

Opiate Addiction Therapies and HIV-1 Tat: Interactive Effects on Glial $[Ca^{2+}]_i$, Oxyradical and Neuroinflammatory Chemokine Production and Correlative Neurotoxicity

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Abstract: Few preclinical studies have compared the relative therapeutic efficacy of medications used to treat opiate addiction in relation to neuroAIDS. Here we compare the ability of methadone and buprenorphine, and the prototypic opiate morphine, to potentiate the neurotoxic and proinflammatory ($[Ca^{2+}]_i$, ROS, H_2O_2 , chemokines) effects of HIV-1 Tat in neuronal and/or mixed-glial co-cultures. Repeated observations of neurons during 48 h exposure to combinations of Tat, equimolar concentrations (500 nM) of morphine, methadone, or buprenorphine exacerbated neurotoxicity significantly above levels seen with Tat alone. Buprenorphine alone displayed marked neurotoxicity at 500 nM, prompting additional studies of its neurotoxic effects at 5 nM and 50 nM concentrations \pm Tat. In combination with Tat, buprenorphine displayed paradoxical, concentration-dependent, neurotoxic and neuroprotective actions. Buprenorphine neurotoxicity coincided with marked elevations in $[Ca^{2+}]_i$, but not increases in glial ROS or chemokine release. Tat by itself elevated the production of CCL5/RANTES, CCL4/MIP-1 β , and CCL2/MCP-1. Methadone and buprenorphine alone had no effect, but methadone interacted with Tat to further increase production of CCL5/RANTES. In combination with Tat, all drugs significantly increased glial $[Ca^{2+}]_i$, but ROS was only significantly increased by co-exposure with morphine. Taken together, the increases in glial $[Ca^{2+}]_i$, ROS, and neuroinflammatory chemokines were not especially accurate predictors of neurotoxicity. Despite similarities, opiates displayed differences in their neurotoxic and neuroinflammatory interactions with Tat. Buprenorphine, in particular, was partially neuroprotective at a low concentration, which may result from its unique pharmacological profile at multiple opioid receptors. Overall, the results reveal differences among addiction medications that may impact neuroAIDS.



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Keywords: Calcium, chemokines, buprenorphine, methadone, morphine, neuro-acquired immunodeficiency syndrome (neuroAIDS), opioid drug abuse, oxidative stress.

INTRODUCTION

Evidence suggests that the severity of neuroAIDS may be markedly exacerbated by chronic opiate drug use. The functional impairment associated with neuroAIDS is underscored by synaptic pruning and neuronal injury [1, 2] and is secondary to an infection that is largely restricted to glia and perivascular macrophages [3-8]. Human immunodeficiency virus type 1 (HIV-1) proteins, such as glycoprotein 120 (gp120) and transactivator of transcription (Tat), are released by infected glia have been implicated as possible mediators of neurotoxicity [9-12] and contribute to the release of glial-derived cellular toxins, including reactive

oxygen species (ROS) [13-15], calcium overload [16], and chemokines such as CCL5/RANTES, TNF- α , IL-6, CCL4/MIP-1 β , and CCL2/MCP-1 [17-24]. Opiates can exacerbate synaptodendritic damage caused by Tat [25, 26] or HIV-1 [27], and significant aspects of the morphine and HIV-1/Tat interactive neurotoxicity are mediated by glia [27, 28], oxygen species (ROS) [13-15], calcium overload [16], and chemokines such as CCL5/RANTES, TNF- α , IL-6, CCL4/MIP-1 β , and CCL2/MCP-1 [17-24]. Opiates can exacerbate synaptodendritic damage caused by Tat [25, 26] or HIV-1 [27], and significant aspects of the morphine and HIV-1/Tat interactive neurotoxicity are mediated by glia [27, 28].

Opiates exacerbate the neuroinflammatory effects of HIV in both astroglia and microglia [29, 30]. Astroglia contribute less to the production of new virions, but they are significant sources of cellular toxins [31-34], and are a key site where opiates act to exacerbate the release of reactive cellular

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products [16, 29, 35-38]. In the case of Tat, morphine potentiates Tat-induced neuronal losses in striatal murine cell cultures [39], and exacerbates Tat toxicity in glial precursors and/or astroglia derived from mouse striatum [40]. Activating μ -opioid receptor (MOR)-expressing astroglia by morphine has been shown to modulate the central nervous system (CNS) response to pathogens such as HIV-1 proteins, resulting in synergistic increases in calcium mobilization [16, 28], increased production in oxyradicals [28, 41] and reduced survival of bystander neurons [28]. Importantly, synergy was lost if glia, but not neurons, lacked MOR, indicating that opiate interactions with HIV converge at the level of MOR-expressing glia [28]. Opiates acting through astroglial intermediates [16, 35-37, 42, 43] release chemokines such as CCL5/RANTES and CCL2/MCP-1 [16, 35-37, 43], which contribute to microglial overactivation and decrease the threshold for neuronal injury or death [36, 42, 44, 45]. In addition, it has been demonstrated that morphine and Tat co-administration synergistically increases ROS in MOR-expressing microglia [46]. Although the mechanism(s) by which morphine further triggers the inflammatory effects of Tat in microglia is unclear, morphine by itself was recently shown to enhance the release of proinflammatory cytokines and nitric oxide *via* a MOR-PKC ϵ -Akt-ERK1/2 signaling pathway in activated microglia [47]. In turn, the viral proteins themselves may intrinsically modulate opioid receptor expression by astroglia and microglia [38], and throughout the CNS [26], suggesting a complex interplay of drug and viral protein actions on glia and neurons.

