (**C**) Relative composition of the mesencephalic neuronal/glial culture was derived from cell counts of 15 fields each that were stained with anti-NeuN/anti-MAP-2, anti-GFAP, or anti-CD68 antibodies and nuclei counterstained with DAPI. Data presented are mean±SD of triplicates of two separate experiments using different brain tissue specimens.

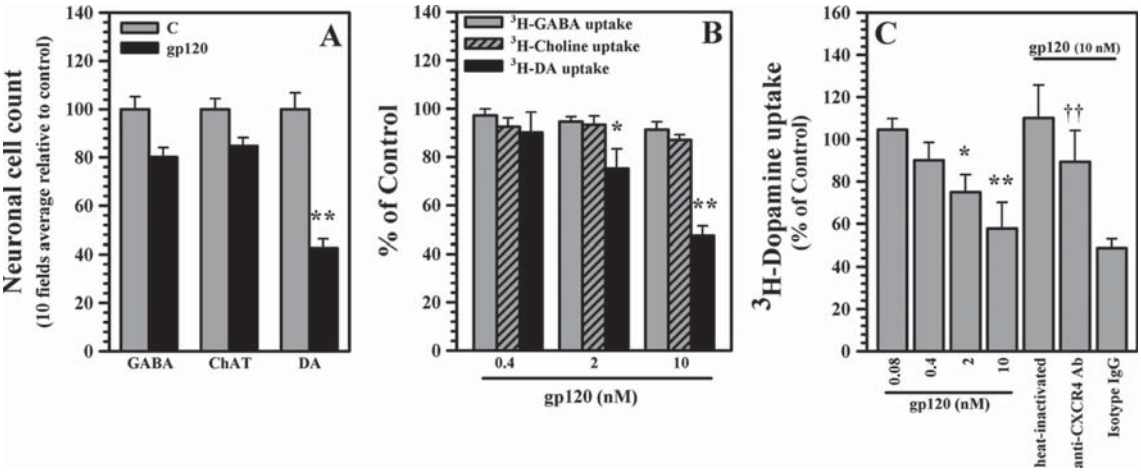

Figure 2 Relative susceptibility of human DAergic neurons to gp120. (**A**) Relative loss of neurons with gp120 exposure (10 nM, 5 days) based on immunostain with anti-NeuN combined with anti-GABA, -ChAT, or -TH antibodies followed by quantification of positively stained cells. (**B**) Mesencephalic neuronal/glial cultures were treated with gp120 at the indicated concentrations for 5 days followed by ^{3}H-GABA, ^{3}H-choline, and ^{3}H-DA addition for 10 min as a measurement of ^{3}H-GABA, ^{3}H-choline, and ^{3}H-DA uptake for GABA transporter, acetylcholine transporter, and DAT activity, respectively. (**C**) Mesencephalic neuronal/glial cultures were treated with gp120 at the indicated concentrations for 5 days followed by ^{3}H-DA addition for 10 min as a measurement of ^{3}H-DA uptake for DAT activity. Heat-inactivated gp120 (10 nM, 85°C, 10 min) was used as a negative control. Data are mean±SD of triplicates of two to four separate experiments using different brain tissue specimens. $^*P$ <.05, $^{**}P$ <.01 versus respective control; ††P <.01 versus gp120.

gp120 for 5 days decreased DA uptake significantly, in a concentration-dependent manner, compared to controls (including heat-inactivated gp120 and anti-CXCR4 controls) (Figure 2B, C). Uptake of GABA and choline by their respective neurons was not significantly impaired (Figure 2B). Additionally, loss of DAergic neurons and morphological changes manifest as blunting of DAergic neurites were observed after exposure to gp120 (Figure 3).

Because neuronal apoptosis is one of the histopathological hallmarks of HAD, the effect of gp120 on mesencephalon viability was assessed. We exposed our mesencephalic neuronal/glial culture to gp120 and assessed the level of apoptosis by measuring histone-associated DNA fragmentation by enzyme-linked immunosorbent assay (ELISA) (Figure 4A, B) and cytochrome *c* levels (Figure 4C). Peak apoptosis occurred at 5 days and with 10 nM of gp120 compared to controls, including heat-inactivated gp120 and anti-CXCR4 controls, thus supporting our hypothesis that gp120 induces apoptosis in our mesencephalic neuronal/glial cultures.

