Morphine potentiates neurodegenerative effects of HIV-1 Tat through actions at \( \mu \)-opioid receptor-expressing glia

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Individuals infected with human immunodeficiency virus-1 who abuse opiates can have a higher incidence of virus-associated neuropathology. Human immunodeficiency virus does not infect neurons, but viral proteins such as transactivator of transcription and glycoprotein 120, originating from infected glia, are neurotoxic. Moreover, functional changes in glial cells that enhance inflammation and reduce trophic support are increasingly implicated in human immunodeficiency virus neuropathology. In previous studies, co-exposure with morphine enhanced transactivator of transcription neurotoxicity towards cultured striatal neurons. Since those cultures contained \( \mu \)-opioid receptor-expressing astroglia and microglia, and since glia are the principal site of infection in the central nervous system, we hypothesized that morphine synergy might be glially mediated. A 60 hour, repeated measures paradigm and multiple co-culture models were used to investigate the cellular basis for opiate-enhanced human immunodeficiency virus neurotoxicity. Morphine co-exposure significantly enhanced transactivator of transcription-induced neuron death when glia were present. Synergistic effects of morphine on transactivator of transcription neurotoxicity were greatest with neuron–glia contact, but also occurred to a lesser extent with glial conditioned medium. Importantly, synergy was lost if glia, but not neurons, lacked \( \mu \)-opioid receptors, indicating that opiate interactions with human immunodeficiency virus converge at the level of \( \mu \)-opioid receptor-expressing glia. Morphine enhanced transactivator of transcription-induced inflammatory effectors released by glia, elevating reactive oxygen species, increasing 3-nitrotyrosine production by microglia, and reducing the ability of glia to buffer glutamate. But neuron survival was reduced even more with glial contact than with exposure to conditioned medium, suggesting that noxious elements associated with cell contact augment the toxicity due to soluble factors. Similar morphine–transactivator of transcription synergy was also observed in studies with the clade C sequence of HIV-1 transactivator of transcription, which did not cause neuron death unless morphine was present. Several paradoxical observations related to opiate effects were noted when \( \mu \)-opioid receptors were specifically ablated from either glia or neurons. This suggests that \( \mu \)-opioid receptor loss in isolated cell types can fundamentally distort cell-to-cell signalling, revealing opponent processes that may exist in individual cell types. Our findings show the critical role of glia in orchestrating neurotoxic interactions of morphine and transactivator of transcription, and support the emerging concept that combined exposure to opiates and human immunodeficiency virus drives enhanced pathology within the central nervous system.
Introduction

Interactions between neurons and glia are critical in development and ongoing function of the CNS. There is substantial evidence in patients and experimental models that glial signalling is disrupted in the HIV-1 infected CNS. These disruptions likely contribute critically to neurodegenerative changes and neurological sequelae. Productive HIV infection in the CNS is almost exclusively restricted to microglia, which release toxic viral proteins, as well as typical inflammatory toxins including chemokines, cytokines and reactive species (Glass et al., 1995; Nath et al., 1999; Yadav and Collman, 2009). Astroglia may also be infected, and although their infection does not produce new virus, both infected astroglia and astroglia activated by viral and cellular toxins released from microglia are potent sources of secreted inflammatory products (Nath et al., 1999; Kramer-Hammerele et al., 2005; Li et al., 2007). Critical astroglial functions, such as glutamate uptake and neurotrophic activity, may also be compromised after exposure to HIV-1 proteins (Wang et al., 2003, 2004; Nosheny et al., 2004, 2006). In sum, the effects of HIV on glia, through infection or indirectly, create a CNS environment less able to support neuron function and survival.

A substantial subset of patients with HIV are exposed to opiates, through the abuse of illicit opiates or legitimate use of opiates for chronic pain syndromes. In the case of most injection drug abusers, exposure to opiates occurs prior to HIV infection, and needle sharing may play a role in acquiring infection. A higher incidence of HIV encephalitis and neuropathology has been reported among HIV-infected individuals who are injection drug abusers, some of whom preferentially abuse opiates relative to other drugs (Bouwman et al., 1998; Donahoe and Vlahov, 1998; Dougherty et al., 2002; Bell et al., 2002, 2006; Kopinsky et al., 2007; Anthony et al., 2008). While opiate exposure probably modifies HIV-1 neuropathogenesis through direct peripheral immune system interactions (Donahoe and Falek, 1988; Peterson et al., 1998; Rogers and Peterson, 2003), effects of opiates in the periphery differ from their actions in brain (Hauser et al., 2007). There is increasing experimental evidence that opiates also exacerbate neurologic deficits and HIV-associated neurocognitive disorders or HIV encephalitis directly through interactions with virus or viral proteins on CNS cells and tissues (Li et al., 2003; Hauser et al., 2005, 2007, 2009; Hu et al., 2005; El-Hage et al., 2006a, b; Kumar et al., 2006; Noel and Kumar, 2006; Bruce-Keller et al., 2008; Turchan-Cholewo et al., 2008, 2009; Bokhari et al., 2009; Malik et al., 2011). Subsets of astroglia and microglia (Ruzicka et al., 1995; Sheng et al., 1997; Stiene-Martin et al., 2001), as well as neurons, express functional opioid receptors during development and in adult CNS.

We have hypothesized that enhanced CNS neurotoxicity with concurrent opiate-HIV exposure may be due to interactions of opiates and viral proteins on glia. To test this hypothesis, we established a culture paradigm in which individual neurons were repeatedly examined over a 60-h period, in the presence or absence of co-cultured glial cells. Morphine did not enhance the toxic effect of transactivator of transcription (Tat) in pure neuron cultures. However, co-exposure to morphine and HIV-1 Tat resulted in synergistic increases in striatal neuron death when glia or glial conditioned media were present. Importantly, co-culture with glia deficient in μ-opioid receptors completely mitigated morphine–Tat interactions. The presence of μ-opioid receptors on neurons was not a factor in Tat–morphine synergy, as long as μ-opioid receptor-positive glia were present. Morphine also enhanced the degeneration of neurons in co-cultures exposed to clade C Tat, which alone was not neurotoxic. Thus, interactions between HIV-1 Tat and morphine that enhance neurodegeneration appear to be mediated by toxic events occurring in μ-opioid receptor-expressing glia. They may derive from morphine’s ability to interfere with glutamate uptake and to enhance Tat-induced glial reactivity in vitro and in vivo. Taken together, our findings suggest a fundamental role for microglia, astroglia or both, in orchestrating neurodegenerative changes in HIV-infected patients exposed to opiates.

Materials and methods

Experiments were conducted in accordance with procedures reviewed and approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee.