Considering that opiate abuse, HIV-1 infection and their comorbidity are global problems, an understanding of opiate addiction therapies in this context is of critical importance. Not only do addiction therapies limit the spread of infection by restricting needle sharing among injection drug users or *via* exchange of sex for drugs [48, 49], they also improve long-term health and “virological success” of drug abusers infected with HIV-1 [50-53]. Treatment protocols include the use of full MOR agonists such as methadone [54, 55] and levo- α acetylmethadol, or partial MOR agonists such as buprenorphine [53], or opioid-receptor antagonists such as naltrexone [48, 51]. Buprenorphine is a more recent, alternative approach to methadone—initially reported to have fewer side effects, less abuse liability, and greater safety because of its actions as a partial MOR agonist [56-59]. Despite reported advantages in treating opiate addiction [48, 49, 60-62], there is clear abuse potential with buprenorphine [63-65]. However, multiple reports suggest that supra-therapeutic doses of buprenorphine can have variable effects on neurogenesis [66, 67]. If administered prenatally, supraphysiological amounts of buprenorphine can cause depressive-like syndromes in rats [68] and reductions in myelin basic protein and the number of myelinated axons [69]. The “atypical” myelin patterns seen with prenatal exposure to higher concentrations of buprenorphine are attributed to buprenorphine’s actions as a partial antagonist at κ -opioid receptors [70] and/or agonist actions at nociceptin/orphanin FQ (NOP or OLR1) receptors [69, 70]. Despite benefits from opiate addiction therapy in HIV-1-infected individuals, few studies have directly assessed the impact of methadone or buprenorphine-HIV-1 interactions in the CNS.

As past research indicates that opiate addiction treatments (e.g. methadone and buprenorphine) appear to limit the systemic consequences and spread of HIV-1 infection, it is hypothesized that opiate substitution therapies may intrinsically differ in their ability to potentiate HIV encephalitis (HIVE) and neuronal injury. Glia are the principal site of opiate drug abuse and Tat/HIV-1 interactions that trigger subsequent neuronal injury. Thus, the present study examined the effects of opiate drugs and drug therapies on isolated glia, and on neuron-glia co-cultures. Our goal was to assess the potential of opiate addiction medications to affect neurotoxic or inflammatory outcomes related to neuroAIDS. First, we systematically examined neurotoxic effects of morphine, buprenorphine, and methadone on Tat-treated striatal spiny neurons cultured with mixed glia. Second, we examined the concentration-dependent effects of opiates on intracellular calcium ($[Ca^{2+}]_i$) levels and on intracellular ROS formation in mixed-glia cultures. Third, we evaluated the effects of opiate drugs on chemokine production in combination with Tat protein. Elucidating the comparative pathophysiological effects of chronic exposure to morphine is critically important for understanding opiate addiction and for understanding how opiates contribute to neuroAIDS. Our findings suggest fundamental differences in the neuropathogenic potential of various opiate drug treatment options, with buprenorphine at lower concentrations actually reducing Tat-induced neurotoxicity. Effects on $[Ca^{2+}]_i$, ROS and chemokine production in mixed-glia appear to be less relevant for neuronal survival.

MATERIALS AND METHODS

Cell Culture

To assess the effects of opiate drugs and/or Tat on the survival of striatal spiny neurons, we co-cultured neurons with mixed glia enriched in astrocytes. This was followed by experiments using mixed-glia cultures to evaluate the pharmacological mechanisms of inflammation-related effects produced by opiate drugs and viral proteins on glial cells. All experiments were conducted in accordance with procedures reviewed and approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee (IACUC).

Mixed-glia cultures. Mixed-glia cultures were derived from striata of day 0-1 postnatal ICR (Charles River Laboratories, Charles River, MA) pups as previously described [16]. Striata were aseptically isolated, minced in media, incubated with trypsin/DNase (37 °C, 30 min, 2.5 mg/ml), triturated through a series of decreasing bore pipettes, and filtered twice through 100 μ m and 40 μ m pore nylon mesh. Growth medium favoring astroglial enrichment consisted of Dulbecco’s Modified Eagle’s Medium (DMEM; GIBCO, Invitrogen) supplemented with glucose (27 mM; Sigma), Na₂HCO₃ (6 mM; Invitrogen), HEPES (10 mM; Invitrogen), and 10% (v/v) fetal bovine serum (FBS; JRH Biosciences or Hyclone, Logan, UT). Cells for each experiment were pooled from a single culture of two brains and plated at 50,000–150,000 cells/cm² in 96-well plates (Costar, Corning Life Sciences, Acton, MA). Mixed-glia cultures were grown for 10-14 days *in vitro* (DIV) at 37 °C

in 5% CO₂/95% air at high humidity until 70–80% confluent. Previous research has shown that these cultures consist of approximately 91% astroglia and 8% microglia [28], mimicking the balance of glial types seen in the mouse striatum.

Neuron-glia co-culture. For the neurotoxicity studies, mixed-glia cultures were cultured first as described above and plated in poly-L-lysine-coated 24-well plates. Cultured striatal neurons from embryonic day 15-16 (E15-16) ICR embryos were prepared as described previously [39]. Briefly, striata from 4-6 embryos for each experiment were dissected free from the brain, minced, and incubated (30 min, 37 °C) with 10 ml trypsin (2.5 mg/ml; Sigma) and DNase (0.015 mg/ml; Sigma) in Neurobasal medium (NBM; Invitrogen) supplemented with B-27 additives (Invitrogen), L-glutamine (0.5 mM; Invitrogen), glutamate (25 μM; Sigma), and an antibiotic mixture (penicillin, streptomycin, amphotericin B; Invitrogen). Tissue was triturated, resuspended in 10 ml medium, filtered twice through a 70-μm pore nylon mesh. Cells (2.5 × 10⁵ neurons per well) were plated on a bedlayer of mixed glia grown in 10% FBS for 6 DIV. Neurons were allowed to mature for 6-8 DIV prior to treatment with Tat ± opiates. At 6-8 DIV, neurons have elaborated axons and dendrites, and express many characteristics of mature neurons, including relatively mature patterns of opioid receptor expression.

Tat/Opiate Treatments

Cells were treated with HIV-1 Tat₁₋₈₆ (ImmunoDiagnostics; clade B), which has a Cys 30-Cys 31 motif that is critical for exciting the NMDA receptor, not present in clade C Tat [71]. As such, clade B Tat acts to inhibit β-catenin signaling, a shared target of inflammatory factors, thereby promoting viral replication within astrocytes [72-75]. Negative controls were treated with HIV-1 Tat_{A31-61} (100 nM mutant Tat), a deletion mutation that lacks the excitotoxic core and basic domains of clade B Tat [76].

