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Synergistic increases in intracellular Ca^{2+} , and the release of MCP-1, RANTES, and IL-6 by astrocytes treated with opiates and HIV-1 Tat[‡]

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Abstract

Recent evidence suggests that injection drug users who abuse heroin are at increased risk for CNS complications from human immunodeficiency virus (HIV) infection. Opiate drugs may intrinsically alter the pathogenesis of HIV by directly modulating immune function and by directly modifying the CNS response to HIV. Despite this, the mechanisms by which opiates increase the neuropathogenesis of HIV are uncertain. Herein we describe the effect of morphine and the HIV-1 protein toxin Tat₁₋₇₂ on astroglial function in cultures derived from ICR mice. Astroglia maintain the blood brain barrier and influence inflammatory signaling in the CNS. Astrocytes can express μ -opioid receptors, and are likely targets for abused opiates, which preferentially activate μ -opioid receptors. While Tat alone disrupts astrocyte function, when combined with morphine, Tat causes synergistic increases in $[\text{Ca}^{2+}]_i$. Moreover, astrocyte cultures treated with morphine and Tat showed exaggerated increases in chemokine release including monocyte chemoattractant protein-1 (MCP-1) and regulated on activation, normal T cell expressed and secreted (RANTES), as well as interleukin-6 (IL-6). Morphine-Tat interactions were prevented by the μ -opioid receptor antagonist β -funaltrexamine, or by immunoneutralizing Tat₁₋₇₂ or substituting a non-toxic, deletion mutant (Tat₃₁₋₆₁). Our findings suggest that opiates may increase the vulnerability of the CNS to viral entry (via recruitment of monocytes/macrophages) and ensuing HIV encephalitis by synergistically increasing MCP-1 and RANTES release by astrocytes. The results further suggest

[‡]**Abbreviations:** alpha chemokine receptor (CXCR); beta chemokine ligand (CCL); beta chemokine receptor (CCR); β -funaltrexamine (β -FNA); calcium-induced calcium release (CICR); excitatory amino acid transporter-2 (EAAT2); granulocyte macrophage colony stimulating factor (GM-CSF); granulocyte-colony stimulating factor (G-CSF); human immunodeficiency virus (HIV); human immunodeficiency virus encephalitis (HIVE); inositol trisphosphate (IP₃); interferon (IFN); interleukin (IL); intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$); monocyte chemoattractant protein (MCP); nor-binaltorphimine (nor-BNI); phosphatidylinositol 3-kinase (PI3-kinase); phospholipase C- γ (PLC γ); regulated on activation, normal T cell expressed and secreted (RANTES); soluble TNF receptor subunit (sTNFR1); stem cell factor (SCF); thrombopoietin (TPO); transactivator of transcription (Tat); tumor necrosis factor- α (TNF- α); vascular endothelial growth factor (VEGF).

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that astrocytes are key intermediaries in opiate-HIV interactions and disruptions in astroglial function and inflammatory signaling may contribute to an accelerated neuropathogenesis in HIV infected individuals who abuse opiates.

Keywords

AIDS; chemokines; μ -opioid receptors; drug abuse; neuroimmunology; cytokine arrays

INTRODUCTION

Opiate drug abuse contributes to the spread of HIV (Petito et al., 1999) through needle sharing among injection drug users or through the exchange of sex for drugs. In addition, the drugs themselves may intrinsically affect the course and severity of the infection (Nath et al., 2002). Thus, the enhanced pathogenesis is likely caused by both indirect (e.g., immune dysfunction) and direct neurotoxic mechanisms (Donahoe and Falek, 1988; Arora, 1990; Rouveix, 1992; Peterson et al., 1993; Nath et al., 2002; Rogers and Peterson, 2003).

“Opiates”, plant alkaloids derived from the opium poppy, mimic endogenous “opioid” peptides by preferentially activating μ -opioid receptors and intrinsically modify immune function. Morphine, the major metabolite of heroin in the CNS (Sawynok, 1986), can modulate the production of cytokines, as well as the function of cytokine receptors in neural cells (Chang et al., 1998; Rogers and Peterson, 2003), including microglia (Chao et al., 1994) and astroglia (Mahajan et al., 2002). Effects include increases in tumor necrosis factor- α (TNF- α) production (Chao et al., 1994) and upregulation of CCR5 and CXCR4 [see (Rogers and Peterson, 2003)], heterologous cross-sensitization of CCR5 (McCarthy et al., 2001; Rogers and Peterson, 2003), and alterations in HIV propagation in lymphocytes and monocytes/macrophages [reviewed in (McCarthy et al., 2001; Rogers and Peterson, 2003)]. Although the prevalence of HIV infection among opiate abusers is substantial, and opiates may accelerate the frequency and severity of HIV encephalitis (HIVE) (Bell et al., 1998; Nath et al., 2000; Nath et al., 2002), other evidence suggest that opiates can allay systemic HIV infection (Donahoe and Vlahov, 1998; Overall, 2004; Donahoe, 2004). The deleterious effects may be particularly evident in the CNS (Donahoe, 2004) and whether opiates are good, bad or act indifferently likely varies among cell types and depends on the parameter measured (Khurdayan et al., 2004).

Chemokines are small proteins of 5 to 12 kDa in size, that serve as chemoattractants for either NK cells, T cells, monocytes, neutrophils, fibroblasts and endothelial cells. Chemokines consist of four major subfamilies, and of those, the CXC and CC families are classified according to the position of the first two cysteines linked by a sulfide bond. It is well accepted that both chemokines and their receptors play a key role in HIV infection and progression. In addition, studies have shown that chemokines and their receptors are involved in the neuropathogenesis of HIV-1 infection in the CNS (Rappaport et al., 1999; Petito et al., 1999; McManus et al., 2000; Wesselingh and Thompson, 2001; Toborek et al., 2003). Chemokine receptors are important HIV-1 co-receptors, in combination with the T lymphocyte receptor, CD4. HIV-1 isolates utilize several members of the chemokine

receptors as co-receptors for infection in the CNS (McManus et al., 2000). The major receptor types reportedly involved in HIV-1 infection are CCR3, CCR5, CXCR4, and CCR2. All these receptor types can be expressed by astrocytes, microglia, and neurons (McManus et al., 2000), as well as by neural progenitors (Tran et al., 2004; Ji et al., 2004; Peng et al., 2004), suggesting that astroglia, as well as other cell types, are significant targets of the inflammatory events associated with HIV pathogenesis.

The striatum is vulnerable to HIV infection and an important target for opiate abuse (Mansour et al., 1988; Masliah et al., 1992a; Glass et al., 1993; Mansour et al., 1994; Berger and Nath, 1997; Nestler and Aghajanian, 1997; Kreek and Koob, 1998; Nath et al., 2000; Bansal et al., 2000; Koob, 2000; Nath et al., 2002). Neurons and astrocytes in the striatum (Gurwell et al., 2001; Stiene-Martin et al., 2001; Nath et al., 2002), as well as neural progenitors in the striatum and elsewhere (Reznikov et al., 1999; Eisch et al., 2000; Stiene-Martin et al., 2001; Persson et al., 2003), widely express μ -opioid receptors and morphine can exacerbate the neurotoxic effects of HIV-derived proteins in neurons. Potential opiate-HIV interactions in astroglia are of interest because of the importance of astroglia in providing metabolic and trophic support for neurons and because of their emerging importance in neuroimmunology. Although the astrocytes themselves can be latently infected with HIV-1 (Bagasra et al., 1996; Nath and Geiger, 1998; Brack-Werner, 1999), viral proteins such as Tat and gp120, which are released from infected macrophages/microglia in the CNS, can also interact with uninfected astrocytes thereby disrupting their function. Tat is a transactivating, nonstructural viral regulatory protein, which is present in the extracellular fluid within the CNS of infected individuals (Hudson et al., 2000) and is intrinsically neurotoxic (Sabatier et al., 1991; Jones et al., 1998; Nath, 1999; Nath, 2002). Extracellular Tat is a potent inducer of diverse genes in a variety of cell types, and has the capacity to activate the pro-inflammatory cytokines IL-6, TNF- α and chemokines interleukin-8 (IL-8) and MCP-1.

Tat or gp120 can destabilize intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) in astroglia (Haughey et al., 1999; Holden et al., 1999), which is similar to effects seen with acute μ -opioid receptor activation (Hauser et al., 1998). Deficits in excitatory amino acid transporter-2 (EAAT2) glutamate transporter expression by human astrocytes have also been noted in response to gp120 or HIV exposure (Wang et al., 2003). Astroglial impairment may contribute to the dysfunction or death of naïve bystanders including neighboring neurons and uninfected glia (Lipton, 1994; Bagasra et al., 1996; Lipton, 1998; Nath and Geiger, 1998; Kolson et al., 1998; Haughey et al., 1999; Kaul et al., 2001). Despite evidence that astroglia are important in the pathogenesis of HIV and findings that κ -opioid receptor activation can modulate the response of astroglia to HIV (Sheng et al., 2003), few studies have explored the effects of opiate drugs in this glial type. For this reason, we examined the interactive effects of morphine, an opiate drug with abuse liability, and HIV Tat₁₋₇₂ on several key astroglial functions. Our results show that opiates act synergistically with Tat₁₋₇₂ to disrupt $[\text{Ca}^{2+}]_i$ homeostasis and the expression and release of critical inflammatory signaling molecules in cultured mouse astrocytes. Opiate-induced alterations in ion homeostasis and inflammatory signaling in astroglia may contribute to the accelerated neuropathology seen with HIV.

METHODS

Materials

Morphine sulfate, β -funaltrexamine hydrochloride (β -FNA), nor-binaltorphimine (nor-BNI), naloxone hydrochloride, glucose, dimethyl sulfoxide (DMSO), MgCl_2 , dantrolene, and HEPES were purchased from RBI/Sigma-Aldrich (Sigma; St. Louis, MO). Trypsin and DNase were obtained from Worthington Biochemical Corporation (Lakewood, NJ). The specific phosphatidylinositol 3-kinase (PI3-kinase) inhibitor (LY294002), calcium ionophore (A23187), and thapsigargin were purchased from Alomone Laboratories (Jerusalem, Israel).

