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ORIGINAL ARTICLE

Morphine and gp120 Toxic Interactions in Striatal Neurons are Dependent on HIV-1 Strain

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Abstract A rigorously controlled, cell culture paradigm was used to assess the role of HIV-1 gp120 ± morphine in mediating opioid-HIV interactive toxicity in striatal neurons. Computerized time-lapse microscopy tracked the fate of individual neurons co-cultured with mixed-glia from mouse striata during opioid and gp120 exposure. Subpopulations of neurons and astroglia displayed μ-opioid receptor, CXCR4, and CCR5 immunoreactivity. While gp120 alone was or tended to be neurotoxic irrespective of whether X4-tropic $gp120_{IIIB}$, R5-tropic $gp120_{ADA}$, or dual-tropic $gp120_{MN}$ was administered, interactive toxicity with morphine differed depending on HIV-1 strain. For example, morphine only transiently exacerbated $gp120_{IIIB}$-induced neuronal death; however, in combination with $gp120_{MN}$, morphine caused sustained increases in the rate of neuronal death compared to $gp120_{MN}$ alone that were prevented by naloxone. Alternatively, $gp120_{ADA}$ significantly increased the rate of neuron death, but $gp120_{ADA}$ toxicity was unaffected by morphine. The transient neurotoxic interactions between morphine and $gp120_{IIIB}$ were abrogated in the absence of glia suggesting that glia contribute significantly to the interactive pathology with chronic opiate abuse and neuroAIDS. To assess how mixed-glia might contribute to the neurotoxicity, the effects of morphine and/or gp120 on the production of reactive oxygen species (ROS) and on glutamate buffering were examined. All gp120 variants, and to a lesser extent morphine, increased ROS and/or decreased glutamate buffering, but together failed to show any interaction with morphine. Our findings indicate that HIV-1 strain-specific differences in gp120 are critical determinants in shaping both the timing and pattern of neurotoxic interactions with opioid drugs.

Keywords Neuro-acquired immunodeficiency syndrome (neuroAIDS) · Opiate drug abuse · Oxidative stress · Regulated cell death · CXCR4, CCR5, astroglia, microglia, basal ganglia

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Introduction

Aberrant glia-neuron signaling is revealed in the pathogenesis of neuro-acquired immunodeficiency syndrome (neuroAIDS) in human immunodeficiency virus type-1 (HIV-1) infected individuals, which is underscored by neuronal injury or death secondary to an infection that is largely restricted to glia. Infected microglia, and to a lesser extent reactive astroglia (Kaul et al. 2001; Gonzalez-Scarano and Martin-Garcia 2005), release products that can be broadly defined as "virotoxins" consisting of toxic viral proteins and inflammatory "cellular toxins" (Nath 2002). Glial-

derived cellular toxins include cytokines such as TNF-α, IL-1β and IL-6, excess glutamate (Wang et al. 2003), platelet activating factor (Bellizzi et al. 2005), and reactive oxygen (ROS) or nitrogen species (RNS). Within the confined extracellular space of the central nervous system (CNS) (Sykova 2005), glial-derived viral proteins and cellular toxins establish deleterious autocrine/paracrine neurochemical feedback loops, spiraling inflammation, and reactive gliosis, the combination of which inevitably leads to neuronal injury and death (Navia et al. 1986; Gonzalez-Scarano and Martin-Garcia 2005; Ellis et al. 2007; McArthur et al. 2010). While astroglia contribute less than microglia to the production of new virus, astroglia are significant sources of cellular toxins (Benos et al. 1994a; Conant et al. 1998; Nath et al. 1999; Kramer-Hammerle et al. 2005), and are key sites where opiates exacerbate the CNS effects of HIV-1 (El-Hage et al. 2005; Hauser et al. 2007; El-Hage et al. 2008b; Zou et al. 2011b).

Not only does opioid drug abuse spread the virus through needle sharing or exchange of sex for drugs, but opioid drug use appears to modify HIV-1 pathogenesis through direct interactions with the immune system (Adler et al. 1993; Peterson et al. 1998; Donahoe and Vlahov 1998). More recently, we and others have proposed that opioids can selectively exacerbate the CNS consequences of HIV-1 infection (Bell et al. 1998; Nath et al. 2000; Gurwell et al. 2001; Nath et al. 2002; Kumar et al. 2004; Hauser et al. 2005; Bell et al. 2006; Turchan-Cholewo et al. 2006; Anthony et al. 2008; Banerjee et al. 2011). Despite in vitro evidence that opioid abuse per se worsens the neurotoxicity, identifying the cellular and molecular sites where opioids first trigger the damage is challenging. Neurons, astroglia, and microglia can all possess μ opioid receptors (MOR), and activating MOR in each cell type appears to modify the disease process uniquely (Peterson et al. 1990; Chao et al. 1994; Peterson et al. 1998; El-Hage et al. 2005; Hu et al. 2005; Turchan-Cholewo et al. 2006; Zou et al. 2011b). To add to the complexity, opioids appear to interact with each viral protein differently (Hauser et al. 2005; Hauser et al. 2007). In the case of gp120, opioids potentiate gp120-induced cytokine release and/or receptor expression from an astroglial-derived cell line (Mahajan et al. 2002; Mahajan et al. 2005) and exacerbate gp120 toxicity in human neurons (Hu et al. 2005). Morphine withdrawal disrupts normal homeostatic synaptic repair mechanisms in gp120-expressing transgenic mice (Bandaru et al. 2011). Lastly, the viral proteins themselves appear to intrinsically modulate opioid receptor expression by astroglia and microglia (Turchan-Cholewo et al. 2008), and throughout the CNS (Fitting et al. 2010), while morphine alone can alter the expression of HIV-1 co-receptors (Steele et al. 2003), suggesting a complex interplay of drug and viral actions in glia.

Gp120 can be directly or indirectly neurotoxic through a variety of mechanisms. Soluble gp120 can interact with CXCR4 or CCR5 chemokine receptors in human and murine neural cells (Meucci et al. 1998; Kaul et al. 2007), via CD4-dependent or -independent mechanisms to induce CNS toxicity and inflammation (Jordan et al. 1991; Moore 1997; Kaul and Lipton 1999; Khan et al. 2004; Misse et al. 2005; Kaul et al. 2007). Although intrinsically neurotoxic, gp120 activates/overactivates macrophages/microglia imparting a generalized state of excitotoxicity (Dreyer et al. 1990; Lipton et al. 1991; Kaul et al. 2007). In addition, gp120 can disrupt ion homeostasis in astroglia, which likely compromises neuron function (Pulliam et al. 1993; Benos et al. 1994a; Benos et al. 1994b; Holden et al. 1999). Furthermore, gp120 can inhibit glutamate uptake by astrocytes through reductions in excitatory amino acid transporter-2 (EAAT-2) mRNA and protein levels (Wang et al. 2003). Less is known about how opioids might exacerbate neuroAIDS through interactions with gp120.

Recently, we found basic differences in morphine interactions with X4-versus R5-tropic strains of HIV-1 in an infectious model of hepatitis C virus (El-Hage et al. 2011). Prompted by these findings, we questioned whether morphine would interact with HIV-1 gp120 to cause neurotoxicity in a strain-dependent manner and posed this hypothesis in the present study. Moreover, influenced by new findings that glia largely mediate morphine and HIV-1 Tat interactive neurotoxicity (Zou et al. 2011b), the studies herein begin to consider whether glia might play a similar role in mediating morphine and HIV-1 gp120 neurotoxic interactions in the CNS.

