Published in final edited form as:
Eur J Neurosci. 2004 June ; 19(12): 3171–3182. doi:10.1111/j.0953-816X.2004.03461.x.

Preferential vulnerability of astroglia and glial precursors to combined opioid and HIV-1 Tat exposure *in vitro*

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Abstract

Human immunodeficiency virus-1 (HIV) infection can cause characteristic neural defects such as progressive motor dysfunction, striatal pathology, and gliosis. Recent evidence suggests that HIV-induced pathogenesis is exacerbated by heroin abuse and that the synergistic neurotoxicity is a direct effect of heroin on the CNS, an alarming observation considering the high incidence of HIV infection with injection drug abuse. Although HIV infection results in neurodegeneration, neurons themselves are not directly infected. Instead, HIV affects microglia and astroglia, which subsequently contributes to the neurodegenerative changes. Opioid receptors are widely expressed by macroglia and macroglial precursors, and the activation of μ-opioid receptors can modulate programmed cell death, as well as the response of neural cells to cytotoxic insults. For this reason, we questioned whether opioid drugs might modify the vulnerability of macroglia and macroglial precursors to HIV-1 Tat protein. To address this problem, the effects of morphine and/or HIV $Tat_{1–72}$ on the viability of macroglia and macroglial precursors were assessed in mixed-glial cultures derived from mouse striatum. Our findings indicate that sustained exposure to morphine and $Tat_{1–72}$ viral protein induces the preferential death of glial precursors and immature oligodendroglia. Moreover, the increased cell death is mediated by μ-opioid receptors and accompanied by the activation of caspase-3. Our results imply that opiates can enhance the cytotoxicity of HIV-1 Tat through direct actions on glial precursors and/or astroglia, suggesting novel cellular targets for HIV-opiate interactions.

Keywords

Human immunodeficiency virus; opioid receptors; striatum; drug abuse; morphine; glial precursors

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Introduction

Drug abuse contributes to the spread of the human immunodeficiency virus (HIV) pandemic (Petito et al., 1999). Besides needle sharing among injection drug users, or the exchange of sex for drugs, as modes for viral spread, the drugs themselves may intrinsically affect the pathogenesis of HIV (Nath et al., 2000; Gurwell et al., 2001; Turchan et al., 2001; Nath et al., 2002). Heroin, morphine, and other opiate drugs of abuse promote HIV infection and the progression to AIDS (Arora 1990; Peterson et al., 1991; Rouveix 1992; Peterson et al., 1993), and appear to accelerate the frequency and severity of HIV encephalitis (HIVE) (Bell et al., 1996; Bell et al., 1998). The enhanced pathogenesis is likely caused by both indirect (e.g., immune suppression) and direct neurotoxic mechanisms (Donahoe & Falek., 1988; Arora 1990; Peterson et al., 1991; Rouveix 1992; Peterson et al., 1993).

Drugs derived from the opium poppy (referred to as “opiates”) mimic endogenous opiate peptides (referred to as “opioids”) through preferential actions at μ-opioid receptors. Opioids modify immune function including HIV propagation in lymphocytes and monocytes/macrophages (Carr & Serou., 1995; Nair et al., 1997; Stoll-Keller et al., 1997; Peterson et al., 1998; Houghtling et al., 2000; Gekker et al., 2001; Suzuki et al., 2001; Suzuki et al., 2002a; Suzuki et al., 2002b; Rogers & Peterson., 2003). Morphine increases transforming growth factor-α production in human and murine microglia (Chao et al., 1994). Despite the prevalence of HIV infection among injection drug users and the importance of the opioid system in the pathogenesis of HIV, it is uncertain how opiate abuse augments the neuropathology of HIV (Nath et al., 2000; Nath et al., 2002).

Many brain regions that are preferentially disrupted by HIV are also enriched in μ-opioid receptors. The basal ganglia are vulnerable to HIV infection and are important targets for opiate abuse (Mansour et al., 1988; Masliah et al., 1992a; Glass et al., 1993; Mansour et al., 1994; Berger & Nath., 1997; Nestler & Aghajanian., 1997; Kreek & Koob., 1998; Nath et al., 2000; Bansal et al., 2000; Koob 2000). Morphine potentiates the toxic effects of HIV-derived proteins (Gurwell et al., 2001; Nath et al., 2002),which are intrinsically neurotoxic (Epstein & Gendelman., 1993; Masliah et al., 1996; Scorziello et al., 1998; Rappaport et al., 1999; Wesselingh & Thompson., 2001; Kaul et al., 2001; Nath 2002; Garden et al., 2002; Haughey & Mattson., 2002). However, because subpopulations of striatal glia (and glia from other brain regions) express μ-opioid receptors (Eriksson et al., 1991; Stiene-Martin et al., 1998; Gurwell et al., 2001), and opioids can modulate programmed cell death normally or following exposure to an apoptotic insult (Meriney et al., 1985; Singhal et al., 2002), we assessed opiates might modify the pathogenesis of HIV by affecting macroglial survival directly. Our results show that morphine acts synergistically with HIV-1 $Tat_{1–72}$ to increase the rate of death to glial precursors and their progeny, which may contribute to the accelerated neurodegenerative changes seen with HIV and opiate abuse.

Materials and methods

Cell culture medium consisted of Dulbecco’s Modified Eagle Medium (DMEM without phenol red) supplemented with antibiotic-antimycotic (100x), L-glutamine, and Dulbecco’s phosphate buffered saline (Gibco Life Technologies; Gaithersburg, MD, USA). Morphine

sulfate, β-funaltrexamine hydrochloride (β-FNA), naloxone hydrochloride, glucose, dimethyl sulfoxide, MgCl2, and HEPES were purchased from RBI/Sigma-Aldrich (St. Louis, MO, USA). Trypsin and DNAse were obtained from Worthington Biochemical Corporation (Lakewood, NJ, USA).

Cell Culture

Mixed glia were isolated from newborn or 1-day-old ICR mice (Charles River) as previously described (Stiene-Martin et al., 1998). All experiments conformed to the local Institutional Animal Care and Use Committee (IACUC) and national (PHS) guidelines on the care and ethical use of animals. All experiments were conducted to minimize the number of mice used and their suffering. Briefly, mice were anesthetized by gas anesthesia and euthanized by decapitation as previously described (Stiene-Martin et al., 1998). Striata were aseptically isolated and cells pooled from 2–3 striata. Growth medium favoring mixed glia consisted of DMEM supplemented with glucose (27 mM), Na_2HCO_3 (6 mM), and 10% (v/v) Fetal Bovine Serum (FBS; JRH Biosciences; Lenexa, KS, USA). Cells were plated at 100,000 cells/well in poly-L-lysine-coated 24-well plates (Costar, Corning Life Sciences; Acton, MA, USA). Mixed glial cultures contained differentiated astrocytes and oligodendrocytes, as well as undifferentiated glial precursors. In the present study, we define “glial precursors” as $nestin^+/A2B5^+/GD3^+$ and did not attempt to further distinguish among glial restricted precursor (GRP) types [e.g., GRP1 versus GRP2; see (Liu et al., 2002)], or attempt to distinguish among GRP1 and oligodendrocyte-type 2 astrocyte (O-2A) progenitors, which some investigators consider to be similar (Liu et al., 2002). Less than 0.4% microglia are present in striatal cultures (Stiene-Martin et al., 1998; Gurwell et al., 2001) (Hauser, unpublished). Cells were maintained for 6–10 days *in vitro* at 35–36°C in 5% CO_2/ 95% air at high humidity.

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, USA). Recombinant active $Tat_{1–72}$ was prepared as described previously (Ma & Nath., 1997) with minor modifications (Gurwell et al., 2001). Inactive Tat ($Tat_{\Delta 31–61}$) was generated from a deletion mutant of the active Tat plasmid, which lacked the sequence encoding the neurotoxic epitope (amino acids 31–61) of $Tat_{1–72}$ (Nath et al., 1996). $Tat_{1–72}$ 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.