The opiate treatments, morphine sulfate, buprenorphine, and methadone were obtained from NIDA Drug Supply System (Rockville, MD). Naloxone (1.5 μM; Sigma) was used as a broad-spectrum opioid receptor antagonist and added 1 h before other treatments. Cell cultures were treated with Tat (100 nM) with time of exposure depending on the experimental assessment. For the concentration-response study, Tat treatment ranged from 0.001 nM to 100 nM. Similarly, depending on the experimental assessment, opiate treatments were added using various concentrations (ranging from 0.01 to 1000 nM) and added concurrently with Tat treatment. Details about the concentrations used and exposure durations are specified in the experimental assessment section for each individual assessment test.

Assessment of Neuronal Viability in Neuron-Glia Co-Culture

To study the neurotoxic effects of opiate drugs and Tat interactions, neuronal survival was measured by conducting repeated measures studies in neuron-glia-co-cultures. Computer-assisted, time-lapse imaging was used to track the same neurons for 48 h during treatment with equimolar concentrations of opiates (morphine 500 nM, buprenorphine

ranging from 5 to 500 nM, and methadone 500 nM) and/or Tat (100 nM) exposure as has been previously described [77]. Briefly, 24-well plates were transferred to a heat insert MXX holder (PeCon Instruments, Houston, TX) and set on the scanning stage of a Zeiss Axio Observer Z.1 inverted microscope (Carl Zeiss Inc., Thornwood, NY). For each well, 6-10 non-overlapping fields were selected. A total of 8-10 individual medium spiny striatal neurons were identified in each field on the basis of their distinctive morphology in digital images. Time-lapse images of the same series of fields were recorded at 30 min intervals for 48 h after concurrent HIV-1 Tat and/or opiate treatment, using an automated, computer-controlled stage encoder and Axiovision 4.6 software (Carl Zeiss, Inc.). During the course of the experiment, cells were maintained in an XL S1 environment incubator (PeCon) at 37 °C in 5% CO₂/95% air at high humidity. At the end of each experiment, we assessed all preselected neurons for viability in digital images taken at each time point during the entire treatment period. Death was assessed using rigorous morphological criteria, including the disintegration of neurites, as well as either the involution or complete fragmentation of the cell body. Neuronal death was confirmed by staining with ethidium monoazide [78]. To compare treatment effects on the survival of individual neurons, repeated assessments of the viability of individual neurons were analyzed at 4 h intervals throughout the 48 h experiment. Findings were recorded as the mean percentage of surviving neurons, relative to pretreatment numbers ± SEM from *n* = 3 independent experiments.

Assessment of Intracellular Calcium ([Ca²⁺]_i) in Mixed-Glia Cultures

The dynamics of [Ca²⁺]_i transients in astrocytes is an important issue because calcium is established as an important second messenger and can trigger multiple sequences of events. Levels of [Ca²⁺]_i production in mixed-glia cultures were measured using a fluorescent microplate reader (NovoStar, BMG Labtech, Inc., Cary, NC). Mixed-glia cultures were plated on special UV-transparent, opaque-walled 96-well cell culture plates (Costar #3603, Corning Life Sciences, NY). Cells were loaded with 4 μM fura-2AM (Molecular Probes, Eugene, OR) for 45 min at 37 °C in Hank's balanced salt solution (HBSS) supplemented with 10 mM HEPES buffer (pH 7.2). After three washes, the cells were further incubated for an additional 30 min at 37 °C to ensure de-esterification of the AM group of the fluorophores. To determine the effects of opiates and/or Tat on glial [Ca²⁺]_i, cultured mixed glia were pretreated for 30 min with Tat (100 nM) and/or morphine, buprenorphine, or methadone (ranging from 0.1 nM to 1000 nM). [Ca²⁺]_i data were acquired at 340/380 nm excitation and 510 nm emission wavelengths. Conversion to [Ca²⁺]_i was calculated according to an equation described previously [79]. Data represent percent of control values ± SEM from *n* = 6 independent experiments.

Assessment of Intracellular Reactive Oxygen Species (ROS) Production in Mixed-Glia Cultures

Glia-derived cellular toxins, including ROS production have been implicated as possible mediators of the opiate-

enhanced neurotoxicity driven by activation of MOR in astrocytes [16, 36-38, 41]. Because astroglia are a key site where opiates act to exacerbate the neurotoxic effects of HIV-1, we measured general ROS production in mixed-glia cultures. For drug assessments, we also looked at more specific hydrogen peroxide (H_2O_2) production.

Measurement of overall ROS production. Levels of ROS production were measured by using the indicator 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM- H_2 DCFDA, Invitrogen, Carlsbad, CA), which is de-acetylated to dichlorofluorescein (DCF). Cells were loaded with 10 μ M CM- H_2 DCFDA in warm HBSS for 45 min according to the manufacturer's protocol, then washed twice before treatments were applied. Two concentration-response experimental designs were used. In one design, cells were treated with Tat (100 nM) and opiates (morphine, buprenorphine, methadone) ranging in concentration from 0.1 nM to 1000 nM. In another design, Tat treatment ranged from 0.001 nM to 100 nM and morphine was held at 500 nM. After treatment, cells were incubated for 45 min at 37 °C before fluorescence was measured at $\lambda_{ex} = 485$ nm and $\lambda_{em} = 520$ nm using a PHERAstar FS (BMG Labtech, Inc., Cary, NC) microplate reader. ROS levels were normalized by cell viability to calculate the ROS/cell viability arbitrary unit \pm SEM at the 45 min time point from $n = 6$ independent experiments.

Measurement of H_2O_2 production. To look at ROS in greater detail, levels of H_2O_2 production were assessed over a range of opiate concentrations as previously described [80]. Briefly, cells were washed once with warm HBSS, and then 50 μ l of HBSS was added to each well, followed by 50 μ l of control (HBSS) or opiates (ranging from 0.01 nM to 1000 nM; morphine, buprenorphine, methadone) and/or 100 nM Tat treatments. To each well, 100 μ l of assay mix (200 μ M homovanillic acid, 10 U/ml horseradish peroxidase, 2 mM HEPES, pH 7.5) with or without catalase (10,000 U/ml), was added. After treatment, cells were incubated for 3 h at 37 °C. Following 3 h incubation at 37 °C, 16 μ l of stop solution (0.1 M glycine, pH 10.0) was added to each well, and the plates were read at $\lambda_{ex} = 321$ nm and $\lambda_{em} = 421$ nm using a PHERAstar FS (BMG Labtech, Inc., Cary, NC) microplate reader. Results were calculated as catalase-inhibitable fluorescence and reported as percent of control values \pm SEM at the 3 h time point from $n = 9-12$ independent experiments.

