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GSK3 β -Activation is a Point of Convergence for HIV-1 and Opiate-Mediated Interactive Neurotoxicity

Ruturaj R. Masvekar^a, Nazira El-Hage^b, Kurt F. Hauser^{b,c}, and Pamela E. Knapp^{a,b,c,*}

^aDepartment of Anatomy and Neurobiology, Virginia Commonwealth University, Richmond, VA 23298, USA

^bDepartment of Pharmacology and Toxicology, Virginia Commonwealth University, Richmond, VA 23298, USA

^cInstitute for Drug and Alcohol Studies, Virginia Commonwealth University, Richmond, VA 23298, USA

Abstract

Infection of the CNS with HIV-1 occurs rapidly after primary peripheral infection. HIV-1 can induce a wide range of neurological deficits, collectively known as HIV-1-associated neurocognitive disorders. Our previous work has shown that the selected neurotoxic effects induced by individual viral proteins, Tat and gp120, and by HIV⁺ supernatant are enhanced by co-exposure to morphine. This mimics co-morbid neurological effects observed in opiate-abusing HIV⁺ patients. Although there is a correlation between opiate drug abuse and progression of HIV-1-associated neurocognitive disorders, the mechanisms underlie interactions between HIV-1 and opiates remain obscure. Previous studies have shown that HIV-1 induces neurotoxic effects through abnormal activation of GSK3 β . Interestingly, expression of GSK3 β has shown to be elevated in brains of young opiate abusers indicating that GSK3 β is also linked to neuropathology seen with opiate abusing patients. Thus, we hypothesize that GSK3 β activation is a point of convergence for HIV- and opiate-mediated interactive neurotoxic effects. Neuronal cultures were treated with supernatant from HIV-1_{SF162}-infected THP-1 cells, in the presence or absence of morphine and GSK3 β inhibitors. Our results show that GSK3 β inhibitors, including valproate and small molecule inhibitors, significantly reduce HIV-1-mediated neurotoxic outcomes, and also negate interactions with morphine that result in cell death, suggesting that GSK3 β -activation is an important point of convergence and a potential therapeutic target for HIV- and opiate-mediated neurocognitive deficits.

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*Corresponding author: Pamela E. Knapp, Department of Anatomy and Neurobiology, P.O. Box: 980709, Virginia Commonwealth University, Richmond, VA 23298, USA., Tel: +1 804 628 7570; Fax: +1 804 827 9974, peknapp@vcu.edu.
N. El-Hage present address: Department of Immunology, Florida International University, Miami, FL 33199, USA

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Keywords

NeuroAIDS; HIV-1; morphine; neurodegeneration; synaptodendritic injury; GSK3 β

INTRODUCTION

Infection of the central nervous system (CNS) with human immunodeficiency virus-1 (HIV-1) occurs rapidly after primary peripheral infection (An et al., 1996; An et al., 1999b; Davis et al., 1992; Kramer-Hammerle et al., 2005). HIV-1 can induce a wide range of CNS deficits, collectively known as HIV-1-associated neurocognitive disorders (HAND); nearly 50% of HIV-1-infected individuals suffer from HAND (Fischer-Smith and Rappaport, 2005; McArthur, 2004; McArthur et al., 2005; McArthur et al., 2003). Infected and/or activated glial and immune cells in the CNS release various viral and cellular factors that drive direct and indirect neuronal toxicity, leading to HAND (Buescher et al., 2007; Epstein and Gendelman, 1993; Gonzalez-Scarano and Martin-Garcia, 2005; Kaul et al., 2005; Kramer-Hammerle et al., 2005). The advent of combination antiretroviral therapy (cART) has reduced the severity of HAND, but the disease prevalence remains the same (Cysique and Brew, 2009; Cysique et al., 2004; Ellis et al., 2007; Robertson et al., 2007; Robinson-Papp et al., 2009; Sacktor, 2002; Sacktor et al., 2002), likely due to longer patient survival (Dore et al., 2003) and the relatively poor CNS penetrance of many antiretroviral drugs (Ellis et al., 2007; Kerza-Kwiatecki and Amini, 1999).

Drug abuse is a major risk factor for HIV infection; nearly 30% of HIV⁺ patients have a history of drug abuse involving opiates (Bokhari et al., 2009; Robertson et al., 1994). A large fraction of HIV⁺ patients are also exposed to opiates for treatment of AIDS-related chronic pain syndromes. Opiate drugs of abuse modulate immune function (Novick et al., 1989; Rogers and Peterson, 2003; Sheng et al., 1997), and also promote HIV-1 replication *in vitro* (Peterson et al., 2004; Peterson et al., 1990). Since opiates by themselves promote outcomes involved in CNS dysfunction, such as blood-brain barrier breakdown, immune and glial cell activation, and neuronal damage (Bell et al., 2006; Hauser et al., 2005; Hu et al., 2002; Sheng et al., 1997), it can be predicted that opiate drug abuse might exacerbate HIV-1 pathogenesis in the CNS. Our previous work has shown that certain neurotoxic effects induced by the individual HIV-1 proteins, trans-activator of transcription (Tat) and glycoprotein 120 (gp120) (Fitting et al., 2010a; Gurwell et al., 2001; Podhaizer et al., 2012; Suzuki et al., 2011; Zou et al., 2011b), and by HIV⁺ supernatant (HIV⁺_{sup}) (Masvekar et al., 2014), are enhanced by co-exposure to morphine, the major metabolite of heroin in the CNS (Sawynok, 1986). This mimics co-morbid neurological effects observed in opiate-abusing HIV⁺ patients (Anthony et al., 2008; Bell et al., 2002; Byrd et al., 2011; Meijerink et al., 2014; Robinson-Papp et al., 2012; Smith et al., 2014). Although there is a correlation between opiate drug abuse and HAND progression, the mechanisms that underlie interactions between HIV-1 and opiates remain obscure; the main aim of this work was to identify point(s) of convergence for HIV-1 and morphine signaling in neurons.