Primary neuron and glial cultures

Mixed glial cultures were derived from striata of Days 0 and 1 postnatal imprinting control region (CD1) or μ-opioid receptor knockout (Loh et al., 1998) pups as published (El-Hage et al., 2005). Striata were dissected, minced and incubated with trypsin (2.5 mg/ml) and DNase (0.015 mg/ml) in Dulbecco’s Modified Eagle’s Medium (DMEM) (30 min, 37°C), filtered through 70-μm filters and triturated, filtered twice through 45-micron and 135-micron filters (Hyclone), then incubated (30 min, 37°C). Striata were minced and incubated with trypsin (2.5 mg/ml) and DNase (0.015 mg/ml) in Dulbecco’s Modified Eagle’s Medium (DMEM) (30 min, 37°C, Invitrogen). Tissue was triturated, resuspended in medium containing 10% defined foetal bovine serum (Hyclone), filtered twice through 135 and 45-micron pore nylon mesh, then plated.

Striatal neurons were cultured from embryonic Days 15 and 16 imprinting control region or μ-opioid receptor knockout mouse embryos as described (Gurwell et al., 2001). Striata were dissected, minced and incubated (30 min, 37°C) with trypsin (2.5 mg/ml) and DNase (0.015 mg/ml) in neurobasal medium (Invitrogen) supplemented with B-27 additives (Invitrogen), L-glutamine (0.5 mM; Invitrogen), glutamate (25 μM; Sigma) and antibiotic mixture (Invitrogen). Tissue was triturated, filtered through 70-μm pore nylon mesh and then plated. Cultures were almost exclusively neurons when assessed by neuronal nuclear or microtubule associated protein 2 immunostaining; glial contamination during experiments was <1%. Twenty five thousand cells were plated per well in 24-well plates coated with poly-L-lysine.

We used cell-specific antibodies to characterize cells in cultures after Tat and opiate treatments. Cells were grown on coverslips and treated
as described below with Tat ± morphine ± naloxone. Duplicate coverslips prepared for n = 3 cultures were immunostained with cell specific antibodies described below and in Table 1. On each coverslip 200 (astroglia and microglia) or 500 (neuron, glial progenitor, oligodendrocytes) Hoechst-stained nuclei were identified in random fields, then assessed for immunostaining. Vehicle-treated cultures were ~91% astroglia and 8% microglia. Progenitors and oligodendrocytes constituted <1:500 cells (<0.2%). Neurons were not seen. Approximately 1% of cells were not identified. These may include immature astroglia lacking glial fibrillary acidic protein (GFAP)-positive intermediate filaments, nestin-positive progenitors, ciliated ependymal cells or endothelial cells. Immunostaining protocols are described in later sections. Treatment with Tat ± morphine significantly increased the percentage of microglia (Table 1). This parallels our findings in vivo where Tat injections or induction of Tat expression in transgenic mice increased microglial populations (El-Hage et al., 2006b; Bruce-Keller et al., 2008).

Co-culture paradigms
All cultures were prepared in 24-well plates. To establish co-cultures between neurons and glia, two deep midline grooves were made with a hot scalpel into the culture surface to prevent movement of cells between sides. Gial cells (3 × 10^5 per well) were plated with the multi-well tilted at an angle to sequester adhering cells to one side of the grooves (Fig. 1A). The grooves effectively inhibited cell crossing to the other side. Even at 2 weeks post plating, glia had not migrated past the midline (Fig. 1A). After 6 days, when glia were confluent on the right half, 2.5 × 10^5 neurons derived from embryonic Days 15/16 striata were plated over the entire surface. Using this technique, only half of the neurons directly contact glia, while all cells are exposed to the right half, 2.5 × 10^5 neurons directly contact glia, while all cells are exposed to the same glial conditioned medium. In other wells, purified neurons were plated in the absence of glia. Neurons were allowed to mature for 7 and 8 days prior to treatment with Tat ± opiates. At this time, they have established axons and dendrites, and express many characteristics of mature neurons, including a full complement of opiate receptors.

Repeated measures assessment
Twenty-four-well plates were transferred to a heat insert MXX holder (PeCon Instruments) and put on the scanning stage of a Zeiss Axio Observer Z1 inverted microscope (Carl Zeiss Inc.). For each well, 6–10 non-overlapping fields were selected. In wells plated with glia and neurons on one half and neurons on the other, 6–10 fields were selected on both right and left halves. A total of 8–10 individual medium neurons for viability in digital images taken at each time point (Fig. 1B). Death was assessed using rigorous morphological criteria, including neurite disintegration, loss of phase brightness and either involution or complete fragmentation of the cell body (Fig. 1B). Neuron death was confirmed by staining with ethidium monoazide (Buch et al., 2007). The viability of individual neuron was analysed at 4 h intervals using a repeated measures ANOVA (Statistica, StatSoft) to compare treatment effects. Findings were recorded as mean percentage of surviving neurons, relative to pre-treatment numbers ± standard error of the mean (SEM) from n = 4–6 separate experiments. The main effects for different treatment groups, irrespective of exposure time, were examined for significant differences. Unless stated, significance was assessed using repeated measures ANOVA and post hoc Duncan’s test when indicated. An alpha level of P < 0.05 was considered significant.

| Table 1 Percentage of cells in mixed glial cultures expressing cell specific markers |
|---|---|---|---|---|---|---|
| GFAP* | Iba-1* | NeuN* (%) | MBP* (%) | Sox2* (%) | Other |
| Control | 90.2 ± 0.4 | 8.8 ± 0.6 | <0.2 | <0.2 | <0.2 | 1.0 ± 0.3 |
| Tat | 84.3 ± 0.9* | 14.7 ± 1.3* | <0.2 | <0.2 | <0.2 | 1.0 ± 0.5 |
| Morphine | 88.8 ± 1.4 | 10.2 ± 1.3 | <0.2 | <0.2 | <0.2 | 1.0 ± 0.3 |
| Tat + morphine | 86.0 ± 1.8 | 13.7 ± 1.7* | <0.2 | <0.2 | <0.2 | 0.3 ± 0.2 |
| Tat + morphine + naloxone | 84.0 ± 0.8* | 15.5 ± 1.0* | <0.2 | <0.2 | <0.2 | 0.5 ± 0.3 |

n = 3 independent cultures; ± SEM; 200 or 500 total cells counted per coverslip; *P < 0.05 versus control. MBP = myelin basic protein; NeuN = neuronal nuclear.

HIV-1 Tat/opiate treatments
Cultures were treated with Tat_{1-86} (100 nM; ImmunoDiagnostics; clade B), morphine sulphate (500 nM; NIDA Drug Supply System), Tat + morphine, or Tat + morphine + naloxone, a broad-spectrum opioid receptor antagonist (1.5 μM; Sigma). Tat and morphine were added concurrently; naloxone was added 1 h before other treatments. Clade C Tat_{1-101} (Diatheva) was used in similar combinations with opiates. The Tat concentration used elicits functional deficits in glia and neurons similar to those occurring in HIV, and is considered to reflect levels seen pathophysiologically (Kruman et al., 1998; Nath et al., 1999; Singh et al., 2004; El-Hage et al., 2005, 2008; Perry et al., 2010). Morphine and naloxone doses were chosen to maximally stimulate and completely block μ-opioid receptors, respectively.