Methods

Mixed-glial cultures Mixed glial cultures (astroglia and microglia) were prepared from P0–P1 ICR (CD-1) mouse striata (Charles River Co., Wilmington, MA). Striata were dissected, minced, and incubated with 10 ml trypsin (2.5 mg/ml) and DNase (0.015 mg/ml) in serum free medium (30 min, 37°C). Tissue was triturated, resuspended in culture medium with 10% fetal bovine serum (FBS), filtered through 135-μm and then 45-μm pore nylon mesh, prior to plating at moderate density (~7.5×10^5 cells/cm^2) on poly-L-lysine coated cell culture plates. The cultures contained 8.8±0.6% microglia, 90.2±0.4% astroglia, and less than 0.2% each of neurons, oligodendroglia, and glial precursors as previously described for our striatal cultures (Zou et al. 2011b).

Neuron cultures: neuron-enriched or co-cultured with mixed glia Striatal neurons were prepared from E15–E16 ICR (CD-1) mouse embryonic striata. Striata were dissected, minced, and incubated with trypsin (2.5 mg/ml) and

DNase (0.015 mg/ml) in Neurobasal Medium with 25 μM glutamate (30 min, 37°C). Tissue was triturated, resuspended, and cells filtered through 70 μm pore nylon mesh. Cultures were established in which (a) isolated neurons were plated alone on poly-L-lysine coated coverslips, or (b) neurons were co-cultured directly on a bedlayer of mixed-glia. Neuron cultures and neuron-glia co-cultures were similarly maintained in neurobasal media supplemented with B27 (Invitrogen, Carlsbad, CA), 0.5 mM L-glutamine, 0.025 mM glutamate, and allowed to mature for about 1 week prior to the start of experiments.

Opioid and HIV-1 gp120 treatments Cultures were treated with X4-tropic gp120 HIV-1_{IIIB}, R5-tropic HIV-1_{ADA}, or dual-tropic $gp120_{MN}$ (500 pM; ImmunoDiagnostics, Woburn, MA); morphine sulfate (500 nM; Sigma, St. Louis, MO); and/or (−)naloxone (1.5 μM, Sigma). The concentration of gp120 was chosen for its significant induction of caspase-3 induced neuronal death (Singh et al. 2004), while the morphine concentration used is able to fully activate MOR and synergistically enhance HIV-1 Tat mediated neurotoxicity (Gurwell et al. 2001; Zou et al. 2011b). Multiple treatments without naloxone were applied simultaneously. Naloxone was added 1 h before morphine when treatments contained both drugs.

MOR, CXCR4, and CCR5 immunofluorescence Striatal neurons or astrocytes were fixed for 15 min in 4% paraformaldehyde, permeabilized for 15 min in 0.1% Triton-X 100 and 0.1% BSA, and subsequently blocked for 1 h at room temperature in PBS supplemented with 0.1% BSA and 1% horse serum. Dual-labeling was performed for 2 h at room temperature using rabbit antisera to MOR (1:2500; Antibodies Incorporated, Davis, CA, 61–204), mouse anti-CXCR4 (1:100; Abcam, Cambridge, MA; ab21555), or mouse anti-CCR5 (1:100; BD Pharmingen, San Diego, CA; 555991), co-localized with antibodies to either anti-microtubule associated protein-2 (MAP2; 1:1000; Millipore, Temecula, CA; MAB378 (mouse) or Abcam; ab32454 (rabbit)), or anti-glial fibrillary acidic protein (GFAP; 1:1000; Millipore; MAB360 (mouse) or AB5804 (rabbit)). Goat anti-rabbit Alexa-488, donkey anti-mouse Alexa-594, goat anti-rabbit Alexa-594 and/or goat anti-mouse Alexa-488 (Invitrogen) secondary antibodies were applied, depending on the primary antibodies, for 1 h at room temperature, followed by counterstaining for 20 min with 0.5 μg/ml Hoechst 33342 (Invitrogen), which labels DNA and identifies all cell nuclei. Coverslips were mounted in ProLong Gold Antifade reagent (Invitrogen). Cells were visualized and digital images were acquired using a Zeiss LSM 700 confocal module configured to an Axio Observer Z.1 microscope and Zen 2010 software (Zeiss Inc., Thornwood, NY).

Assessment of neuron viability Time-lapse digital images of cells were recorded at 20 min intervals for 60 h, using a microscope and automated, computer-controlled stage encoder with environmental control (37°C, 95% humidity, 5% CO_2) that allowed us to repeatedly track the same neurons (Axio Observer Z.1, Zeiss). Neurons possessed an ovoid cell body, extensive nuclear euchromatin, discrete nucleoli, and well-defined dendritic arbor, which was confirmed in some instances by the neuronal markers NeuN and MAP2. Repeated measures ANOVA was used to assess the effect of treatment on the survival of individual neurons followed throughout the course of the 60 h experiment (percentage of pretreatment value) (Statistica, StatSoft, Tulsa, OK). In each experiment, treatments were distributed across cells pooled from the same pups. Approximately 50 healthy neurons with well-defined dendritic and axonal arbors were identified within ≥8 overlapping fields (40× magnification; MosaiX module, Zeiss AxioVision 4.6) within individual culture wells in each experiment prior to treatment (0 h). Using a computerized microscope stage encoder and tracking software (Zeiss, AxioVision 4.6; Mark&Find, MosaiX, and Time Lapse modules), the same neurons were repeatedly observed at 20 min intervals, and the effect of gp120 and/or opioids on the proportion of surviving neurons determined at 4 h intervals and averaged per culture well/treatment per experiment. Neuron death was monitored in individual cells and defined by rigorous criteria, which over time included the dissolution of Nissl substance, transient cytoplasmic swelling and vacuolization, nuclear destruction or pyknosis, and eventual destruction of the cell body (Singh et al. 2004; Singh et al. 2005; Bakalkin et al. 2010; Zou et al. 2011b). In some cases, neuron death was confirmed by viability markers such as ethidium homodimer or trypan blue (not shown); however, these markers all possess some inherent cytotoxicity with prolonged exposure, which precludes their use in experiments wherein cells are monitored continuously. The effect of each treatment on neuron survival was analyzed statistically using a repeated measures ANOVA from n=3–7 experiments (250–350 total neurons per treatment) and reported as mean neuron survival ± the standard error of the mean (SEM).

Astroglial ROS ROS was assessed using the cell-permeant dye 2′,7′-dichlorofluorescein diacetate (DCF-DA), which is hydrolyzed in the cell to DCF and fluoresces when in contact with ROS (such as OH, $ONOO^-$, and H_2O_2). Measurements were performed on a Victor 3 platereader (Perkin Elmer, Waltham, MA) at λ_{ex}=488 nm and λ_{em}= 525 nm after 90 min treatments. To assess the nature of the production of ROS, pharmacological inhibitors were used.