Identified originally as a regulator of glycogen metabolism, glycogen synthase kinase-3 β (GSK3 β) is a central component of various signaling pathways in neurons, including those

affecting neuronal plasticity, gene expression, and cell survival (Frame and Cohen, 2001; Grimes and Jope, 2001; Jacobs et al., 2012). GSK3 β activity appears to be dysregulated in multiple neuropathological conditions, including Alzheimer's disease, Parkinson's disease, schizophrenia, autism, and bipolar mood disorder, and pharmacological inhibition of GSK3 β is effective against some symptomatology in these diseases (Emamian et al., 2004; Frame and Cohen, 2001; Haenisch et al., 2014; Jacobs et al., 2012; Kaytor and Orr, 2002; Koistinaho et al., 2011; Kozikowski et al., 2006; Leroy et al., 2007; Schaffer et al., 2008). In previous studies, HIV-1 neurotoxicity was linked to abnormal activation of GSK3 β (Crews et al., 2009; Dou et al., 2003; Dou et al., 2005; Everall et al., 2002; Maggirwar et al., 1999; Sui et al., 2006), GSK3 β has also been linked to neuropathology seen in opiate-abusing patients (Anthony et al., 2010; Ramage et al., 2005). We therefore tested GSK3 β activation as a point of convergent signaling for interaction between HIV-1 and morphine.

Both lethal and sublethal effects of HIV⁺_{sup} $\pm$ morphine treatments were assessed on neuron populations, and also by time-lapse imaging of individual cells over 72 h. Valproic acid and small molecule GSK3 β inhibitors significantly reduced HIV⁺_{sup}-mediated neurotoxic outcomes. Interactions between HIV and morphine that resulted in neuronal death were also abrogated, implicating GSK3 β as a mediator of specific neurotoxic events initiated by combined exposure to HIV-1 and opiates.

MATERIALS and METHODS

All experimental procedures were reviewed and approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee.

Neuron cultures

As the striatum is a principal, sub-cortical target of HIV-1, and as levels of opioid receptors in the striatum are relatively high (Arvidsson et al., 1995; Berger and Arendt, 2000; Berger and Nath, 1997), it is a region in which HIV-opiate interactions are likely to occur. Thus, our culture model system primarily used murine striatal neurons. We also tested effects of HIV⁺_{sup} and morphine on cortical and hippocampal neurons. Neurons were cultured as previously described (Masvekar et al., 2014; Podhaizer et al., 2012; Zou et al., 2011a; Zou et al., 2011b). In brief, striata or cortices from E15–E16 or hippocampi from P0–P1 ICR (CD-1) mice (Charles River Laboratories International, Inc., Wilmington, MA) were dissected, minced and incubated with trypsin (2.5 mg/ml; Sigma-Aldrich, St. Louis, MO) and deoxyribonuclease (DNase; 0.015 mg/ml; Sigma-Aldrich) in neurobasal medium (Gibco, Grand Island, NY) for 30 min at 37°C. Tissue was resuspended in neurobasal medium supplemented with B-27 additives (Gibco), L-glutamine (0.5 mM; Gibco), glutamate (25 μ M; Sigma-Aldrich) and Antibiotic-Antimycotic (Gibco), triturated, and filtered twice through 70 μ m pore, nylon mesh filters (BD Biosciences, San Jose, CA). Cells were seeded into culture plates (Corning Inc., Corning, NY) pre-coated with poly-L-lysine (0.5 mg/ml; Sigma-Aldrich), in supplemented neurobasal medium. Purity was determined by immunostaining for microtubule-associated protein 2 (MAP-2, a dendrite marker; Abcam, Cambridge, MA; ab32454), and cultures were found to be > 80% neurons.

HIV-1 supernatant

Cells of the acute monocytic leukemia cell line, THP1 (ATCC, Manassas, VA), were cultured at 0.5×10^5 cells/ml in RPMI-1640 medium (Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco) and 100 U/ml Penicillin-Streptomycin (Gibco), and stimulated with interleukin-2 (IL-2; 100 ng/ml; Sigma-Aldrich) and phytohaemagglutinin (PHA; 5 μ g/ml; Sigma-Aldrich), for 48 h. Stimulated cells were treated with polybrene (2 μ g/ml; Sigma-Aldrich) for 30 min at 37°C, resuspended in fresh medium, and exposed to HIV-1_{SF162} (p24 = 50 pg/ml; a R5-tropic HIV-1 strain; from Dr. Jay Levy (Cheng-Mayer and Levy, 1988), through NIH AIDS Research and Reference Reagent Program, Germantown, MD). After 5 d, supernatants (HIV⁺_{sup}) were collected by filtering through a 0.20- μ m filter, and viral infection was confirmed by quantifying HIV-p24 levels using ELISA (Advanced Bioscience Laboratories, Rockville, MD), typically in 5 d a 3–5 fold increase in HIV-p24 levels were observed. Supernatant from untreated/uninfected THP1 cells (Control_{sup}) was used as a control. Cell culture supernatants were aliquoted and stored at –80°C. To minimize variability, the same supernatant was used for all the experiments.