μ-Opioid receptor knockout mice
μ-Opioid receptor-deficient (C57Bl6 background) mice, provided by Dr Sandra Welch, have been well characterized (Loh et al., 1998; Park et al., 2000; Roy et al., 2001). The entire exon 2–3 region was replaced with a Neo cassette. Reverse–transcription polymerase chain reaction was used to verify the identity of knockout mice using primer pairs designed to amplify segments between the mutated exons (exons 1 and 2 primer set: P1: 5’-GACCGCTCAGGCAGGTCCATCC-3’; P2: 5’-CCACGTCCCATCAGGTAGT-3’; Exon 2 and 3 primer set: P3: 5’-CTTGCCTGCGCAGGATGCCT-3’; P4: 5’-GATCTGGCCAGACTTGGAG-3’). Briefly, total RNA was extracted from primary brain cultures obtained from presumed knockout and wild-type mice, using the RNeasy® kit (QIAGEN). Complementary DNA was obtained by computer-controlled stage encoder and Axiovision 4.6 software (Carl Zeiss, Inc.). During 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 (Fig. 1B). Death was assessed using rigorous morphological criteria, including neurite disintegration, loss of phase brightness and either involution or complete fragmentation of the cell body (Fig. 1B).
reverse transcription with 6-bp long random primer sets. Polymerase chain reaction was carried out using complementary DNA as a template, and polymerase chain reaction products were visualized in 2% agarose gels with ethidium bromide (Fig. 3). Littermate μ-opioid receptor-null mice given a high anaesthetic dose of morphine (100 mg/kg) (Dahan et al., 2001) showed no signs of anaesthesia or altered behaviour, indicating functional deletion of the μ-opioid receptor gene.

Figure 1 (A) Image of glial cells plated for a glia–neuron co-culture. A mixed glial culture is plated in the field to the right. Glial movement to the centre field is stopped by one of two deep grooves made in the plate. Glia are completely absent in the left field. When neurons are seeded over the entire plate, there is contact between neurons and glia in the right field; neurons in the left field receive glial conditioned medium but do not contact glia. (B) A time lapse, repeated measures analysis is used to assess neuron death in co-cultures, and to understand the relationship between glia and neurons that underlies HIV-1 Tat ± opiate effects. A single neuron (arrow) was followed in images taken every 20 min; selected time points are shown. The neuron appears quite healthy between 24 and 32 h. At 36 h, some larger neurites have become thinner and/or begun to retract (arrowheads). From 36 to 48 h the cell body and processes undergo further degeneration (arrowheads mark retracting/degenerating neurites). By 56 h the neuron is clearly dead, although many adjacent cells, both neurons and glia, remain alive and occasionally exhibit movement during this time period. Scale bar = 10 μm.
Tat transgenic mice

To determine the effect of Tat and morphine on reactive glial changes in vivo we utilized a Tet-on transgenic model with doxycycline-inducible HIV-1 Tat,1–86 expression in astroglia (Bruce-Keller et al., 2008; Hauser et al., 2009). Male transgenic mice and control littermates expressing only the reverse tetracycline transactivator gene were fed chow containing doxycycline for 12 days. During the final 5 days, subsets of mice received twice-daily injections of morphine intraperitoneally (10 mg/kg). Mice were deeply anaesthetized with isoflurane and perfused with 4% paraformaldehyde. Brains were cryoprotected, embedded, sectioned at 10 μm and immunostained for glial and reactive markers.

Immunohistochemistry

Cells were fixed in 4% paraformaldehyde and permeabilized with Triton-X 100. Neurons were detected using monoclonal antibodies to microtubule associated protein 2 (Chemicon, MAB378, 1:1000) or neuronal nuclei (Chemicon, MAB377, 1:200). Neuroprogenitors were identified by an antibody to sex determining region of Y-box-2 (R&D Systems; MAB2018, 1:200). Oligodendrocytes were identified using a monoclonal myelin basic protein antibody (SMI-99; Covance Inc., 1:500). A polyclonal antibody to ionized calcium binding adaptor molecule 1 (Iba-1) (Wako Chemicals, 1:500) and a polyclonal antibody to GFAP (Millipore, 1:1000) were used to identify microglia and astroglia, respectively. Antibodies used to detect μ-opioid receptor included a rabbit polyclonal (Antibodies Incorporated, 1:2000), and a guinea pig polyclonal to a C-terminal sequence (Chemicon, AB5509, 1:250). A mouse antibody to 3-nitrotyrosine (Santa Cruz Biotechnology, 1:100) was used to detect this reactive species in microglia in vivo. Primary antibodies were detected using appropriate secondary antibodies conjugated to Cy3, Oregon green, Alexa 488 and Alexa 594. Cell nuclei were visualized with Hoechst 33342 dye. In frozen sections, Iba-1+ cells were assessed as a per cent of 200 total Hoechst+ cells in duplicate sections (averaged) in n = 4 mice. The per cent of Iba-1+ cells expressing 3-nitrotyrosine was assessed in 200 Iba-1+ cells in duplicate sections (averaged) in n = 4 mice. Significance was assessed by ANOVA with post hoc Duncan’s test. Confocal, immunofluorescent images were acquired using a Zeiss LSM 700 laser scanning confocal microscope configured to an Axio Observer Z.1 microscope, and processed using Zen 2010 software (Carl Zeiss, Inc.). Multiple z-stacks were acquired and compressed into single projected images to show cells in their entirety.