Assessment of Chemokine Release in Mixed-Glia Cultures

To examine whether opiate drugs and Tat in combination or alone can influence levels of released chemokines, the medium of striatal mixed-glia cultures was evaluated using a mouse cytokine assay system (Bio-Plex; Bio-Rad, Hercules, CA). Cells were incubated for 8 h with equimolar concentrations of opiates (500 nM, buprenorphine, or methadone) and/or Tat (100 nM). Three different chemokines were assayed simultaneously, including MIP-1 β (or CCL4), RANTES (or CCL5), and MCP-1 (or CCL2). Calibration curves were prepared using sequential, four-fold dilutions of recombinant chemokine standards. Samples were measured and blank values were subtracted from all

readings. Bio-Plex assays (Bio-Rad) were carried out directly in 96-well plates at room temperature, and protected from light, according to manufacturer's directions. 50 μ l of multiplex bead working solution was added to the plate and removed by vacuum filtration. 100 μ l of Bio-Plex wash buffer was dispensed to each well and washed twice. Diluted samples or blanks were incubated (30 min, room temperature) with continuous shaking at 300 rpm. Beads were washed, incubated with detection antibody, and measured with streptavidin phycoerythrin. The fluorescence intensity of the beads was measured in a volume of 125 μ l of Bio-Plex assay buffer. Data analyses were performed with Bio-Plex Manager software, version 4.0. Protein levels were measured in all samples using the bicinchoninic acid method (Pierce BCA Protein Assay Kit; Thermo Scientific, Rockford, IL). In samples where chemokine levels were below the detectable limit of the standard curve, data were replaced by the lowest value of the standard curve to avoid missing data points for analyses. The data were then transformed to indicate percent change values. Data represent the percentage of control values \pm SEM from $n = 6$ independent experiments.

Statistical Analyses

Data were analyzed using analysis of variance (ANOVA) techniques (SYSTAT 11.0 for Windows, SYSTAT Inc.) followed by Duncan's post-hoc analyses. For the within-subjects factors (i.e., neurotoxicity time-lapse studies), violations of compound symmetry were addressed by using the Greenhouse-Geisser (1959) degrees of freedom correction factor [81]. An alpha level of $p < 0.05$ was considered significant for all statistical tests used. Data are presented as the mean \pm SEM.

RESULTS

Neurotoxicity of Opiates and/or Tat in Neuron-Glia Co-Culture

Effects of opiates (500 nM) and/or Tat (100 nM) exposure on neuronal survival were examined over a 48 h period (Fig. 1A). A repeated-measures ANOVA demonstrated a significant time x treatment interaction ($p_{GG} < 0.001$). Neuronal survival significantly decreased over the 48 h period (time effect) for all Tat treatments and buprenorphine ($p_{GG} < 0.001$), whereas morphine and methadone alone as well as control displayed no significant effect on neuronal survival over time. Exposure to Tat alone was intrinsically neurotoxic ($p < 0.001$ vs control), as was buprenorphine alone ($p < 0.001$ vs control). Importantly, all three opiates accelerated Tat neurotoxicity significantly above levels seen with Tat alone ($p < 0.001$).

As exposure to 500 nM buprenorphine alone was intrinsically neurotoxic, additional studies were conducted with lower concentrations of buprenorphine (5 and 50 nM, Fig. 1B). A repeated-measures ANOVA revealed a significant time x treatment interaction ($p_{GG} < 0.001$). 50 nM and 500 nM buprenorphine resulted in a significant decrease in neuronal survival over the 48 h period ($p_{GG} < 0.05$), but no time effect was noted for 5 nM buprenorphine alone. For combined treatment, only 500 nM buprenorphine accelerated