Treatments

To avoid the potential confound of morphine increasing HIV replication (Peterson et al., 2004; Peterson et al., 1993; Peterson et al., 1990), morphine was only added to cell-free supernatants. Our previous studies showed that supernatants from HIV-infected cells induce neurotoxic effects in a concentration-dependent manner over a range of p24 levels (10–500 pg/ml), but the interactions with morphine were significant only at lower HIV-exposure levels (p24 = 10 and 25 pg/ml) (Masvekar et al., 2014). Thus, current studies used an HIV⁺_{sup} exposure level of p24 = 25 pg/ml. HIV⁺_{sup} and Control_{sup} were diluted similarly and added to neuronal cultures in the presence or absence of morphine (morphine sulfate; Sigma-Aldrich), at a dose that maximally stimulates neuronal and glial μ -opioid receptors (MORs) *in vitro* (500 nM) (Bruce-Keller et al., 2008; El-Hage et al., 2005; Gurwell et al., 2001; Ikeda et al., 2010; Masvekar et al., 2014; Miyatake et al., 2009; Podhaizer et al., 2012; Zou et al., 2011b). To determine the role of GSK3 β in HIV- and opiate-mediated neurotoxicity, HIV⁺_{sup} $\pm$ morphine treatments were conducted in the presence of a standard GSK3 β inhibitor, valproate (valproic acid, VPA; 1 mM; Sigma-Aldrich), or in the presence of two very selective, small molecule inhibitors, SB415286 (10 μ M; Sigma-Aldrich; a competitive inhibitor of the ATP binding site on GSK3 β and GSK3 α with an IC₅₀ value of 78 nM (Coghlan et al., 2000)) or GSK3 β inhibitor XXVI (XXVI; 1 μ M; EMD Millipore, Billerica, MA; a cell-permeable, pyrazolone GSK3 β inhibitor with an IC₅₀ value of 34 nM).

TUNEL assay

Cells were fixed in 4% paraformaldehyde (Sigma-Aldrich) overnight at 4°C, permeabilized in 0.1% Triton-X 100 (Molecular Probes) and 0.1% BSA (Invitrogen, Grand Island, NY) for 15 min, blocked in 0.1% BSA (Invitrogen, Grand Island, NY) and 1% horse serum (Invitrogen) for 30 min. Cells were subsequently labeled with Hoechst 33342 (Sigma-Aldrich) and TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling; Roche Applied Sciences, Mannheim, Germany). Cells were visualized and digital images were acquired using the Axio Observer Z.1 microscope and Zen 2010 software (Zeiss Inc.,

Thornwood, NY). Neuronal apoptosis was assessed by manually counting the percentage of TUNEL(+) cells.

Assessment of neuron viability

Time-lapse digital images of pre-selected neurons were captured at 1 h intervals for 72 h after treatments using a microscope with a computer-regulated stage (Axio Vision 4.6; Carl Zeiss Inc.) and in a controlled environmental chamber (37°C, 95% humidity, 5% CO₂). In digital images, neurons were assessed for viability at 6 h intervals using rigorous morphological criteria confirming cell death, including cytoplasmic swelling, nuclear destruction, cell body fragmentation, excessive neurite degeneration, and loss of phase brightness (Masvekar et al., 2014; Podhaizer et al., 2012; Singh et al., 2004; Zou et al., 2011a; Zou et al., 2011b). Findings were reported as the average percentage of neuronal survival, with respect to pre-treatment neuron count $\pm$ the standard error of the mean (SEM). The effect of each treatment on neuronal survival was analyzed using a repeated-measure analysis of variance (ANOVA) followed by Duncan's post hoc test (Statistica 8.0; StatSoft, Tulsa, OK) from $n = 3$ –5 experiments in which at least 50 neurons per treatment group were followed per experiment (150–250 neurons total per treatment group).

Assessment of neuritic arborization

Cells were fixed, permeabilized, blocked, and subsequently labeled for MAP-2 (Abcam), TUNEL (Roche Applied Sciences), and Hoechst 33342 (Sigma-Aldrich). Cells were visualized and digital images were acquired. Neuritic arborization was evaluated using Sholl analysis; a 'Sholl score' was measured by counting the number of intersections of MAP-2-positive neurites with equidistant concentric circles of increasing radius, centered on the cell body (Sholl, 1953). To avoid the inclusion of dead cells, we specifically evaluated changes in the arborization only of TUNEL(–) cells.

Immunoblotting

Whole cell extracts were prepared using radioimmunoprecipitation assay (RIPA) buffer (Sigma-Aldrich) with protease and phosphatase inhibitors (Roche Applied Sciences), and total protein concentrations were determined by bicinchoninic acid (BCA) assay (Thermo Fisher Scientific, Rockford, IL). Cell lysates containing equal amounts of total protein (~5–10 μ g) were heated at 100°C for 5 minutes in laemmli buffer (Sigma-Aldrich), electrophoretically separated on 10% SDS-polyacrylamide gels (Bio-Rad, Hercules, CA), and transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad). Membranes were incubated with primary antibodies to phospho-GSK3 β -Ser9 (Cell Signaling Technology, Danvers, MA; 5558), GSK3 β (Cell Signaling Technology; 9832), β -catenin (Cell Signaling Technology; 9562), glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Abcam; ab8245), MAP-2 (Abcam) and postsynaptic density protein 95 (PSD-95; UC Davis/NIH NeuroMab Facility, Davis, CA; 73-028). Appropriate horseradish peroxidase-conjugated secondary antibodies (SouthernBiotech, Birmingham, AL) were used. Membranes were detected using SuperSignal® West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific), and visualized using a Kodak Image Station 440CF.

Statistical Analyses

All data were expressed as average $\pm$ SEM. Unless otherwise indicated, data were analyzed statistically using a one-way ANOVA followed by Duncan's post hoc test using Statistica 8.0 (StatSoft); an α level of $p < 0.05$ was considered significant.