Oxidative stress is considered a major contributor to HIV-1 neuropathogenesis, and this mechanism of damage was examined. In support of the hypothesis that gp120 induces oxidative damage, we quantified 8-isoprostane levels (a measure of lipid peroxidation) in our mesencephalic neuronal/glial cultures and found that exposure to gp120 resulted in a concentration-dependent increase in 8-isoprostane levels, with a 4-fold increase seen with 10 nM gp120 (Figure 5). We then assessed the role of ROS and in this process and demonstrated an increase in intracellular ROS with exposure to gp120 (Figure 6A, B) and identified that superoxide was involved, as demonstrated by its concentration-dependent increase in response to gp120 (Figure 6C) and the partial reversal of gp120-induced impairment of DA uptake by the NADPH oxidase inhibitor diphenyleneiodonium (DPI) (Figure 6D).

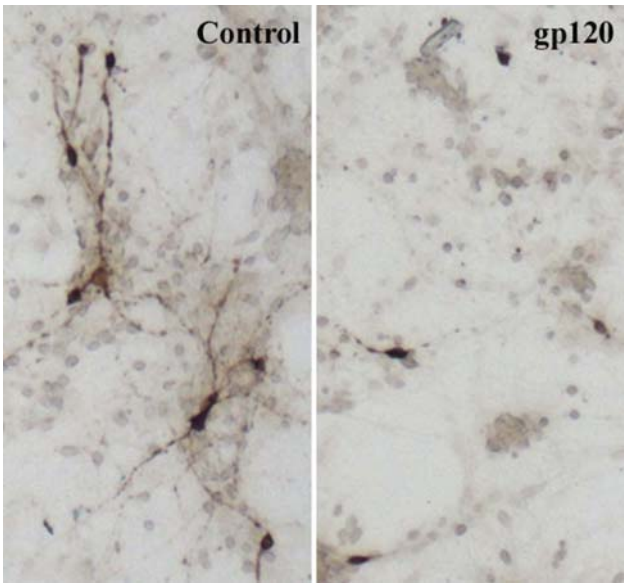

Figure 3 gp120-induces damage in DAergic neurons. Mesencephalic neuronal/glial cultures treated with gp120 (10 nM) for 5 days were immunocytochemically stained with rabbit anti-TH antibody developed with Vector ABC and DAB kit. Untreated Control showed numerous and extensive neurites, whereas gp120-treated cultures exhibited TH-IR cell reduction and marked loss and blunting of neurites (10×).

Discussion

In the present study, we demonstrate for the first time that human DAergic neurons are preferentially sensitive to the neurotoxic effects of the HIV-1 surface glycoprotein gp120 when compared to GABAergic and cholinergic neurons, which are also present in the human mesencephalon. This damage was manifest by gp120-induced cellular loss, blunting of neurites, and impaired ^{3}H-DA uptake. These results parallel the finding that DAergic neurons are reduced in patients with HAD (Itoh *et al*, 2000; Sardar *et al*, 1996), and specifically that dopamine transporters are significantly reduced in these patients compared with seronegative controls (Wang *et al*, 2004). Previous studies have shown that gp120 exposure results in decreased neuronal cellular numbers (Corasaniti *et al*, 2000; Kaul and Lipton, 1999; Meucci *et al*, 1998). Examination of the specific effect of gp120 on DAergic neurons in a rat model has shown a loss of DAergic neurons (Bachis *et al*, 2006; Mocchetti *et al*, 2007; Nath *et al*, 2000; Nosheny *et al*, 2006, 2007) and a decrease in ^{3}H-DA uptake (Bennett *et al*, 1995; Wallace *et al*, 2006). In one study, which did not show a reduction in DAergic neurons, a reduction in dopamine neuron process length was observed (Bennett *et al*, 1995). Furthermore, *in vivo* experiments have shown that this DAergic neuronal loss is specific to the A9 DAergic cell group of the substantia nigra (Bachis *et al*, 2006; Mocchetti *et al*, 2007; Nosheny *et al*, 2006, 2007). The impairment of ^{3}H-DA uptake by DAergic neurons in our study could be the result of either an alteration of K_M or V_{max}. Although definitive studies to resolve this were not performed, a blunting of DAergic neurites was observed in similar fashion to that seen in a rat model (Bennett *et al*, 1995), suggesting that the impaired ^{3}H-DA uptake was due to an altered V_{max}. The unique aspect of our results is the demonstration of the preferential sensitivity of human DAergic neurons to gp120-induced toxicity as compared to other neuronal types of the mesencephalic neuronal/glial cultures. Interestingly, this preferential sensitivity may not be specific for human cells only, as this sensitivity of DAergic neurons to gp120-induced toxicity has also been demonstrated in the pedal ganglia of the bivalve *Mytilus* (Stefano *et al*, 1993).