HIV-1 Tat

The *tat* gene encoding the first 72 amino acids was amplified from HIV_{BRU} obtained from Dr. Richard Gaynor through the AIDS repository at the NIH and inserted into an *E. coli* (PinPoint Xa-2) vector (Promega, Madison, WI). Recombinant active Tat₁₋₇₂ was prepared as described previously (Ma and Nath, 1997) with minor modifications (Gurwell et al., 2001). Tat₁₋₇₂ proteins expressed from this construct are naturally biotinylated and can be purified on a column of soft release avidin resin, cleaved from the fusion protein using factor Xa, eluted from the column followed by desalting on a PD10 column. Although endotoxins are below detectable range (*Limulus* Amebocyte lysate assay; Associates of Cape Cod, MA), both Tat₁₋₇₂ and inactive Tat (Tat₃₁₋₆₁) are incubated in DetoxiGel™ Endotoxin removing Gel (Pierce, Rockford, IL) according to the manufacturer's protocol to remove possible trace amounts below 1 pg/ml.

Tat₁₋₇₂ was used in the present study because it has been used extensively in studies of neural toxicity, enabling us to make direct comparisons with earlier studies (Gurwell et al., 2001; Singh et al., 2004; Khurdayan et al., 2004). The first exon of Tat₁₋₇₂ consists of five distinct domains. Collectively, the first three domains (N-terminal, cysteine-rich and core domains) constitute the activation domain, while the fourth and fifth domains are important for RNA binding and ability of Tat to act as a transcriptional transactivator (Jeang et al., 1999). The second Tat exon consists of two domains, including an RGD domain that binds to specific integrins and a second highly conserved domain whose functional significance is unclear (Brake et al., 1990). Although Tat variants from the first and second exons are both neurotoxic, subtle differences likely exist and further study is warranted. Moreover, the extent that the effects of Tat_{BRU} derived from HIV clade B can be generalized to Tat from other HIV strains such as HIV clade C is uncertain and needs to be explored.

Inactive control Tat (Tat₃₁₋₆₁) was generated and purified as described above for Tat₁₋₇₂ from a deletion mutant of the active Tat plasmid, which lacked the sequence encoding the neurotoxic epitope (amino acids 31-61) of Tat₁₋₇₂ (Nath et al., 1996). Immunoneutralized Tat₁₋₇₂ was generated as follows: 100 μl of diluted rabbit-anti-tat sera was added to 50 μl of washed protein A (Roche, Germany) beads and placed on a rocker at room temperature for 1 h. The mixture was centrifuged at 3,000 rpm for 3 min. The supernatant was removed and the beads washed three times with PBS to remove any remaining unbound sera. Next, 100 nM Tat₁₋₇₂ dissolved in serum free medium was added to the washed protein A beads and

placed on rocker plate for 1h. Afterwards, the mixture was spun down as describe above and the supernatant was carefully removed for further use.

Cell Culture

Astroglial-enriched cultures were prepared using 1-4-day-old ICR mice (Charles River Inc., Charles River, MA) as previously described (Stiene-Martin et al., 1998). Briefly, mice were anesthetized and euthanized according to NIH and IACUC guidelines as previously described (Stiene-Martin et al., 1998), which minimizes the number of animals used and their discomfort. Striata were aseptically isolated and cells pooled from 2-4 striata. Growth medium favoring astroglial enrichment consisted of DMEM (GIBCO) supplemented with glucose (27 mM), Na_2HCO_3 (6 mM), HEPES (10 mM) and 10% (v/v) Fetal Bovine Serum (FBS; JRH Biosciences). Cells were plated at 50,000 cells/cm² in poly-L-lysine-coated 24-well plates (for immunocytochemistry), 6-well plates (for cytokine and chemokine detection), or 48-well (for $[\text{Ca}^{2+}]_i$ measurements) plates (Costar, Corning Life Sciences; Acton, MA). Cells were maintained to near-confluence for at least 7 days *in vitro* at 35-36°C in 5% CO_2 /95% air at high humidity. In order to reduce the number of microglia within the astrocyte cultures, 5 mM of leucine methyl-ester (LME) was added for 1-3 h prior to the onset of the experiments as described by others (Thiele et al., 1983). Representative cultures were also immunostained for CD11b which showed a negligible population of contaminating microglia.

Primary microglia were enriched from mixed brain cell cultures prepared from 1-2 day postnatal mouse cerebral cortex using our standard procedures (Knapp, 1997). Briefly, meninges were removed from the cortical tissue which was subsequently dissociated using both mechanical and enzymatic (trypsin, DNase) methods. Single cell suspensions were prepared by filtration of the resulting dissociated preparation through 30- μM nylon mesh. Cells were plated at a relatively low density (5×10^6 per T-25 flask) to discourage the survival of oligodendrocyte precursors and grown in medium containing 10% fetal bovine serum. After 8-10 days the confluent cultures consisted of a bed layer of flat astrocytes with a small population of microglia either loosely attached to the surface or floating in the medium. The microglia were dislodged by rotary shaking of the flasks (150 rpm, 30 min). 3.5×10^5 microglia were plated per well on poly-L-lysine coated 6-well tissue culture plates in medium containing 1% fetal bovine serum.

Experimental Treatments

Cells were continuously exposed to serum-free medium alone (vehicle-treated controls), 500 nM morphine sulfate (Sigma) and/or 100 nM Tat₁₋₇₂ (or immunoneutralized or mutant Tat) for varied intervals. The concentrations of morphine and Tat₁₋₇₂ protein were chosen based on a concentration-response studied that showed a saturation curve starting at 0.5 μM for morphine and at 100 nM for Tat₁₋₇₂. In addition, ongoing studies in this lab as well as others have shown that these concentrations are clinically relevant. Cultures were exposed with media alone (control), morphine (500 nM) and / or Tat₁₋₇₂ (100 nM) in the presence or absence of naloxone (a μ , δ and κ antagonist), β -FNA (a selective μ -opioid receptor antagonist), or nor-BNI (a selective κ -opioid receptor antagonist). Multiple concentrations of the above antagonists (i.e., 0, 0.5, 1.5, 2.5, 5, 7.5, and 10 μM) were initially screened as

part of the intracellular calcium ($[Ca^{2+}]_i$) studies. Pretreatment with 1.5 μ M amounts of opioid antagonists for 1 h was determined to be an optimal to block the effects of 500 nM morphine in living cells and this concentration was used in subsequent studies. We have found 0.1-1 μ M concentrations of morphine to maximally active μ -opioid receptors in astroglia (Stiene-Martin and Hauser, 1991; Stiene-Martin et al., 1991; Hauser et al., 1996). Antagonists were dissolved in cell culture medium prior to use. Microglia were cultured in 6 well plates containing either control medium, or medium with added Tat (100 nM), morphine (500 nM) or Tat plus morphine. Conditioned medium was collected after 18 h. Details of the array development and quantification are the same as for the astrocytes.

Intracellular calcium ($[Ca^{2+}]_i$)

$[Ca^{2+}]_i$ was measured in individual flat, polyhedral cells within glial-enriched cultures as previously described (Hauser et al., 1996; Stiene-Martin et al., 1998). Briefly, cells were grown on MatTek dishes (Natick, MA). Cells were loaded with 10 μ M fura-2/AM (Molecular Probes, Eugene, OR) for 45 min at 35 °C in growth media that included 10 mM Hepes buffer (pH 7.2) and 2% DMSO. Ratiometric $[Ca^{2+}]_i$ measurements were made using a Dage 72 CCD camera (Michigan City, IN), Hamamatsu Photonics C2400 intensifier (Hamamatsu-City, Japan) (Stiene-Martin et al., 1998), and an Axon Instruments Image Lightening 2000 capture board with Workbench 4 ratiometric software (Union City, CA). $[Ca^{2+}]_i$ was determined from the ratio of fluorescence at 340 and 380 nm excitation wavelengths (Grynkiewicz et al., 1985). Mean $[Ca^{2+}]_i$ levels were determined before and after morphine, naloxone, and/or Tat₁₋₇₂ treatment. About 35 flat, polyhedral astrocytes were sampled per treatment group in each experiment. Data are reported as $[Ca^{2+}]_i$ and represent the mean $\pm$ SEM of 7 experiments (~250 cells were sampled in total per each treatment group).

$[Ca^{2+}]_i$ in astroglia-enriched populations was measured using a fluorescent microplate reader with an integrated injector (PerkinElmer, Wallac Victor³; Wellesley, MA). Cells grown in 48-well plates grown to 80-85% confluence were treated with serum free medium for 24 h prior to further treatment. Cells were loaded with 4 μ M fura-2/AM (Molecular Probes, Eugene, OR) for 45 min at 35 °C in growth media that included 10 mM HEPES buffer (pH 7.2). After three washes, the cells were further incubated for an additional 10-15 min at 35 °C. To determine the mechanism(s) by which morphine and Tat₁₋₇₂ interact to increase Ca^{2+} , cultured astrocytes were incubated with thapsigargin, dantrolene, LY294002, or A23187. For each inhibitor, multiple concentrations were screened to determine the lowest concentration that would blocked increases in $[Ca^{2+}]_i$. The lowest effective concentration was deemed optimal and used in the experiments reported herein. Concentrations screened included: both thapsigargin and LY294002 (0, 1, 2.5, 5, 10, 12.5, 25 μ M); dantrolene (0, 1, 2.5, 5, 10, 25, 50 μ M); and A23187 (0, 0.1, 0.25, 0.5, 1, 1.5, 2-5 μ M). Inhibitors were added to growth medium containing calcium (1.0 mM) and EGTA (1.0 mM) and cultures preincubated for 20 min at 35 °C (5% CO₂/95% air) prior to the start of experiments. Tat₁₋₇₂ (100 nM) was added to the cell cultures while morphine (500 nM, final concentration) was injected into each well. $[Ca^{2+}]_i$ is proportional to the ratio of fluorescence at 340 and 380 nm excitation wavelengths (Grynkiewicz et al., 1985) and reported as the fura-2 ratio. Mean $[Ca^{2+}]_i$ levels were determined before and after injection

of 500 nM morphine into each well. Data represent the mean $\pm$ SEM of 4 independent observations.

Mouse Cytokine Antibody Arrays

Astrocytes were cultured in 6-well plates containing medium alone (Controls), 500 nM morphine and/or 100 mM Tat₁₋₇₂. For negative controls, the inactive Tat (Tat₃₁₋₆₁) and Immuno-neutralized Tat were used. Spent medium containing released cytokines was collected after the indicated times. To detect changes in the levels of cytokines and chemokines released, mouse-specific, TransSignal Cytokine Antibody Arrays (RayBiotech Inc., Norcross, GA) were used. Antibody arrays allowed for simultaneous detection of multiple cytokines and chemokines in a single experiment. Briefly, 2 ml of serum free media collected from cells grown in media, or incubated with 500 nM morphine and or 100 mM Tat₁₋₇₂ for 4 h or 12 h were individually added to a cytokine array membrane. After 2 h of incubation at room temperature, each membrane was thoroughly washed with the provided washing buffers. Each membrane was then incubated for 1-2 h at room temperature with biotin-conjugated anti-cytokine antibodies, followed by the incubation of streptavidin-HRP conjugate. After several washes, cytokine-antibody complexes were detected by chemiluminescence using reagents and procedures provided by RayBiotech Inc. Levels of individual cytokines were assessed semi-quantitatively using a Kodak Image Station 440CF as described by the manufacturer and previously published (Huang, 2001; Huang et al., 2001).