RESULTS

HIV- and morphine-mediated GSK3 β activation

GSK3 β activation/inactivation was assessed at 4 h after treatments. Cell lysates were immunoblotted for phospho-GSK3 β -Ser9 (p-GSK3 β -S9; an inactive form of GSK3 β) (Frame et al., 2001; Grimes and Jope, 2001; Jacobs et al., 2012; Kaytor and Orr, 2002), GSK3 β (total GSK3 β ; t-GSK3 β), β -catenin, and GAPDH (Fig. 1). HIV⁺_{sup}, with or without morphine, significantly decreased p-GSK3 β -S9 (normalized with t-GSK3 β ; p-GSK3 β -S9/t-GSK3 β ; Fig. 1A), suggesting an increase in GSK3 β activation. VPA significantly abrogated HIV⁺_{sup} $\pm$ morphine-mediated effects. β -Catenin is a downstream target of GSK3 β ; active GSK3 β phosphorylates β -catenin and initiates rapid ubiquitin-mediated degradation by the proteasome (Aberle et al., 1997; Jacobs et al., 2012; Kaytor and Orr, 2002). HIV⁺_{sup}, with or without morphine, significantly reduced β -Catenin levels (Fig. 1B), suggesting an enhancement in GSK3 β activity. VPA completely inhibited the effect of HIV⁺_{sup} + morphine and partially inhibited the effect of HIV⁺_{sup} alone, returning both to levels that were not different from control levels.

Significant individual or interactive effects of morphine were not detected at 4 h. Thus, based on the time-dependent effects of opiates observed by others (Dobashi et al., 2010; Xie et al., 2010), we examined HIV⁺_{sup} and morphine-mediated effects at longer time intervals (Fig. 2). At all assessed time points (12, 24, and 72 h), HIV⁺_{sup} significantly reduced p-GSK3 β -S9. At 24 and 72 h, morphine alone also induced significant loss of p-GSK3 β -S9. A significant interaction between HIV⁺_{sup} and morphine was seen only at 24 h.

Role of GSK3 β in HIV $\pm$ morphine-mediated cell death

Death of pre-selected neurons was observed using time-lapse imaging; cells were repeatedly imaged at 1 h intervals throughout the 72 h treatment period (Fig. 3A). Neurons exposed to morphine and/or VPA had survival values equivalent to control, while all groups treated with HIV⁺_{sup} showed significantly reduced neuronal survival (Fig. 3B). Morphine significantly enhanced HIV⁺_{sup}-mediated neuronal death. HIV⁺_{sup} $\pm$ morphine-mediated effects were partially, but significantly, reversed by VPA co-treatment. The survival of neurons treated with 'HIV⁺_{sup} + VPA' and 'HIV⁺_{sup} + morphine + VPA' was not significantly different, showing that VPA effectively negated any HIV⁺_{sup}-morphine interactions. Cell death was additionally confirmed using TUNEL staining (Fig. 3C). HIV⁺_{sup} induced a significant increase in TUNEL(+) neurons, which was augmented by morphine co-treatment. HIV⁺_{sup} $\pm$ morphine-mediated effects were partially, but significantly, reversed by VPA co-treatment. As seen for the time-lapse analyses, VPA also nullified the interactions between HIV⁺_{sup} and morphine.

Role of GSK3 β in HIV $\pm$ morphine-mediated changes in neuritic arborization, MAP-2 and PSD-95

Since neurite degeneration and synapse losses are the major substrate for HAND (Bellizzi et al., 2006; Ellis et al., 2007; Everall et al., 1999; Kim et al., 2008; Masliah et al., 1997) we assessed changes in neuritic arborization using MAP-2 immunostaining (Fig. 4A) followed by Sholl analysis (Fig. 4B). At 72 h, HIV⁺_{sup} induced a significant reduction in neuritic arborization. There was no significant morphine interaction, and the HIV⁺_{sup} effect was partially, but significantly, reversed by VPA co-treatment. Neuritic/synaptic degeneration was additionally confirmed by immunoblotting for MAP-2 and PSD-95 (Fig. 4C and D). At 72 h, HIV⁺_{sup} significantly decreased MAP-2. Similar to the Sholl analysis, there was no significant morphine interaction (Fig. 4C), and VPA significantly abrogated HIV⁺_{sup}-mediated loss of MAP-2. HIV⁺_{sup} also induced a significant loss in PSD-95, but in this case, there was an increased loss with morphine co-treatment (Fig. 4D). VPA partially, but significantly, reversed HIV⁺_{sup} $\pm$ morphine effects on PSD-95. The HIV-morphine interaction on PSD-95 levels was maintained in the presence of VPA.

Neuritic/synaptic degeneration was also assessed in cortical and hippocampal neurons (Fig. 5). In cortical neurons (Fig. 5A), HIV⁺_{sup} significantly reduced MAP-2 and PSD-95, without significant morphine interactions. VPA significantly abrogated HIV⁺_{sup}-mediated loss of MAP-2 and partially, but significantly, abrogated loss of PSD-95. In hippocampal neurons (Fig. 5B), HIV⁺_{sup} induced a significant loss of MAP-2 and PSD-95, which was significantly augmented by morphine co-treatment. VPA significantly reversed HIV⁺_{sup} $\pm$ morphine-mediated effects on MAP-2 and partially, but significantly, reversed effects on PSD-95.