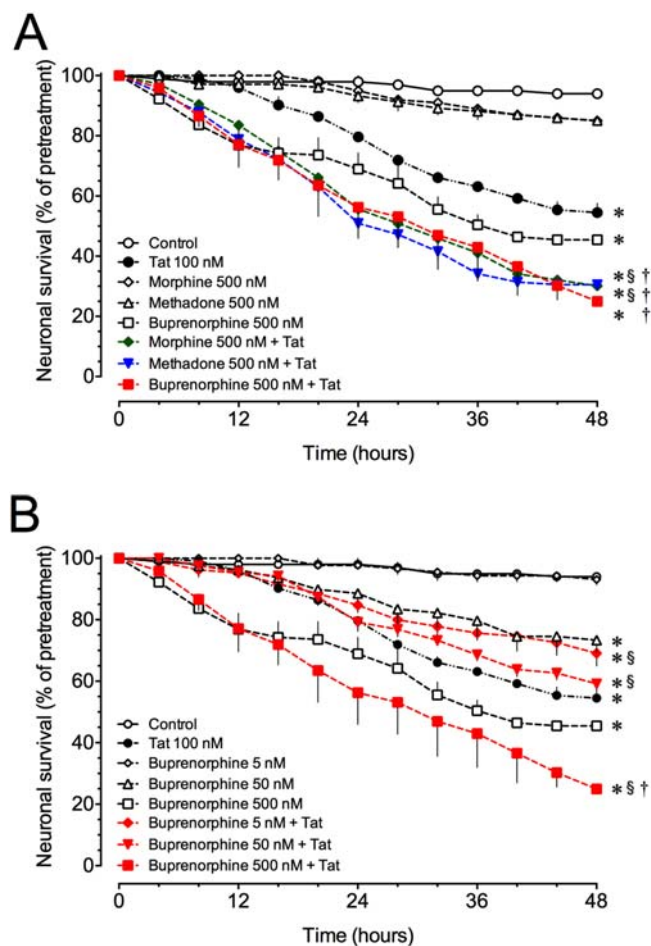


Fig. (1). Time-dependent effects of opiate drugs and/or Tat on the survival of striatal spiny neurons cultured with mixed glia enriched in astrocytes. Results are the mean percentage of surviving neurons, relative to pretreatment number \pm SEM from $n = 3$ experiments. (A) Neurons were assessed before and following exposure to equimolar concentrations of opiates (500 nM) and/or Tat (100 nM). Exposure to Tat and buprenorphine alone is intrinsically neurotoxic ($*p < 0.05$ vs control), whereas morphine and methadone alone have no significant effects on neuronal survival. Co-exposure to Tat and different opiates display neurotoxicity ($*p < 0.05$ vs control) and morphine and methadone accelerate Tat neurotoxicity significantly ($\dagger p < 0.05$ vs Tat; $\S p < 0.05$ vs corresponding drug alone). (B) Exposure to lower concentrations of buprenorphine alone or in combination with Tat indicate no significant effects for 5 nM buprenorphine, but for 50 nM and 500 nM ($*p < 0.05$ vs control). Buprenorphine with Tat exposure increases toxicity significantly ($\S p < 0.05$ vs corresponding concentration alone). Further, 500 nM buprenorphine accelerates Tat neurotoxicity significantly ($\dagger p < 0.05$ vs Tat), whereas 5 nM buprenorphine appears to protect against Tat neurotoxicity ($\# p < 0.05$, buprenorphine 5 nM + Tat vs Tat).

Tat neurotoxicity significantly above levels seen with Tat alone ($p < 0.05$). Interestingly, 5 nM buprenorphine appeared to protect against Tat neurotoxicity at 44 h and 48 h following continuous exposure ($\# p < 0.05$, buprenorphine 5 nM + Tat vs Tat). No significant difference was noted for 50 nM buprenorphine + Tat compared to Tat alone.

Effects of Opiate Drugs \pm Tat (100 nM) on Intracellular Calcium $[Ca^{2+}]_i$ in Mixed-Glial Cultures

The effects of opiates and/or Tat (100 nM) exposure were examined on mixed glia by assessing $[Ca^{2+}]_i$ (Fig. 2). All three opiate drugs showed a concentration-dependent increase in $[Ca^{2+}]_i$ ($p < 0.05$ for each opiate drug), as well as a significant main effect of Tat ($p < 0.05$ for each opiate drug). For morphine and buprenorphine, Tat increased $[Ca^{2+}]_i$ similarly across different concentrations (Fig. 2A, B). In contrast, for methadone, Tat significantly increased $[Ca^{2+}]_i$ production only for the lower concentrations (< 100 nM), but not for 100 nM or 1000 nM methadone (Fig. 2C).

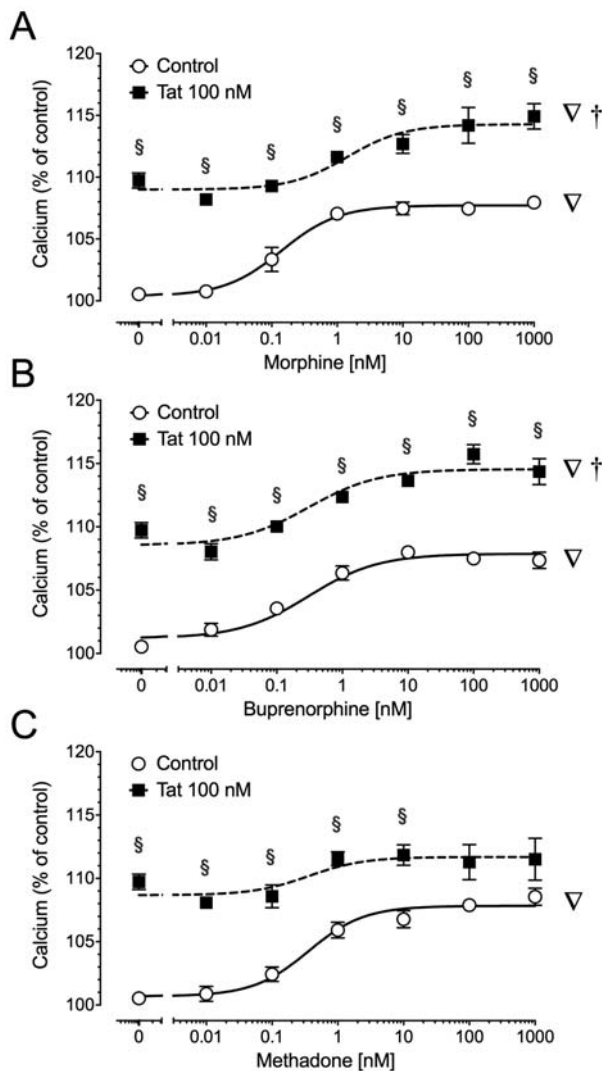


Fig. (2). Effects of Tat (100 nM) \pm different concentrations of opiate drugs on $[Ca^{2+}]_i$ production were assessed by fura-2AM in mixed-glial cultures after 30 min pretreatment. Results represent the percentage of control values and are the mean \pm SEM from $n = 6$ experiments. $[Ca^{2+}]_i$ significantly increases in a concentration-dependent manner for all three opioid drugs, with Tat increasing $[Ca^{2+}]_i$ for (A) morphine, (B) buprenorphine, and (C) partially for methadone. $\S p < 0.05$ vs corresponding drug concentration alone; $\nabla p_{GG} < 0.05$, significant concentration-dependent drug effect; $\dagger p < 0.05$, significant Tat effect.

Effects of Opiate Drugs ± Tat (100 nM) on ROS in Mixed-Glial Cultures

To evaluate the effects of opiate drugs ± Tat on oxidative stress in mixed-glia, ROS production was assessed by DCF reactivity (Figs. 3, 4) and H₂O₂ production (Fig. 5).