RNA isolation and RT-PCR

In each case, total RNA was extracted from cells using TRI reagent (Sigma, St. Louis, MO, USA). First, 2 μ g of RNA was reverse transcribed into cDNA using RETROscript (Ambion Inc, Austin, TX, USA) according to manufacturer's instructions. Since quantitative RT-PCR is such a sensitive technique, possibility of a false positive caused by genomic DNA contamination in the RNA preparation was eliminated by performing a minus-reverse transcriptase (RT) control during RT-PCR. In addition, the following negative controls were performed among the PCR reactions. One negative control contained a minus-template reaction and the second control contained the untreated RNA product. These controls verified that neither the PCR reagents nor the RNA was contaminated with DNA. In the event that the RNA preparation was contaminated with genomic DNA, an acid phenol:chloroform extraction (5:1 phenol: CHCl₃; pH 4.7) was performed. Acid phenol:chloroform extraction partitions DNA into organic phase. The RNA remains in the aqueous phase and can be subsequently recovered by 0.5 M ammonium acetate and ethanol precipitation. For amplification of MCP-1, RANTES, IL-6, TNF- α , μ -opioid receptor and β -actin genes, the following primer combinations were used, TNF- α (5'-ATGAGCACAGAAAGCATGATC-3') (5'-TACAGGCTTGTCACCTCGAATT-3'), RANTES (5'-CAGCTGCCCTCACCATCATCCTCA-3') (5'-GCTGGTTTCTTGGGTTTGCTGTGC-3') MCP-1 (5'-GGGTCTTTGGGAATATAATGTGTA-3') (5'-AGCCCTGTGCCTCTTCTTCT-3') MUOR (5'-CCCCCTGCCTGTATTTGTGGTTT-3') (5'-CATGGCCCTCTATTCTATCGTGTG-3') IL-6 (5'-TGGAATTGGGGTAGGAAGGA-3') (5'-GTTGCCTTCTTGGGACTGATG-3') and β -actin (5'-TGTGATGGTGGGAATGGGTCAG-3') (5'-TTTGATGTACGCACGATTTC-3'). The

β -actin mRNA was used as an internal control. The PCR mixture consisted of 600 mM Tris-SO₄ (pH 8.9), 180 mM ammonium sulfate, 10 mM dNTP mixture, 50 mM MgSO₄, 1 U Platinum Taq DNA polymerase (Invitrogen, Life Technologies), 10 pmol of primer, and reverse transcript template cDNA in a total volume of 50 μ l. PCR products were separated by 1.5% agarose gel electrophoresis, stained with ethidium bromide, and visualized under UV light using a Kodak 440CF Image Station (Rochester, NY).

Statistical Analyses

Results depicted are the average of at least three independent experiments using three to four animals per group in each experiment and are expressed as the mean $\pm$ SEM. Unless specified otherwise, ANOVA followed by Duncan's *post hoc* test were used to determine whether significant changes in cytokine or chemokine proteins, or differences in $[Ca^{2+}]_i$ (ANOVA, Statistica, StatSoft, Tulsa, OK). In cases where noted, the non-parametric Kruskal-Wallis ANOVA was used, which included overall differences in cytokine/chemokine mRNA levels (RT-PCR) and elsewhere when the intergroup variances were non-homogenous. If significant main effects were noted, Kruskal-Wallis multiple comparisons test (Kruskal-Wallis test) was then used to compare intergroup differences (Statistica, StatSoft, Tulsa, OK). Treatment effects were considered significant if $P < 0.05$.

RESULTS

Effects of opiates and Tat₁₋₇₂ on astroglial $[Ca^{2+}]_i$

Morphine and HIV-1 Tat₁₋₇₂ treatment synergistically increased intracellular calcium ($[Ca^{2+}]_i$) in type 1 astroglia as assessed using fura-2 (Fig. 1A-H). In individual cells, morphine (1 μ M) and/or Tat₁₋₇₂ (100 nM) alone significantly increased $[Ca^{2+}]_i$ within seconds following exposure (Fig. 1E,F). The response to morphine and/or Tat₁₋₇₂ differed among individual astrocytes (A-F) as noted previously (Stiene-Martin et al., 1998). Subpopulations of striatal astrocytes do not express μ -opioid receptors (Stiene-Martin et al., 1998; Holden et al., 1999). Therefore, some cells responded synergistically (thick line), while others did not (thin-dashed line) (Fig. 1E-F). Despite the fact that only 30% of striatal astrocytes express μ -opioid receptors (Stiene-Martin et al., 1998), mean increases in $[Ca^{2+}]_i$ levels within the entire population of striatal astrocytes were, nevertheless, evident due to robust increases in the μ receptor-expressing cells (Fig. 1G,H). Pretreatment with Tat₁₋₇₂ (100 nM) exacerbated morphine (1 μ M)-dependent increases in astroglial $[Ca^{2+}]_i$ (Fig. 1E,G), and *visa versa* (Fig. 1F). The antagonist naloxone markedly attenuated morphine's effects but had no apparent effect by itself or in combination with Tat₁₋₇₂ (Fig. 1H, and data not shown). Together, morphine and Tat₁₋₇₂ caused significant interactive increases in $[Ca^{2+}]_i$ ($P = 0.027$; two-way factorial ANOVA). The baseline mean $[Ca^{2+}]_i$ level in untreated type 1 astrocytes was 96.5 ± 6.4 nM.

The time course of morphine-induced $[Ca^{2+}]_i$ increases was assessed in untreated, Tat₁₋₇₂, β -FNA, or Tat₁₋₇₂ plus β -FNA-treated astrocyte populations using fura-2-based ratiometric imaging (Fig. 2A). As noted previously, compared to pretreatment values (mean $[Ca^{2+}]_i$ determined at 5 sec intervals for 25 sec), morphine or Tat₁₋₇₂ alone caused relevant increases in $[Ca^{2+}]_i$ (Fig. 2A, and data not shown), while addition of morphine to the Tat₁₋₇₂

pretreatment exacerbated those increases (Fig. 2A,B). This included both the initial peak increases at the time of morphine injection (Injection), as well as the subsequent mean increases (mean $[Ca^{2+}]_i$ determined at 5 sec intervals for a 50 sec duration). Morphine-induced increases in $[Ca^{2+}]_i$ (either alone or the synergistic increases observed in Tat₁₋₇₂ pretreated cultures) were prevented by pretreatment with the μ -opioid receptor antagonist β -FNA at a concentration of 1.5 μ M. β -FNA alone had no effect on $[Ca^{2+}]_i$ in astrocytes. Collectively, these findings suggested that the synergistic increases in $[Ca^{2+}]_i$ caused by morphine and Tat₁₋₇₂ are mediated by μ -opioid receptors.

To better explore the mechanisms by which morphine and Tat₁₋₇₂ might interact to increase $[Ca^{2+}]_i$, cultures were pretreated for 20 min with thapsigargin, which depletes IP₃-dependent intracellular stores and dantrolene, which attenuates Ca²⁺-induced Ca²⁺ release (CICR). Both thapsigargin at a concentration of 5 μ M and dantrolene at 10 μ M completely blocked synergistic increases in $[Ca^{2+}]_i$ evoked by concurrent morphine and Tat₁₋₇₂ treatment (Fig. 2B). Disruption of Ca²⁺ signaling with the Ca²⁺ ionophore, A23187 at a concentration of 1 μ M, also negated morphine and/or Tat₁₋₇₂-evoked $[Ca^{2+}]_i$ increases. However, because there was evidence of frank toxicity after ~30 min exposure to A23187, the data were not interpreted further (data not shown). Collectively, the findings suggest that morphine and Tat₁₋₇₂-signaling events converge at the level of IP₃-dependent Ca²⁺ release and undergo subsequent feedback amplification of the IP₃-induced Ca²⁺ signal via CICR. Because PI3-kinase is thought to be a major downstream effector of the μ -opioid receptor and modulator of $[Ca^{2+}]_i$, we additionally examined the effects of PI3-kinase on combined morphine-Tat₁₋₇₂-induced synergistic increases in $[Ca^{2+}]_i$. The PI3-kinase inhibitor, LY294002 at a concentration of 5 μ M, also completely blocked synergistic increases in $[Ca^{2+}]_i$. Lastly, because many of the Ca²⁺ modulators alone effect $[Ca^{2+}]_i$ resting levels (e.g., LY294002 and thapsigargin alone caused some elevations in $[Ca^{2+}]_i$), we did not make systematic comparisons of their effects with and without Tat₁₋₇₂ (prior to morphine injection). However, it is interesting to note that dantrolene pretreatment abolished Tat₁₋₇₂-induced increases in $[Ca^{2+}]_i$. Studies are underway to explore more systematically the role of Ca²⁺ in morphine-Tat₁₋₇₂ interactions, and how $[Ca^{2+}]_i$ may potentially augment the release of cytokines/chemokines (discussed below).

Secretion of chemokine proteins by astrocytes following morphine and Tat₁₋₇₂ exposure

The medium from cells incubated with media alone (control), morphine (500 nM) and/or Tat₁₋₇₂ (100 nM) was evaluated for released chemokines and cytokines using cytokine arrays (Figs. 3-4). Tat₁₋₇₂ alone caused significant increases in MCP-1 and RANTES levels in the medium at 4 h or 12 h following exposure. Importantly, however, cells treated with both morphine and Tat₁₋₇₂ showed synergistic increases in amounts of MCP-1, RANTES, and IL-6 released (^b*P* < 0.05; compared to treatment with Tat₁₋₇₂ alone), indicating a marked interaction. Unlike MCP-1 and RANTES, significant increases in IL-6 protein were evident following combined Tat₁₋₇₂ and morphine exposure, while Tat or morphine alone had no effect (Figs. 3B; 4A,B). Slight increases in TNF- α were noted following Tat₁₋₇₂ exposure (Table 1) and accompanied by increases in TNF- α mRNA transcripts (described below); however, Tat-induced elevations were not increased further by concurrent morphine administration. Additional cytokine proteins, which were assayed as part of the array, did

not show significant Tat₁₋₇₂ and/or morphine interactions at 4 h or 12 h with the exception of MCP-5, which displayed a similar albeit attenuated response compared to MCP-1 (Fig. 3B). MCP-5 is a mouse homologue to MCP-1, which is typically co-regulated with and acts similarly to MCP-1 (mice produce both MCP-1 and MCP-5). A human homologue to MCP-5 has not been identified and MCP-5 was not characterized further in the present study. Increases in IL-12 were noted with Tat or Tat and morphine exposure at 12 h (Figs. 3B; Table 1); however, the IL-12 response was less consistent relative to other cytokines—perhaps suggesting 4 or 12 h were not the optimal times to measure the peak response. A summary of our findings at 12 h following morphine (500 nM) and/or Tat (100 nM) exposure are presented in Table 1. With the exception of TNF- α , mRNA levels were not assayed unless there were marked changes in cytokine protein levels in the medium. The negative controls, which included 100 nM deletion (Tat₃₁₋₆₁) mutant Tat [Tat(mut)] and 100 nM immunoneutralized Tat (Neutral Tat) did not increase the release of any cytokines or chemokines alone or in the presence of morphine (Figs. 3-4).