Opioid Treatments

Cells were treated with medium alone (vehicle-treated controls), morphine sulfate (500 nM) (Sigma) with or without naloxone (1.5 μM) (a μ-, δ-, and κ-opioid receptor antagonist) or β-FNA (1.5 μM) (a selective μ-opioid receptor antagonists). Opioid drugs and Tat were prepared in basal cell culture medium before use; concentrations and treatment intervals varied as noted.

Viability

Mixed glial cultures were continuously exposed to opioids and/or HIV-1_{BRU} $Tat_{1–72}$ (or $Tat_{\Delta 31–61}$) for 24 or 96 h, while vehicle-treated cultures served as vehicle-treated controls. Following treatments, cultures were rinsed and incubated in Dulbecco's phosphate buffered saline (DPBS) containing ethidium homodimer (3.5 μM) and calcein-AM (4 μM) for 30 min at 35°C in 5% CO_2/95% air using the Live/Dead assay kit (Molecular Probes, Eugene, OR, USA) as previously described (Hauser et al., 2000). The proportion of dead/dying flat, polyhedral (type 1) astroglia, as well as undifferentiated glial precursors and their progeny, were sampled and determined as previously described (Hauser et al., 2000). As noted before, the identity of glial precursors was confirmed by $nestin^+$/$A2B5^+$/$GD3^+$ immunoreactivity. However, because the expression of a cell type specific marker might be attenuated or lost in dying cells, studies using viability assays and morphologic criteria (not relying on immunocytochemical markers criteria) were also performed. Approximately 400–600 glia were counted per experimental group. Independent determinations were made from 4–5 experiments and reported as the mean ± SEM.

To detect active caspase-3, phospho-specific rabbit anti-human/mouse caspase-3 antisera was obtained from R & D Systems, Inc (Minneapolis, MN, USA) and used at a concentration of 1:400. Anti-phosphospecific caspase-3 antibodies were detected using goat anti-rabbit conjugated to Cy3 (Jackson ImmunoResearch, West Grove, PA, USA; 1:300 dilution). Caspase-3 becomes activated in the cytoplasm before translocating into the nucleus during apoptosis. Because caspase-3 activation precedes apoptotic cell death, caspase-3 was assayed at 24 h following opioid and/or Tat exposure. Some cell cultures were pretreated with opioid antagonists (β-FNA and naloxone) for 15 min prior to morphine treatment. The proportion of undifferentiated glia displaying active caspase-3 positive in their nuclei versus non-labeled cells was determined using a Nikon Diaphot (Melville, NY, USA) fluorescent microscope with 100x–fluorecent objective.

To determine the phenotypic identity of the dying cells, cultures were incubated with 0.5 μg/ml ethidium monoazide (EMA; Molecular Probes) in DPBS at 36°C. EMA was photoaffinity linked (exposure at a 20 cm distance from a 60 W fluorescent lamp for 30 min) to the DNA of dying cells and combined with immunocytochemical detection of phenotypic determinants as described below.

Immunocytochemistry

Immature neural cells were detected using anti-nestin monoclonal IgG1 [1:1 dilution; Developmental Studies Hybridoma Band (DSHB), University of Iowa, Iowa City, USA], and mouse anti-GD3 monoclonal IgG_3 (1:50; National Cancer Institute BRB Preclinical Repository, Bethesda, MD, USA) (Goldman & Reynolds., 1996) antibodies. Mouse anti-A2B5 IgM (1:200, Chemicon) was used to detect oligodendrocyte-type 2 astrocyte bipotential (O-2A) progenitors (Raff et al., 1983; Raff et al., 1984; Gard & Pfeiffer., 1990). As mentioned, some investigators consider O-2A progenitors to be similar to oligodendrocyte precursors (Dietrich et al., 2002) or GRP 1 cells (Liu et al., 2002). Neurons were detected using anti-polysialyated neural cell adhesion molecule (E-NCAM) IgM (1:1; DSHB), mouse anti-neuronal nuclear (NeuN) monoclonal IgG1 (1:100; Chemicon,

Temecula, CA, USA), or rabbit anti-protein gene product (PGP) 9.5 antisera (1:1800 dilution; Chemicon). PGP 9.5 is a neuronal ubiquitin carboxyl-terminal hydrolase (Wilkinson et al., 1989), which may additionally label some non-neuronal neural crest derivatives and endocrine cells (Thompson et al., 1983). Astrocytes were detected using mouse anti-glial fibrillary acidic protein (GFAP) monoclonal antibodies (1:300, Boehringer Mannheim, Indianapolis, IN, USA) or rabbit anti-GFAP antisera (1:600 dilution; Chemicon). Oligodendrocytes were identified using anti-O4 rat IgM monoclonal antibodies [1:2 dilution from ascites fluid; gift from Dr. M. Schachner (Sommer & Schachner., 1981; Bansal et al., 1989)]. Immunodetection of μ-opioid receptors (μOR) was performed using rabbit anti-μOR1 (MOR1) (1:1000) affinity purified, polyclonal antisera (PharMingen; San Diego, CA, USA). In some instances, cells were counterstained with Hoechst 33342 dye (Sigma).

Primary antibodies were detected using appropriate species and/or immunoglobulin-specific secondary antibodies conjugated to fluorescent tags. Secondary anti-rabbit antibodies conjugated to CyDyes™ (Cy3 or Cy5, 1:250 dilution; Amersham Biosciences, Pittsburgh, PA, USA), or Alexa (Alexa 488 or 350; Molecular Probes) fluorochromes. Viability was measured in distinct cell types by combining cell-type-selective immunocytochemical markers with a functional assay for viability (EMA photoaffinity labeling).

Statistical Analysis

Effects of opioids and/or Tat on glial viability were assessed using ANOVA and followed by Duncan's *post hoc* test when significant ANOVA treatment effects were noted (Statistica 6.0, StatSoft, Tulsa, OK, USA). Concentration-dependent differences in Tat toxicity ± morphine were compared using Student's *t* test. Data are reported as the mean ± SEM of 4-6 experiments. Treatment effects were considered significant if $P < 0.05$.

Results

Effects of opiates & Tat_{1-72} on glial cell viability

Flat, polyhedral (type 1) astroglia were identified by distinct morphologic and immunologic characteristics ($GFAP^+/A2B5^-$). Type 1 astrocytes were readily discerned from, immature neural precursors, which are round and undifferentiated, smaller diameter, and morphologically distinct (arrows; Fig. 1A,B) (Table 1). μ-Opioid receptors are expressed by a subpopulation of type 1 astrocytes in the striatum (Stiene-Martin et al., 1998; Stiene-Martin et al., 2001), as well as $nestin^+/GFAP^-$ less differentiated glial precursors. Most of the small, undifferentiated neural precursors (91.0 ± 1.3%) expressed μ receptor immunoreactivity (Fig. 1C,D).

Astroglia

Fluorescently labeled living (calcein+) and dying (ethidium homodimer+) cells were characterized based on morphologic criteria as (i) type 1 astrocytes—flat and polyhedral with a single, large ovoid nuclei containing multiple nucleoli or as (ii) small, round, process-bearing (or without processes), and undifferentiated cells (Fig. 2). Further characterization of the glial subpopulations using EMA and cell-type-specific antigenic markers confirmed

that the calcein/ethidium homodimer labeled cells were GFAP-immunoreactive type 1 astrocytes (Table 2).

At 24 h following exposure, neither morphine (500 nM) nor $Tat_{1–72}$ (100 nM) alone or in combination induced significant increases in astroglial death compared to vehicle-treated controls (data not shown), confirming previous findings (Gurwell et al., 2001).