Effects of small molecule GSK3 β -inhibitors

The role of GSK3 β activation in HIV⁺_{sup} $\pm$ morphine-mediated neurotoxicity was confirmed additionally using the small molecule GSK3 β -inhibitors, SB415286 or XXVI (Fig. 6), both of which have very high specificity for GSK3 β compared to VPA. Both small molecule inhibitors partially, but significantly, reduced HIV⁺_{sup} $\pm$ morphine-mediated neuronal death, and also abrogated HIV⁺_{sup}-morphine interactions (Fig. 6A and B). Both inhibitors reversed the effect of HIV⁺_{sup} $\pm$ morphine on neurite losses, although SB415286 only resulted in partial recovery (Fig. 6C).

DISCUSSION

Multiple neurodegenerative processes, including HAND, involve dysregulation of GSK3 β pathways (Crews et al., 2009; Dou et al., 2003; Dou et al., 2005; Everall et al., 2002; Maggirwar et al., 1999; Sui et al., 2006). The present work supports those findings, and importantly also demonstrates that GSK3 β -activation is a point of convergence for specific interactions between HIV and opiates that lead to neurodegenerative outcomes. We used multiple GSK3 β -inhibitors, and found that inhibition entirely negated the ability of morphine to enhance HIV⁺_{sup}-mediated striatal neuron death. GSK3 β -inhibitors did not entirely disrupt HIV⁺_{sup}-morphine interactive effects on PSD95 in striatal neurons, and only

partially reversed certain effects of HIV⁺_{sup} ± morphine, indicating the likely involvement of other signaling pathways in various aspects of HIV ± opiate-related neurotoxicity.

Though HIV-1 induces profound changes in neuronal morphology, function, and survival, the virus itself rarely, if ever, infects neurons (An et al., 1999a; Bagasra et al., 1996; Kramer-Hammerle et al., 2005; Takahashi et al., 1996); instead, infected and activated glial cells produce cellular and viral products that drive secondary, “bystander” toxicity in neurons (Buescher et al., 2007; Epstein and Gendelman, 1993; Gonzalez-Scarano and Martin-Garcia, 2005; Kaul et al., 2005; Kramer-Hammerle et al., 2005). Our previous studies have shown that glia are crucial in determining the extent of neurotoxic interactions between HIV-1 proteins (Podhaizer et al., 2012; Zou et al., 2011b) or HIV-infective supernatant (Masvekar et al., 2014), and opiates. Since the present studies focused on understanding pathway(s) that underlie HIV-1 and opiate interactions within neurons, glia were excluded from our culture system. Still, the importance of glial-to-neuron signaling in HIV- and opiate-mediated neurotoxicity should be considered and not to be underestimated.

In accord with many previous studies (Crews et al., 2009; Dou et al., 2003; Dou et al., 2005; Everall et al., 2002; Maggirwar et al., 1999; Sui et al., 2006), our results show that HIV⁺_{sup} induces GSK3β-activation, and that treatments to inhibit GSK3β activation ameliorate neuron death, and neurite and synaptic damage caused by HIV⁺_{sup}. Previous studies have shown that the HIV-1 proteins Tat and gp120 abnormally activate GSK3β (Crews et al., 2009; Dewhurst et al., 2007; Everall et al., 2002; Maggirwar et al., 1999; Sui et al., 2006). But as our model system uses supernatant from HIV-infected cells, which includes various viral proteins and cytotoxic factors, it is hard to predict which proteins/factors play a role in GSK3β-activation. Presumably, our results may reflect the combined effects of multiple proteins and factors present in the supernatant. Some previous studies have shown that viral proteins induce abnormal GSK3β activation by reducing expression of neurotrophic factors such as fibroblast growth factors (FGFs), which otherwise inhibit GSK3β activity via phosphorylation at Ser 9 residue through activation of phosphatidylinositol-3-kinase (PI3K)/Akt (Crews et al., 2009; Everall et al., 2002).

Abnormally activated GSK3β can promote neuronal damage by dysregulating the function/stability of various structural, metabolic, and signaling proteins, including tau, β-catenin, MAP-2, activator protein 1 (AP-1), cyclic AMP response element binding protein (CREB), nuclear factor-kappa B (NF-κB), heat shock factor-1 (HSF-1), and others (Frame and Cohen, 2001; Grimes and Jope, 2001; Kaytor and Orr, 2002; Plyte et al., 1992). GSK3β inhibitors only partially reversed certain neurotoxic outcomes induced by HIV⁺_{sup}, suggesting the involvement of signaling molecules/pathways other than GSK3β. Of interest in this regard are kinases such as mitogen-activated protein kinase 10 (MAPK10), double-stranded RNA-activated protein kinase (PKR), and cyclin-dependent kinase 5 (CDK5), all of which are known to play important role in HIV-mediated neurodegenerative processes (Alirezai et al., 2007; Crews et al., 2009; Patrick et al., 2011).

Studies from the brains of opiate-abusing patients have indirectly implicated the role of GSK3β-activation in opiate-associated neuropathology (Anthony et al., 2010; Ramage et al., 2005), showing increased levels of phosphorylated tau, a target of GSK3β, in the brains of

HIV⁻, opiate abusers at several ages. However, other studies have disagreed on whether opiates induce GSK3 β activation (Dobashi et al., 2010; Xie et al., 2010) or inactivation (Li et al., 2010; Polakiewicz et al., 1998); this may be because the effects of opiates on GSK3 β -activity occur in a dose- and time-dependent manner (Xie et al., 2010). Acute activation of MORs tends to inhibit GSK3 β activity via PI3K/Akt signaling pathway (Polakiewicz et al., 1998), while chronic activation of MORs tends to elevate GSK3 β activity (Dobashi et al., 2010; Xie et al., 2010), perhaps through increased intracellular calcium (Dobashi et al., 2010; Hartigan and Johnson, 1999; Quillan et al., 2002). In accord with these studies, our results also show that morphine exerts time-dependent effects on GSK3 β activation (Figs. 1 and 2). At earlier time points (4 and 12 h) morphine effects were not observed, but at later time points (24 and 72 h) morphine significantly induced GSK3 β activation.