Reactive oxygen species and glutamate measurements

Intracellular reactive oxygen species production was measured using dihydrofluorescein diacetate (Invitrogen) for glia treated with Tat alone (100 nM), or with increasing concentrations of morphine (10–10–5 M). Cells were treated with 10 μM of cell-permeant dihydrofluorescein diacetate for 45 min at 37°C in Hank’s balanced salt solution, washed and exposed to Tat and/or morphine for 90 min. Reactive oxygen species production was measured using a Victor 3 plate reader (PerkinElmer, Inc.) at \( \lambda_{\text{excitation}} = 488 \) and \( \lambda_{\text{emission}} = 525 \) nm. Glutamate uptake was measured in confluent glial cultures grown in 24-well plates (n = 3–4). Medium was aspirated from each well; adherent cells were washed with Na+-free Hank’s balanced salt solution. Cells were pre-incubated for 45–60 min at 37°C with 500 μl of Hank’s balanced salt solution alone or with 500 nM morphine and/or 100 nM Tat. Glutamate was added to each well from a stock solution to a final concentration of 1.2 mM. Sample medium was removed from individual wells at 0–240 min; glutamate values were quantified using a colorimetric detection kit (BioVision) with absorbance at 450 nm. Additional experiments were conducted in the presence of glutamate transporter inhibitors to assess morphine and Tat affects on transporter function. DL-threo-β-benzyloxyaspartate (DL-TBOA; TOCRIS Biosciences; 10 μM) is extensively used to inhibit excitatory amino acid transporter (EAAT) 1–5-mediated transport, but has relatively low affinity. Thus we additionally employed (2S, 3S)-3-[3-[(trifluoromethyl) benzoylamino] benzoyloxy]aspartate (TFB-TBOA; TOCRIS Biosciences; 1 μM), a recently developed EAAT 1–3 inhibitor. Both inhibitors should target glial cultures that almost exclusively express EAAT1 and EAAT2. Cells were pre-incubated in inhibitors for 45–60 min at 37°C either alone or combined with morphine and Tat prior to glutamate addition. Significance was assessed using repeated measures ANOVA with post hoc Duncan’s testing.

Results

Synergistic effect of morphine on HIV-1 Tat-induced neurotoxicity requires glia

In previous studies, co-exposure to morphine caused dose dependent, synergistic increases in striatal neuron death caused by HIV-1 Tat (Gurwell et al., 2001). Since those cultures contained 30% glia, it was unclear whether morphine effects were directly on neurons or mediated through glia. To distinguish between direct and indirect effects of Tat alone, and combined Tat and morphine exposure, the present studies compared survival of individual neurons over 60 h using a repeated measures analysis paradigm in three different types of culture: (i) isolated striatal neurons (99% pure); (ii) neurons exposed to glial conditioned medium; and (iii) neurons in contact with a glial bedlayer (Fig. 1). We first examined for main effects of treatment, time and the presence of glia using a one-way ANOVA with post hoc Duncan’s test. Significant differences were found for all interactions.

Roughly 95% of untreated (control) neurons grown in isolation survived for 60 h (Fig. 3A). Similar to numerous reports of Tat neurotoxicity, our results show that exposure to HIV-1 Tat,1–86 (100 nM) decreased isolated neuron survival. There was a significant main effect for morphine and Tat treatments (P < 0.001). For both Tat and Tat + morphine groups, neuron survival was significantly less than control at each time after 16 h (Fig. 3A, P < 0.05). At no time were there differences between the effect of Tat alone or Tat + morphine. Although there was no overall main effect of morphine alone, neuron survival was less than control specifically at 60 h (Fig. 3A, P < 0.05).

The presence of glial cells profoundly influenced both the overall survival of striatal neurons and the effects of combined Tat and morphine exposure. When neurons were grown either in glial conditioned medium (Fig. 3B) or with glial contact (Fig. 3C), but without opiate or Tat treatment, their survival over 60 h was 80–85%, a significant reduction compared with 95% survival in isolated cultures (P < 0.05; compare black circles in Fig. 3A–C). Morphine did not reduce survival at any time under either
condition of glial exposure. Tat by itself induced modest neuron death over control values at 40–60 h with glial contact ($P < 0.05$ versus control; Fig. 3C). Importantly, under both conditions of glial exposure co-exposure to Tat + morphine profoundly reduced neuron survival. In neurons exposed to glial conditioned medium, Tat + morphine resulted in greater toxicity than Tat alone at every time after 20 h, and <40% of neurons that were tracked survived after 60 h co-exposure ($P < 0.05$ versus Tat alone; Fig. 3B). Glial contact accelerated this effect since combined Tat and morphine was toxic at 8 h and thereafter; <30% of neurons in direct contact with glia survived after 60 h co-exposure ($P < 0.05$ versus all others; Fig. 3C). The synergistic effect of morphine was reversible by concurrent administration of naloxone, confirming that effects were mediated by opioid receptors (Fig. 3B and C). Interestingly, when neurons were in contact with glia, naloxone brought neuron survival completely back to control levels, blocking not only synergy between morphine and Tat, but also toxicity of Tat alone.

### Synergistic neurotoxicity of HIV-1 Tat and morphine mediated by glia requires $\mu$-opioid receptor

While morphine is a preferential $\mu$-opioid receptor agonist, it can have activity at both $\kappa$- and $\delta$-opioid receptors and effects unrelated to anti-nociception. Our experimental design utilized morphine instead of a more selective $\mu$-opioid receptor agonist because morphine is the major bioactive metabolite of heroin in the CNS, and thus of great relevance to drug abuse. Since subsets of both glia and neurons in our system express $\mu$-opioid receptors (Fig. 2), morphine might have actions at any or all of these cell types. Therefore, additional studies used cells derived from $\mu$-opioid receptor-null mice (Fig. 3D) to determine unambiguously whether $\mu$-opioid receptor on glia and/or neurons was necessary for morphine to enhance Tat-mediated death. In untreated cultures, the survival of neurons co-cultured in contact with glia was ~80–85% after 60 h irrespective of the phenotype of either glia or neurons (Fig. 3E and F) and similar to cultures of wild-type neurons and glia (Fig. 3C). To determine the role of neuronal $\mu$-opioid receptor in Tat–morphine toxicity, co-cultures were established between $\mu$-opioid receptor-null neurons and wild-type glia. Co-exposure to Tat and morphine resulted in the same, consistent and significant interactive toxicity observed with both wild-type neurons and glia (Fig. 3D, $P < 0.01$ versus Tat alone). Neurotoxicity followed a similar temporal pattern (significantly higher after 8 h) and reached the same approximate level as in wild-type cultures. These findings strongly suggested that $\mu$-opioid receptor expression by neurons was not a major determinant of synergistic toxicity. Morphine by itself was not toxic at any time. Tat alone was neurotoxic at 24 h and thereafter (Fig. 3D, $P < 0.05$ versus control and appeared slightly more toxic in these cultures than in wild-type (Fig. 3C) or $\mu$-opioid receptor-null glial co-cultures (Fig. 3F).

To test whether $\mu$-opioid receptor expression by glia plays a role in interactive Tat–morphine toxicity, $\mu$-opioid receptor-null glia and wild-type neurons were co-cultured. In this case, the outcome was quite different. The synergistic effect of morphine on Tat-induced death was eliminated. Similar to other conditions, morphine by itself had no effect, and Tat by itself caused modest toxicity at 32 h and afterwards (Fig. 3E, $P < 0.05$ versus control). These findings, together with those for $\mu$-opioid receptor-null neurons, strongly support the hypothesis that interactive or synergistic neurotoxicity caused by morphine and Tat is largely mediated through effects on glia.