We also showed that gp120 induces apoptosis within the human mesencephalic neuronal/glial

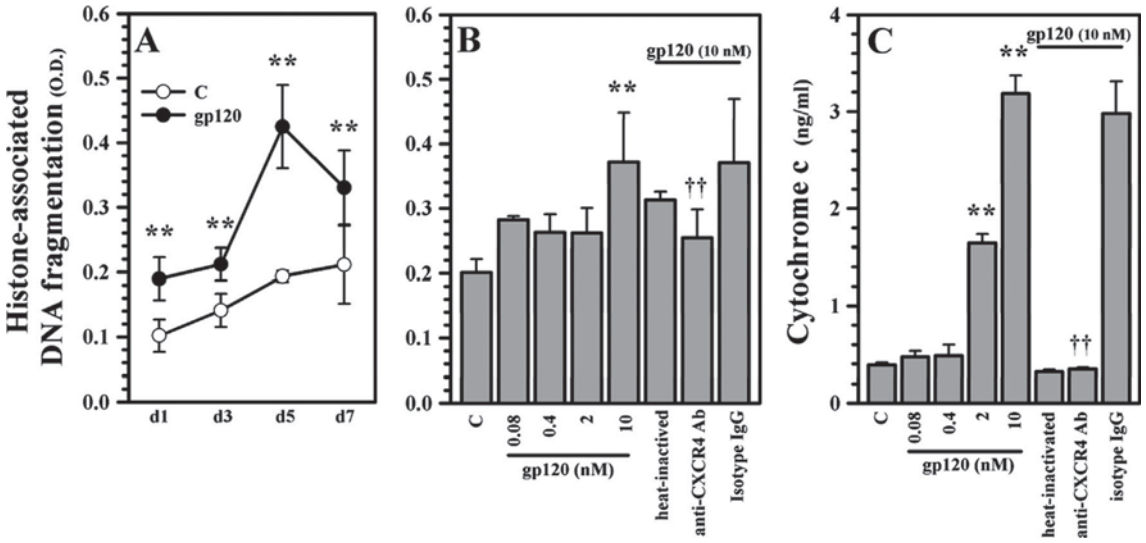

Figure 4 gp120 induces apoptosis in human mesencephalic neuronal/glial cultures. (**A**) Treatment with gp120 (10 nM) over 7 days induced apoptosis of mesencephalic neuronal/glial cultures measured by cell death ELISA. (**B**) Mesencephalic neuronal/glial cultures exposed to gp120 for 5 days at the indicated concentrations or treated with the indicated antibodies for 1 h prior to gp120 (10 nM) for 5 days were measured by cell death ELISA. Heat-inactivated gp120 (10 nM, 85°C, 10 min) was used as negative control. (**C**) Cell lysates collected from mesencephalic neuronal/glial cultures treated as in **B** were used in a cytochrome *c* assay. Data are mean±SD of triplicates of two separate experiments using different brain tissue specimens. $^{**}P$ <.01 versus control (C); $^{††}P$ <.01 versus gp120.

culture as measured by cell death ELISA and cytochrome *c* assay. Other investigators have reported apoptosis of rodent DAergic neurons after gp120 exposure, which appears to be caspase-3 related (Mocchetti *et al*, 2007).