Time-course of chemokine mRNA induction by morphine and HIV-1 Tat₁₋₇₂ in mouse astrocytes

To understand better the combined effects of HIV and opiate drugs and to extend the studies of released proteins using cytokine arrays, the effects of morphine (500 nM) and/or Tat₁₋₇₂ (100 nM) on cytokine mRNA expression was examined at 30 min, 4 h, and 12 h following continuous exposure using semi-quantitative RT-PCR (Figs. 5-6). RNA was extracted and reverse transcribed, and the corresponding cDNA amplified by PCR using primers as described in the experimental procedures.

In general, morphine and/or Tat₁₋₇₂-treatment had minimal effects on cytokine mRNA levels at 30 min with the exception of TNF- α and IL-6 mRNAs, which showed significant increases following Tat₁₋₇₂ exposure (irrespective of whether morphine was co-administered) (Fig. 6). Minimally detectable amounts of MCP-1 and RANTES mRNA transcripts were present in vehicle-treated control cultures at every time point, while transcript levels of both increased markedly with Tat₁₋₇₂ or morphine and Tat₁₋₇₂ exposure at 4 h and 12 h following treatment. Levels of both MCP-1 (Fig. 5D) and RANTES mRNA (Fig. 5E) were significantly greater with combined morphine plus Tat₁₋₇₂ exposure compared to Tat₁₋₇₂ alone at 4 h or 12 h following exposure, but not at 30 min (Fig. 5B,C). In the continued presence of Tat₁₋₇₂, the mRNA of the pro-inflammatory cytokines TNF- α (Fig. 6A,C) or IL-6 (Fig. 6B,D) continued to increase from ~2-2.5-fold increases at 30 min to ~5-15-fold increases at 4 h or 12 h compared to vehicle-treated controls. Concurrent treatment with morphine had no additional effects on TNF- α or IL-6 mRNA levels compared to exposure to Tat₁₋₇₂ alone (Fig. 6A-D). In general, increases in cytokine mRNA levels coincided with increases in proteins, although the relative magnitude of morphine-evoked increases in Tat₁₋₇₂-induced cytokine production was greater at the protein level. The amplification products for β -actin, MCP-1, RANTES, TNF- α or IL-6, and IL respectively, migrated as expected to 500, 481, 431, 275 and 478 bp positions on agarose gels.

Morphine modulates immune function in astrocytes through actions on μ -opioid receptors

To determine whether the effects of morphine on Tat₁₋₇₂-exposed astroglia were mediated through μ opioid receptors, astrocyte-enriched cultures were incubated with or without the irreversible selective μ receptor antagonist, β -FNA (1.5 μ M), morphine (500 nM), and/or Tat₁₋₇₂ (100 nM) for 4 h and 12 h (Fig. 5D,E). Treatment with β -FNA plus morphine and/or Tat₁₋₇₂ did not effect the expression of β -actin (data not shown). β -FNA significantly attenuated the ability of morphine to exacerbate Tat₁₋₇₂-induced increases on MCP-1 and RANTES mRNA levels at 4 and 12 h, indicating that the effects of morphine are mediated by μ opioid receptors.

Although morphine is a preferential μ -opioid receptor agonist, it can also have significant immunomodulatory effects through actions at κ -opioid receptors. To determine whether morphine might be affecting cytokine expression through actions at κ -opioid receptors, experiments identical to those described above were additionally performed using the κ opioid antagonist, nor-BNI (1.5 μ M), instead of β -FNA. Morphine and/or Tat₁₋₇₂-induced changes in cytokine mRNA levels were not prevented by κ -opioid receptor blockade, further indicating the affects of morphine on astrocytes were mediated by μ -opioid receptors (data not shown).

Microglia

For comparison, we tested whether primary microglial cells treated with Tat and/or morphine had a similar cytokine response (Fig. 7). Microglia are not abundant in murine cortical cultures, and we were limited in terms of the number of total cells available for plating. In preliminary array studies, we collected conditioned medium after 12 hrs; however, many signals were below the limit of detection. By 18 h of incubation, sufficient cytokines had accumulated to be reliably detected. Unlike astrocytes, unstimulated microglia produced MCP-1, MCP-5, and RANTES. Exposure to 500 nM morphine did not change the cytokine secretion profile or the amounts of cytokines secreted (Fig. 7). Similar to results in astroglia, 100 nM Tat₁₋₇₂ significantly increased the level of secretion of MCP-1 irrespective of morphine treatment. But whereas combined exposure to morphine and Tat synergistically increased MCP-1 output in astrocytes, morphine did not induce additional MCP-1 secretion in microglia. Likewise, Tat alone significantly elevated IL-6 secretion by microglia, but morphine and Tat did not synergize to enhance IL-6 secretion. Microglia exposed to Tat also secreted significant amounts of granulocyte colony stimulating factor (G-CSF) but again the secretion was not enhanced by concomitant exposure to morphine. Neither Tat alone, nor Tat and morphine in combination, altered the levels of secreted RANTES or MCP-5 above unstimulated secretion rates. Thus, although microglia and astrocytes showed some similarities in terms of their profile of cytokine secretion, microglia had higher basal production of MCP-1, MCP-5, and RANTES and showed no synergistic responses to exposure of Tat and morphine together. Microglia exposed to Tat also secreted significantly elevated levels of G-CSF, a cytokine not detected in astrocyte-conditioned medium.

DISCUSSION

Our findings provide novel evidence that exposure to morphine can dramatically exaggerate key astroglial responses to HIV-1 Tat. This includes marked alterations in $[Ca^{2+}]_i$ homeostasis and an exaggerated release of the critical inflammatory cytokines MCP-1, RANTES, and IL-6. If astroglia in the infected CNS respond similarly, then astrocytes may act as gatekeepers by triggering early pro-inflammatory events including the recruitment of monocytes/macrophages, and opiates act by exacerbating these early astroglial-mediated events. Monocyte/macrophages are a vector for viral entry into the CNS and thought to be key mediators of neurodegeneration (Nath, 1999; Kaul et al., 2001; Garden, 2002). The results suggest that astroglia are important intermediaries in neuroAIDS and infer a mechanism by which opiates enhance the neuropathogenesis of HIV.

HIV is a multisystems disease that acts through multiple toxic events and signaling cascades. HIV is characterized by astrogliosis, the presence of multinucleate giant cells and viral products, and neuronal degeneration (neuronal death and/or dendritic pruning) (Navia et al., 1986; Masliah et al., 1992a; Masliah et al., 1992b; Nath, 1999; Petit et al., 1999). Astroglial apoptosis is evident in the CNS of individuals with HIV (Petit and Roberts, 1995; Thompson et al., 2001), and the severity of HIV dementia correlates positively with the proportion of apoptotic astrocytes (Thompson et al., 2001). In addition to maintaining the blood brain barrier and influencing pro-inflammatory events in the CNS, astroglia provide essential metabolic and trophic support for neurons. Alterations in astroglial function are likely to profoundly impact CNS function.

Our results indicate that opiates and Tat₁₋₇₂ act synergistically to destabilize astroglial function (Ca^{2+} regulation) and suggest that the interaction is mediated through opioid receptors. Activation of astroglial μ -opioid receptors cause $[Ca^{2+}]_i$ overloading, increased oxyradical (ROS) production, and cellular hypertrophy (Hauser et al., 1996; Hauser et al., 1998), although the increases in mean $[Ca^{2+}]_i$ and ROS disappeared at 72 h and seemingly did not result in cytotoxicity (Hauser et al., 1998). Furthermore, the finding that the selective μ -opioid receptor antagonist, β -FNA significantly attenuated morphine's effects on $[Ca^{2+}]_i$ or cytokine expression suggests that opiate-HIV Tat₁₋₇₂ glial interactions are mediated specifically by μ -opioid receptors. Heroin, which readily crosses the blood brain barrier, is rapidly hydrolyzed to morphine, which is present at tissue concentrations exceeding 1 μ M in drug-tolerant individuals (Gurwell et al., 2001). Although additional study is needed to confirm the role of μ -opioid receptor types, morphine is a preferential μ receptor agonist and μ receptors are widely expressed by subsets of astrocytes (Stiene-Martin et al., 1998; Stiene-Martin et al., 2001).

In the present study, synergistic increases in $[Ca^{2+}]_i$ were markedly suppressed by inhibiting IP₃-dependent increases in $[Ca^{2+}]_i$ and by inhibiting CICR from internal stores. Thapsigargin, which depletes IP₃-dependent intracellular stores and dantrolene, which attenuates CICR, both completely blocked increases in $[Ca^{2+}]_i$ evoked by concurrent morphine and Tat₁₋₇₂ treatment. Disruption of Ca^{2+} signaling with the Ca^{2+} ionophore, A23187, also negated morphine and/or Tat₁₋₇₂-evoked $[Ca^{2+}]_i$ increases. As noted, however, A23187 caused cytotoxicity after ~30 min exposure making the results difficult to interpret.