### Clade C Tat becomes toxic to striatal neurons in the presence of morphine

The clade B Tat protein used in our studies is generally recognized to have neurotoxic qualities. Tat protein from clade C HIV-1 results in far less, if any, neurotoxicity in vitro (Li et al., 2008; Mishra et al., 2008; Campbell et al., 2010), in general agreement with lower incidence of cognitive effects historically reported in individuals infected with clade C virus (Satishchandra et al., 2000; Wadia et al., 2001). We conducted co-culture studies to determine whether morphine might also enhance the neurotoxic properties of clade C Tat. Murine striatal neurons were co-cultured for 72 h in direct contact with wild-type astroglia, in the absence or presence of morphine, at concentrations used in studies with clade B Tat. Individual cells were again followed throughout the experiment, but survival was assessed only at 24 h intervals. While clade C Tat by itself did not affect striatal neuron survival, co-exposure to morphine caused significant neuron loss at both 48 and 72 h as measured by main effects for the conducted ANOVA (Fig. 4). Even though these experiments were 12 h longer than studies with clade B Tat, the overall toxicity for clade C Tat + morphine was considerably less (30% loss with clade C; 70% loss with clade B).

### Morphine enhances the toxic milieu induced by Tat

If morphine and Tat converge on glia to produce bystander effects in neurons, cytotoxic products should be demonstrable from the glia used for co-culture. Not surprisingly, Tat alone (100 nM) significantly increased reactive oxygen species production (Fig. 5B; $P < 0.05$ versus vehicle-treated controls). Moreover, morphine by itself caused modest, but nevertheless significant, concentration-dependent increases in reactive oxygen species in glial cultures (Fig. 5B; $P < 0.05$ versus vehicle-treated controls). Importantly, in combination with 100 nM Tat, the effect of morphine on reactive oxygen species was exacerbated in a concentration-dependent manner and large increases in reactive oxygen species were apparent even with low concentrations of morphine (Fig. 5B). In all cases morphine effects were fully reversible by naloxone, as well as by the selective $\mu$-opioid receptor antagonist d-Phe–Cys–Tyr–o-Trp–Arg–Thr–Pen–Thr–NH$_2$ (CTAP; data not shown). To test whether morphine and/or Tat limit the ability of astroglia to buffer glutamate, glial cultures were challenged with excess l-glutamate and residual glutamate was measured over 240 min. Starting at 30 min, glutamate levels were higher with all treatments compared with vehicle (Fig. 5A). Glutamate levels were
higher in Tat and combined morphine + Tat groups than with morphine alone. The trend for a greater effect with combined morphine and Tat than with Tat alone was not significant. Two different inhibitors of glial EAAT1 and EAAT2 transporters completely stopped glutamate buffering. The inhibitors also eliminated effects of Tat and morphine (Fig. 5A), suggesting that Tat and morphine interfered with glutamate transporter function. Separate analyses for each treatment revealed no inhibitor time effect; glutamate levels remained at pre-treatment levels with no measurable glutamate release over 240 min.

Figure 2  Immunohistochemistry was used to demonstrate the cellular content of glial cultures and the expression of μ-opioid receptor in glia and neurons. All images are confocal; multiple z-stacks are compressed into a single image to show localization through entire cell. A and C are shown at lower magnification to give a representative view of glial cultures used for the co-culture system. Smaller panels to the right of each figure show colour separation. (A and B) Cells were immunostained for GFAP (green) and Iba-1 (red), and counterstained with Hoechst 33342 nuclear marker (blue). Note that the majority of glia in A are GFAP + astrocytes, with ~8% Iba-1 + microglia. Astroglia take a variety of morphological forms, some of which are shown at higher magnification in B and D. They have varying levels of GFAP staining intensity. B shows both a ramified (arrow) and a more activated, amoeboid (arrowhead) Iba-1 + microglial cell at higher magnification, with surrounding astroglia. Ramified microglia tend to have a much lower intensity of Iba-1 immunostaining. (C and D) Cells were immunostained for GFAP (red) and μ-opioid receptor (green) and counterstained with Hoechst 33342 (blue). Again, the majority of cells in C are astrocytes, and many are μ-opioid receptor-positive independent of their morphology. D shows two GFAP + astroglia at higher magnification where μ-opioid receptor localization can be appreciated in detail. In most cells, μ-opioid receptor exists both in distinct cytoplasmic regions and more superficially as a punctate distribution at the cell surface and along processes. E shows two neurons with microtubule associated protein-2b (red) and μ-opioid receptor (green) immunostaining in a low density region of a mixed culture, with similar intracellular and surface μ-opioid receptor localization. The microglial cell in F is largely amoeboid with only a few short, thin processes. Immunostaining intensity for both Iba-1 (red) and μ-opioid receptor (green) is intense, and μ-opioid receptor localization is similar to that in astroglia and neurons. MOR = μ-opioid receptor. Scale bars: A and C = 50 μm; B, D, E and F = 10 μm.
Figure 3  Neurotoxic interactions between Tat and morphine are mediated by glia. (A–C) Results from cultures with wild-type cells. (E and F) Results from cultures with μ-opioid receptor-deficient cells. (A–C) Wild-type neurons were grown in isolation (A) or co-cultured with wild-type glia (B and C). In A, Tat treatment for 60 h significantly reduced the survival of isolated striatal neurons as compared with untreated neurons at each time point after 16 h (*P < 0.05, double lines versus black circles); morphine by itself was not toxic except at the 60 h time point (#P < 0.05, open circles), and morphine did not enhance Tat-mediated toxicity (red line). In B and C, neurons were grown in the presence of glial conditioned medium (CM) or in contact with glia, respectively. With glia present, there was a reduction in survival even without treatment (compare black circles in A–C). Morphine by itself did not affect survival at any time under either condition of glial exposure (open circles); Tat by itself caused significant toxicity compared with untreated controls at 40–60 h (*P < 0.05; double lines). When Tat and morphine exposure were combined, the reduction in neuron survival was dramatic. In the presence of glial conditioned medium (B), Tat + morphine enhanced neuron loss at every time point after 20 h, and <40% of neurons survived through 60 h co-exposure (**P < 0.05 versus all other groups; Fig. 2B, red line). Glial contact accelerated the time of death; the combination of Tat + morphine was toxic at 8 h and at all time points thereafter versus all other groups (**P < 0.05; Fig. 2C, red line). Scale bar in each = 10 μm. Survival was also significantly less overall in the neurons with glial contact versus those bathed in glial conditioned medium at all points after 8 h (C, **P < 0.05 versus red lines in B). In both B and C, naloxone blocked the synergistic effects of morphine. In the case of glial contact (C) naloxone brought survival to control levels. (D and E) Loss of μ-Opioid receptor on glia, but not on neurons, eliminated the synergistic effect of morphine on Tat toxicity. (D) μ-Opioid receptor gene deletion was confirmed by polymerase chain
Measures of inflammation were also made in vivo, in transgenic mice induced to express Tat. Transgenic mice ± morphine had elevated populations of fba-1 microglia, and mice receiving concurrent morphine administration showed an increase in reactive (3-nitrotyrosine *) microglia (Fig. 5C–G).