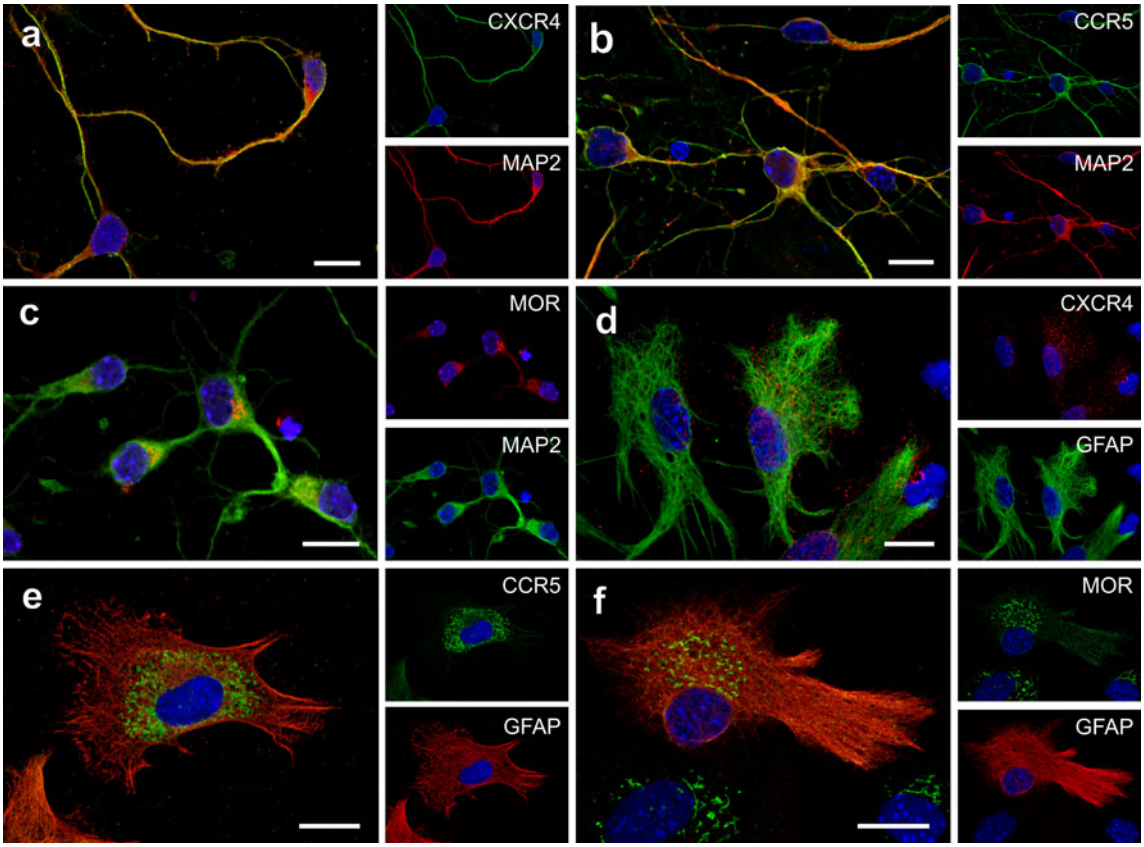


Fig. 1 Cellular localization of CXCR4, CCR5, and/or MOR immunofluorescence in striatal neurons and astrocytes. Receptors were colocalized in subsets of neurons (**a-c**) or astrocytes (**d-f**), respectively, using antibodies against MAP2 or GFAP; cells were counterstained with Hoechst 33342 (*blue*). In neurons, CXCR4 (**a**), CCR5 (**b**), and MOR (**c**) immunoreactivity was associated with the cell body and dendrites, while CCR5 antigenicity appeared to extend into some axons (**b**). In GFAP-immunolabeled astrocytes, CXCR4 (**d**), CCR5 (**e**), and MOR (**f**) displayed a punctate pattern of labeling with some faint reactivity extending into the cell processes (**d-f**); scale bar= 10 μm

N-acetylcysteine (NAC) acts as a precursor to glutathione, which quenches reactive species; while diphenyleneiodonium chloride (DPI) reportedly inhibits NADPH oxidase, thereby reducing the conversion of O_2 to superoxide ($O_2^{-\bullet}$) (Williams and Griendling 2007).

Glutamate measurements in mixed-glial cultures Glutamate uptake was measured in confluent mixed glial cultures as previously described (Zou et al. 2011b). Briefly, adherent cells were washed once with 0.5 ml/well of 37°C Na^+-free medium containing 10 mM Hepes, 5 mM D-glucose (pH 7.4) and supplemented with 10% fetal bovine serum (FBS). Prior to assay, the cells were pre-incubated for 45–60 min at 37°C in 500 μl of Na^+-free Hank's balanced salt solution (HBSS) alone or in buffer with 500 nM morphine and/or 500 pM $gp120_{MN}$. Glutamate was added to each well to a final concentration of 1.0 mM (Sigma). Sample medium was removed from individual wells at 0, 15, 30, 45, 60, 120 and 240 min. Glutamate concentration was determined using a colorimetric detection kit (BioVision, Mountain View, CA) according to the manufacturer's instructions. Absorbance was assessed at 450 nM on a Victor 3 plate reader (Perkin-Elmer, Waltham, MA).

Statistical analyses Statistical analyses were conducted by repeated measures, one-way, and/or two-way analysis of variance (ANOVA) followed by Duncan's post-hoc testing (StatSoft, Statistica, Tulsa, OK). In the time-lapse microscopy (survival) studies, the advantage of using a repeated measures (block randomized) design is that individual neurons are compared to themselves prior to and during treatment. Since the design eliminates inter-subject variability, it is a fundamentally more sensitive approach to examining cell responses. Comparing proportional changes in the same neuron before and during treatment permits the use of repeated measures ANOVA rather than regular multiway ANOVA, and is more sensitive than statistical assessment across cell populations (Bakalkin et al. 2010; Zou et al. 2011b). For the neuron survival and glutamate determinations, we first examined for main effects of treatment, time, and/or the presence of glia, using a repeated measures ANOVA. When interaction effects were

significant ($p<0.05$), treatment effects and temporal differences were further analyzed by ANOVA and/or Duncan's post hoc testing.

Results

MOR and chemokine receptor immunoreactivity on neurons and glia MOR, CXCR4, and CCR5 immunofluorescence was colocalized in untreated striatal neurons and astrocytes by confocal microscopy to assess the extent to which the principal molecular targets of opioid drugs and gp120 were present and to assess their cellular distribution in each cell type. Receptors were colocalized in neurons (Fig. 1a-c) or astrocytes (Fig. 1d-f), respectively, using antibodies against MAP2 or GFAP; cells were counterstained with Hoechst 33342. In neurons, CXCR4, CCR5, and MOR immunoreactivity was associated with the cell body and dendrites, while CCR5 immunofluorescence often continued into the axolemma (Fig. 1a-c). The proportion of CXCR4 and CCR5 immunoreactive neurons was 69.3±0.7% and 77.4±1.7%, respectively, which also infers that a subset of neurons co-express both chemokine receptors. Astrocytes tended to display punctate patterns of CXCR4, CCR5, and MOR immunolabeling (Fig. 1d-f); faint, diffuse receptor reactivity was occasionally evident at the cell membrane (e.g., CCR5 in Fig. 1e). The proportion of CXCR4 and CCR5 immunofluorescent astrocytes was 45.2±1.6% and 43.8±2.4%, respectively.

Neurotoxicity induced by $gp120_{IIIB}$ and morphine Analyzing the response of individual neurons repeatedly throughout the experiment eliminates inter-subject variability and permits subtle treatment effects to be revealed through a repeated measures ANOVA (Singh et al. 2004; Singh et al. 2005; Bakalkin et al. 2010; Zou et al. 2011b). The use of computer-aided, near-continuous tracking of large numbers of neurons (and glia) greatly facilitates this task, while increasing the sensitivity of the assay (Fig. 2). This strategy has been used by us to examine neuroprotective effects of silencing the phosphatase and tensin homolog on chromosome 10 (PTEN) on HIV-1 $gp120_{IIIB}$-induced neurotoxicity (Zou et al. 2011a), to demonstrate the role of glia in morphine-induced exacerbation of Tat neurotoxicity (Zou et al. 2011b), and to show that mutations in dynorphin A underlie spinocerebellar ataxia type 23 (Bakalkin et al. 2010). In the present work, tracking the responses of individual neurons has revealed sometimes subtle differences in the cytotoxic effects of gp120 from different HIV-1 strains, as well as strain-specific gp120 interactions with morphine.