Collectively, the findings suggest that morphine and Tat₁₋₇₂-induced signaling events converge at the level of $[Ca^{2+}]_i$. This appears to involve events converging at IP₃-dependent Ca^{2+} release and subsequent feedback amplification of the IP₃-induced Ca^{2+} signal via CICR as described in other systems (Berridge, 2002). This was anticipated, because both morphine and Tat can act independently through this pathway (Hauser et al., 1996; Haughey et al., 1999). Finally, the μ -opioid receptor antagonist, β -FNA, and the PI3-kinase inhibitor, LY294002, also completely blocked synergistic increases in $[Ca^{2+}]_i$. Again, the results were not surprising, because β -FNA is a μ -opioid receptor antagonist and PI3-kinase is thought to be a major downstream effector of the μ -opioid receptor (Polakiewicz et al., 1998; Persson et al., 2003). PI3-kinase can influence $[Ca^{2+}]_i$ by modulating PLC γ activity or by regulating Ca^{2+} influx (Barker et al., 1999). The present findings are important because they suggest that opioid and Tat signals converge at the level of $[Ca^{2+}]_i$ and reveal a potential target for therapeutic intervention. Studies in progress are further exploring the role of Ca^{2+} in morphine-Tat interactions.

In addition to affecting $[Ca^{2+}]_i$, the present results show that Tat caused marked increases in several key inflammatory cytokines that could be even further elevated by co-administering morphine. Prior studies report that μ -opioid receptor activation regulates the expression of α - and β -chemokines and their receptors in Tat-exposed astrocytes (Mahajan et al., 2002). Reactive astroglial changes are also noted in the brains of chronic opiate abusers. In particular, diffuse, reactive astrocytosis, with regressive astrocytic changes, is a common postmortem finding in the brains of both HIV-seropositive and seronegative intravenous heroin abusers (Gosztonyi et al., 1993; Oehmichen et al., 1996; Buttner et al., 2000), and the reactive astroglial changes accompanying heroin abuse may be exaggerated by concurrent HIV infection (Makrigeorgi-Butera et al., 1996).

Chemokines have the ability to participate in both the recruitment of immune cells into the brain or by altering cell function. The CC chemokines MCP-1 and RANTES, which are major attractants for monocyte/macrophage entry into the CNS and key initiators of pro-inflammatory cascades (Luo et al., 2002a), have been found in the brain tissue and cerebrospinal fluid of patients with HIV-1 encephalitis and AIDS dementia complex (Kelder et al., 1998; McManus et al., 2000). Although the cytokine array is sensitive and can reportedly detect protein levels as low as 4 pg/ml (compared to 40 pg/ml with ELISA), we did not see increases in TNF- α release at 4 or 12 h. Increases in TNF- α were anticipated because Tat has been previously reported to increase TNF- α expression in astrocytes (Chen et al., 1997) and TNF- α can induce the expression of IL-6 (Norris et al., 1994), MCP-1 (Oh et al., 1999; Luo et al., 2003), and RANTES (Oh et al., 1999; Meeuwsen et al., 2003) in human and/or mouse astrocytes. Importantly, we found large relative increases in TNF- α mRNA levels at 30 min and declining levels thereafter in response to Tat₁₋₇₂ exposure. This suggests that Tat might have induced rapid and transient increases in TNF- α release prior to 4 h when we assayed released cytokines. TNF- α exists as an anchored type II transmembrane protein and is rapidly released from the parent protein by a cell surface matrix metalloproteinase, TNF- α converting enzyme (TACE or ADAM-17) (Gearing et al., 1994), which is expressed by astrocytes (Goddard et al., 2001). The half life of TNF- α protein is 4.6 min in mouse serum (Kamada et al., 1999) and of TNF- α mRNA is about 30 min (Ng et al., 1994), suggesting its presence is short lived.

In our studies, morphine by itself failed to increase levels of cytokines by astrocytes at 4 h or 12 h. When combined with Tat, morphine caused marked increases in MCP-1 and RANTES mRNA levels compared to Tat alone, and markedly increased levels of MCP-1, RANTES, and IL-6 proteins in the medium of exposed astroglia. Although morphine exposure greatly exaggerated Tat-induced cytokine production, it did not induce novel patterns of cytokine expression suggesting that morphine was modulating the response of astrocytes to Tat. Based on evidence that TNF- α can induce MCP-1, RANTES and IL-6 production (Ng et al., 1994; Oh et al., 1999; Luo et al., 2003), it was interesting that morphine failed to increase its production beyond increases seen with Tat alone. Morphine-induced increases in Tat-evoked MCP-1, RANTES, and IL-6 production are not preceded by synergistic increases in TNF- α , suggesting that morphine's effects are not mediated by further increases in TNF- α levels beyond those seen with Tat alone. Morphine-induced increases in MCP-1, RANTES, and IL-6 seemingly occur through actions that are downstream of TNF- α . Although the mechanisms by which morphine might affect the production of these chemokines is uncertain, elevated $[Ca^{2+}]_i$ appears to be an integral part of the signaling pathway. Morphine's effects on chemokine production appear to be mediated through μ -opioid receptors since β -FNA, but not nor-BNI, significantly attenuated the effects of morphine plus Tat on MCP-1 and RANTES mRNA levels at 4 h and 12 h. The potential role of δ -opioid receptors in mediating morphine's actions was not explored in the present study. We previously found that, unlike astrocytes from other brain regions, confluent striatal astrocytes do not show increases in $[Ca^{2+}]_i$ in response to δ -opioid receptor activation and fewer than 10% of cultured striatal astrocytes possess δ -opioid receptor immunoreactivity (Stiene-Martin et al., 1998). This suggests that the δ receptor phenotype or astroglia subpopulation expressing δ receptors are lost with progressive maturation in vitro, and δ receptors unlikely to mediate morphine's effects, although this needs to be confirmed in future studies.

For a number of reasons, it is extremely unlikely that any of our results are due to contamination by microglia, which are a potential source of secreted cytokines in CNS cultures. To address this issue, we physically removed the superficial microglia from the astrocyte cultures by orbital rotation, and then treated the cultures with leucine methyl-ester for 1-3 h prior to the onset of the experiments to deplete any remaining microglia. Selected cultures were also immunostained for CD11b, which showed a negligible population of contaminating microglia. We also tested for cytokine production by murine microglia, and found a distinct pattern of secretion that differed from our astroglial cultures (Fig. 7). Notably, morphine and Tat did not additively increase production of any microglial cytokine and stimulated microglia routinely produced G-CSF, which was never detected in astrocyte cultures. Thus, our results likely reflect cytokine output from astrocytes in response to morphine and/or Tat.

Another potential source of cytokines is from neural precursors, which are present in our astrocyte cultures in significant numbers as very small, undifferentiated glial precursors (Khurdayan et al., 2004). Neural stem cells (NSC) and/or glial progenitors express cytokines (Luo et al., 2002b; Klassen et al., 2003), and the pattern of expression is developmentally regulated, cell-type- (Luo et al., 2002b) and species-specific (Klassen et al., 2003). Moreover, these glial precursors express μ -opioid receptors, and combined morphine and

Tat exposure cause marked increases in their death, inferring that the glial precursors are affected directly (Khurdayan et al., 2004). Importantly, IL-6, which increases dramatically in response to Tat and especially morphine plus Tat in the present study, is not detected in mouse (Klassen et al., 2003) or rat (Luo et al., 2002b) neural progenitors, though IL-6 is expressed by adult hippocampal rat progenitors (Klassen et al., 2003). Although this may suggest that increases in IL-6 in the present did not originate from glial progenitors, an alternative explanation is that Tat and/or morphine induce its expression in neural progenitors. Even less is known about the extent to which neural progenitors express MCP-1 and RANTES. Lastly, further highlighting their potential importance in HIV neuropathogenesis, recent findings indicate that neural progenitors can be a reservoir for HIV and differentiation toward an astroglial phenotype promotes marked increases in viral production (Lawrence et al., 2004). Collectively, the above findings suggest that glial precursors are novel targets for HIV, and additionally suggest that opioids play a significant role in modulating that pathogen-target relationship.

Astrocyte viability is unaffected by continuous combined morphine and Tat exposure at 24 h (Gurwell et al., 2001), and astroglia recover from opioid-induced increases in $[Ca^{2+}]_i$ and oxyradicals after 72 h despite continuous exposure (Hauser et al., 1998). This suggests that morphine and Tat-induced $[Ca^{2+}]_i$ overload, and cytokine production do not result in overt losses in astrocyte viability. However, significant disruptions in astroglial function may occur without cell losses, and these might limit their ability to buffer extracellular glutamate and potassium from neighboring neurons (Kaul et al., 2001; Nath, 2002). In addition, increases in cytokine production by affected astrocytes would likely augment the direct neurotoxic actions of opiates and/or Tat (Gurwell et al., 2001) or via monocyte/macrophage recruitment.

Synergistic effects of opioids and HIV on the expression of chemokines by astroglia have not been previously demonstrated. Assuming that astrocytes within the CNS behave similarly to astroglia in vitro, this might in part explain how opiate drug use accelerates HIV. By exacerbating the production of proinflammatory chemokines in HIV infected individuals; heroin would likely accelerate an influx in monocyte and T-cell infiltration into the CNS parenchyma and potentially exacerbate neurodegenerative events. This includes disruption of astrocyte function and potential damage to the astrocytes themselves. While numerous studies have provided insight into the development of HIV-associated inflammatory responses, it is still not clear how changes in chemokines and their receptors affect HIV. The release of chemokines and the expression of their receptors by astrocytes could be a consequence of the inflammation, or it could be an adaptive host response to try to compete with Tat for chemokine receptor binding sites. Control, morphine and/or Tat exposed astrocytes continued to express CCR2 and CCR5 mRNAs, the receptor for the chemokines MCP-1 and RANTES, respectively (El-Hage, unpublished data). We propose that morphine potentiates Tat-induced increases in MCP-1 and RANTES, while concurrently increasing HIV-1 CCR2 and CCR5 co-receptors, which may promote viral binding, the trafficking of HIV-1 infected monocytes across the blood brain barrier into the CNS, thus accelerating disease progression.