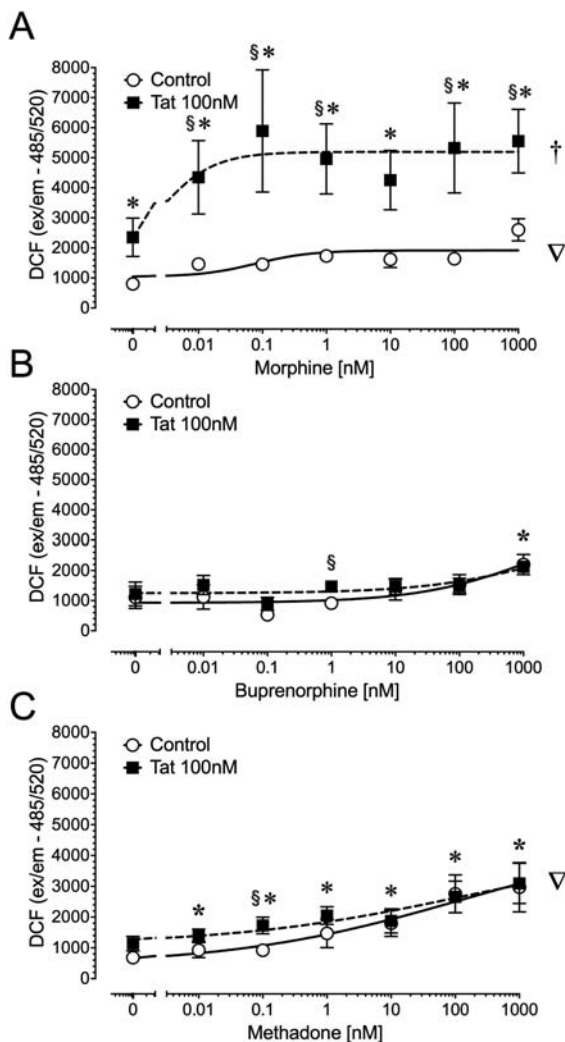


Fig. (3). ROS production was assessed by dichlorofluorescein diacetate fluorescence (DCF) at 45 min following continuous exposure *in vitro*. Results are the mean ± SEM from $n = 6$ experiments. Findings in mixed glia reveal significant concentration-dependent effects for (A) morphine and (C) methadone ($p_{GG} < 0.05$, significant concentration-dependent drug effect), but not (B) buprenorphine. A significant main effect for Tat is only noted for morphine ($\dagger p < 0.05$). Buprenorphine and methadone yield similar responses in mixed glia when co-exposed with Tat. * $p < 0.05$ vs control; $\S p < 0.05$ vs corresponding drug concentration alone.

DCF assessments. A significant concentration-dependent drug effect was noted for morphine (Fig. 3A, $p < 0.05$) and methadone (Fig. 3C, $p < 0.05$), but not buprenorphine (Fig. 3B). In combination with Tat, only morphine increased ROS across different concentrations (Fig. 3A, $p < 0.05$), but no Tat effect was noted for

buprenorphine or methadone (Fig. 3B, C). When assessing different concentrations of Tat in combination with or without morphine (Fig. 4), only Tat concentrations ≤ 0.1 nM showed enhanced DCF values in combination with morphine. This suggests that lower Tat concentrations may have greater potential for interacting with opiate drugs.

H₂O₂ assessments. To evaluate the pharmacological mechanisms of inflammation-related effects produced by opiate drugs more in detail, full concentration effect curves for each opiate on the production of H₂O₂ were determined (Fig. 5). Morphine caused a significant concentration-dependent effect ($p < 0.05$) with 3-fold elevations in H₂O₂ at the highest concentration of morphine (Fig. 5A). Co-administering 1.5 μ M naloxone significantly decreased the effect of 100 nM morphine on H₂O₂ production ($p < 0.05$). No significant concentration-dependent effect was noted for buprenorphine (Fig. 5B) or methadone (Fig. 5C). Naloxone (1.5 μ M) significantly decreased H₂O₂ production at 100 nM for buprenorphine ($p < 0.05$). Importantly, a significant Tat effect was noted for H₂O₂ production compared to controls ($p < 0.05$).

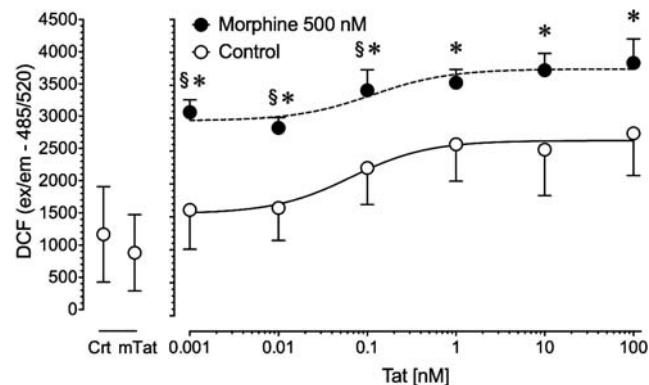


Fig. (4). Concentration-dependent effects of Tat ± morphine (500 nM) on ROS formation measured by DCF in mixed-glia at 45 min following continuous exposure *in vitro*. Tat at concentrations ≥ 0.1 nM significantly increases ROS (* $p < 0.05$ vs control); while a non-toxic Tat deletion mutant (mTat) (100 nM) fails to increase ROS. Morphine exposure lowers the threshold for Tat-induced ROS production at lower doses of Tat (500 nM) ($\S p < 0.05$ vs corresponding Tat concentration alone). Results are the mean ± SEM from $n = 6$ experiments. Crt = control.

Effects of Tat and/or Buprenorphine and Methadone on Chemokine Production in Mixed-Glial Cultures

MIP-1 β , RANTES, and MCP-1 production was assessed in striatal mixed glia for Tat (100 nM) and/or buprenorphine and methadone (500 nM) at 8, 24, and 72 h (Fig. 6). The findings demonstrated similar effects at 8, 24, and 72 h and for this reason only the 8 h time is shown. Tat treatment alone significantly elevated RANTES from ($p < 0.05$). None of the opiates alone had a significant effect. Co-exposure to Tat and methadone significantly increased RANTES production compared to Tat alone ($p < 0.05$) and naloxone (1.5 μ M) was able to reverse this effect.