Not surprisingly, $gp120_{IIIB}$ alone was toxic to striatal neurons (Figs. 2 and 3) as previously published by our lab (Singh et al. 2004; Singh et al. 2005), which is in agreement with its neurotoxic effects in neurons from other brain regions (Meucci and Miller 1996; Digicaylioglu et al. 2004; Kaul et al. 2007; Robinson et al. 2007). Moreover, exposure to $gp120_{IIIB}$ was neurotoxic irrespective of whether glia were present or absent, although there was a slight trend toward greater relative neuron losses (compared to vehicle-treated controls) when neurons were co-cultured with glia. Our analysis also revealed modest background losses of neurons in control cultures treated with vehicle alone during 60 h. When neurons were cultured in isolation, morphine did not increase neuron death beyond levels seen in controls (Fig. 3a), while in the presence of glia, morphine alone did cause significant neuron death (compare Fig. 3b and a). Thus, our findings that morphine and Tat neurotoxic interactions are mediated by glia (Zou et al. 2011b), may also pertain to morphine and gp120, suggesting a broad role for glia in mediating opiate neurotoxicity. However, as this study's aim was to assess strain differences in gp120-morphine interactions, and because morphine did not consistently show interactive neurotoxicity with some gp120 strain variants, the role of glia was not systematically assessed against $gp120_{ADA}$ or $gp120_{MN}$.

When morphine was combined with $gp120_{IIIB}$ in the presence of glia, it potentiated the neurotoxic effects of gp120. This interaction, although highly significant, was transient in nature, consisting of accelerated neuronal losses at 12 h following continuous exposure to both agents. Since neither $gp120_{IIIB}$ nor morphine by itself was toxic at 12 h or 16 h, the interaction was noteworthy. The mechanisms by which morphine exacerbates gp120 neurotoxicity are uncertain, but clearly they reside in either the astroglia, the microglia, or in some interactive crosstalk that occurs between these glial types since exacerbation was not observed when glia were absent (Fig. 3a). The interactive morphine and gp120-induced neurotoxicity was prevented by concurrent treatment with naloxone, suggesting the effect is mediated by opioid receptor-expressing glia (Fig. 3b), while naloxone alone has been previously shown not to effect striatal neuronal survival in this co-culture system (Gurwell et al. 2001; Zou et al. 2011b). Finally, exposure to denatured or deglycosylated gp120 (500 pM; data not shown) was not neurotoxic suggesting the toxic effects of gp120 were selective as previously published (Singh et al. 2004; Singh et al. 2005).

Neurotoxicity induced by $gp120_{ADA}$ and $gp120_{MN}$, and morphine To assess whether opiate-gp120 interactions were influenced by X4 versus R5 tropism, the effects of $gp120_{ADA}$, a R5-tropic strain, and $gp120_{MN}$, a dual-tropic strain, were tested in the co-culture paradigm. Similar to $gp120_{IIIB}$, $gp120_{ADA}$ (Fig. 4) and $gp120_{MN}$ (Fig. 5a) alone were toxic to neurons in the presence of glia, although

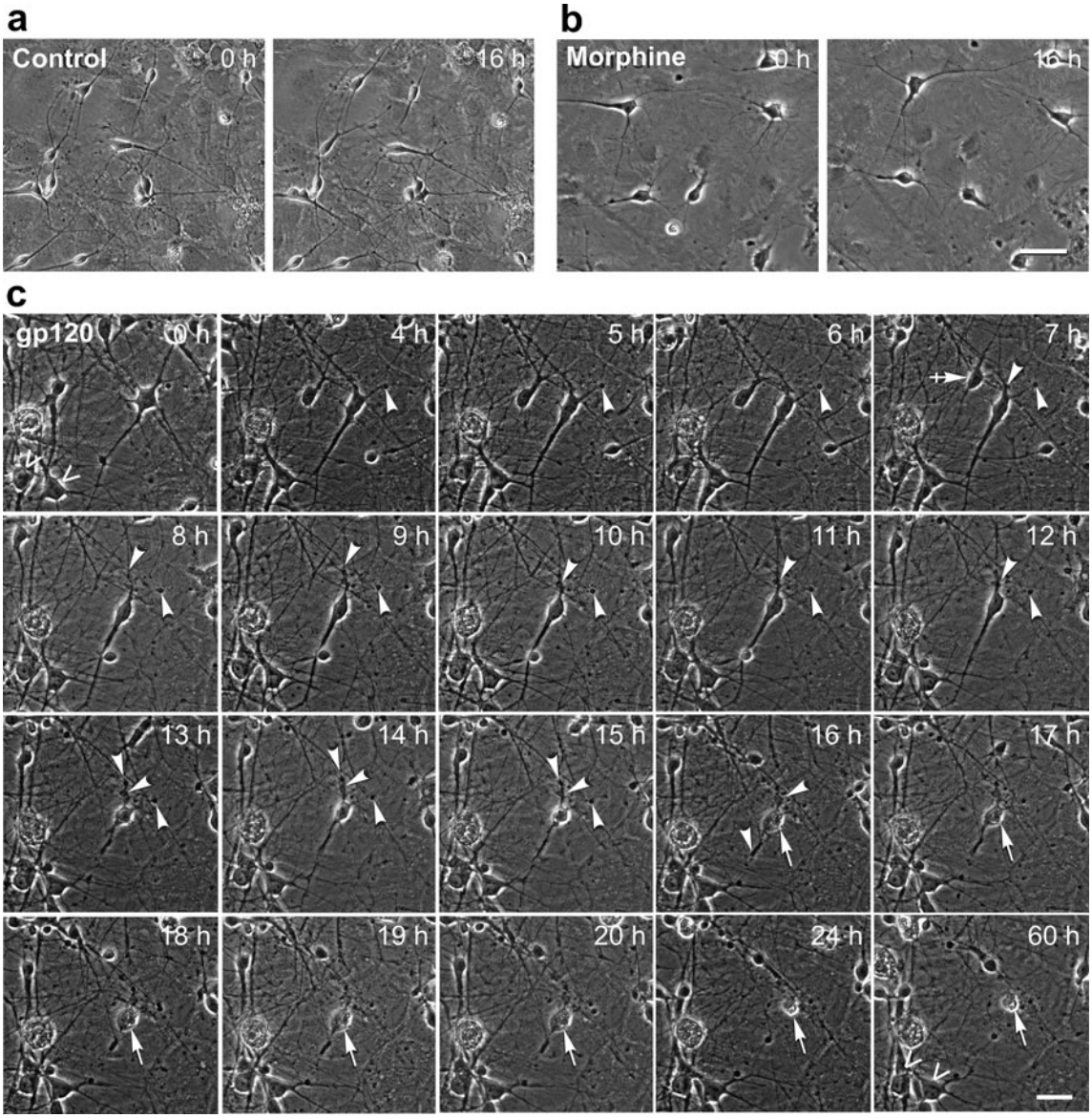


Fig. 2 Phase-contrast images showing time-lapse tracking of neuronal injury and death in the same neurons before and during exposure to vehicle-control medium (**a**), morphine (**b**), or $gp120_{IIIB}$ (**c**). Although there are some small changes in the morphology of cells in (**a**) and (**b**), there is no neuron death over this 16 h time course. A neuron that dies over the same time course during $gp120_{IIIB}$ treatment is followed in (**c**). Neuron death is often preceded by the systematic loss/degeneration of neurites (dendrite pruning; *arrowheads*) over a prolonged period. Death per se occurs more rapidly, as denoted here by a slight swelling and some loss of birefringence (*arrow*; 16 h) and DNA fragmentation thereafter (24 h and 60 h). Neuron death can be confirmed by annexin V reactivity, and the inability to exclude viability markers such as ethidium homodimer, ethidium monoazide, or trypan blue (see text). Some neurons remain viable despite gp120 exposure (*open arrowheads* at 0 h and 60 h) (**c**); some surrounding cells, including astroglia, immature glial precursors and macrophages/microglia, are motile and can display sporadic movement, as indicated by the progenitor which moves to the top of the field between 7 h and 8 h (*hatched arrow*) (**c**). Scale bar=20 μm