Oxidative stress plays a role in HIV-1 neuropathogenesis (Aksenov *et al*, 2003, 2006; Minghetti *et al*, 2004; Mollace *et al*, 2001; Pocernich *et al*, 2005; Sheng *et al*, 2000; Turchan-Cholewo *et al*, 2006; Wallace *et al*, 2006). Microglia are the most capable brain cell type for generating large quantities of the free radical (Chao *et al*, 1995). Murine models of Parkinson's disease have also demonstrated that selective toxicity of DAergic neurons to lipopolysaccharide (LPS) or 1-methyl-4-phenylpyridinium (MPP^+) involve microglial-derived O_2^- (Block *et al*, 2004; Gao *et al*, 2003). In the current study, oxidative damage in the mesencephalic neuronal/glial culture was demonstrated by enhanced levels of 8-isoprostanes (stable byproducts of oxidative damage to lipids), increased intracellular ROS, as well as evidence of the involvement of O_2^-. Although many oxidative products may be involved in oxidative damage in our mesencephalic neuronal/glial cultures, it is likely that free radicals generated downstream from have important deleterious effects on DAergic neurons.

The effects observed in our experiments were most robust at the 10 nM concentration of gp120. Several other investigators have found that similar concentrations were necessary to elicit responses, including the use of in human microglia (gp120, 100 nM) (D'Aversa *et al*, 2005) and in rat neurons (gp120, 10^{-13} to 10^{-8} M [Bennett *et al*, 1995]; gp120, 2 nM [Yao *et al*, 2009]; and gp120, 5 nM [Bachis *et al*, 2006]). Our laboratory has also demonstrated

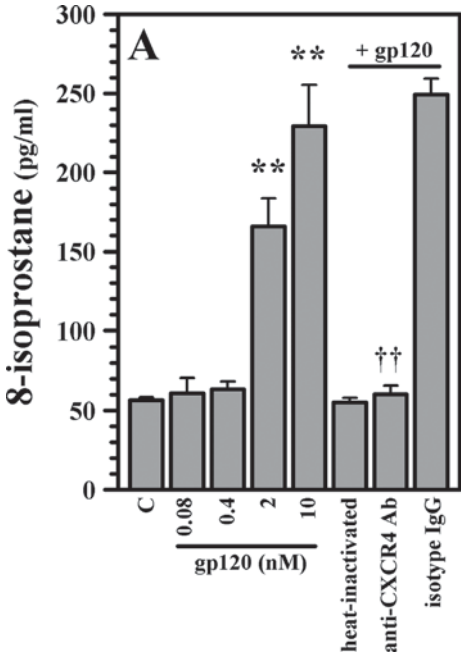

Figure 5 gp120 induces lipid peroxidation in human mesencephalic neuronal/glial cultures. (**A**) Mesencephalic neuronal/glial cultures were treated with gp120 at the indicated concentrations for 5 days or treated with the indicated antibodies for 1 h prior to gp120 (10 nM) for 5 days, followed by supernatant collection for 8-isoprostane assay. Heat-inactivated gp120 (10 nM, 85°C 10 min) was used as a negative control. Data are mean±SD of triplicates of two separate experiments using different brain tissue specimens. $^{*}P$ <.05, $^{**}P$ <.01 versus control (c); $^{††}P$ <.01 versus gp120.