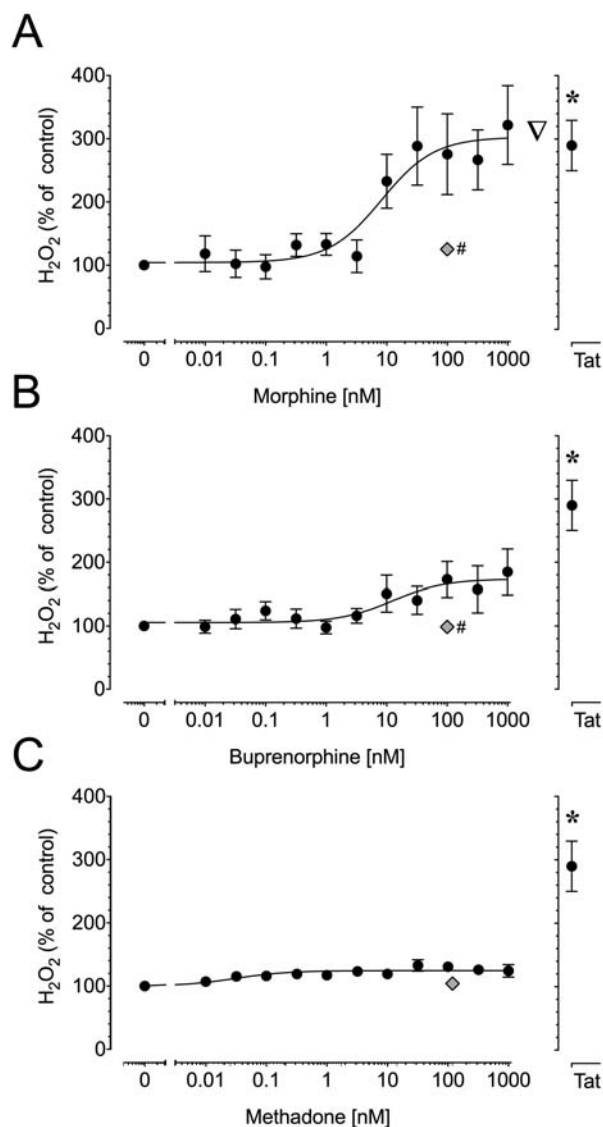


Fig. (5). Concentration-dependent effects of different opiates on ROS assessed by H_2O_2 in a mixed-glia cultures. Results represent the percentage of control values and are the mean \pm SEM from $n = 9-12$ experiments. **(A)** Morphine indicates a significant concentration-dependent response ($^*p < 0.01$) with a 3-fold elevation in H_2O_2 at the highest drug concentration. Naloxone (1.5 μ M) significantly decreases H_2O_2 production at 100 nM ($^{\#}p < 0.05$ vs control). **(B)** No significant concentration-dependent effect is noted for buprenorphine. Naloxone (1.5 μ M) significantly decreases H_2O_2 production at 100 nM ($^{\#}p < 0.01$ vs control). **(C)** No effects are noted for methadone. Importantly, Tat increases H_2O_2 production significantly ($^*p < 0.01$ vs control).

DISCUSSION

Despite widespread use of, and benefits from, opiate addiction therapy in HIV-1-infected individuals, preclinical studies have not directly compared the relative effects of buprenorphine and methadone or their potential interactions with HIV-1 in the CNS. The findings of the present study indicate there are inherent pharmacological differences in the action of opiate addiction treatments on neuronal survival. Morphine and methadone by themselves cause minimal

neuronal death, but both exacerbate neurotoxicity in combination with Tat. This suggests that they may have potential negative side effects as opiate substitution therapies for HIV-1-infected patients. In contrast, buprenorphine at low concentrations was actually protective against Tat-induced neurotoxicity, although higher concentrations were toxic by themselves and exacerbated Tat-induced toxicity. The protective effect of 5 nM buprenorphine needs to be assessed more systematically in future studies. In patients receiving intravenous buprenorphine (0.3 mg) the buprenorphine plasma level has been shown to vary from 0.96 ± 0.15 ng/ml initially after 30 min to 0.10 ± 0.02 ng/ml after 13 h [82]. These levels are equivalent to 2 – 0.21 nM, indicating that 5 nM is more applicable to the clinical setting compared to 500 nM that shows neurotoxicity in our present study. This finding suggests basic differences in the actions of each opiate drug on neuronal injury induced by drug treatment alone.

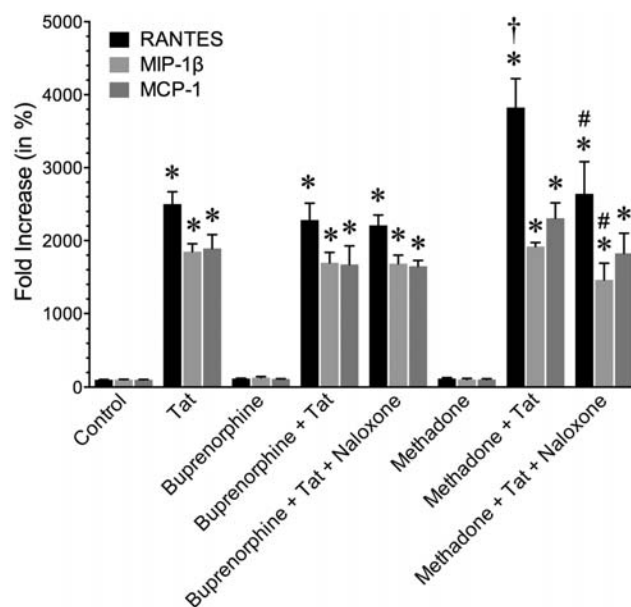


Fig. (6). Effects of buprenorphine or methadone \pm Tat on chemokine production in mixed glia following 8 h of continuous exposure. Exposure to Tat alone significantly increases RANTES, MIP-1 β , and MCP-1 production ($^*p < 0.05$ vs control levels), while neither buprenorphine (500 nM) nor methadone (500 nM) alone affect the release of these three chemokines. In combination, methadone + Tat markedly increase the release of RANTES/CCL5, but not MIP-1 β or MCP-1, beyond levels seen with Tat alone ($^{\dagger}p < 0.05$) and the methadone-Tat interaction is prevented by naloxone (1.5 μ M) ($^{\#}p < 0.05$ vs methadone + Tat). By contrast, Tat-induced chemokine release is unaffected by buprenorphine. Results are expressed as percent of control values and are the mean \pm SEM from $n = 6-12$ experiments.