$gp120_{ADA}$ was less toxic, displaying significant increases in the rate of neuron death (time x treatment) compared to controls rather than mean differences at any particular time (Fig. 4). In contrast to a very transient exacerbation of $gp120_{IIIB}$ toxicity by morphine, morphine failed in enhance $gp120_{ADA}$-induced cell death (Fig. 4). Interestingly, a significant exacerbation of $gp120_{MN}$ toxicity was apparent at both early and later phases of the experiment (Fig. 5a). Lastly, the effects of morphine were reversed by naloxone (Fig. 5a).

Viral protein effects on glutamate uptake To explore whether morphine and/or gp120 might restrict the ability of astroglia within the mixed-glial cultures to buffer glutamate, an exogenous L-glutamate challenge was applied and residual glutamate was measured in the medium

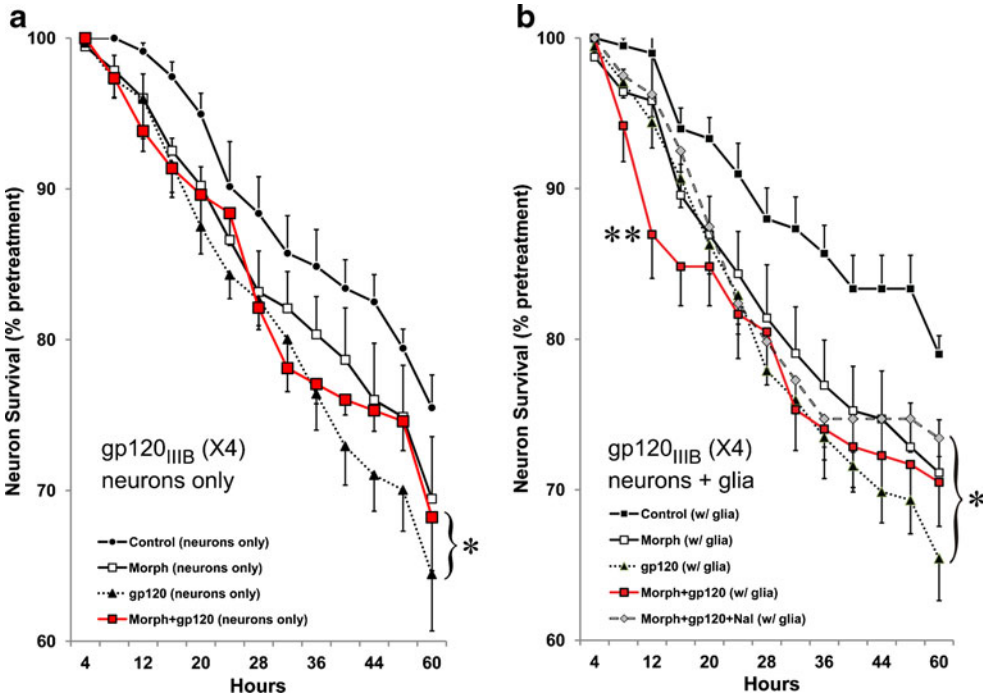


Fig. 3 Time-dependent effects of morphine and/or HIV-1 $gp120_{IIIB}$ on the survival of medium spiny striatal neurons cultured alone (**a**) or with a glial bedlayer (**b**). There were main effects of both time and treatment, as well as a significant interaction effect (time × treatment) (**a**), which were further examined using Duncan's post-hoc analysis. Exposure to $gp120_{IIIB}$ ± morphine was intrinsically neurotoxic, irrespective of whether neurons were cultured ± glia (**a**-**b**) (*$P<0.05$ vs. control) (**a**). Morphine transiently and significantly accelerated $gp120_{IIIB}$ neurotoxicity above control levels, but only when the neurons were co-cultured with glia (**$P<0.05$ vs. control, but not morphine or gp120 treatment, at 12 h; $n=6$ experiments) (**b**). Concurrent exposure to naloxone (Nal) negated the combined neurotoxic effects of morphine and $gp120_{IIIB}$ at 12 h (**b**). Interestingly, morphine alone displayed modest, but significant neurotoxicity, but again, only in the presence of high-density glia (*$P<0.05$ vs. control) (**b**). Neuron death is reported as the percentage dying relative to pretreatment numbers; data are the mean number of surviving neurons ± SEM from $n=4$–6 experiments

over 240 min (Fig. 5b). $Gp120_{MN}$ showed the greatest neurotoxic interaction with morphine, and was therefore anticipated to interact with morphine to affect extracellular glutamate. Extracellular glutamate was rapidly depleted during the first 45 min in untreated controls, but glutamate buffering was significantly attenuated in morphine, gp120, or morphine plus gp120-treated mixed-glial cultures at 45 min, or gp120 and/or morphine plus gp120 exposed cultures thereafter (Fig. 5b). Note that in a previous study two different inhibitors of the major glial EAAT1 and EAAT2 transporters completely blocked glutamate uptake (Zou et al. 2011b). Moreover, in the presence of the EAAT inhibitors, glutamate levels remained at pretreatment levels with no measurable glutamate release over 240 min irrespective of Tat and morphine exposure (Zou et al. 2011b). This suggests that net levels of glutamate in the medium in the present study reflect morphine and gp120-induced changes in glutamate transporter function and not glutamate release.

Effects of morphine and $gp120_{IIIB}$ on astroglial ROS The effects of gp120 from different HIV-1 strains on ROS have not been systematically explored in our neuron mixed-glial co-culture system. Moreover, differences in gp120 neurotoxicity provided impetus to further evaluate the relationship between gp120 concentration, viral strain, and ROS (Fig. 6a-c). Morphine has previously been shown to cause concentration-dependent increases in ROS in an identical mixed-glial striatal culture model as used in the present study (Zou et al. 2011b) and was not reassessed. In all instances, gp120 caused significant, concentration-dependent increases in DCF fluorescence; however, the extent of the increase differed among gp120 variants among HIV-1 strains (Fig. 6a-c). The EC_{50} values for X4 $gp120_{IIIB}$, R5 $gp120_{ADA}$, and bitropic $gp120_{MN}$ were 8.4 nM, 0.54 nM, and 0.07 nM, respectively. Interestingly, the increases in ROS caused by a particular gp120 variant did not correlate well with neurotoxicity or a propensity for the variant to interact with morphine.