Unlike at 24 h, significant increases in type 1 astrocyte death were apparent in a small proportion of astrocytes following combined morphine (500 nM) and $Tat_{1–72}$ (100 nM) exposure at 96 h (Fig. 2A–C). Morphine and $Tat_{1–72}$ in combination showed significantly greater toxicity compared to morphine ($P < 0.01$) or Tat alone ($P < 0.025$). Neither morphine nor Tat alone was toxic compared to vehicle-treated controls. The interactive toxicity was significantly attenuated by naloxone (1.5 μM) ($P < 0.0025$, β-FNA (1.5 μM) ($P < 0.025$), or when morphine was coadministered with a non-toxic deletion mutant of Tat ($Tat_{\Delta 31–61}$) ($P < 0.005$). Although there were significant increases in the death of type 1 astrocytes, it is noteworthy that the dying cells only represent a small proportion of the total astrocyte population (Fig. 2). Only 1.2 ± 0.3% of cells with type 1 astroglial morphology normally die in vehicle-treated cultures. Therefore, a vast majority of type 1 astrocytes (∼95%) survived irrespective of morphine-$Tat_{1–72}$ treatment and displayed no cytological evidence of impending death such as plasmalemmal blebbing, nonuniform calcein-AM distribution, and cytoplasmic vacuoles. Although morphine alone failed to increase astroglial death, morphine appeared to induce cellular hypertrophy as previously described (Stiene-Martin et al., 1991; Hauser et al., 1996). Moreover, similar types of changes were observed in astrocytes with combined morphine and $Tat_{1–72}$ exposure, although cellular hypertrophy was not measured directly.

Glial precursors/Immature glia

At 24 h, few small, undifferentiated glia were observed in mixed-glial cultures derived from the striatum. At this time, few dying cells were evident using viability assays in vehicle-control or experimental treatment groups.

By contrast, at 96 h, greater numbers of undifferentiated glia were present suggesting the rapid growth and proliferation of glial precursors during the first few days in vitro. Using the ethidium homodimer and calcein viability assay, significant increases in the death of small, undifferentiated glia were readily seen following combined morphine (500 nM) and $Tat_{1–72}$ (100 nM) exposure ($P < 0.001$ vs. vehicle-treated controls), that were not apparent with morphine or Tat treatment alone (Fig. 2D). To further explore the interactive toxicity between morphine and Tat, mixed glial were exposed to a saturating concentration of morphine (500 nM), and increasing concentrations of Tat (Fig. 3). In the presence of morphine, Tat caused significant, concentration-dependent losses in glial precursors at 1 nM ($P < 0.025$), 10 nM ($P < 0.005$) or 100 nM ($P < 0.001$) concentrations, but not at a 0.1 nM concentration (Fig. 3). Cytotoxicity was not evident with 10 nM or 100 nM Tat alone (Fig. 3). To better identify the type(s) of cells that were dying following combined morphine and $Tat_{1–72}$ exposure, cells were photoaffinity labeled with EMA and assessed immunocytochemically. The findings showed that many of the cells displaying significant increases in EMA^+-labeling were dying glial precursors based on morphologic (small

diameter, undifferentiated cells) and immunologic (e.g., the cells did not express GFAP) criteria (see Table 2).

Although large numbers of dying undifferentiated glia were not seen at 24 h using cell viability assays (ethidium homodimer or EMA), significant increases in caspase-3 activation were noted at 24 h in combined morphine- (500 nM) and $Tat_{1–72}$- (100 nM) treated cultures compared to cultures treated with vehicle ($P < 0.01$), morphine ($P < 0.001$), or $Tat_{1–72}$ ($P < 0.05$) alone (Fig. 4). In the vehicle-treated controls, 15.4 ± 1.6 of the undifferentiated glia were caspase-3 immunopositive. Interestingly, morphine exposure alone caused significant reductions in caspase-3 activation compared to vehicle-treated controls suggesting that morphine is anti-apoptotic ($P < 0.005$) (Fig. 4). In a few cases, cells expressing activated caspase-3 were EMA^+ (Fig. 4A–C); however, most caspase-3-positive cells did not yet display EMA suggesting that caspase-3 activation precedes cell death by a significant amount of time, which has been observed in striatal neurons (Singh et al., 2003).

Further examination indicated that a majority of the undifferentiated cells exhibited an antigenic profile indicative of GRPs or O-2A cells, which included immunoreactivity for nestin (55.7 ± 2.9%), GD3 (81.7 ± 3.3%), and A2B5 (65.1 ± 1.8%), but failed to display immunoreactivity for neuronal (NeuN or E-NCAM), oligodendroglial (O4), or astroglial (GFAP) phenotypic markers (Fig. 5). A subset of undifferentiated cells (1.2 ± 0.3%) expressed PGP 9.5, which labels neurons, as well as some non-neuronal neural crest-derivatives and endocrine cells (Thompson et al., 1983; Wilkinson et al., 1989). However, because NeuN and E-NCAM immunoreactivity were not evident, and the PGP 9.5-labeled cells did not have a neuronal morphology, suggested that PGP 9.5 was not labeling neurons. A smaller proportion of the dying cells displayed O4 immunoreactivity, but also expressed immature markers (e.g., nestin, A2B5, and GD3), suggesting they were immature oligodendria (Fig. 5). Although there was a trend toward increased death of O4-immunoreactive oligodendrocytes following combined morphine and $Tat_{1–72}$ exposure compared to vehicle-treated controls, the effect was not significant (Table 2). The proportion of ethidium $homodimer^+$, undifferentiated glia (12.4 ± 1.6%; Fig. 2D) or $EMA^+/GFAP^-$ undifferentiated glia (11.6 ± 1.9; Table 2) in vehicle-treated control cultures was similar. Some cell death in vehicle-treated control cultures was not surprising since some glial precursors die during normal development (Raff et al., 1993).

Each glial subtype responded differently to combined morphine and $Tat_{1–72}$ exposure (Table 2; Fig. 6). This includes glial precursors, as well as immature type 1 astrocytes (Knapp et al., 1998) Khurdayan and Hauser, unpublished). By contrast, increased rates of oligodendrocyte and type 2 astrocyte deaths were not seen in the present study (Table 2). It was anticipated that type 2 astrocytes would not be responsive to morphine or morphine plus $Tat_{1–72}$, because they do not express opioid receptors or respond functionally to opioids (Hauser & Stiene-Martin., 1991) (Hauser, unpublished observations). By contrast, immature oligodendrocytes express μ-opioid receptors and activating opioid receptor can affect oligodendrocyte cell death (Knapp et al., 2001). However, despite expressing μ-opioid receptors, morphine did not alter $Tat_{1–72}$ toxicity in $O4^+/nestin^-$ oligodendrocytes, although as noted, concurrent morphine and $Tat_{1–72}$ is cytotoxic to oligodendrocyte precursors.

Discussion

Our findings indicate that opiates modify the effects of $Tat_{1–72}$ in astroglia and glial precursors, and suggest that the interaction is mediated through opioid receptors. Moreover, the ability to attenuate morphine's effects significantly using the selective antagonist, β-FNA, suggests that opiate-HIV $Tat_{1–72}$ glial interactions are mediated by μ-opioid receptors in particular. 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 astrocytes, oligodendrocytes, and glial precursors (Stiene-Martin et al., 1998; Knapp et al., 1998; Stiene-Martin et al., 2001; Persson et al., 2003a; Persson et al., 2003b) (Khurdayan, Buch, and Hauser, unpublished). An interesting observation was that the cytotoxic effects of morphine and $Tat_{1–72}$ differed depending on the particular type of glial cell involved. Although our initial goal was to characterize the effects of opioids and $Tat_{1–72}$ on type 1 astrocytes, as the studies progressed, it became apparent that in combination morphine and $Tat_{1–72}$ were toxic to glial precursors/immature glia. For this reason, viability was assessed within several glial types.

HIV is a multisystem disease that acts through multiple toxic events and signaling cascades. HIVE is characterized by astrocytosis, 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; Petito et al., 1999). Astroglial apoptosis is evident in the CNS of individuals with HIVE (Petito & Roberts., 1995; Thompson et al., 2001), and the severity of HIV dementia correlates positively with astrocytic cell death (Thompson et al., 2001).

Tat is a transactivating, nonstructural viral regulatory protein encoded by two exons (Atwood et al., 1993). Several alternatively processed forms of Tat are released by infected lymphoid and glial cells including Tat formed from the first ($Tat_{1–72}$) and second ($Tat_{1–86}$, $Tat_{1–101}$) exons (Malim & Cullen., 1991; Tardieu et al., 1992; Atwood et al., 1993; Ensoli et al., 1993; Ma & Nath., 1997; Chang et al., 1997). All forms of Tat are present extracellularly in the CNS of infected individuals (Hudson et al., 2000) and all are neurotoxic (Sabatier et al., 1991; Nath & Geiger., 1998; Jones et al., 1998; Nath 1999; Nath 2002). Potential molecular targets of Tat include CXCR4 (Marechal et al., 1999; Xiao et al., 2000; Ghezzi et al., 2000), αv integrin subunit-containing receptors (Etienne-Manneville & Hall., 2001; Milner et al., 2001), vascular endothelial growth factor-1 receptor (VEGF-1 receptor or flt-1) (Krum & Rosenstein., 1998), and low density lipoprotein receptors (Liu et al., 2000).