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References

- Arora PK. Morphine-induced immune modulation: does it predispose to HIV infection? *NIDA Res Monogr.* 1990; 96:150–165. [PubMed: 2233993]
- Bagasra O, Lavi E, Bobroski L, Khalili K, Pestaner JP, Tawadros R, Pomerantz RJ. Cellular reservoirs of HIV-1 in the central nervous system of infected individuals: identification by the combination of in situ polymerase chain reaction and immunohistochemistry. *AIDS.* 1996; 10:573–585. [PubMed: 8780811]
- Bansal AK, Mactutus CF, Nath A, Maragos W, Hauser KF, Booze RM. Neurotoxicity of HIV-1 proteins gp120 and tat in the rat striatum. *Brain Res.* 2000; 879:42–49. [PubMed: 11011004]
- Barker SA, Lujan D, Wilson BS. Multiple roles for PI 3-kinase in the regulation of PLCgamma activity and Ca²⁺ mobilization in antigen-stimulated mast cells. *J Leukoc Biol.* 1999; 65:321–329. [PubMed: 10080534]
- Bell JE, Brettle RP, Chiswick A, Simmonds P. HIV encephalitis, proviral load and dementia in drug users and homosexuals with AIDS. Effect of neocortical involvement. *Brain.* 1998; 121:2043–2052. [PubMed: 9827765]
- Berger JR, Nath A. HIV dementia and the basal ganglia. *Intervirology.* 1997; 40:122–131. [PubMed: 9450229]
- Berridge MJ. The endoplasmic reticulum: a multifunctional signaling organelle. *Cell Calcium.* 2002; 32:235–249. [PubMed: 12543086]
- Brack-Werner R. Astrocytes: HIV cellular reservoirs and important participants in neuropathogenesis [editorial] [In Process Citation]. *AIDS.* 1999; 13:1–22. [PubMed: 10207540]
- Brake DA, Debouck C, Biesecker G. Identification of an Arg-Gly-Asp (RGD) cell adhesion site in human immunodeficiency virus type 1 transactivation protein, tat. *J Cell Biol.* 1990; 111:1275–1281. [PubMed: 2202737]
- Buttner A, Mall G, Penning R, Weis S. The neuropathology of heroin abuse. *Forensic Sci Int.* 2000; 113:435–442. [PubMed: 10978659]
- Chang SL, Wu GD, Patel NA, Vidal EL, Fiala M. The effects of interaction between morphine and interleukin-1 on the immune response. *Adv Exp Med Biol.* 1998; 437:67–72. [PubMed: 9666258]
- Chao CC, Gekker G, Sheng WS, Hu S, Tsang M, Peterson PK. Priming effect of morphine on the production of tumor necrosis factor- α by microglia: Implications in respiratory burst activity and human immunodeficiency virus-1 expression. *J Pharmacol Exp Ther.* 1994; 269:198–203. [PubMed: 8169825]
- Chen P, Mayne M, Power C, Nath A. The Tat protein of HIV-1 induces tumor necrosis factor- α production. Implications for HIV-1-associated neurological diseases. *J Biol Chem.* 1997; 272:22385–22388. [PubMed: 9278385]
- Donahoe RM. Multiple ways that drug abuse might influence AIDS progression: clues from a monkey model. *J Neuroimmunol.* 2004; 147:28–32. [PubMed: 14741423]
- Donahoe RM, Falek A. Neuroimmunomodulation by opiates and other drugs of abuse: relationship to HIV infection and AIDS. *Adv Biochem Psychopharmacol.* 1988; 44:145–158. [PubMed: 3041744]
- Donahoe RM, Vlahov D. Opiates as potential cofactors in progression of HIV-1 infections to AIDS. *J Neuroimmunol.* 1998; 83:77–87. [PubMed: 9610676]
- Eisch AJ, Barrot M, Schad CA, Self DW, Nestler EJ. Opiates inhibit neurogenesis in the adult rat hippocampus. *Proc Natl Acad Sci U S A.* 2000; 97:7579–7584. [PubMed: 10840056]
- Everall IP. Interaction between HIV and intravenous heroin abuse? *J Neuroimmunol.* 2004; 147:13–15. [PubMed: 14741419]
- Garden GA. Microglia in human immunodeficiency virus-associated neurodegeneration. *Glia.* 2002; 40:240–251. [PubMed: 12379911]

- Gearing AJ, Beckett P, Christodoulou M, Churchill M, Clements J, Davidson AH, Drummond AH, Galloway WA, Gilbert R, Gordon JL. Processing of tumour necrosis factor- α precursor by metalloproteinases. *Nature*. 1994; 370:555–557. [PubMed: 8052310]
- Glass JD, Wesselingh SL, Selnes OA, McArthur JC. Clinical-neuropathologic correlation in HIV-associated dementia. *Neurology*. 1993; 43:2230–2237. [PubMed: 8232935]
- Goddard DR, Bunning RA, Woodroffe MN. Astrocyte and endothelial cell expression of ADAM 17 (TACE) in adult human CNS. *Glia*. 2001; 34:267–271. [PubMed: 11360299]
- Gosztonyi G, Schmidt V, Nickel R, Rothschild MA, Camacho S, Siegel G, Zill E, Pauli G, Schneider V. Neuropathologic analysis of postmortal brain samples of HIV-seropositive and -seronegative i.v. drug addicts. *Forensic Sci Int*. 1993; 62:101–105. [PubMed: 8300019]
- Gryniewicz G, Poenie M, Tsien RY. A new generation of calcium indicators with greatly improved fluorescence properties. *J Biol Chem*. 1985; 260:3440–3450. [PubMed: 3838314]
- Gurwell JA, Nath A, Sun Q, Zhang J, Martin KM, Chen Y, Hauser KF. Synergistic neurotoxicity of opioids and human immunodeficiency virus-1 Tat protein in striatal neurons in vitro. *Neuroscience*. 2001; 102:555–563. [PubMed: 11226693]
- Haughey NJ, Holden CP, Nath A, Geiger JD. Involvement of inositol 1,4,5-trisphosphate-regulated stores of intracellular calcium in calcium dysregulation and neuron cell death caused by HIV-1 Protein Tat. *J Neurochem*. 1999; 73:1363–1374. [PubMed: 10501179]
- Hauser KF, Harris-White ME, Jackson JA, Opanashuk LA, Carney JM. Opioids disrupt Ca^{2+} homeostasis and induce carbonyl oxyradical production in mouse astrocytes in vitro: transient increases and adaptation to sustained exposure. *Exp Neurol*. 1998; 151:70–76. [PubMed: 9582255]
- Hauser KF, Stiene-Martin A, Mattson MP, Elde RP, Ryan SE, Godleske CC. μ -Opioid receptor-induced Ca^{2+} mobilization and astroglial development: Morphine inhibits DNA synthesis and stimulates cellular hypertrophy through a Ca^{2+} -dependent mechanism. *Brain Res*. 1996; 720:191–203. [PubMed: 8782912]
- Holden CP, Nath A, Haughey NJ, Geiger JD. Involvement of Na^+/H^+ exchangers, Ca^{2+} channels, and excitatory amino acid receptors in intracellular Ca^{2+} responses to HIV-1 gp120 in cultured human fetal brain cells. *Neuroscience*. 1999; 91:1369–1378. [PubMed: 10391443]
- Huang RP. Detection of multiple proteins in an antibody-based protein microarray system. *J Immunol Methods*. 2001; 255:1–13. [PubMed: 11470281]
- Huang RP, Huang R, Fan Y, Lin Y. Simultaneous detection of multiple cytokines from conditioned media and patient's sera by an antibody-based protein array system. *Anal Biochem*. 2001; 294:55–62. [PubMed: 11412006]
- Hudson L, Liu J, Nath A, Jones M, Raghavan R, Narayan O, Male D, Everall I. Detection of human immunodeficiency virus regulatory protein tat in CNS tissues. *J Neurovirology*. 2000; 6:145–155. [PubMed: 10822328]
- Jeang KT, Xiao H, Rich EA. Multifaceted activities of the HIV-1 transactivator of transcription, Tat. *J Biol Chem*. 1999; 274:28837–28840. [PubMed: 10506122]
- Ji JF, He BP, Dheen ST, Tay SS. Expression of chemokine receptors CXCR4, CCR2, CCR5 and CX3CR1 in neural progenitor cells isolated from the subventricular zone of the adult rat brain. *Neurosci Lett*. 2004; 355:236–240. [PubMed: 14732474]
- Jones M, Olafson K, Del Bigio MR, Peeling J, Nath A. Intraventricular injection of human immunodeficiency virus type 1 (HIV-1) tat protein causes inflammation, gliosis, apoptosis, and ventricular enlargement. *J Neuropathol Exp Neurol*. 1998; 57:563–570. [PubMed: 9630236]
- Kamada H, Tsutsumi Y, Tsunoda S, Kihira T, Kaneda Y, Yamamoto Y, Nakagawa S, Horisawa Y, Mayumi T. Molecular design of conjugated tumor necrosis factor- α : synthesis and characteristics of polyvinyl pyrrolidone modified tumor necrosis factor- α . *Biochem Biophys Res Commun*. 1999; 257:448–453. [PubMed: 10198233]
- Kaul M, Garden GA, Lipton SA. Pathways to neuronal injury and apoptosis in HIV-associated dementia. *Nature*. 2001; 410:988–994. [PubMed: 11309629]
- Kelder W, McArthur J, Nance-Sproson T, McCleron D, Griffin D. Beta-Chemokines MCP-1 and RANTES are selectively increased in the cerebral spinal fluid with human immunodeficiency virus-associated dementia. *Ann Neurol*. 1998; 44:831–835. [PubMed: 9818943]