Why might morphine, methadone, and buprenorphine act differently? Several possibilities exist and all are likely to be operative to varying extents. First, similar to other G protein coupled receptors (GPCRs) [83-85], distinct opiate drugs can have differential actions in cells at MOR by coupling to one or more downstream effectors including $G\alpha$, $G\beta\gamma$, and/or β -arrestin. Second, morphine, methadone, and buprenorphine

can activate MOR, δ -opioid (DOR), and κ -opioid (KOR), receptors. MOR and KOR stimulation can have opposing effects [86]. For example, MOR activation increases HIV-1 replication [87-90], while KOR downregulates HIV-1 expression in human microglia [87]. To add to the intricacy, morphine and methadone are both preferential MOR agonists, while buprenorphine has complex actions as a partial MOR agonist, a partial KOR antagonist [58, 91-94], and as a NOP agonist at high concentrations [69, 95, 96]. NOP activation has been considered as a possible underlying mechanism for the bell-shaped dose-response curve of buprenorphine [97]. Leukocytes including macrophages can express NOP [86] and buprenorphine preferentially activates NOP at high concentrations. Although the reported anti-inflammatory role for NOP activation in glia [98] and in spinal cord in a neuropathic pain model [99] seems contrary to the observed increases in neuronal death in the present study, it is uncertain to what extent HIV-1 Tat exposure might reprogram microglia/perivascular macrophages to uniquely respond to NOP stimulation. Third, GPCR heteromeric/oligomeric complexes may create novel actions that differ in cell-type and in context-specific manners [100]. MOR activation induces heterologous desensitization of CCR5, but not CXCR4 [101, 102]. Selectively targeting CCR5-MOR heteromeric complexes affects HIV entry *via* CCR5 HIV co-receptors [103, 104]. KOR activation can inhibit or trigger the release of specific proinflammatory cytokines [105], and KOR and CXCR4 can undergo cross-desensitization, suggesting additional potentially novel modes of buprenorphine action.

The finding that morphine exacerbates Tat neurotoxicity confirms prior studies [25, 39, 106], and we have now shown that equimolar concentrations of morphine, methadone, and buprenorphine show similar, neurotoxic interactions with Tat. As 100 nM Tat alone is significantly neurotoxic, the combinations of Tat and opiates may have reached a “ceiling effect” of maximal toxicity, which did not allow us to discriminate interactions between the opiates. The ability to vary opiate concentrations during Tat exposure allowed us to see the rate of neuronal losses (accelerated/decelerated). Further investigations will vary concentrations of Tat, using lower efficacious dosing [26] to further delineate the thresholds for opiate interactions on neuronal survival. The present study utilized Tat at 100 nM (and opiates at 500 nM) to permit comparisons with past experiments.

Although ROS production by microglia and astrocytes is implicated as a contributor to neurotoxicity [16, 36-38, 41], we did not find a clear-cut correlation since the three opiates by themselves were similar in their influence on ROS production in mixed glia. Alternatively, elevations in $[Ca^{2+}]_i$ in mixed-glia seemed to better coincide with neuronal losses. All three drugs alone caused marked increases in $[Ca^{2+}]_i$ at the highest concentrations, and while only buprenorphine alone caused neuronal losses—there was a tendency toward increased neuronal loss with morphine or methadone treatment and morphine-induced neuronal losses have occasionally been found [28]. We suspect that $[Ca^{2+}]_i$ dysregulation and heightened ROS in glia partially compromises neurons, but these events alone seem insufficient to trigger significant neuronal death. Alternatively, if the glial dysregulation coincides with an

alternative insult—such as direct neuronal damage [26] or indirect insults such as other viral or cellular toxins—the compromised glia may be unable to provide sufficient metabolic/trophic support to prevent neuronal demise [26].

Besides increasing $[Ca^{2+}]_i$ mobilization [16] and the production of oxyradicals [41], HIV-1 proteins also significantly increase the release of chemokines, such as MCP-1 and RANTES from astrocytes [16, 36-38, 107]. RANTES, MCP-1, and MIP-1 β have been demonstrated to be elevated in CNS tissues and/or the cerebrospinal fluid of individuals with AIDS or HIV-1-associated neurocognitive disorders [32, 108-110]. Past research from our laboratory has already shown that morphine can dramatically exaggerate key astroglial responses to Tat [16]. Marked alterations in $[Ca^{2+}]_i$ homeostasis and an exaggerated release of the critical inflammatory chemokines MCP-1 and RANTES, and the cytokine IL-6 was reported for combined Tat and morphine treatment. This is in contrast to the present findings, where the combination of Tat and buprenorphine or methadone, did not exaggerate the release of MCP-1. Instead, Tat and methadone yielded an increase only in the release of RANTES, and Tat and buprenorphine treatment had no interactive effect. Astrocytes express CCR5 and CCR1, which are key receptor targets of RANTES [111], and RANTES is a potent activator of astrocytes. Findings that methadone or morphine [16], but not buprenorphine, in combination with Tat exacerbate the release of RANTES from mixed glia, suggest basic differences in the actions of each opiate drug on inflammatory responses induced by Tat proteins and additionally suggest that buprenorphine is intrinsically toxic to neurons in high concentrations.

Overall, our findings suggest that opiate drugs by themselves act similarly to modify proinflammatory ROS, $[Ca^{2+}]_i$ and the signature of the chemokine/cytokines released by glia. However, the ability to induce particular glial inflammatory factors does not necessarily coincide with the ability of each of these opiate addiction treatments to exacerbate Tat-induced neurotoxicity. Buprenorphine, in particular, shows the greatest range of interactive effects with Tat on neurotoxicity, presumably due to the drug's complex actions at multiple non-MOR sites. At lower doses, buprenorphine actually limits the neurotoxicity of HIV-1 Tat. Thus, despite a potentially narrow therapeutic index, buprenorphine may have distinct advantages in the treatment of opiate addiction or pain management in HIV-1-infected patients.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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