Statistically significant increases in astroglial toxicity were apparent at 96 h, although the total proportion of dying astrocytes was relatively small (about 5%) compared to those astrocytes not displaying cytopathology. In combination, morphine and $Tat_{1–72}$ can synergistically alter intracellular Ca^{2+} homeostasis, reactive oxygen species, and cytokine production in astrocytes at 1–4 h following exposure (Gurwell, El-Hage and Hauser, unpublished observations). Whether this contributes to moderate increases in astrocyte death at 96 h is uncertain and is being investigated; however, the disruptions in astroglial function are probably unrelated to astrocyte cell death. The reasons for this include: (i) an inexplicably long delay (at least 72 h) between dysfunction and death; (ii) the proportion of

dying astrocytes is relatively low (∼5%) compared to viable cells; and (iii) astrocytes can adapt to disruptions in ion homeostasis caused by opioids (Hauser et al., 1998).

Synergistic death of glial precursors

Few studies have characterized mixed glial cultures derived from the neonatal striatum. Because the subventricular zone comprises a considerable proportion of the striatum of newborn or P1 mice, it was anticipated that a disproportionately large number of cells in our cultures would include glia precursors. Interestingly, our results show that about 40% of the cells in the striatal cultures are undifferentiated glia/glial precursors (Table 1), which may exceed the proportion of immature glia in neonatal cortical or whole forebrain cultures.

Glial cell death was accompanied by chromatin condensation and nuclear shrinkage (pyknosis), and/or cellular fragmentation characteristic of apoptosis. Tat has been shown to activate apoptotic cascades involving caspase-3 and/or Par-4 in primary neurons in culture (Kruman et al., 1998; Kruman et al., 1999; Singh et al., 2004). The present findings suggest that morphine and $Tat_{1–72}$ in combination kill glial precursors through a caspase-3-dependent apoptotic mechanism similar to neurons, although additional studies are needed to confirm this. Morphine alone significantly reduced the proportion of active-caspase-3-immunoreactive immature glia, although morphine exposure did not result in enhanced survival as assessed by EMA at 96 h. The reason why morphine reduced the proportion of active caspase-3 positive immature glia is uncertain. Morphine can prevent apoptosis (Meriney et al., 1985) and this may involve the activation of PI-3-kinase and Akt (Polakiewicz et al., 1998; Persson et al., 2003a), which has been proposed as a pathway mediating neuroprotection downstream of the μ opioid receptor (Polakiewicz et al., 1998). Studies in progress are addressing how morphine modulates apoptosis.

By co-localizing cell type-specific markers in EMA-photoaffinity-labeled cells, we found a majority of the dying cells were glial precursors having characteristics of O-2A glial progenitors [$A2B5^+$, $GFAP^-$ and $O4^-$ (Raff et al., 1983; Fulton et al., 1992)] or GRP1s [$A2B5^+$/$nestin^+$/$GD3^+$ (Liu et al., 2002)], while some immature oligodendrocytes ($A2B5^+$/$O4^+$) and immature astrocytes (nestin+/GFAP+) were also preferentially lost. Since our cell culture conditions encourage glial precursors to develop into oligodendroglia and many precursors would have become oligodendroglia with further maturation, we speculate that cells committed to an oligodendroglial fate may be preferentially vulnerable to combined opiate-HIV-1 toxicity. Interestingly, there was a trend toward greater numbers of dying oligodendrocytes with combined morphine-$Tat_{1–72}$ exposure compared to vehicle-treated cells (Table 2), although oligodendrocyte losses were not statistically significant at 96 h. As mentioned elsewhere, since morphine and Tat in combination are killing O-2A cells, significant oligodendrocyte losses might become apparent as precursor pools become depleted with more prolonged exposure (Fig. 6). Also, as noted earlier, the expression of some cell type specific markers might be selectively lost or attenuated in dying cells and this should be considered when interpreting the results. A more detailed assessment of the effects of opioids-HIV in oligodendrocytes at different stages of development using multiple immunological markers for oligodendrocyte in addition to O4 might reveal particular stages during development when oligodendria are especially vulnerable to opioids and HIV.

Assuming glial precursors and immature oligodendria are affected *in vivo* similarly, chronic opiate abuse is likely to have devastating consequence on the long-term maintenance of glial populations and CNS function.

White matter pathology has been reported with heroin use (Rizzuto et al., 1997; Buttner et al., 2000; Zheng & Zhang., 2001; Barnett et al., 2001). Immature oligodendria express μ-opioid receptors when they are proliferating (Knapp et al., 1998; Stiene-Martin et al., 2001), but levels of μ- receptor expression decrease with oligodendrocyte differentiation (Tryoen-Toth et al., 2000). Oligodendrocytes do not express δ-opioid receptors *in vitro*, while κ-opioid receptors are expressed by more mature, non-dividing cells (Knapp et al., 1998). This infers that opiate drugs, which preferentially activate μ-opioid receptors, are more likely to affect immature oligodendrocytes.

The effects of HIV on oligodendria have been less well characterized than astrocytes. Oligodendrocytes can reportedly become infected (An et al., 1999) and may subsequently undergo degenerative and/or apoptotic changes (Schmidbauer et al., 1992; Cosenza et al., 2002). HIV-1 encodes two sequences with considerable homology to the response element of the hnRNP A2-RNA trafficking factor present in oligodendrocytes (Mouland et al., 2001). Although white mater pallor is seen frequently in HIV infected patients, it is commonly thought to be secondary to the breakdown of the blood brain barrier (Petito & Cash., 1992; Power et al., 1993; Buttner et al., 1996).

Depending on dosage, opioids can have opposing actions through the same receptor type (Crain & Shen., 1990; Gintzler & Xu., 1991). Similarly, as a function of dose, μ-opioid agonists can prevent or exacerbate cell death (Meriney et al., 1985; Singhal et al., 1997; Singhal et al., 1998; Polakiewicz et al., 1998). Opioid drug abusers reportedly tolerate opioid blood levels that are several to 100-fold greater than those seen therapeutically (Gurwell et al., 2001). Restated, the morphine concentrations and pharmacodynamic parameters used to exacerbate $Tat_{1–72}$ toxicity in the present study are likely to be achieved with chronic drug abuse, but are unlikely to be realized with therapeutic dosages. Unlike drug abuse, therapeutic dosages of opiates are likely to parallel the actions of endogenous opioid peptides whose role in the pathogenesis of neuro-AIDS is not understood.

Our findings indicate that opiate drug abuse can exacerbate the pathogenesis of HIV through independent actions in separate glial subpopulations *in vitro*. Although our in vitro studies represent an important first step in identifying potential targets of opiate-HIV interactions in the CNS, additional studies need to be performed before the present findings can be generalized to the adult brain. The consequences of destroying glial precursors are uncertain. Fated glial precursors may possess life spans lasting years, suggesting that deficits in gliogenesis would take time to become evident. A speculative notion is that the progressive destruction of glial precursors and ensuing predicted loss in total glial numbers might contribute to the accelerated neurocognitive defects seen in chronic opiate abusing-HIV infected individuals. Interestingly, disruptions in neurogenesis have been implicated in the etiology of Alzheimer's disease (Haughey et al., 2002), traumatic brain injury (Braun et al., 2002; Chirumamilla et al., 2002), seizure disorders (Ribak & Dashtipour., 2002; Parent & Lowenstein., 2002), and neuropsychiatric disorders (Jacobs et al., 2000; Kempermann 2002;

McEwen 2002), and may accompany opiate exposure (Dodge Miller et al., 1982; Eisch et al., 2000; Stiene-Martin et al., 2001; Duman et al., 2001).