- Khurdayan VK, Buch S, El-Hage N, Lutz SE, Goebel SM, Singh IN, Knapp PE, Turchan-Cholewo J, Nath A, Hauser KF. Preferential vulnerability of astroglia and glial precursors to combined opioid and HIV-1 Tat exposure *in vitro*. *Eur J Neurosci*. 2004; 19:3171–3182. [PubMed: 15217373]
- Klassen HJ, Imfeld KL, Kirov II, Tai L, Gage FH, Young MJ, Berman MA. Expression of cytokines by multipotent neural progenitor cells. *Cytokine*. 2003; 22:101–106. [PubMed: 12849709]
- Knapp PE. Injury stimulates outgrowth and motility of oligodendrocytes grown *in vitro*. *Exp Cell Res*. 1997; 234:7–17. [PubMed: 9223365]
- Kolson DL, Lavi E, Gonzalez-Scarano F. The effects of human immunodeficiency virus in the central nervous system. *Adv Virus Res*. 1998; 50:1–47. [PubMed: 9520995]
- Koob GF. Neurobiology of addiction. Toward the development of new therapies. *Ann N Y Acad Sci*. 2000; 909:170–185. [PubMed: 10911930]
- Kreek MJ, Koob GF. Drug dependence: stress and dysregulation of brain reward pathways. *Drug Alcohol Depend*. 1998; 51:23–47. [PubMed: 9716928]
- Lawrence DM, Durham LC, Schwartz L, Seth P, Maric D, Major EO. Human immunodeficiency virus type 1 infection of human brain-derived progenitor cells. *J Virol*. 2004; 78:7319–7328. [PubMed: 15220405]
- Lipton SA. HIV-related neuronal injury. Potential therapeutic intervention with calcium channel antagonists and NMDA antagonists. *Mol Neurobiol*. 1994; 8:181–196. [PubMed: 7999315]
- Lipton SA. Neuronal injury associated with HIV-1: approaches to treatment. *Annu Rev Pharmacol Toxicol*. 1998; 38:159–77. 159–177. [PubMed: 9597152]
- Luo Y, Berman MA, Abromson-Leeman SR, Dorf ME. Tumor necrosis factor is required for RANTES-induced astrocyte monocyte chemoattractant protein-1 production. *Glia*. 2003; 43:119–127. [PubMed: 12838504]
- Luo Y, Berman MA, Zhai Q, Fischer FR, Abromson-Leeman SR, Zhang Y, Kuziel WA, Gerard C, Dorf ME. RANTES stimulates inflammatory cascades and receptor modulation in murine astrocytes. *Glia*. 2002a; 39:19–30. [PubMed: 12112372]
- Luo Y, Cai J, Liu Y, Xue H, Chrest FJ, Wersto RP, Rao M. Microarray analysis of selected genes in neural stem and progenitor cells. *J Neurochem*. 2002b; 83:1481–1497. [PubMed: 12472902]
- Ma M, Nath A. Molecular determinants for cellular uptake of Tat protein of human immunodeficiency virus type 1 in brain cells. *J Virol*. 1997; 71:2495–2499. [PubMed: 9032389]
- Mahajan SD, Schwartz SA, Shanahan TC, Chawda RP, Nair MP. Morphine regulates gene expression of alpha- and beta-chemokines and their receptors on astroglial cells via the opioid mu receptor. *J Immunol*. 2002; 169:3589–3599. [PubMed: 12244149]
- Makrigeorgi-Butera M, Hagel C, Laas R, Puschel K, Stavrou D. Comparative brain pathology of HIV-seronegative and HIV-infected drug addicts. *Clin Neuropathol*. 1996; 15:324–329. [PubMed: 8937778]
- Mansour A, Fox CA, Burke S, Meng F, Thompson RC, Akil H, Watson SJ. Mu, delta, and kappa opioid receptor mRNA expression in the rat CNS: An *in situ* hybridization study. *J Comp Neurol*. 1994; 350:412–438. [PubMed: 7884049]
- Mansour A, Khachaturian H, Lewis ME, Akil H, Watson SJ. Anatomy of CNS opioid receptors. *Trends Neurosci*. 1988; 11:308–314. [PubMed: 2465635]
- Masliah E, Achim CL, Ge N, DeTeresa R, Terry RD, Wiley CA. Spectrum of human immunodeficiency virus-associated neocortical damage. *Ann Neurol*. 1992a; 32:321–329. [PubMed: 1416802]
- Masliah E, Ge N, Achim CL, Hansen LA, Wiley CA. Selective neuronal vulnerability in HIV encephalitis. *J Neuropathol Exp Neurol*. 1992b; 51:585–93. [PubMed: 1484289]
- McCarthy L, Wetzel M, Sliker JK, Eisenstein TK, Rogers TJ. Opioids, opioid receptors, and the immune response. *Drug Alcohol Depend*. 2001; 62:111–123. [PubMed: 11245967]
- McManus CM, Weidenheim K, Woodman SE, Nunez J, Hesselgesser J, Nath A, Berman JW. Chemokine and chemokine-receptor expression in human glial elements: induction by the HIV protein, Tat, and chemokine autoregulation. *Am J Pathol*. 2000; 156:1441–1453. [PubMed: 10751368]

- Meeuwse S, Persoon-Deen C, Bsibsi M, Ravid R, Noort JM. Cytokine, chemokine and growth factor gene profiling of cultured human astrocytes after exposure to proinflammatory stimuli. *Glia*. 2003; 43:243–253. [PubMed: 12898703]
- Nath A. Human Immunodeficiency Virus (HIV) Proteins in Neuropathogenesis of HIV Dementia. *J Infect Dis*. 2002; 186(Suppl 2):S193–8. S193-S198. [PubMed: 12424697]
- Nath A. Pathobiology of human immunodeficiency virus dementia. *Semin Neurol*. 1999; 19:113–127. [PubMed: 10718533]
- Nath A, Geiger J. Neurobiological aspects of human immunodeficiency virus infection: neurotoxic mechanisms. *Prog Neurobiol*. 1998; 54:19–33. [PubMed: 9460791]
- Nath A, Hauser KF, Wojna V, Booze RM, Maragos W, Prendergast M, Cass W, Turchan JT. Molecular basis for interactions of HIV and drugs of abuse. *J Acquir Immune Defic Syndr*. 2002; 31(Suppl 2):S62–S69. [PubMed: 12394784]
- Nath A, Jones M, Maragos W, Booze R, Mactutus C, Bell J, Hauser KF, Mattson M. Neurotoxicity and dysfunction of dopamine systems associated with AIDS dementia. *Psychopharmacol*. 2000; 14:222–227.
- Nath A, Psooy K, Martin C, Knudsen B, Magnuson DS, Haughey N, Geiger JD. Identification of a human immunodeficiency virus type 1 Tat epitope that is neuroexcitatory and neurotoxic. *J Virol*. 1996; 70:1475–1480. [PubMed: 8627665]
- Navia BA, Cho ES, Petito CK, Price RW. The AIDS dementia complex: II Neuropathology. *Ann Neurol*. 1986; 19:525–535. [PubMed: 3014994]
- Nestler EJ, Aghajanian GK. Molecular and cellular basis of addiction. *Science*. 1997; 278:58–63. [PubMed: 9311927]
- Ng SB, Tan YH, Guy GR. Differential induction of the interleukin-6 gene by tumor necrosis factor and interleukin-1. *J Biol Chem*. 1994; 269:19021–19027. [PubMed: 8034659]
- Norris JG, Tang LP, Sparacio SM, Benveniste EN. Signal transduction pathways mediating astrocyte IL-6 induction by IL-1 beta and tumor necrosis factor-alpha. *J Immunol*. 1994; 152:841–850. [PubMed: 7506738]
- Oehmichen M, Meissner C, Reiter A, Birkholz M. Neuropathology in non-human immunodeficiency virus-infected drug addicts: hypoxic brain damage after chronic intravenous drug abuse. *Acta Neuropathol (Berl)*. 1996; 91:642–646. [PubMed: 8781664]
- Oh JW, Schwiebert LM, Benveniste EN. Cytokine regulation of CC and CXC chemokine expression by human astrocytes. *J Neurovirol*. 1999; 5:82–94. [PubMed: 10190694]
- Peng H, Huang Y, Rose J, Erichsen D, Herek S, Fujii N, Tamamura H, Zheng J. Stromal cell-derived factor 1-mediated CXCR4 signaling in rat and human cortical neural progenitor cells. *J Neurosci Res*. 2004; 76:35–50. [PubMed: 15048928]
- Persson AI, Thorlin T, Bull C, Eriksson PS. Opioid-induced proliferation through the MAPK pathway in cultures of adult hippocampal progenitors. *Mol Cell Neurosci*. 2003; 23:360–372. [PubMed: 12837621]
- Peterson PK, Gekker G, Schut R, Hu S, Balfour HH Jr. Chao CC. Enhancement of HIV-1 replication by opiates and cocaine: The cytokine connection. *Adv Exp Med Biol*. 1993; 335:181–188. [PubMed: 8237594]
- Petito CK, Kerza-Kwiatecki AP, Gendelman HE, McCarthy M, Nath A, Podack ER, Shapshak P, Wiley CA. Review: neuronal injury in HIV infection. *J Neurovirol*. 1999; 5:327–341. [PubMed: 10463855]
- Petito CK, Roberts B. Evidence of apoptotic cell death in HIV encephalitis [see comments]. *Am J Pathol*. 1995; 146:1121–30. [PubMed: 7747806]
- Polakiewicz RD, Schieferl SM, Gingras AC, Sonenberg N, Comb MJ. mu-Opioid receptor activates signaling pathways implicated in cell survival and translational control. *J Biol Chem*. 1998; 273:23534–23541. [PubMed: 9722592]
- Rappaport J, Joseph J, Croul S, Alexander G, Del Valle L, Amini S, Khalili K. Molecular pathway involved in HIV-1-induced CNS pathology: role of viral regulatory protein, Tat. *J Leukoc Biol*. 1999; 65:458–465. [PubMed: 10204574]

- Reznikov K, Hauser KF, Nazarevskaja G, Trunova Y, Derjabin V, Bakalkin G. Opioids modulate cell division in the germinal zone of the late embryonic neocortex. *Eur J Neurosci.* 1999; 11:2711–2719. [PubMed: 10457167]
- Rogers TJ, Peterson PK. Opioid G protein-coupled receptors: signals at the crossroads of inflammation. *Trends Immunol.* 2003; 24:116–121. [PubMed: 12615205]
- Rouveix B. Opiates and immune function. Consequences on infectious diseases with special reference to AIDS. *Therapie.* 1992; 47:503–512. [PubMed: 1301643]
- Sabatier JM, Vives E, Marbrouk K. Evidence for neurotoxicity of tat from HIV. *Journal of Virology.* 1991; 65:961–967. al e. [PubMed: 1898974]
- Sawynok J. The therapeutic use of heroin: a review of the pharmacological literature. *Can J Physiol Pharmacol.* 1986; 64:1–6. [PubMed: 2420426]
- Sheng WS, Hu S, Lokensgard JR, Peterson PK. U50,488 inhibits HIV-1 Tat-induced monocyte chemoattractant protein-1 (CCL2) production by human astrocytes. *Biochem Pharmacol.* 2003; 65:9–14. [PubMed: 12473373]
- Singh IN, Goody RJ, Dean C, Ahmad NM, Lutz SE, Knapp PE, Nath A, Hauser KF. Apoptotic death of striatal neurons induced by HIV-1 Tat and gp120: differential involvement of caspase-3 and endonuclease G. *J Neurovirol.* 2004; 10:141–151. [PubMed: 15204919]
- Stiene-Martin A, Gurwell JA, Hauser KF. Morphine alters astrocyte growth in primary cultures of mouse glial cells: Evidence for a direct effect of opiates on neural maturation. *Dev Brain Res.* 1991; 60:1–7. [PubMed: 1914143]
- Stiene-Martin A, Hauser KF. Glial growth is regulated by agonists selective for multiple opioid receptor types *in vitro*. *J Neurosci Res.* 1991; 29:538–548. [PubMed: 1665190]
- Stiene-Martin A, Knapp PE, Martin KM, Gurwell JA, Ryan S, Thornton SR, Smith FL, Hauser KF. Opioid system diversity in developing neurons, astroglia, and oligodendroglia in the subventricular zone and striatum: impact on gliogenesis *in vivo*. *Glia.* 2001; 36:78–88. [PubMed: 11571786]
- Stiene-Martin A, Zhou R, Hauser KF. Regional, developmental, and cell cycle-dependent differences in μ , δ , and κ -opioid receptor expression among cultured mouse astrocytes. *Glia.* 1998; 22:249–259. [PubMed: 9482211]
- Thiele DL, Kurosaka M, Lipsky PE. Phenotype of the accessory cell necessary for mitogen-stimulated T and B cell responses in human peripheral blood: Delineation by its sensitivity to the lysosomotropic agent, L-leucine methyl ester. *J Immunol.* 1983; 131:2282–2290. [PubMed: 6605384]
- Thompson KA, McArthur JC, Wesselingh SL. Correlation between neurological progression and astrocyte apoptosis in HIV-associated dementia. *Ann Neurol.* 2001; 49:745–752. [PubMed: 11409426]
- Toborek M, Lee YW, Pu H, Malecki A, Flora G, Garrido R, Hennig B, Bauer HC, Nath A. HIV-Tat protein induces oxidative and inflammatory pathways in brain endothelium. *J Neurochem.* 2003; 84:169–179. [PubMed: 12485413]
- Tran PB, Ren D, Veldhouse TJ, Miller RJ. Chemokine receptors are expressed widely by embryonic and adult neural progenitor cells. *J Neurosci Res.* 2004; 76:20–34. [PubMed: 15048927]
- Wang Z, Pekarskaya O, Bencheikh M, Chao W, Gelbard HA, Ghorpade A, Rothstein JD, Volsky DJ. Reduced expression of glutamate transporter EAAT2 and impaired glutamate transport in human primary astrocytes exposed to HIV-1 or gp120. *Virology.* 2003; 312:60–73. [PubMed: 12890621]
- Wesselingh SL, Thompson KA. Immunopathogenesis of HIV-associated dementia. *Curr Opin Neurol.* 2001; 14:375–379. [PubMed: 11371763]