Exposing mixed-glial cultures to morphine and/or HIV-1 $gp120_{IIIB}$ significantly elevated ROS production as determined by DCF (Fig. 6d). Combined morphine plus gp120 failed to elevate ROS beyond levels seen with either substance alone. The ROS inhibitors NAC and DPI were used to confirm experimentally that morphine and/or HIV-1 gp120-induced increases in ROS were driving DCF

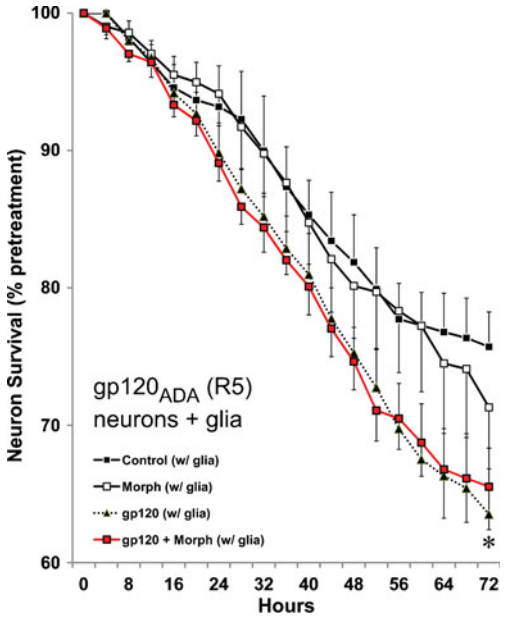


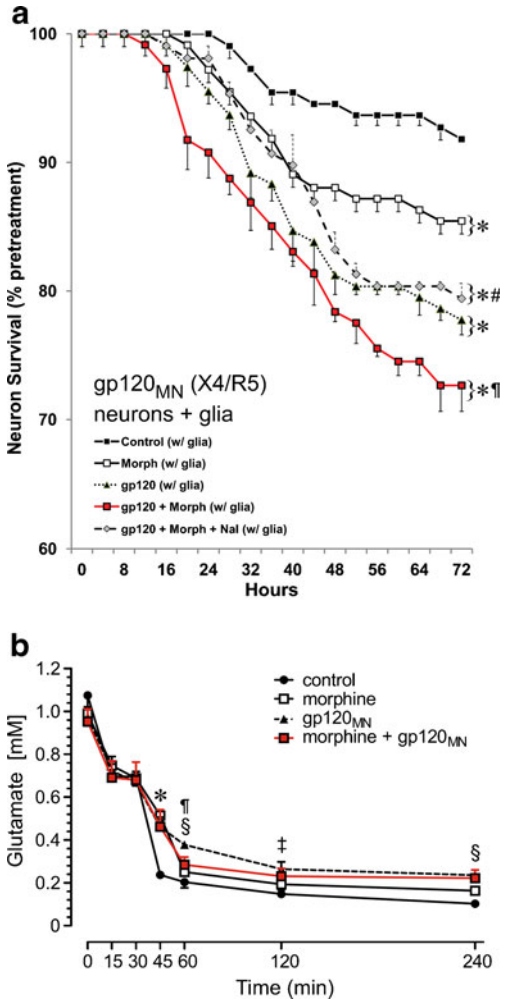
Fig. 4 Time-dependent effects of exposure to morphine (Morph) and/or R5-tropic HIV-1 $gp120_{ADA}$ in neuron and mixed-glial co-cultures for 72 h. Analyses for R5-tropic $gp120_{ADA}$ showed a main effect for time, but not for treatment (**a**). Although there was a significant interaction effect (time × treatment), Duncan's post-hoc testing did not reveal significance at particular times. When the analysis was simplified by eliminating the morphine group, there was decline in survival for R5-tropic $gp120_{ADA}$ exposed neurons at the last time point of assessment (*P<0.05 vs. control at 72 h) (**a**). Otherwise, values for $gp120_{ADA}$ treatment alone vs. control (e.g., P=0.056 at 68 h) or $gp120_{ADA}$ + morphine (e.g., P=0.054 at 56 h) were close, but not significant; data are the mean number of surviving neurons ± SEM from n=4–6 experiments

Fig. 5 Time-dependent effects of exposure to morphine (Morph), X4/R5-tropic HIV-1 $gp120_{MN}$ and/or naloxone (Nal) in neuron and mixed-glial co-cultures for 72 h (**a**). There were significant time and treatment effects for dual-tropic $gp120_{MN}$, as well as a significant interaction effect. Post-hoc analyses showed that all treatment groups were different from control values starting at 20 h following continuous exposure, except for morphine whose effects began at 40 h (*P<0.05 vs. control). Additionally, morphine co-treatment enhanced the effects of $gp120_{MN}$ at both early time points (20–28 h), and at 56 h and thereafter ($^{¶}P\leq0.05$), and the effects of morphine were prevented by naloxone (Nal) ($^{\#}P\leq0.05$ vs. morphine + $gp120_{MN}$; *P<0.05 vs. control). Unlike $gp120_{ADA}$, co-administering morphine exacerbated the neurotoxicity of $gp120_{MN}$ suggesting that morphine interacts differently with gp120 variants from different HIV-1 strains. Data are the mean number of surviving neurons ± SEM from n=4–6 experiments (**a**). Effects of morphine (500 nM) and/or dual-tropic $gp120_{MN}$ (500 pM) on glutamate buffering (**b**). Mixed-glial cultures were challenged with 1 mM glutamate and the levels were assessed at 0, 15, 30, 45, 60, 120 and 240 min. Exogenously administered glutamate was rapidly depleted during the first 60 min and continued to slowly decrease thereafter in untreated controls. $gp120_{MN}$ treated cultures ± morphine, demonstrated a significant deficit in the ability to uptake glutamate beginning at 45 min which persisted throughout the experiment. Values indicate the amount of glutamate (mM) remaining in the media over the 240 min time course ± SEM of n=3 experiments (*P<0.05 vs. control; $^{§}P$<0.05 control vs. gp120 alone or morphine + gp120; $^{¶}P$<0.05 gp120 vs. morphine + gp120; $^{‡}P$<0.05 control vs. gp120 alone) (**b**)

fluorescence, rather than auto-oxidation of the probe (Bonini et al. 2006) (Fig. 6d). Pretreatment with NAC eliminated gp120 -induced increases in ROS production. Pretreatment with DPI also eliminated or significantly reduced ROS production, suggesting a specific role for NADPH oxidase in the production of ROS caused by morphine and $gp120_{IIIB}$ in mixed glia.

Discussion

The results provide the first direct evidence suggesting that the neurotoxic interactions of morphine and gp120 differ among HIV-1 strains. The findings additionally suggest that the timing, extent, and pattern of the morphine and gp120 insults are likely to be critical in determining the neurotoxic outcome. Lastly, our results continue to support the notion that opioids acting via MOR-expressing glia per se mediate key aspects of the neurotoxic effects of opioids alone or in combination with gp120, a concept that has gained considerable support from our recent work examining morphine and HIV-1 Tat coexposure (Zou et al. 2011b) Fig. 3. While the nature of the opioid-induced toxic signal (s) is uncertain, clearly some aspects of opioid drug actions are not due to a direct effect on neurons, but instead originate from glia. The notion of opioid actions in astroglia (Shinoda et al. 1989; Hauser et al. 1990; Stiene-Martin and Hauser 1991; Stiene-Martin et al. 1993; Hauser et al. 1996; Stiene-Martin et al. 1998) and microglia (Chao et al. 1994; Peterson et al. 1995; Chao et al. 1996; Turchan-Cholewo et al. 2008) or monocytic precursors (Przewlocki et al. 1992; Gaveriaux et al. 1995; Grimm et al. 1998; Rogers et al. 2000) alone or in relation to HIV-1 is not novel (Hauser et al. 2007). Indeed, it has long-been suggested that opioids modify CNS plasticity or disease through actions in astroglia (Ronnback and Hansson 1988; Stiene-Martin and Hauser 1990; Stiene-Martin and Hauser 1991; Deleo et al. 2004; Hauser et al. 2005; Narita et al. 2006; Hansson 2006; Kim et al. 2006; Hauser et al. 2007; Hutchinson et al. 2011). However, it has been quite difficult to separate, especially in vivo, the actions of opioids on glia from their effects on neurons, since all neural cell types examined thus far can express functional opioid receptors including MOR (Stiene-Martin et al. 1991; Eriksson et al. 1991; Bem et al. 1991; Stiene-Martin and Hauser 1991; Barg et al. 1992; Hauser et al. 1996; Stiene-Martin et al. 1998; Knapp et al. 1998; Miyatake et al. 2009). These cell types include neurons, astroglia, microglia, oligodendroglia, and glial progenitors (Hauser et al. 2009; Hahn et al. 2010), as well as support cells within the CNS such as endothelial cells and the connective tissue stroma surrounding large blood



vessels, which can also express opioid receptors (see Hauser et al. 2007).