Acknowledgements

This work was supported by NIH DA13559. Monoclonal antibodies developed by Drs. Thomas M. Jessell and Jane Dodd (E-NCAM), and Dr. Susan Hokfield (nestin), were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHHD and maintained by the University of Iowa, Department of Biological Sciences. GD3 monoclonal IgG3 were obtained from the National Cancer Institute BRB Preclinical Repository, Bethesda, MD). We thank Mr. Kenneth Martin for valued technical assistance and Dr. Robin J. Goody for helpful discussions.

Abbreviations

Akt	protein kinase B
β-FNA	β-funaltrexamine hydrochloride
DPBS	Dulbecco's phosphate buffered saline
E-NCAM	polysialyated neural cell adhesion molecule
GFAP	glial fibrillary acidic protein
GRP	glial restricted precursor
HIV	Human Immunodeficiency Virus
μOR	μ-opioid receptor
Mor	morphine
Nal	naloxone
NeuN	neuronal nuclear marker
O-2A	oligodendrocyte-type 2 astrocyte precursor
PI3-kinase	phosphoinositide-3 kinase

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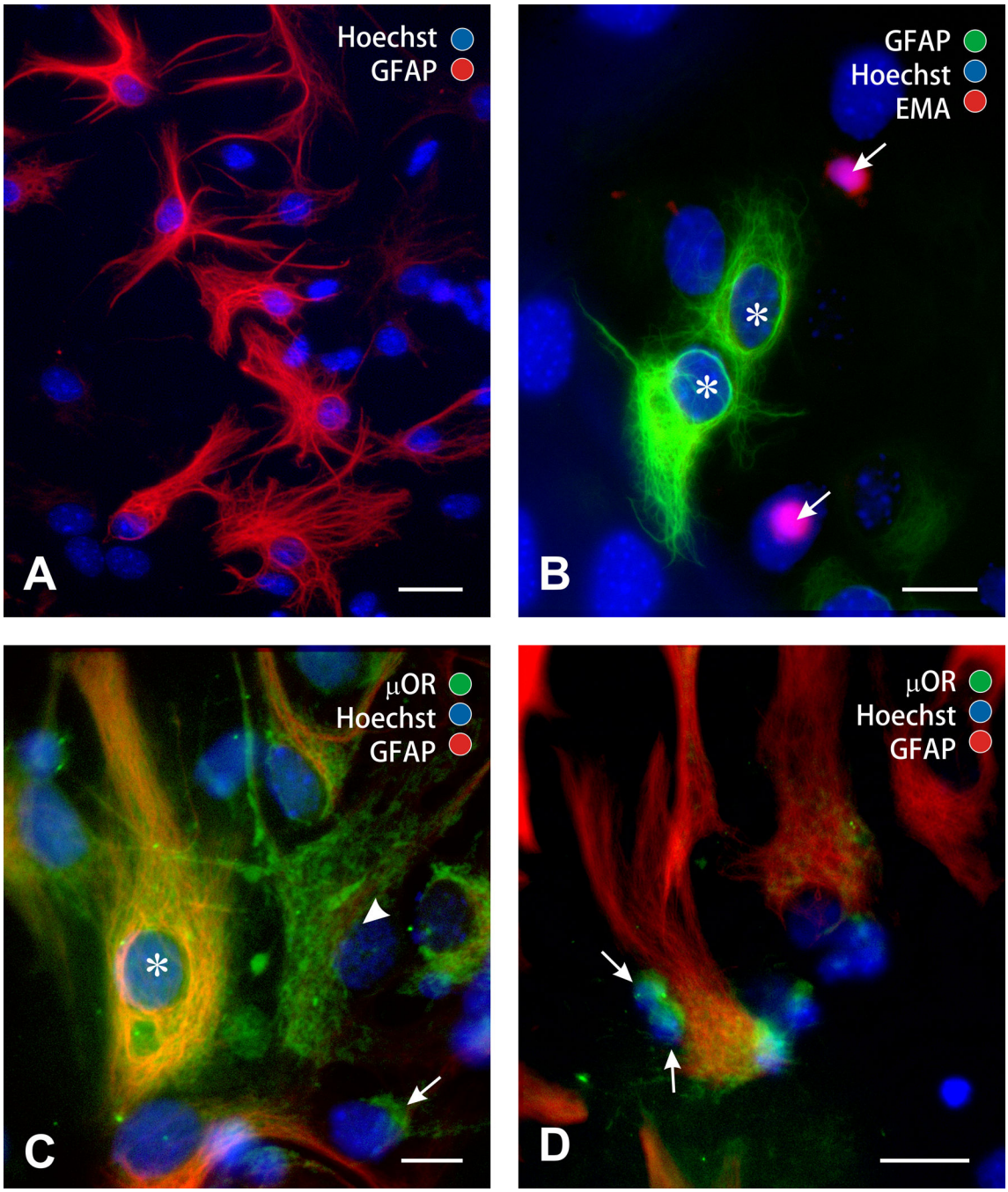

Figure 1.

Phenotypic characterization of neural cell populations in mixed-glial cultures. Mixed- glial cultures contained large numbers of flat, polyhedral (type 1) astrocytes that were glial fibrillary acidic protein ($GFAP^+$) immunoreactive (A); scale bar = 25 μm. Besides type 1 astrocytes (asterisks), mixed glial cultures also contained large numbers of undifferentiated glia/glial precursors (arrows) (B). The undifferentiated variants were round, small-diameter, and morphologically distinct lacking mature cytoplasmic processes (arrows) from type 1 astrocytes (asterisks); scale bar 15 = μm (B). μ-Opioid receptors (μOR) are expressed by

subpopulations of type 1 astrocytes ($\mu OR^+/GFAP^+$; asterisk), as well as less (arrow) and partially (arrowhead) differentiated flat, polyhedral cells presumed to be type 1 astrocyte precursors ($\mu OR^+/GFAP^-$) (C). Small, undifferentiated glial precursors almost uniformly expressed μ-opioid receptors (arrows) (D); scale bar = 20 μm.