Discussion

Models of HIV neurotoxicity

HIV is a human specific disease; modelling in other species presents a challenge. While non-human primates infected with simian immunodeficiency virus (SIV) are invaluable for assessing preclinical issues and vaccine efficacy (Haigwood, 2004; Silvestri, 2008; Valentine and Watkins, 2008), their limited availability and the difficulty of culturing simian CNS tissue makes testing cellular mechanisms unrealistic in SIV models. Mouse striatal neuron cultures have been well characterized by our laboratories (Gurwell et al, 2001; Singh et al., 2004; Bakalkin et al., 2010), and are a reasonable choice for modelling interactive effects of HIV-1 proteins and opiates. In the present studies, post-mitotic striatal neurons are allowed to mature for 7 and 8 days in vitro, at which time they express many characteristics of mature CNS neurons, including N-methyl-D-aspartate (NMDA), AMPA [2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl)propanoic acid], and dopamine receptors, GABA (γ-aminobutyric acid), neuropeptides typical for striatum, and opiate receptors (Freese et al., 1990; Vaysse et al., 1990; Mao and Wang, 2001; Goody et al., 2003; Singh et al., 2003; Cepeda et al., 2008). Certainly, one must be cautious when extrapolating from murine to human systems. However, the model used here permits a degree of experimental control that is impossible in vivo, or in human cultures, where characteristics such as age/developmental stage, purity and brain region are not well controlled.

Tat neurotoxicity may be largely due to direct effects

It is generally accepted that HIV-1 Tat can directly induce neuron death. Purified neurons exposed to exogenous viral proteins show cell loss, caspase-3 activation, mitochondrial membrane potential changes and other measures of toxicity (Cheng et al., 1998; Kruman et al., 1998; Gurwell et al., 2001; Singh et al., 2004; Akensenova et al., 2009). The direct toxicity due to Tat in our isolated neurons (Fig. 2A) was less than reported by others, which may reflect purity of various neuron preparations, type of neurons, their age and relative maturity, and/or to repeated sampling of individual neurons versus population-based analyses. Different Tat sequences may also vary in toxicity. The survival curves for untreated neurons show that both types of glial-containing cultures create conditions that compromise neurons. Our glial preparation was predominantly astroglial, but also contained 8–10% microglia. Although astroglia in the brain have protective effects, culture manipulations and conditions cause some activation of both glial types, which likely reduces baseline neuron survival. In co-cultures with glial contact (Fig. 3C), Tat induced 10% additional loss of neurons over 60 h. This paralleled the loss in isolated cultures, suggesting that Tat by itself may act largely through direct mechanisms to induce toxicity in striatal neurons.

Direct effects of HIV-1 Tat can be mediated by excitotoxic interactions of Tat with NMDA receptors (Song et al., 2003; Akensenova et al., 2009; Eugenin et al., 2011). The Cys 30–Cys 31 motif in Tat is reported to be critical for activating NMDA receptors (Li et al., 2008), and the Cys 31 → Ser 31 mutation in clade C Tat may account for lower toxicity compared with clade B Tat, reported by others (Li et al., 2008; Mishra et al., 2008; Rao et al., 2008; Campbell et al., 2010) and also found in this study (Fig. 4). While clade C infection has historically been stated to result in lower incidence or severity of CNS involvement than clade B infection, more recent studies have detected a range of neurocognitive deficits (Satishchandra et al., 2000; Riedel et al., 2006; Yepthomi et al., 2006; Gupta et al., 2007). Alternatively, Tat may also be endocytosed after interaction with a low density lipoprotein receptor-related protein (LRP) (Liu et al., 2000) or heparin sulphate proteoglycan (Rusnati et al, 1997) on neuron membranes and may interfere with events critical for neuron stability/function including amyloid precursor protein metabolism (Liu et al., 2000), histone acetylation and neurotrophin signalling (Wong et al., 2005). The Tat C-terminus also contains a tripeptide Arg–Gly–Asp (RGD) domain which may disrupt neuron function by activating integrin receptors (Brake et al., 1990; Barillari et al., 1993; Ma and Nath, 1997).

Figure 3 Continued

reaction for exons 2 and 3. Bands 1 and 2 show amplified product using primers for exons 2 and 3, respectively, in cells from wild-type mice. Bands 3 and 4 show that both primers failed to amplify detectable product from µ-opioid receptor-null cells. Co-cultures containing either µ-opioid receptor-deficient neurons and wild-type glia (E) or wild-type neurons and µ-opioid receptor-deficient glia (F) were treated with HIV-1 Tat and opiates over 60 h to determine the cells on which morphine acted to enhance Tat-induced neurotoxicity. In both types of co-cultures, Tat by itself was toxic to striatal neurons (*P < 0.05 versus untreated control, double lines), while morphine alone showed no toxicity (open circles). Tat and morphine co-treatment resulted in a synergistic neurotoxic effect when µ-opioid receptor was absent on neurons but present on glia (E, **P < 0.01 versus all other groups, red line). The synergy was completely abolished when wild-type neurons were co-cultured with µ-opioid receptor-deficient glia (F, red line). Interactive morphine–Tat toxicity was also abolished by the broad-spectrum opiate antagonist naloxone, which by itself was not toxic (E). These findings strongly support the concept that the enhanced toxicity is opiate specific, and that µ-opioid receptor expression on glia mediates neurotoxicity Tat + morphine interactions. MOR = µ-opioid receptor; WT = wild-type.
Morphine enhances Tat-induced neurotoxicity in the presence of glia

Our results show that glia are required for the interactive neurotoxicity driven by HIV-1 Tat and morphine. This was apparent from several perspectives. In isolated neurons, addition of morphine did not augment Tat-induced neuron loss (Fig. 2A). However, neuron–glial co-cultures exposed to Tat and morphine had dramatically elevated neuron death. The onset of neuron death was also temporally accelerated. Since morphine by itself was minimally toxic, the increased neuron loss represents a synergistic effect. Synergistic toxicity was observed when neurons were in contact with glia or surrounded by glial conditioned medium. However, the contact-mediated toxicity was significantly greater than with soluble factors. Morphine potentiates release of a number of toxic effectors, including tumour necrosis factor-α, nitrites, interferon-γ and glutamate, from glia and other cells co-exposed to Tat, HIV or other inflammatory agents (Chao et al., 1994; Kapasi et al., 2000; El-Hage et al., 2005; Bokhari et al., 2009; Turchan-Cholewo et al., 2009; Gupta et al., 2010). Other factors similarly upregulated in glia, such as monocyte chemoattractant protein-1, regulated upon activation, normal T cell expressed and secreted, macrophage inflammatory protein-1α and β (Mahajan et al., 2005), are not directly neurotoxic but may secondarily drive toxicity by activating microglia. The data additionally show that morphine enhances Tat-induced reactive oxygen species and reactive nitrogen species production in vitro, and intrinsically compromises the ability of astroglia to buffer glutamate. In vivo, transgenic mice expressing Tat had microgliosis, and morphine enhanced microglial reactivity (Fig. 5C and D). Overall, the combined effects of HIV-1 Tat and morphine produce a less supportive and more reactive, deleterious cellular milieu. The greatest neuron loss occurred with neuron–glia contact, suggesting that morphine and Tat may jointly interfere with signalling pathways involving membrane bound components, such as chemokine (C-X3-C motif) ligand 1/chemokine (C-X3-C motif) receptor 1 (Meucci et al., 2000; Meuth et al., 2008).