MOR and CXCR4 or CCR5 may interact at multiple levels to modify HIV-1 pathogenesis. In both human and murine models of HIV-1 encephalitis (HIVE), the neurotoxicity driven by opioids and/or gp120 appears to be mediated through the differential activation of death-promoting mixed-lineage kinases, including p38 MAPK (Yi et al. 2004; Hu et al. 2005; Singh et al. 2005; Wan et al. 2006; Kaul et al. 2007) and perhaps other cell death (Lannuzel et al. 1997; Yi et al. 2004; Bodner et al. 2004; Singh et al. 2005) or interference with pro-survival pathways (Polakiewicz et al. 1998; Kolson 2002; Kaul et al. 2007) and ERK (Belcheva et al. 2005; Miyatake et al. 2009). Combined exposure to morphine and gp120 exacerbates apoptosis in both human peripheral blood mononuclear cells and neurons through mechanisms that likely involve β-arrestin 2 (Moorman et al. 2009) and a p38 MAPK-dependent pathway (Hu et al. 2005), respectively. Gp120, for example, has been shown to upregulate MOR in HL-60 human promyelocytic leukemia cells (Beltran et al. 2006); while morphine can increase CCR5 and CCR3 expression in U373 astrocytoma cells (Mahajan et al. 2005). This phenomenon is not restricted to gp120, as we have shown that HIV-1 Tat expression in vivo dysregulates opioid receptors, including MOR, in a regional and cell specific manner (Fitting et al. 2010). Bidirectional heterologous interactions between G-protein coupled chemokine receptors including CXCR4 or CCR5, and opioid receptors, including MOR (Rogers and Peterson 2003; Chen et al. 2004; Finley et al. 2008), are also possible. Evidence for CXCR4-MOR heterodimers exists, although the functional significance of such direct molecular interactions is unclear (Toth et al. 2004).

Morphine alone caused modest but sustained toxicity in neurons co-cultured with glia (Fig. 3), which has been previously reported by other investigators (Hu et al. 2002; Malik et al. 2011). Interestingly, morphine appeared to interact differently with gp120 from each viral strain. Morphine transiently accelerated the neurotoxic effects of X4 $gp120_{IIIB}$, had no affect on R5 $gp120_{ADA}$-induced neuron death, but caused a more sustained enhancement of X4/R5 $gp120_{MN}$ cytotoxicity. Such findings suggest that the timing and/or duration of the opioid insult, as well as strain of HIV-1 gp120 involved, may be critical in determining the extent and pattern of neuron loss. Moreover, similar proportions of striatal neurons (and astrocytes) express both chemokine receptors, suggesting the expression of CXCR4 versus CCR5 by a disproportionate number of cells does not underlie differences in neurotoxicity. There was no clear-cut relationship between X4/R5 tropism and morphine interfacing, although further study is needed to address this issue fully. Morphine increases expression of intracellular ferritin-heavy chain, which attenuates protective CXCR4-signals in neurons (Sengupta et al. 2009); however, this mechanism is less likely to be operative in the present study since morphine had minimal interactions with X4 $gp120_{IIIB}$. Although it is uncertain how morphine transiently increases gp120 neurotoxicity in the present study, the temporal selectivity and likely dependence on glia might offer some clues as to possible mechanisms and might partially explain why interactive morphine and gp120 neurotoxicity has not been consistently reported in past studies (Hu et al. 2005; Turchan-Cholewo et al. 2006). It is well established that key aspects of gp120 neurotoxicity can be mediated through microglia (Lipton et al. 1994; Kaul et al. 2001; Gonzalez-Scarano and Martin-Garcia 2005; Kaul et al. 2007) by activation of either CXCR4 (Meucci et al. 1998; Zheng et al. 1999; Kaul and Lipton 1999; Kaul et al. 2007), or CCR5 (Kaul et al. 2007). Gp120 can also affect astroglia and neurons directly through CD4-independent mechanisms, and X4 and R5 gp120 HIV-1 strain variants differ in their neurotoxic interactions among cells (Koller et al. 2002; Khan et al. 2004; Kaul et al. 2007; Medders et al. 2010; Bachis et al. 2010).