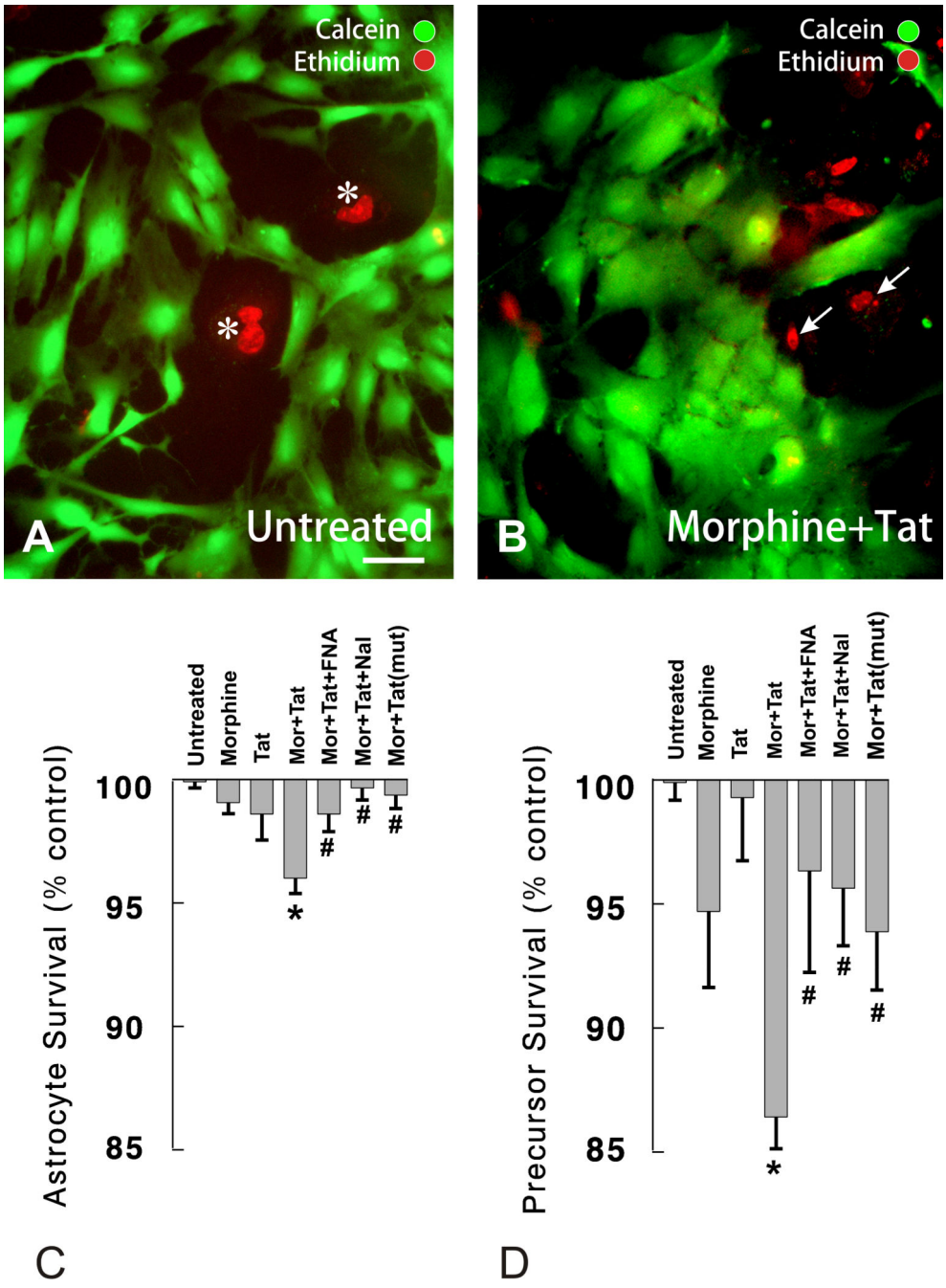

Figure 2.

Effects of morphine and/or $Tat_{1–72}$ on cell survival at 96 h was assessed in mixed-glial cultures by fluorescently labeling living (calcein^{+}) and dying (ethidium homodimer^{+}) cells. Cells were characterized morphologically as flat and polyhedral with single, large ovoid nuclei containing multiple nucleoli [morphologically identical to (type 1) astrocytes] (A) or as small, round or process-bearing, and undifferentiated cells. Combined morphine (Mor) (500 nM) and $Tat_{1–72}$ (Tat) (100 nM) exposure, increased the proportion of dying type 1 astrocytes (asterisks in A; C), as well as the proportion of dying small-diameter,

undifferentiated glia/glial precursors (arrows in B; D). Combined morphine and $Tat_{1–72}$ toxicity was significantly attenuated by coadministering naloxone (Nal; 1.5 μM) or β-funaltrexamine (β-FNA; 1.5 μM) (C, D), or by substituting $Tat_{\Delta 31–61}$ [Tat (mut)] instead of $Tat_{1–72}$ (Tat) (C,D); $^{*}P < 0.05$ vs. vehicle-treated cultures, or cultures treated with morphine or $Tat_{1–72}$ alone; $^{\#}P < 0.05$ vs. morphine + $Tat_{1–72}$-treated cultures; A, B; same magnification; scale bar = 15 μm.

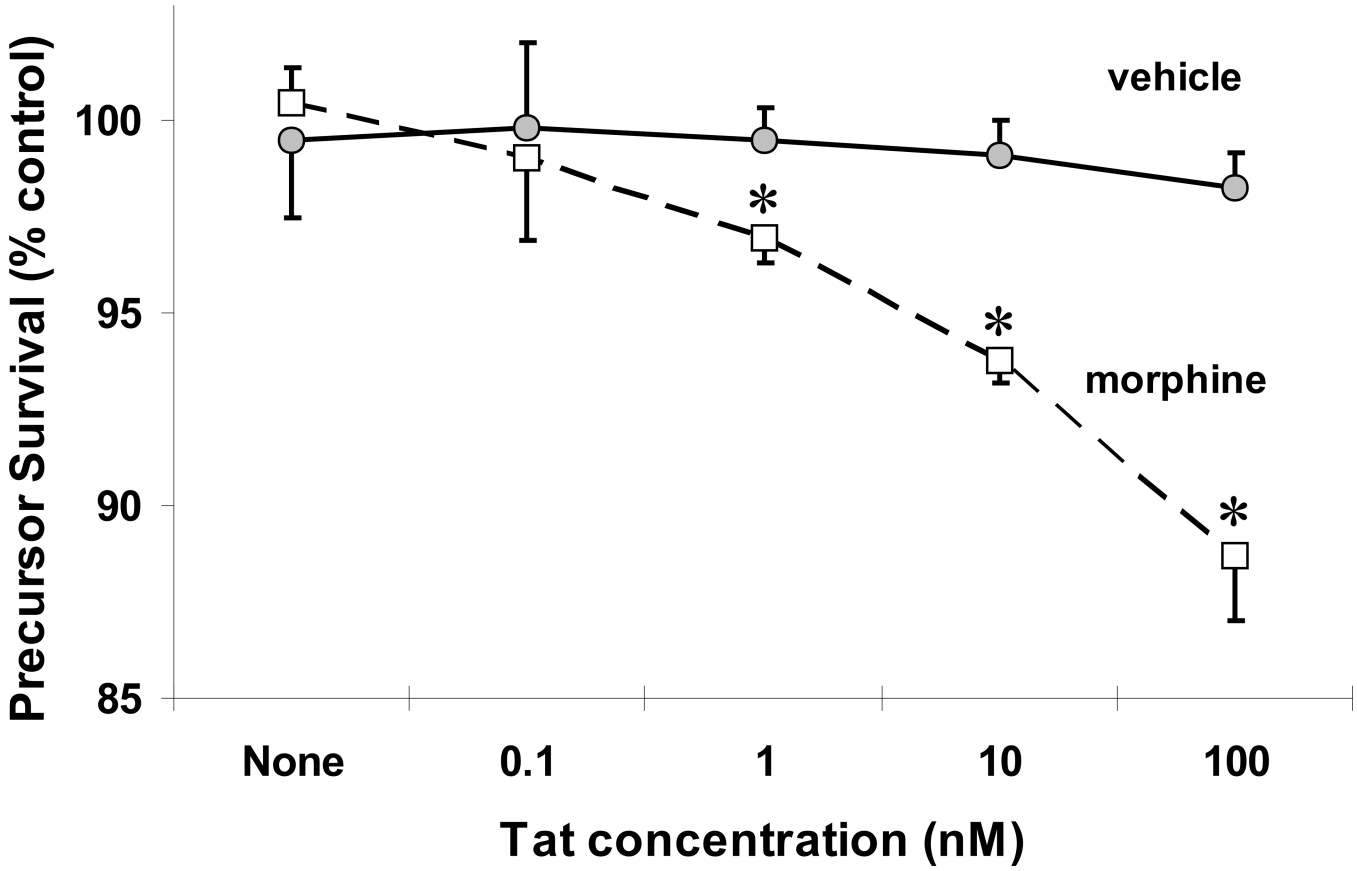

Figure 3.

Effect of $Tat_{1–72}$ concentration on the survival of glial precursors in the presence and absence of morphine. Mixed glia were exposed to increasing concentrations of Tat with or without morphine (500 nM) and the viability of undifferentiated glia was assessed in $calcein^{+}$ and ethidium $homodimer^{+}$-labeled cells. In the presence of morphine, Tat caused significant, concentration-dependent losses in glial precursors at 1 nM, 10 nM or 100 nM concentrations (*$P < 0.025$ vs. treatment without morphine), but not at 0.1 nM. Cytotoxicity was not evident with when cells were exposed to Tat alone at 0.1 to 100 nM concentrations.