Morphine was marginally toxic in our studies, perhaps related to the finding that morphine reduced glutamate buffering (Fig. 5A). Morphine-induced toxicity has occasionally been observed in other systems (Hu et al., 2002; Turchan-Cholewo et al., 2006; Malik et al., 2011) and might be a greater factor with chronic drug exposure. The concept that chronic opiate exposure can by itself be neurotoxic is supported by studies in human brain where opiate abuse was associated with premature hyperphosphorylation and deposition of Tau, and correlative increases in microglial activation (major histocompatibility complex class II expression) (Anthony et al., 2010).

Morphine effects are mediated through μ-opiate receptors

Morphine acts at multiple opioid receptors, any of which might contribute to synergistic toxicity. We used cells from transgenic mice lacking μ-opiate receptors to define the cellular origin of Tat–morphine synergy. Co-cultures between μ-opiate receptor-null neurons and wild-type glia showed unabated neurotoxicity due to Tat and morphine, while toxicity was completely abolished when μ-opiate receptor was absent from glia. Thus, it appears that μ-opiate receptor on glia are both necessary and sufficient for morphine to synergistically increase HIV-1 Tat-induced neuron death. When mixed populations of human neurons and glia were treated with morphine + Tat, death was accelerated in neurons with an ApoE4 allele (Turchan-Cholewo et al., 2006), suggesting that results in the murine system may reflect a more general phenomenon. Similarly, morphine potentiated glycoprotein 120-induced neurotoxicity in human neuron–glia cultures (Hu et al., 2005) suggesting that a glial-dependent phenomena may extend to additional HIV proteins.

The opiate–Tat synergy observed in vitro mirrors certain findings made by our lab and others in animal models. In an inducible transgenic mouse with HIV-1 Tat1–86 produced in astroglia, morphine co-exposure augmented degenerative processes in neurite structure within the striatum (Fitting et al., 2010). No increased neuron death was observed in either striatum (Fitting et al., 2010) or hippocampus (unpublished data). This could relate to differences in delivery/titre of Tat or morphine, or to different neuron susceptibility in vivo. Toxicity in vivo may also require longer exposure times. Interestingly, co-exposure to morphine did increase Terminal deoxynucleotidyl transferase dUTP nick end labelling-positive oligodendroglia in the striatum/corpus callosum of these mice over the same time period (Hauser et al., 2009), and also

Figure 4 Effect of clade C Tat and morphine on striatal neuron survival. Cultures containing both glia and neurons were treated with clade C Tat (Tat[C], 100 nM) with or without morphine (Morph) or naloxone (Nal). Individual neurons were selected prior to treatment and reassessed at 14 h intervals. There was no effect of any treatment on survival at 24 h. Although clade C Tat was not toxic at any time point, simultaneous exposure to both Tat and morphine induced significant toxicity at 48 and 72 h. *P < 0.05 versus control and Tat; #P < 0.05 versus clade C Tat + morphine, suggesting that the synergistic effect is opiate specific.

a A significant main effect versus all other groups at this time.
Synergistic effects of Tat and morphine on inflammation in vitro (A and B) and in vivo (C–G). (A) An enzymatic assay was used to determine whether morphine and Tat affect the ability of astroglia to buffer excess glutamate. Glial cultures were challenged with 1.2 mM L-glutamate and residual glutamate in the medium assessed at 15–240 min. Glutamate buffering was reduced in all treatment groups versus vehicle at 30 min and thereafter (*P < 0.05 versus control). Further, at 30 min and thereafter, the effect of Tat + morphine was greater than the morphine effect alone (b, P < 0.05 versus Tat and Tat + morphine groups). The consistent trend for Tat + morphine to be higher than Tat alone was not significant. Two different EAAT inhibitors completely eliminated glutamate uptake and there was no significant main effect for time, indicating that glutamate levels did not change from baseline (§). Morphine + Tat treatment had no effect on glutamate levels in the presence of inhibitor. (B) Reactive oxygen species production was assessed by dichlorofluorescein diacetate fluorescence (DCF) at 45 min following continuous treatment of mixed glia with morphine (10–10–10 M) either with or without co-exposure to HIV-1 Tat (100 nM). Tat alone significantly increased reactive oxygen species production (*P < 0.05 versus vehicle-treated controls). Morphine by itself also caused modest but significant concentration dependent increases in reactive oxygen species (P < 0.05 versus vehicle-treated controls). In the presence of Tat there was a synergistic elevation in reactive oxygen species at every morphine concentration. In all cases, morphine effects were reversible by naloxone, showing opiate specificity. Results are mean ± SEM from six experiments. (C and D) Inducible, transgenic mice expressing HIV-1 Tat (Tat+) and control littermate (Tat−) mice were exposed to doxycycline (DOX) for 12 days. A subset received morphine (Morph) twice daily subcutaneously for the final 5 days. Frozen sections were immunostained using antibodies to detect the microglial marker Iba-1 and 3-nitrotyrosine (3-NT), a reactive nitrosyl product typically detected in activated microglia. C shows that doxycycline induction of the Tat transgene increased Iba-1+ microglia as a per cent of (continued)
had synergistic effects on Tat-induced microglial and astroglial reactivity (Bruce-Keller et al., 2008). An additional consideration in these experiments is that Tat expression can affect the endogenous opioid system, and vice versa. For example, chronic morphine treatment reportedly increased activity of the tat gene, but not activity of other HIV-1 genes, e.g. nef, rev and vpr in HIV-transgenic rats (Sultana et al., 2010). Our data have shown that induction of Tat in transgenic mice significantly alters expression of genes for receptors and peptides in all major opioid systems (Fitting et al., 2010). Similar interactions between Tat and endogenous opioid signalling must also occur in co-cultures, and may result in occasional paradoxical findings. For example, naloxone reversed Tat and morphine synergy, but also eliminated the modest Tat toxicity observed with neuron–glial contact (Fig. 3C).