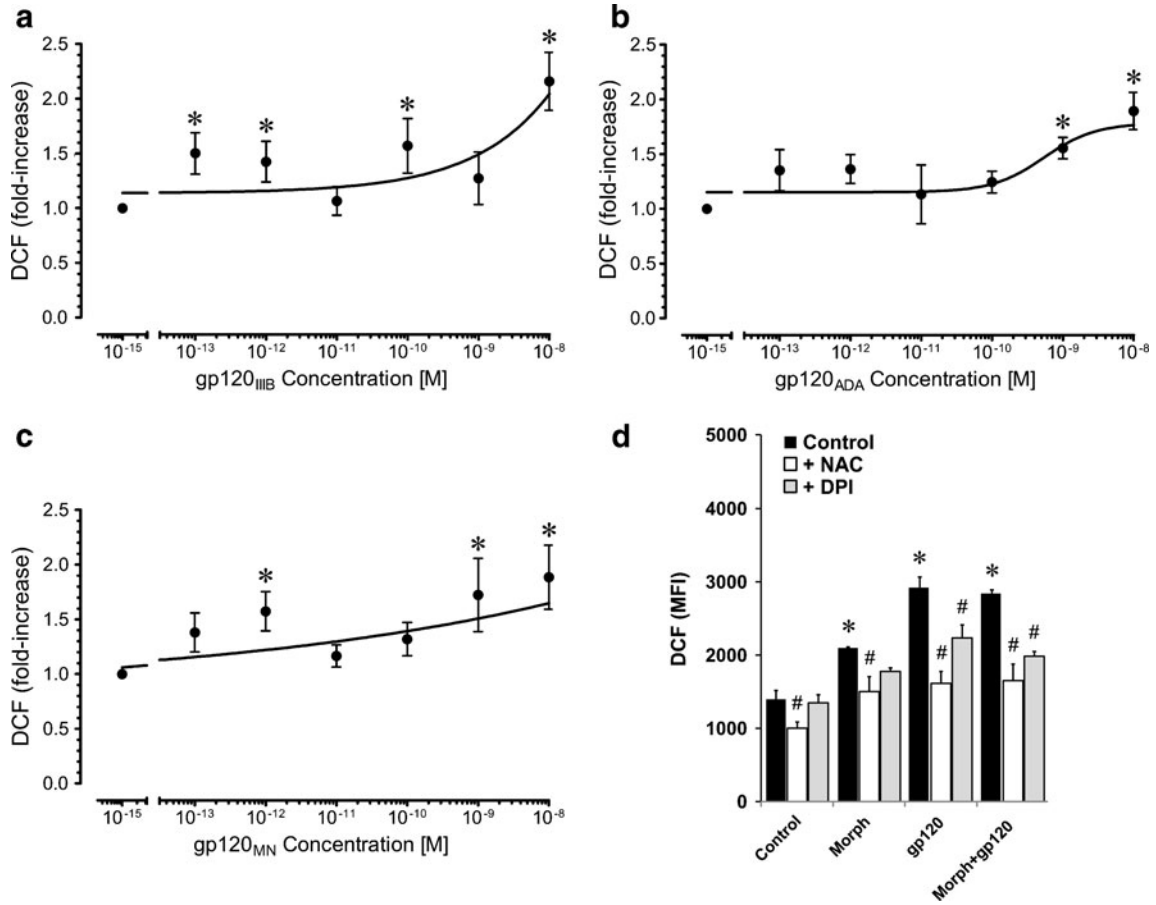


Fig. 6 Effects of gp120 and morphine on ROS production in mixed-glia assessed by DCF fluorescence. A concentration-effect curve revealed significant increases in DCF relative fluorescence following 45 min exposure to $gp120_{IIIB}$ with an $EC_{50}=8.4\times10^{-9}$ (*$P<0.05$) (**a**). Similar concentration-effect curves for $gp120_{ADA}$ (**b**) and $gp120_{MN}$ (**c**) also show ROS elevation, but with somewhat different kinetics. $Gp120_{IIIB}$ ± morphine significantly increased ROS formation in mixed-glial cultures but without any additive or synergistic effect (**d**). Cells were pretreated with vehicle, NAC or DPI for 20 min before incubation with DCF-DA and subsequently treated with $gp120_{IIIB}$ (500 pM) and/or morphine (500 nM) for 40 min prior to assay. Mean DCF relative fluorescence units (MFI) were used as an estimate of ROS and were compared for each treatment. $Gp120_{IIIB}$ and morphine both markedly increased ROS production (*$P<0.05$ vs. control). NAC and/or DPI significantly decreased $gp120_{IIIB}$ and/or morphine-induced ROS production ($^{\#}P<0.05$ vs. treatment with gp120 and/or morphine alone); data are the mean ± SEM of $n=3$ experiments (**d**)

The modest neurotoxicity seen with morphine was not seen in our initial studies of mixed striatal neuron-glia cultures following exposure for 16 h (Gurwell et al. 2001). However, our current time-lapse, repeated measures assessments are made over an expanded time course, and are more sensitive to subtle treatment effects than previous strategies (Bakalkin et al. 2010; Zou et al. 2011b). Opioids have diverse actions that are contextual (Akil et al. 1984; Carr and Serou 1995; Hauser and Mangoura 1998). Despite many studies acknowledging cytoprotective actions, there are nevertheless numerous reports that MOR agonists, including morphine, can intrinsically promote regulated cell death (Fuchs and Pruett 1993; Maneckjee and Minna 1994; Nair et al. 1997; Yin et al. 1997; Goswami et al. 1998; Singhal et al. 1999; Malik et al. 2002; Hu et al. 2002; Madden et al. 2002; Kapasi et al. 2004; Tegeder and Geisslinger 2004). We also previously noted a modest neurotoxicity due to morphine alone in a time-lapse study designed to examine Tat-opiate comorbidity (Zou et al. 2011b). More recently, Alzheimer's-like hyperphosphorylation of Tau and correlative increases in microglial activation have been reported in the brains of chronic opioid drug abusers without detectable co-infection or other disease comorbidity (Anthony et al. 2010).

Exposure to HIV-1 virus or gp120 (Wang et al. 2003), as well as morphine (Zou et al. 2011b), can inhibit glutamate uptake by astrocytes or mixed-glia, respectively. Conversely, CXCL12 (SDF-1α), the principal endogenous CXCR4 agonist, enhances glutamate release from human and murine astrocytes (reviewed by Cali and Bezzi 2010). Together, these findings prompted an assessment of the effects of gp120 ± morphine on the time-dependent uptake of glutamate from mixed-glial cultures. The ability of morphine to exacerbate Tat-induced glutamate release from

microglia (Gupta et al. 2010) provided further impetus. Although the present studies do not discern glutamate uptake from release, a previous study using this culture model revealed deficits in recovery from a glutamate challenge that were largely due to reduced uptake (Zou et al. 2011b). However, despite the ability of $gp120_{MN}$ and to a lesser extent morphine, to inhibit glutamate buffering by mixed-glia, the lack of significant $gp120_{MN}$-morphine interactions suggests that the enhanced neuron losses are not caused by excessive extracellular glutamate. Alternatively, it is possible that gp120 variants from other HIV-1 strains might interact with morphine to affect glutamate buffering differently compared to $gp120_{MN}$.

ROS increases are an important proinflammatory trigger and evident in a variety of cell types. We questioned whether morphine and/or $gp120_{IIIB}$-induced increases in ROS in mixed-glia might contribute to the pro-inflammatory response (Mahajan et al. 2005). Because of reported differences in the neurotoxic effects of X4 versus R5 gp120 (Kaul et al. 2007; Medders et al. 2010; Bachis et al. 2010), and our present findings, we initially questioned whether the individual gp120 variants might differentially trigger ROS. Interestingly, despite some differences in the EC_{50} values, the maximum ROS response was only increased about 2-fold above untreated control values, was comparable among gp120 variants throughout the concentration-effect curve, and showed considerable variability. Similarly, at the 500 pM concentration used to examine neurotoxicity, $gp120_{IIIB}$, $gp120_{ADA}$, or $gp120_{MN}$ caused equivalent increases in ROS, suggesting that it would be difficult to differentiate among gp120 effects using ROS as an outcome measure. Next, to see whether morphine might exacerbate gp120-induced increases in ROS, we examined interactions with $gp120_{IIIB}$, which showed the greatest relative increase in ROS. Despite a trend toward a near-additive increase, co-administration of morphine and $gp120_{IIIB}$ failed to cause even an additive increase in ROS, suggesting that increases in ROS are unlikely to play a significant role in regulating opioid drug actions in HIV-1 gp120-exposed glia. Importantly, unlike gp120, morphine + Tat show interactive increases in ROS (Zou et al. 2011b), indicating fundamental differences in the interactions of morphine with different HIV proteins in terms of ROS production. There may also be interactive effects of gp120 and morphine on ROS signaling in each of the individual cell types that are diluted in the present co-culture system. Lastly, while ROS is certainly an important component of glial inflammation, there are many other intracellular events, such as signaling through $[Ca^{2+}]_i$ (El-Hage et al. 2005; El-Hage et al. 2008a), and bioactive intercellular compounds with potential neurotoxic effects that might be released by interactions between gp120 and morphine.

Finally, integrating prior examinations of morphine and HIV-1 Tat interactions (Nath et al. 2002; Hauser et al. 2005; Hauser et al. 2007; Zou et al. 2011a; Zou et al. 2011b), and the present findings regarding morphine-gp120 interactive neurotoxicity, several conclusions can be drawn. First, that there is considerable selectivity in morphine's actions. Secondly, morphine interacts uniquely with each viral component resulting in distinct pathophysiologic consequences for the host. Lastly, the unique interactions with morphine extend not only to the level of the particular viral protein involved, but also to differences among viral strains, as is illustrated with gp120 in the present study. Moreover, an alternative outcome measure besides neurotoxicity, or varying the pharmacological parameters of morphine exposure or withdrawal (e.g., Bandaru et al. 2011), might reveal a different pattern of gp120-opiate drug interactions. Together with previous findings from our lab and others, the results underscore the complexity of interactions between opiate drug abuse and neuroAIDS, and the need for systematic approaches for understanding regional, temporal, cell-specific, and genotypic differences in opioid drug-HIV-1 interactions in CNS.

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