At 24 h, morphine significantly augmented HIV⁺_{sup}-mediated GSK3 β activation (Fig. 2), but this transient interactive effect of morphine was not seen at 72 h. This loss in interaction may be due to selective loss of MOR-expressing neurons, as nearly 50% of cells have died by this time (Figs. 3 and 6). Even though there was no significant interaction between HIV and morphine at 72 h, both HIV and morphine alone induced significant GSK3B activation at that time.

Although there was no interactive effect of morphine on the relative expression of p-GSK3 β at 72 h (Fig. 2), morphine did exacerbate the effects of HIV⁺_{sup} on both PSD-95 and neuron death at that time (Figs. 3 and 4). This discordance may be due to temporal difference in the 'cause' and the 'effect'. The interactive elevation in GSK3 β activity seen prior to 72 h is the 'cause', which results in the aggravation of neurotoxic outcomes, an 'effect' seen later.

In terms of HIV⁺_{sup}-morphine interactions, VPA co-treatment negated the interaction that augmented striatal neuron death (Fig. 3), but had no effect on interactive reduction in PSD-95 (Fig. 4D). This discrepancy likely reflects the role(s) of signaling molecules/ pathways other than GSK3 β , and is understandable given that multiple downstream pathways and events are triggered by opiates, HIV virions, and factors secreted from HIV-infected/activated cells. For example, CDK5, like GSK3 β , is known to be involved in both HIV- and opiate-associated neuropathology (Anthony et al., 2010; Crews et al., 2009; Parkitna et al., 2006; Patrick et al., 2011; Wang et al., 2007), and might be a likely candidate in this regard.

The studies with striatal neurons used isolated cells lacking excitatory presynaptic inputs that would normally originate from trophic, glutamatergic corticostriatal afferents. Although isolated striatal neurons do not elaborate excitatory PSD-95-containing dendritic spines in normal numbers, they express relatively high levels of PSD-95. Interestingly, HIV⁺_{sup}-morphine interactions were not observed to affect neurite length (Fig. 4B) or the expression of the neurite marker protein, MAP-2 (Fig. 4C); however, morphine significantly augmented the HIV-mediated loss of PSD-95 in striatal neurons (Fig. 4D). This suggests that HIV-opiate interactions induce adverse effects on synaptic stability instead on neurite outgrowth/pruning.

HIV and opiate neurotoxicity frequently show regional specificity in the brain (Fitting et al., 2010b; Pang et al., 2013). This is partly due to variability in expression of receptors important for HIV binding and entry, as well as opiate receptors (Arvidsson et al., 1995; Berger and Arendt, 2000; Berger and Nath, 1997; Mansour et al., 1995; Mansour et al., 1988; Nath, 2014; Podhaizer et al., 2012; Stiene-Martin et al., 1998; Turchan-Cholewo et al., 2008). However, studies presented here also showed different interactive effects of HIV and morphine in isolated neuron cultures derived from different brain regions. For example, in all neuron cultures (striatal, cortical and hippocampal; Figs. 4 and 5), HIV⁺_{sup} induced loss of PSD-95, but only in striatal and hippocampal neurons were these effects augmented by morphine co-treatment. Thus, the regional specificity of HIV and morphine interactions in the brain likely has both neuronal and glial contribution.

In previous studies, VPA significantly ameliorated HIV-mediated neurotoxic outcomes both in experimental models (Crews et al., 2009; Dewhurst et al., 2007; Dou et al., 2003) and clinically (Schifitto et al., 2006). Though VPA is a potent GSK3 β inhibitor, it is not a specific inhibitor; VPA targets additional molecules/pathways, including histone deacetylase (HDAC), Na⁺ channels, Ca²⁺ channels, voltage-gated K⁺ channels, GSK3 α , and others (Chateauvieux et al., 2010). Therefore, we also tested the effects of the small molecule GSK3 β -inhibitors, SB415286 and XXVI, which have much greater target specificity. Both small molecule inhibitors ameliorated the individual or combined neurotoxic outcomes due to HIV⁺_{sup} and morphine (Fig. 6), providing confirmation for a role of GSK3 β .

HIV⁺ patients who abuse opiates show more serious neuropathologies and behavioral and cognitive deficits even though they receive anti-retroviral therapy (Anthony et al., 2008; Bell et al., 2002; Byrd et al., 2011; Meijerink et al., 2014; Robinson-Papp et al., 2012; Smith et al., 2014). Patients with HIV-related pain syndromes, for whom opiates are usually prescribed, might also be at risk for such effects over chronic treatment times. And although recent studies suggest that certain aspects of HIV-related neuropathologies may be reversible (Bellizzi et al., 2006; Ellis et al., 2007; Kim et al., 2008), the situation may be less positive for opiate-exposed patients. Our recent study showed that neurite recovery from an HIV insult, normally robust after return to control conditions, was limited in the continued presence of morphine (Masvekar et al., 2014). This raises the prospect that neurologic symptoms may be more difficult to reverse in opiate-exposed patients even when viral titers are controlled. As the cellular basis of opiate-enhanced HIV-1 neuropathology is not understood, it is not clear whether a different, or additional, therapeutic course would be helpful for patients with dual exposure. The current findings predict that therapeutics specifically limiting GSK3 β -activation might be particularly useful as an adjunctive therapy for reducing HAND symptomatology in HIV patients who abuse or are otherwise exposed to opiates.