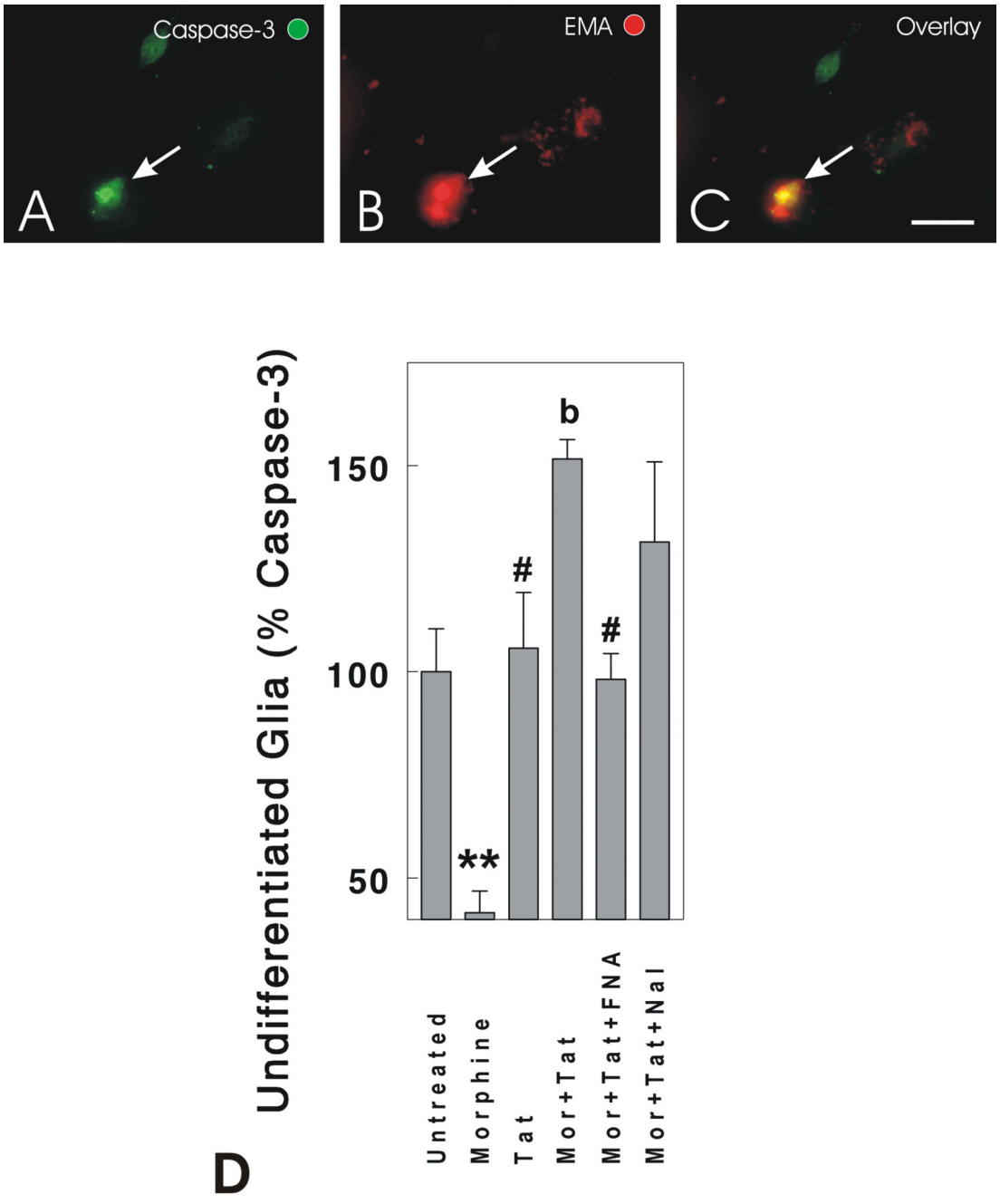

Figure 4.

Effects of opioids and/or $Tat_{1–72}$ on caspase-3 activation in undifferentiated glia at 24 h following exposure. Phospho-specific antibodies were used to detect activated caspase-3 in the nuclei of dying cells using fluorescence microscopy (A,C). Caspase-3 activation preceded cell death, although a subset of ethidium monoazide positive cells (EMA) also possessed activated caspase-3 (arrow shows the same dying cell in A-C). Morphine (Mor) caused a significant reduction in the proportion of undifferentiated glia possessing active caspase-3, while significant increases in caspase-3 activation and translocation into cell

nuclei were seen when morphine and Tat were combined (D). The combined effects of morphine + Tat were blocked by 15 min preexposure to the non-competitive, selective μ-opioid receptor antagonist β-FNA, but not by the competitive μ, δ, and κ-opioid receptor antagonist naloxone (Nal) (D); $^{**}P < 0.01$ vs. other treatment groups; $^{\#}P < 0.05$ vs. morphine + $Tat_{1–72}$-treated cultures; $^{b}P < 0.05$ vs. other treatment groups—except Mor+Tat +Nal; scale bar = 10 μm).

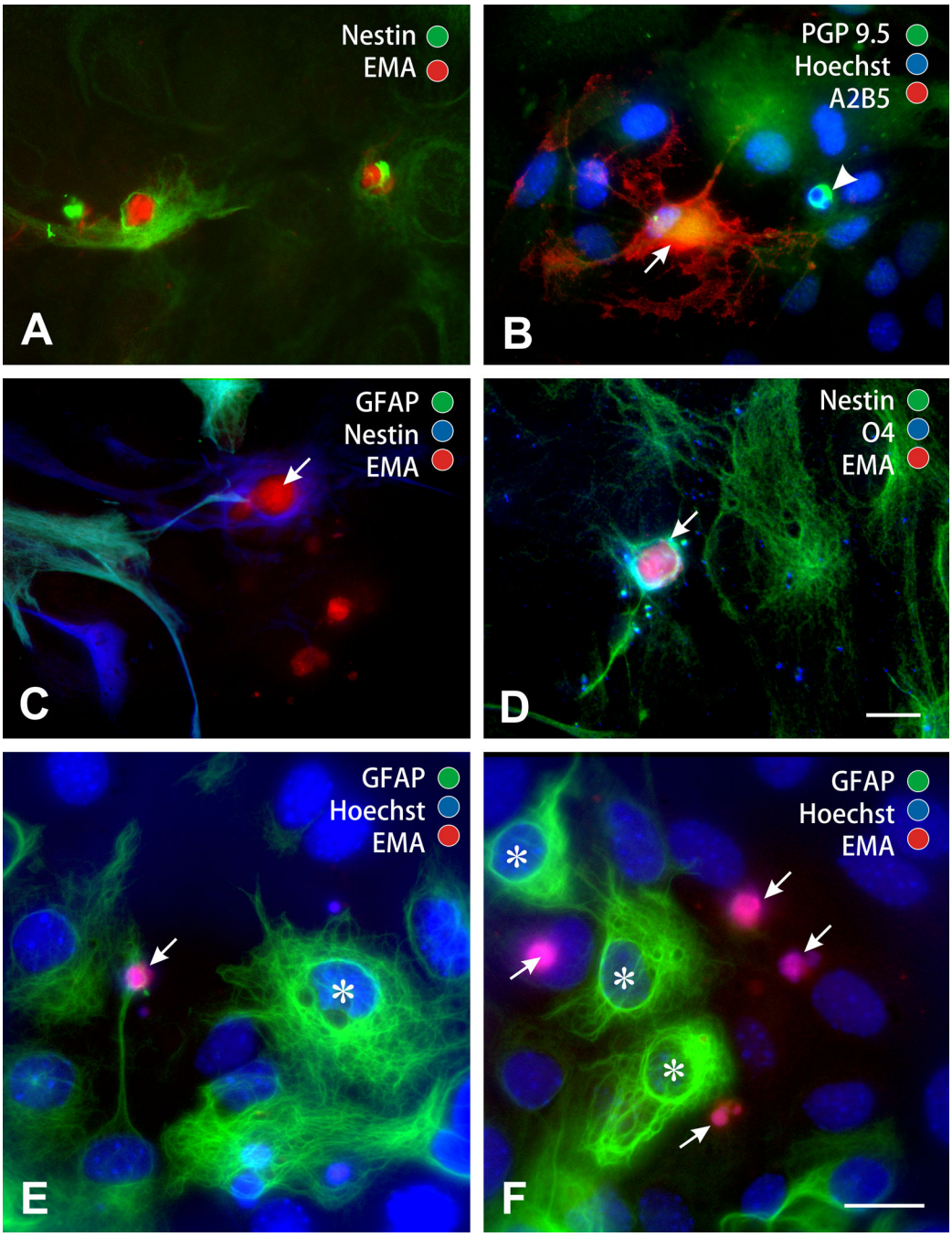

Figure 5.