In cultures where glia lacked µ-opioid receptor, not only was synergy between morphine and Tat blocked, but morphine and Tat together were less toxic than Tat alone (Fig. 3B; compare open circles with red line). In cultures where neurons lacked µ-opioid receptor, effects of Tat alone were more pronounced than in wild-type cultures (Fig. 3E). Loss of µ-opioid receptor in one cell type may create fundamental imbalances in cell-to-cell signalling, and may reveal opponent processes that exist between individual cell types. Alternatively, in the absence of µ-opioid receptor, effects of morphine or naloxone on α- and δ-opioid receptors may emerge. All CNS cell types have endogenous opioid peptides and receptors. Many opioids are promiscuous in terms of receptor utilization, and changes in expression of individual receptors can drive compensatory changes in other receptors and peptides. Even in isolated cells, these complex relationships make full experimental control impossible. Our approach has been to target parameters most critical for a specific question. Removing µ-opioid receptor from glia or neurons allows us to determine the importance of each cell in driving interactive neurotoxicity. However, it is an artificial situation that likely alters the balance and responses to other receptors/peptides within co-cultures, as observed in studies for CXCR4 and opioid receptors (Burbassi et al., 2010).

Does the morphine enhancement of Tat-mediated neurotoxicity involve microglia or astroglia? Since glial cultures were heterogeneous, we cannot make this determination. Astroglia and microglia both show synergistic inflammatory/reactive responses to combined morphine–Tat exposure. It is likely that crosstalk between cell types is critical in driving milieu changes that limit neuron function and/or survival. One scenario, based on synergistic dysregulation of intracellular calcium concentration homeostasis and upregulated chemokine (regulated upon activation, normal T cell expressed and secreted/CC chemokine ligand-5, monocyte chemoattractant protein-1/CC chemokine ligand-2) release from astroglia by morphine–Tat co-exposure (El-Hage et al., 2005), involves astroglia as initial targets of Tat or HIV and opiates. Subsequent release of chemokines that activate resident microglia and attract infected and uninfected monocytes from the periphery may set up a chain of events that culminates in neuron dysfunction.

Correlates of disease process in human tissues

Even in the era of combined anti-retroviral therapy, the CNS harbours virus due to poor blood–brain barrier penetration of therapeutic compounds (Langford et al., 2006), and viral titer can differ substantially between CSF and serum (Canestri et al., 2010; Bogoch et al., 2011). Owing to persistant virus and longer patient survival, ~50% of patients with HIV will eventually develop CNS neurological complications (Kaul et al., 2005; Mattson et al., 2005; Antinori et al., 2007; Ellis et al., 2007; Haughey et al., 2008). Although HIV-associated dementia is now a less frequent manifestation of HIV-associated neurocognitive disorder, the prevalence of less severe cognitive dysfunctions including asymptomatic neurocognitive impairment and minor or moderate cognitive disabilities has not been reduced or has increased (Sacktor et al., 2002; McArthur et al., 2003; Kaul et al., 2005; Rumbaugh et al., 2008). At a microscopic level, CNS tissues show numerous signs of chronic immune activation and encephalitis, including increased infiltration of monocyte-derived macrophages and enhanced release of oxidative and other cellular toxins (Cherner et al., 2002; Kraft-Terry et al., 2010). Although reactive gliosis and myelin degeneration are common, cognitive impairment in combined anti-retroviral therapy-treated individuals correlates most closely with synaptodendritic pathology (Everall et al., 1999; Ellis et al., 2007).

There is substantial evidence that exposure to opiates can exacerbate CNS pathology in patients with HIV (Bouwman et al., 1998; Donahoe and Vlahov, 1998; Bell et al., 2002, 2006; Dougherty et al., 2002; Kopinsky et al., 2007; Anthony et al., 2008). Observations in HIV-infected patients are supported by confirmatory work in rodent models detailed above. Morphone–viral synergism has also been observed in non-human primates infected with either SIV or chimeric SIV–HIV. In such studies, chronic opiate exposure routinely increases viral load, reactive oxygen species and pro-inflammatory markers in the CNS and/or CSF (Chuang et al., 2005; Perez-Casanova et al., 2007; Bokhari et al., 2011). Somewhat differently, Marcario and colleagues (2008) found that morphine increased histopathology in SIV-infected brains, although cell-mediated immune responses

Figure 5 Continued

total cells; co-exposure to morphine did not enhance the effect. However, morphine did enhance the doxycycline induced increase in Iba-1+/microglia with reactive 3-nitrotyrosine in their cytoplasm (D). (E–G) Confocal, immunofluorescent images of cells that are either positively or negatively stained for Iba-1, with fluorescent signals separated in smaller panels to the right. The Iba-1+/3-nitrotyrosine+ microglial cell from a control (Tat−) mouse (F) is highly ramified and shows a relatively low level of 3-nitrotyrosine expression. The section in (G) is from a Tat+ mouse treated with doxycycline and morphine. Several microglia in various stages of activation are shown, and the level of 3-nitrotyrosine is noticeably higher. Arrowheads indicate cells with marginal 3-nitrotyrosine staining. Arrows indicate cells with robust 3-nitrotyrosine expression. The cell in (E) has an amoeboid microglial morphology and is µ-opioid receptor-positive/3-nitrotyrosine+. Scale bars in (E–G) = 10 μm.
were reduced and viraemia/CSF viral titres were unaffected. A more limited set of studies show that opiates hasten disease progression, brain entry and/or neuropathology in SIV (Kumar et al., 2006; Noel et al., 2006; Perez-Casanova et al., 2007; Marcario et al., 2008; Bokhari et al., 2011), possibly related to the finding that opiate exposure can alter viral evolution (Noel and Kumar, 2006; Rivera-Amill et al., 2010). Controversy on the effects of morphine in non-human primate models remains (Burdo et al., 2006; Donahoe et al., 2009), perhaps related to opiate delivery regimens and/or different abilities of SIV/chimeric SIV–HIV strains to penetrate the brain. Opiate timing relative to HIV exposure is a potentially important variable; our studies used a simultaneous delivery paradigm for technical reasons and to avoid issues of opioid tolerance and dependence that are best assessed in vivo.

The work presented here starts to address the question of how opiate exposure accelerates HIV-mediated CNS damage. Since subsets of all cell types in both human and murine brains express µ-opioid receptors, it is essential to understand whether accelerated neuropathogenesis is through a direct opiate effect on neurons, and/or indirectly mediated by surrounding glia. Our findings argue strongly that interactions between opiates and HIV-1 Tat that reduce neuron survival are driven primarily by glial cells expressing µ-opioid receptors, either astrocytes or microglia, or perhaps both acting in concert.

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