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Abbreviations

CNS	central nervous system
HIV-1	human immunodeficiency virus-1
HAND	HIV-1-associated neurocognitive disorders
cART	combination antiretroviral therapy
Tat	trans-activator of transcription
gp120	glycoprotein 120
GSK3β	glycogen synthase kinase-3 β
MAP-2	microtubule-associated protein 2
MORs	μ -opioid receptors
VPA	valproate
p-GSK3β-S9	phospho-GSK3 β -Ser9
PSD-95	postsynaptic density protein 95
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
FGFs	fibroblast growth factors (FGFs)
PI3K	phosphatidylinositol-3-kinase
AP-1	activator protein 1
CREB	cyclic AMP response element binding protein
NF-κB	nuclear factor-kappa B
HSF-1	heat shock factor-1
MAPK10	mitogen-activated protein kinase 10
PKR	double-stranded RNA-activated protein kinase
CDK5	cyclin-dependent kinase 5
HDAC	histone deacetylase

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HIGHLIGHTS

- We demonstrate that GSK3 β -activation is a point of convergence for specific interactions between HIV and opiates that lead to neurodegenerative outcomes.
- Multiple GSK3 β -inhibitors, including valproic acid and small molecule inhibitors, were used to determine GSK3 β pathway involvement.
- GSK3 β -inhibitors entirely negated the ability of morphine to enhance the HIV - mediated death of striatal neurons.
- GSK3 β -inhibitors partially reversed effects of HIV and HIV+morphine on PSD95 levels and neurite reduction, and did not negate interactive effects, implying the involvement of additional signaling pathways in these outcomes.

Figure 1. HIV^{sup} ± morphine-mediated GSK3β activation

At 4 h after treatments, cells were lysed and protein levels were detected by immunoblotting. Findings were reported as average normalized protein levels (% control) ± SEM. Significance was analyzed by one-way ANOVA and Duncan's post hoc test; $n = 3$ separate experiments. **(A)** Immunoblotting for phospho-GSK3β-Ser9 (p-GSK3β-S9) and total GSK3β (t-GSK3β); levels of p-GSK3β-S9 were normalized with t-GSK3β (p-GSK3β-S9/t-GSK3β). HIV^{sup} significantly reduced p-GSK3β-S9 ($*p < 0.05$ vs. Control), without any significant morphine interaction. VPA significantly abrogated HIV^{sup} ± morphine-mediated effects ($\#p < 0.05$). **(B)** Immunoblotting for β-catenin and GAPDH; levels of β-catenin were normalized to GAPDH (β-catenin/GAPDH). HIV^{sup} significantly decreased β-catenin ($*p < 0.05$ vs. Control) without any morphine interaction. VPA restored β-catenin levels to control values ($\#p < 0.05$), although in the case of neurons treated with HIV^{sup} alone the effect was less significant (HIV + VPA was not different from either HIV or control). Control = control_{sup}; HIV = HIV^{sup}; Mor = morphine; VPA = valproate.

Figure 2. HIV⁺_{sup}- and morphine-mediated interactive effects on GSK3β-activation

At 12, 24, and 72 h after treatments, cells were lysed and immunoblotted for p-GSK3β-S9 and t-GSK3β. Findings were reported as average levels of p-GSK3β-S9 normalized with t-GSK3β (p-GSK3β-S9/t-GSK3β) as a percentage of control values $\pm$ SEM. Significance was analyzed by one-way ANOVA and Duncan's post hoc test; $n = 4$ separate experiments. At all time points, HIV⁺_{sup} significantly reduced p-GSK3β-S9 ($*p < 0.05$ vs. Control). At 24 and 72 h, morphine alone also induced significant loss of p-GSK3β-S9, but a significant interaction with HIV⁺_{sup} was seen only at 24 h ($^{\$}p < 0.05$). Control = control_{sup}; HIV = HIV⁺_{sup}; Mor = morphine.

Figure 3. Role of GSK3 β in HIV⁺_{sup} ± morphine-mediated cell death

(A) Cells were repeatedly imaged for 72 h after treatments. Digital images show the same cells/fields at 0, 24 and 72 h (white arrowheads indicate dead cells that were alive in previous image). (B) Cells were assessed for viability at 6 h intervals in digital images. Findings were reported as the average percentage of neuronal survival as a proportion of pre-treatment neuron count $\pm$ SEM. Significance was analyzed by repeated measures ANOVA and Duncan's post hoc test; $n = 3$ separate experiments (at least 150 neurons per treatment group). HIV⁺_{sup} significantly reduced neuronal survival (* $p < 0.05$ vs. Control), which was significantly augmented by morphine co-treatment (\$ $p < 0.05$). HIV⁺_{sup} ± morphine-mediated effects were partially, but significantly, reversed by VPA (# $p < 0.05$). VPA also effectively negated HIV⁺_{sup}-morphine interactions. (C) At 72 h after treatments, cells were fixed, permeabilized, and labeled for TUNEL and Hoechst 33342. The rate of neuronal apoptosis was reported as the average percentage of TUNEL(+) cells $\pm$ SEM. Significance was analyzed by one-way ANOVA and Duncan's post hoc test; $n = 3$ separate

experiments. $\text{HIV}^+_{\text{sup}}$ significantly increased the percentage of TUNEL(+) cells ($*p < 0.05$ vs. Control), and morphine augmented the effect ($^{\$}p < 0.05$). $\text{HIV}^+_{\text{sup}} \pm$ morphine-mediated effects were partially, but significantly, reversed by VPA co-treatment ($^{\#}p < 0.05$), and VPA negated interactions between $\text{HIV}^+_{\text{sup}}$ and morphine. Control = control_{sup}; HIV = $\text{HIV}^+_{\text{sup}}$; Mor = morphine; VPA = valproate.