Identification of dying cells in morphine and HIV-1 $Tat_{1–72}$ exposed mixed glial cultures at 96 h. Dead cells were photoaffinity labeled with ethidium monoazide (EMA^+) in combination with cell-type selective immunocytochemical markers (A–F). Most dying cells were undifferentiated or partially differentiated glia (e.g., arrows in F), which expressed immature (e.g., nestin) but not mature phenotypic markers (e.g., GFAP or O4). Dying cells included: immature neural cells ($nestin^+/EMA^+$) (A); immature glia ($A2B5^+/PGP\ 9.5^-/EMA^+$) with oligodendroglial morphology (arrow) (B); small numbers of immature neural

cells were morphologically similar to type 1 astrocytes but lacked GFAP (arrow) (C); immature oligodendroglia (nestin$^+$/O4$^+$/EMA$^+$) (arrow) (D); astroglia (GFAP$^+$) with process-bearing (type 2) morphology (GFAP$^+$/EMA$^+$; arrow) (E) (asterisk; a type 1 astrocyte for comparison) (E); and astroglia (GFAP$^+$) with flat, polyhedral (type 1) morphology (asterisks) (F). Neurons (arrowhead) were absent or rarely present (B). The scale bar in D = 10 μm (A-D, same magnification); the scale bar in f = 10 μm (E-F, same magnification).

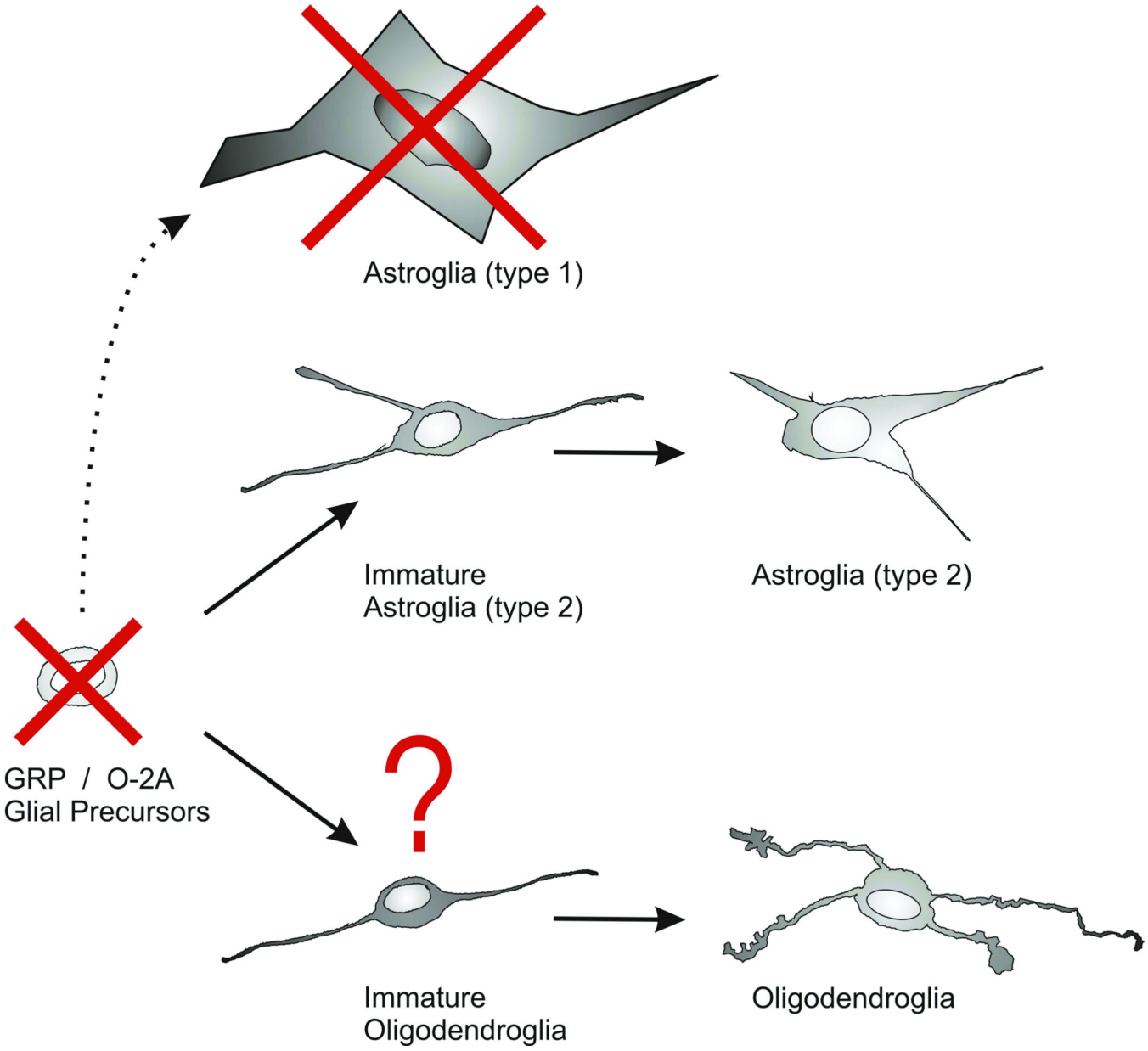

Figure 6.

Summary of the immature glial types that are preferentially vulnerable to combined morphine plus $Tat_{1–72}$ exposure. Glial precursors [glial restricted precursors (GRPs) or oligodendrocyte-type 2 astrocyte (O-2A) progenitors] are preferentially destroyed by combined morphine and Tat exposure (red X). Type 1 astrocytes (dashed arrow) also showed significant losses (red X), while the viability of oligodendroglia and type 2 astrocytes was unaffected by combined morphine-$Tat_{1–72}$ exposure. It is noteworthy that most GRPs in our cultures will develop into oligodendroglia and there was a trend toward a reduction in the number of immature oligodendrocytes (red ?).

Table 1

Characterization of the cell types present within striatal mixed glial cultures.

Cell Type	Percentage of the Total Cells (%)
Astrocytes	
Type 1 (GFAP$^+$ / A2B5$^-$; large diameter, flat polyhedral)	61.0 ± 3.1
Type 2 (GFAP$^+$ / A2B5$^+$; small diameter, process-bearing)	0.4 ± 0.1
Glial precursors/Immature oligodendroglia* (non-GFAP) (A2B5$^+$ / Nestin$^+$ / GD3$^+$)	38.7 ± 3.0
Neurons (NeuN$^+$ / E-NCAM$^+$) (GFAP$^-$)	ND

* Glial precursor/immature oligodendroglia expressed immature markers (A2B5, nestin, and GD3) and were morphologically undifferentiated (small diameter, round, and lacking cytoplasmic processes). Mean ±SEM from 4 experiments.

ND = Not Detected; NeuN$^+$ or E-NCAM$^+$ cells were not detected

Table 2

The cytotoxic effects of concurrent morphine and $Tat_{1–72}$ exposure varied among different glial types. Combined morphine and $Tat_{1–72}$ treatment increased the proportion of ethidium monoazide-labeled (EMA^+) dying glial precursors and flat, polyhedral (type 1) astrocytes.

Cell Type	Vehicle-treated controls (% dying)[b]	Combined morphine-$Tat_{1–72}$ exposed (% dying)[b]
Undifferentiated Neural Cells (non-GFAP) (small, morphologically undifferentiated)	11.6 ± 1.9	24.0 ± 3.5*
Immature Oligodendrocytes ($O4^+$)	14.9 ± 1.4	20.7 ± 4.7
Astrocytes ($GFAP^+$)		
Type 1 (flat, polyhedral)	1.4 ± 0.6	6.2 ± 0.8*
Type 2 (process-bearing)	6.3 ± 3.2	8.8 ± 6.8

*$P < 0.05$ versus vehicle-treated controls (Student's t test).

[b]Values are the number of dying cells as a percentage of the total cells of a particular phenotype in striatal mixed glial cultures. For example, % dying oligodendrocytes = [(EMA^+ oligodendrocytes/total oligodendrocytes) x 100] and are presented as the mean ± SEM from 4 experiments.