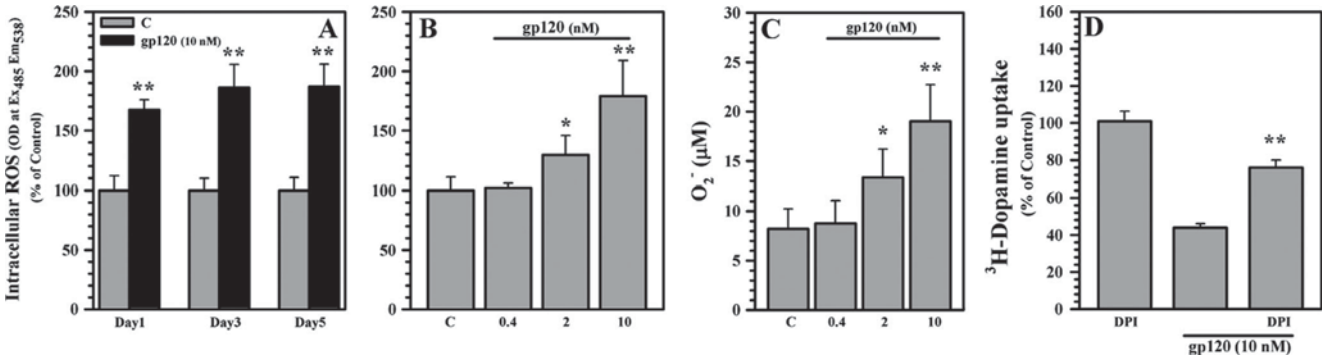

Figure 6 gp120 induces ROS and production in human mesencephalic neuronal/glial cultures. (**A**) Treatment with gp120 (10 nM) over 5 days demonstrated increased intracellular ROS production in mesencephalic neuronal/glial cultures. (**B**) Mesencephalic neuronal/glial cultures exposed to gp120 for 5 days at the indicated concentrations were evaluated for intracellular ROS. (**C**) Mesencephalic neuronal/glial cultures exposed to gp120 for 3 days at the indicated concentrations were evaluated for production. (**D**) Mesencephalic neuronal/glial cultures were treated with gp120 (10 nM) for 5 days with or without the NADPH oxidase inhibitor diphenyleneiodonium (DPI), followed by ^{3}H-DA addition for 10 min as a measurement of ^{3}H-DA uptake for DAT activity. Data are representative of mean±SD of triplicates of three to four separate experiments using different brain tissue specimens. $^*P < .05$, $^{**}P < .01$ versus control.

that similar concentrations of gp120 induced apoptosis in human cortical neuronal cultures (Hu *et al*, 2005). Differences in gp120 sources and species (rodent versus human) used likely account for the diversity of concentrations used in various publications.

Results reported here show that gp120 preferentially damages DAergic neurons in a human mesencephalic neuronal/glial culture model, elicits apoptosis, and induces oxidative damage in a manner that involves the participation of O_2^-, and this human mesencephalic neuronal/glial culture model could be used in future studies to investigate involvement of such processes in damage to DAergic neurons and understand the relative sensitivity of DAergic neurons to gp120.

Methods

Human fetal brain tissue was obtained under protocol approved by the Human Subjects Research Committee at our institution.

Reagents
The following reagents were purchased from the indicated sources: Recombinant HIV-1 $gp120_{LAV}$ (IIIB), which has clade B origins (Protein Sciences, Meriden, CT); Dulbecco's modified Eagle's medium (DMEM), Hanks' balanced salt solution (HBSS), penicillin, streptomycin, trypsin, glucose, bovine serum albumin, polyoxyethylenesorbitan monolaurate (Tween 20), phosphate-buffered saline (PBS), Krebs-Ringer buffer, paraformaldehyde, uridine, fluorodeoxyuridine, poly-D-lysine, superoxide dismutase (SOD), mazindol, goat or donkey serum, rabbit anti-γ-aminobutyric acid (GABA) antibody (Sigma-Aldrich, St. Louis, MO); rabbit anti-tyrosine hydroxylase (TH) antibody (Pel-Freez, Rogers, AR); fetal bovine serum (FBS) (Hyclone Laboratories, Logan, UT); mouse anti-microtubule-associated protein-2 (MAP-2), mouse anti-neuron-specific nuclear protein (NeuN), goat anti-choline acetyltransferase (ChAT) and rabbit anti-dopamine β-hydroxylase (DBH) antibodies (Chemicon, Temecula, CA); rabbit anti-glial fibrillary protein (GFAP) and mouse anti-CD68 antibodies (DAKO, Carpinteria, CA); streptavidin Northern Lights(NL)637 secondary antibody, donkey anti-rabbit NL557 and anti-mouse NL493 antibodies (R&D Systems, Minneapolis, MN); donkey anti-rabbit, anti-mouse, or anti-goat immunoglobulin G (IgG) rhodamine- or fluorescein isothiocyanate (FITC)-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA); propidium iodide (PI) and Hoechst stain 33342 (Roche, Indianapolis, IN); [7,8-^{3}H]dopamine (DA, 50 Ci/mmol), 4-amino-*n*-[2,3-^{3}H]butyric acid (GABA, 80 Ci/mmol)) and [methyl-^{3}H]choline chloride (66.7 Ci/mmol) (GE Healthcare, Piscataway, NJ).