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Figure 4. Role of GSK3 β in HIV⁺_{sup} ± morphine-mediated changes in neuritic arborization, MAP-2 and PSD-95

(A) At 72 h after treatments, cells were fixed, permeabilized, and labeled for MAP-2 (green), TUNEL (red) and Hoechst 33342 (blue) (B) Neurite arborization was measured by Sholl analysis in digital images of TUNEL(–) neurons. The findings were reported as average Sholl score $\pm$ SEM. HIV⁺_{sup} significantly reduced the Sholl score (* p < 0.05 vs. Control), without any significant morphine interaction. HIV⁺_{sup} ± morphine-mediated effects were partially, but significantly, reversed by VPA co-treatment (# p < 0.05). (C) At 72 h after treatments, cells were lysed and immunoblotted for MAP-2 and GAPDH. Findings were reported as average normalized MAP-2 (% control) $\pm$ SEM. HIV⁺_{sup}, with or without morphine, significantly reduced MAP-2 (* p < 0.05 vs. Control). VPA significantly inhibited HIV⁺_{sup} ± morphine-mediated effects (# p < 0.05). (D) Cell lysates were immunoblotted for PSD-95 and GAPDH. Findings were reported as average normalized PSD-95 (% control) $\pm$ SEM. HIV⁺_{sup} induced a significant loss of PSD-95 (* p < 0.05 vs. Control), which was augmented by morphine co-treatment (\$ p < 0.05). HIV⁺_{sup} ± morphine-mediated effects were partially, but significantly, reversed by VPA co-treatment (# p < 0.05). Even in the presence of VPA, morphine significantly augmented the effects of HIV⁺_{sup} (\$ p < 0.05). In all studies, significance was analyzed by one-way ANOVA and Duncan's post hoc test, from n = 3 separate experiments. Control = control_{sup}; HIV = HIV⁺_{sup}; Mor = morphine; VPA = valproate.

Figure 5. Role of GSK3 β in HIV⁺_{sup} ± morphine-mediated changes in MAP-2 and PSD-95 in cortical and hippocampal neurons

At 72 h after treatments, cells were lysed and immunoblotted for MAP-2, PSD-95 and GAPDH. Findings were reported as average normalized protein levels (% control) $\pm$ SEM. Significance was analyzed by one-way ANOVA and Duncan's post hoc test; $n = 3$ separate experiments. **(A) Cortical Neurons. MAP-2:** HIV⁺_{sup} ± morphine induced significant loss of MAP-2 ($*p < 0.05$ vs. Control), VPA significantly inhibited HIV⁺_{sup}-mediated effects ($^{\#}p < 0.05$). **PSD-95:** HIV⁺_{sup}, with or without morphine, significantly reduced PSD-95 ($*p < 0.05$ vs. Control). HIV⁺_{sup}-mediated loss of PSD-95 was partially, but significantly, reversed by VPA co-treatment ($^{\#}p < 0.05$). **(B) Hippocampal Neurons. MAP-2:** HIV⁺_{sup} induced a significant loss of MAP-2 ($*p < 0.05$ vs. Control), which was augmented by morphine co-treatment ($^{\$}p < 0.05$). HIV⁺_{sup} ± morphine-mediated loss of MAP-2 was significantly reversed by VPA co-treatment ($^{\#}p < 0.05$). **PSD-95:** HIV⁺_{sup} induced a significant loss of PSD-95 ($*p < 0.05$ vs. Control), which was augmented by morphine co-treatment ($^{\$}p < 0.05$). HIV⁺_{sup} ± morphine-mediated effects were partially, but significantly, reversed by VPA co-treatment ($^{\#}p < 0.05$). Control = control_{sup}; HIV = HIV⁺_{sup}; Mor = morphine; VPA = valproate.

Figure 6. Effects of small molecule GSK3 β -inhibitors

(A) Cell viability was assessed by time-lapse imaging analysis. Neurons exposed to 'Control + SB', 'Control + XXVI', 'Mor', 'Mor + SB' and 'Mor + XXVI' treatments had survival equivalent to 'Control', but are not shown here in order to highlight other groups. HIV^{sup} significantly reduced neuronal survival ($*p < 0.05$ vs. Control), with a significant morphine interaction ($\$p < 0.05$). The effects of HIV^{sup} were partially, but significantly, reversed by both small molecule inhibitors ($\#p < 0.05$ vs. respective HIV or HIV + Mor). Both SB415286 and XXVI also effectively negated interactions between HIV^{sup} and morphine. (B) Cell death was examined using TUNEL-staining. HIV^{sup} significantly increased the percentage of TUNEL(+) cells ($*p < 0.05$ vs. Control), with a significant morphine interaction ($\$p < 0.05$). Both small molecule inhibitors partially reversed HIV^{sup} $\pm$ morphine-mediated effects ($\#p < 0.05$) and also eradicated the HIV^{sup}-morphine interaction. (C) Neuritic arborization was evaluated using MAP-2 immunostaining and Sholl analysis. HIV^{sup} significantly reduced the Sholl score ($*p < 0.05$ vs. Control), without a morphine interaction. All losses in arborization were partially, but significantly, reversed by both small molecule inhibitors ($\#p < 0.05$), except that XXVI completely reversed the effect of HIV^{sup}. Control = control_{sup}; HIV = HIV^{sup}; Mor = morphine; SB = SB415286; XXVI = GSK3 β inhibitor XXVI.