Figure 1.

Effect of morphine (Morph) and/or Tat₁₋₇₂ on intracellular calcium $[Ca^{2+}]_i$ in flat, polyhedral (type 1) astroglia as assessed by ratiometric analysis using fura-2 (A-H). Morphine (1 μM) and/or Tat (100 nM) alone significantly increased $[Ca^{2+}]_i$ in individual type 1 astrocytes (A-B,E-G), compared to mean baseline levels (96.5 ± 6.4 nM). Alternatively, pretreatment with Tat₁₋₇₂ (100 nM) exacerbated the subsequent increases in astroglial $[Ca^{2+}]_i$ to morphine (1 μM) (E), and visa versa (F). The responses differed dramatically among individual astrocytes; some cells responded synergistically (thick line),

while others did not (thin dashed-line). In type 1 astroglial populations, morphine (1 μM) ($*P < 0.05$) or Tat₁₋₇₂ (100 nM) ($P < 0.05$) alone significantly increased mean $[\text{Ca}^{2+}]_i$ levels; however, in combination mean $[\text{Ca}^{2+}]_i$ levels increased synergistically ($^bP < 0.027$; ANOVA, two-way interaction) (G). The interactive increases in $[\text{Ca}^{2+}]_i$ caused by morphine and Tat₁₋₇₂ ($^bP < 0.0024$, pretreatment vs. morphine + Tat post-treatment) were significantly attenuated by naloxone pretreatment (3 μM for 1 h) ($^{\#}P < 0.05$) (h). Data in G & H are the mean $\pm$ SEM from 7 experiments; in each experiment ~ 35 astrocytes were arbitrarily sampled (~ 250 astrocytes total).

Figure 2.

Inhibition of morphine (Morph) and/or Tat₁₋₇₂-induced increases in intracellular calcium $[Ca^{2+}]_i$ by thapsigargin, dantrolene, and the PI3-kinase inhibitor, LY294002. Cells were incubated with serum free medium or stimulated with 500 nM morphine $\pm$ 100 nM Tat₁₋₇₂ in the presence or absence of 1.5 μ M β -FNA. Cells were pre-incubated with the specific inhibitor of phosphatidylinositol 3-kinase (LY294002) (5 μ M), the Ca^{2+} -ionophore A23187 (1 μ M), thapsigargin (5 μ M) (thaps), or dantrolene (10 μ M) (dantr) for 20 min before stimulation of the cells with morphine and/or Tat₁₋₇₂. Bars represent the mean $\pm$ SEM of

four independent experiments (* $P < 0.05$ versus vehicle-treated control cultures; # $P < 0.05$ versus morphine + Tat₁₋₇₂-treated cultures; ^b $P < 0.05$ versus both vehicle-treated control and morphine + Tat₁₋₇₂-treated cultures).

Figure 3.

Effects of morphine (500 nM) and/or Tat₁₋₇₂ (100 nM) on cytokine release by astrocytes at 4 h (A) and 12 h (B) following continuous exposure. Controls for Tat₁₋₇₂ included 100 nM deletion (Tat₃₁₋₆₁) mutant Tat [Tat(mut)] and 100 nM immunoneutralized Tat₁₋₇₂ (Neutral Tat) administered in the presence or absence of morphine. Supernatants were collected and analyzed for cytokines using TranSignal™ RayBiotech mouse cytokine antibody arrays; the rectangles in the upper left and lower right portions of the arrays indicate positive and

negative controls; while the centrally positioned rectangles indicate several cytokines that showed specific responses to morphine and/or Tat (IL-6, MCP-1, and RANTES).

Figure 4.

Effects of morphine (500 nM) and/or Tat₁₋₇₂ (100 nM) on cytokine release by astrocytes at 4 h (A) and 12 h (B) following continuous exposure. Neither Tat₃₁₋₆₁ [Tat(mut)] (100 nM) nor immunoneutralized Tat₁₋₇₂ (NeuTat) alone or in combination with morphine (100 nM) affected the release of cytokines.

Figure 5.

Time-dependent effects of morphine and Tat₁₋₇₂ on the expression of MCP-1 and RANTES mRNA levels in astrocytes assayed by RT-PCR. Cells were cultured with or without morphine (500 nM) (Morph), Tat₁₋₇₂ (100 nM), or morphine plus Tat₁₋₇₂ for 30 min, 4 or 12 h. RNA was extracted, reverse transcribed, and amplified by PCR with the primers as indicated in material and methods. PCR products were separated on a 1.5 % agarose gel. β -actin was used as a housekeeping gene and its expression remained unchanged. The expression levels of different chemokine mRNAs were normalized to β -actin mRNA, and

fold-induction was calculated relative to cells incubated in culture medium (vehicle) alone as described in the methods. The μ -opioid receptor antagonist β -FNA reversed morphine-induced exacerbation of Tat₁₋₇₂ effects on MCP-1 and RANTES gene expression in cultured astrocytes suggesting the effects of morphine were mediated through μ -opioid receptors; while β -FNA had no effect on Tat-induced changes in the absence of morphine. Cells were incubated $\pm$ morphine (Morph or Mor), Tat₁₋₇₂, and/or β -funaltrexamine (FNA) for 4 or 12 h. Bars represent the mean $\pm$ SEM from at least three independent experiments (* P < 0.05 versus vehicle-treated controls; # P < 0.05 versus morphine + Tat₁₋₇₂-treated cultures; ^b P < 0.05 versus both vehicle-treated control and Tat₁₋₇₂-treated cultures; Kruskal-Wallis test).

Figure 6.

Time-dependent effects of morphine and Tat₁₋₇₂ on the expression of TNF- α and IL-6 mRNA levels in astrocytes assayed by RT-PCR at 30 min (A,B) or 4 h or 12 h (C,D). RNA was analyzed by RT-PCR, normalized to β -actin mRNA levels, and fold induction was calculated in comparison to the cells in cultured medium alone as described in Fig. 5. β -actin expression was unaffected by any of the treatments. Bars represent the mean $\pm$ SEM from at least three independent experiments (* P < 0.05 versus vehicle-treated controls; # P < 0.05 versus morphine + Tat₁₋₇₂-treated cultures; ^b P < 0.05 versus both vehicle-treated control and Tat₁₋₇₂-treated cultures; Kruskal-Wallis test).

Figure 7.

Effects of morphine (500 nM) and/or Tat₁₋₇₂ (100 nM) on cytokine release by microglia at 18 h following continuous exposure. Tat alone caused significant increases in MCP-1 (* $P > 0.05$), and in IL-6 and G-CSF levels (* $P > 0.05$ versus vehicle-treated controls; Kruskal-Wallis test), while the addition of morphine had no added effect beyond that seen with Tat alone. Unlike findings at 18 h, microglia showed minimal release of cytokines in response to Tat and/or morphine at 12 h following continuous exposure (see text). Therefore, the

response of microglia to morphine and/or Tat differed markedly from the response of astroglia.

Table 1

Summary of the effects of morphine and/or Tat on cytokines/chemokines detected in the medium of astrocyte cultures at 12 h.

	Control	Morph	Tat	Morph + Tat
G-CSF	-	-	-	-
GM-CSF	-	-	-	-
IL-2	-	-	-	-
IL-3	-	-	-	-
IL-4	-	-	+	+
IL-5	-	-	-	-
IL-6	-	-	+	++
IL-9	-	-	-	-
IL-10	-	-	-	-
IL-12	-	-	+	+
IL-12 P70	-	-	-	-
IL-13	-	-	-	-
IL-17	-	-	-	-
IFN- γ	-	-	-	-
MCP-1	-	-	++	++++
MCP-5	-	-	+	++
RANTES	-	-	++	++++
SCF	-	-	-	-
sTNFR1	-	-	-	-
TNF- α	-	-	+	+
TPO	-	-	-	-
VEGF	-	-	-	-

Vehicle- or morphine-treated astrocytes showed no detectable increases in cytokine/chemokine levels in the medium using TranSignal™ RayBiotech antibody arrays, while Tat or morphine (Morph) and Tat (Morph+Tat) caused marked increases in specific cytokines.

- not detected

+ slight levels detected

++ moderate levels detected

+++ high levels detected

++++ very high levels detected

Granulocyte macrophage colony stimulating factor (GM-CSF); granulocyte-colony stimulating factor (G-CSF); interleukin (IL); interferon (IFN); monocyte chemoattractant protein (MCP); regulated on activation, normal T cell expressed and secreted (RANTES); soluble TNF receptor subunit (sTNFR1); stem cell factor (SCF); thrombopoietin (TPO); tumor necrosis factor (TNF); vascular endothelial growth factor (VEGF).