Assay kits
The following kits were purchased from the indicated sources: ApopTag *in situ* apoptosis detection kit (Chemicon); cell death detection kit (Roche); 8-isoprostane EIA kit (Cayman Chemical, Ann Arbor, MI); Vectastain ABC kit (Vector Laboratories, Burlingame, CA).

Mesencephalic neuronal/glial culture
Human fetal brain tissue was obtained from women undergoing elective abortion, in accordance with informed-consent guidelines and a protocol approved by the Human Subjects Research Committee at our institution. Mesencephalic neuronal/glial cultures were prepared from 8- to 12-week-old aborted human fetal brain tissues as described previously (Dong *et al*, 1993; Silani *et al*, 1994) with modification. In brief, ventral mesencephalon tissues were dissected and incubated in 0.1% trypsin for 15 min at 37°C, followed by washing twice with DMEM containing

10% FBS. After gentle mechanical trituration with pasture pipette, single-cell suspensions in DMEM containing 10% FBS and penicillin/streptomycin (100 U/ml/100 μg/ml) were stained with trypan blue to confirm cell viability before being plated onto poly-D-lysine–coated culture plates (5×10^5 cells/well or 1×10^5 cells/well in 24- or 96-well plates) or 4-well chamber slides (4×10^5 cells/well). On day 5, cell cultures were treated with uridine (33.6 μg/ml) and fluorodeoxyuridine (13.6 μg/ml), followed by medium replacement with DMEM and 10% FBS on day 6 and every 4 days thereafter. Presence of dopaminergic neurons was verified by ^{3}H-DA uptake and immunocytochemical staining of tyrosine hydroxylase (TH). Twelve-day-old mesencephalic neuronal/glial cultures were used for all experiments. Because the nigrostriatal pathway expresses CXCR4, we elected to use the CXCR4-specific $gp120_{LAV}$ (IIIB) for all experiments (Banisadr *et al*, 2002).

Immunocytochemical staining

For neuronal staining, monoclonal anti-NeuN or anti-MAP-2 antibodies were used. To identify dopaminergic, GABAergic or cholinergic neurons, polyclonal anti-TH, -GABA, or -ChAT antibodies were used, respectively. Astrocytes and microglia were detected with anti-GFAP and -CD68 antibodies, respectively. Following previously described procedures (Bennett *et al*, 1995; Hu *et al*, 2005), mesencephalic neuronal/glial cell cultures grown in 24-well plates or 4-well chamber slides were fixed with 4% paraformaldehyde for 20 min, followed by washing with PBS and incubation with 10% normal goat or donkey serum containing 0.3% Triton X-100 in PBS for 1 h at room temperature (RT). After washing, primary antibodies were added and incubated overnight at 4°C (anti-NeuN/anti-MAP2 1:200, anti-TH/anti-GABA 1:1000, anti-ChAT 1:500, anti-GFAP 1:1000, and anti-CD68 1:100). After washing 4 times, secondary antibodies were added (donkey anti-mouse, -rabbit, or -goat FITC- or rhodamine-conjugated) for 1 h at RT. For triple staining, cell cultures were first stained with rabbit anti-GFAP and mouse anti-CD68 antibodies, labeled with donkey anti-rabbit NL557 and anti-mouse NL493 antibodies, and then incubated with biotinylated mouse anti-MAP-2 antibody (Leinco Technologies, St Louis, MO), followed by streptavidin NL637 secondary antibody, and nuclei counterstained with DAPI. To visualize gp120-induced morphology damage, cell cultures stained with anti-TH antibody were incubated with biotinylated goat anti-rabbit IgG for 60 min, followed by addition of Vectastain ABC reagents and color development with 3,3′-diaminobenzidine (DAB) (Vector Laboratories).

Dopamine, GABA, and choline transporter activity assays

High-affinity transporter activity assays were performed as previously described (Bennett *et al*, 1995; Chao *et al*, 1992). After treatment, mesencephalic neuronal/glial cell cultures were washed 3 times with DMEM or Krebs-Ringer buffer. For DA and GABA uptake, cultures were incubated with ^{3}H-dopamine (50 nM) or ^{3}H-GABA (100 nM) in DMEM for 10 min at 37°C. For choline uptake, ^{3}H-choline (100 nM) in Krebs-Ringer buffer was added for 10 min at 37°C. After washing 3 times with cold DMEM or Krebs-Ringer buffer, cells were lysed in 2 N NaOH (300 μl), followed by lysate collection into scintillation cocktail for ^{3}H radioactivity counting. Nonspecific dopamine, GABA, or choline uptake was observed in the presence of mazindol (10 μM), β-alanine (100 nM), or unlabeled choline (1 μM), respectively.

Assessment of oxidative tissue damage

All reactive species themselves are short-lived due to their highly reactive nature. For this reason, oxidative tissue damage is generally analyzed through measurement of secondary products indicative of oxidative damage. We assessed the level of oxidative damage to lipids using an 8-isoprostane enzyme immunoassay (EIA) kit to quantify the level of lipid peroxidation in samples and performed according to the manufacturer's instructions (Cayman).

Cell death detection ELISA

Quantitative *in vitro* determination of cytoplasmic histone-associated DNA fragments after induced cell death was performed according to manufacturer's protocol. To measure apoptosis, cell lysates from untreated control or treated cell cultures in 24-well plates were added to the streptavidin-coated 96-well ELISA plates together with anti-histone–biotinylated and anti-DNA–peroxidase antibodies. After incubation and washing, DNA fragments were captured and detected by a chromogenic enzyme-substrate reaction (Hu *et al*, 2006).

Quantikine Cytochrome c Immunoassay

The Quantikine Cytochrome c Immunoassay was purchased from R&D Systems. Briefly, a monoclonal antibody specific for cytochrome *c* was used in the precoated microplate. Standards and cell lysate samples were pipetted into the wells and any cytochrome *c* present was bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific for cytochrome *c* was added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells and color develops in proportion to the amount of cytochrome *c* bound in the initial step. The color development was stopped and the intensity of the color was measured with a plate reader at 450 nm. The minimum detectable dose of cytochrome *c* is typically less than 0.31 ng/ml.

TUNEL staining
The TUNEL (deoxynucleotidyltransferase dUTP nick end labeling) technique used has been previously described (Hu *et al*, 2002). After treatment, cells were fixed in 4% paraformaldehyde for 20 min. ApopTag *in situ* apoptosis detection kit was used to label fragmented DNA ends with digoxigenin-11-UTP using terminal deoxynucleotidyl transferase. Fluorescein-labeled anti-digoxigenin antibody was then used to detect the labeled ends in the nuclei. After washing and air drying, the slides were examined under a fluorescence microscope.

Intracellular ROS assay
The production of intracellular ROS in human mesencephalic neuronal/glial cultures treated with gp120 was measured by 2′,7′-dichlorofluorescin diacetate (DCFH-DA; Invitrogen, Carlsbad, CA) as previously described (Block *et al*, 2004). Human mesencephalic neuronal/glial cultures (1×10^5 cells/well) were incubated with gp120 (0.4 to 10 nM) for 1, 3, or 5 days, followed by washing and addition of 20 μM DCFH-DA in HBSS (with Ca^{2+}) for 30 min before being read at 485 nm (excitation) and 530 nm (emission) with a fluorescence micro plate reader (Molecular Devices, Sunnyvale, CA). The results were presented as the ratio of the fluorescence of gp120-treated samples to that of the control samples.

Superoxide assay
The production of by human mesencephalic neuronal/glial cultures treated with gp120 was determined by measuring the superoxide dismutase (SOD)-inhibitable reduction of ferricytochrome *c*, as previously described (Chao *et al*, 1994) with slight modification. Human mesencephalic neuronal/glial cultures (1×10^5 cells/well) were incubated with gp120 (0.4 to 10 nM) for 3 days, followed by washing and addition of HBSS with or without SOD (500 U/ml) and then ferricytochrome *c*. Plate was read at 550 nm with a micro plate reader.

Statistical analysis for functional assays
For comparison of the means of two groups, Student's *t* test was used. For comparison of means of multiple groups, analysis of variance (ANOVA) was performed, followed by either Fisher's protected least significant difference (PLSD) test or Scheffe test.

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