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Synergistic Neurotoxicity of Opioids and Human Immunodeficiency Virus-1 Tat Protein in Striatal Neurons *In Vitro*

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Abstract

Human immunodeficiency virus (HIV) infection selectively targets the striatum, a region rich in opioid receptor-expressing neural cells, resulting in gliosis and neuronal losses. Opioids can be neuroprotective or can promote neurodegeneration. To determine whether opioids modify the response of neurons to human immunodeficiency virus type 1 (HIV-1) Tat protein-induced neurotoxicity, neural cell cultures from mouse striatum were initially characterized for μ and/or κ opioid receptor immunoreactivity. These cultures were continuously treated with morphine, the opioid antagonist naloxone, and/or HIV-1 Tat (1-72) protein, a non-neurotoxic HIV-1 Tat deletion mutant (TatΔ31-61) protein, or immunoneutralized HIV-1 Tat (1-72) protein. Neuronal and astrocyte viability was examined by ethidium monoazide exclusion, and by apoptotic changes in nuclear heterochromatin using Hoechst 33342. Morphine (10 nM, 100 nM or 1 μM) significantly increased Tat-induced (100 or 200 nM) neuronal losses by about two-fold at 24 h following exposure. The synergistic effects of morphine and Tat were prevented by naloxone (3 μM), indicating the involvement of opioid receptors. Furthermore, morphine was not toxic when combined with mutant Tat or immunoneutralized Tat. Neuronal losses were accompanied by chromatin condensation and pyknosis. Astrocyte viability was unaffected.

These findings demonstrate that acute opioid exposure can exacerbate the neurodegenerative effect of HIV-1 Tat protein in striatal neurons, and infer a means by which opioids may hasten the progression of HIV-associated dementia.

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Keywords

morphine; heroin; drug abuse; acquired immunodeficiency syndrome (AIDS); basal ganglia; apoptosis

The human immunodeficiency virus (HIV) epidemic in North America and Western Europe is in part being driven by drug abuse.[52] However, very little is known about the potential interaction of these drugs with HIV infection in the genesis of cerebral dysfunction. The observation of an increased frequency and severity of HIV encephalitis in an opiate abusing cohort suggests a relationship between HIV and drug abuse.[7]

HIV preferentially targets the basal ganglia.[10,56] Interestingly, the basal ganglia also express high levels of opioid receptors.[3,46,76] Based on this and other evidence, we recently proposed that drugs of abuse potentiate the effects of HIV by direct actions on neurons and glia.[52] Despite the impairment in neuronal function and neuronal losses, the virus itself rarely targets neurons.[5,36,53] Instead, HIV infects non-neuronal intermediates causing a productive infection in microglia and infiltrating monocytes. Recent evidence suggests that products released from HIV infected cells are neurotoxic (reviews[20,36,40,53]). These products can be broadly classified into two groups. “Virotoxins” are encoded by the viral genome, while “cellular toxins” are encoded by the host genome.[53] Most evidence suggests that the virotoxins can induce the expression of cellular toxins initiating a spiraling cascade of events.[53] Of the virotoxins produced, Tat is highly neurotoxic,[23,39,44,58] secreted extracellularly from infected cells,[13] and can be detected in the brains of patients with HIV encephalitis.[29,31,38]

Although there is a correlation between opioid drug abuse and accelerated HIV-induced damage to the brain,[8] the nature of potential synergistic interactions is uncertain. Both opioids[25,26,28] and Tat[30,39,54,58] can disrupt cellular function or induce toxicity in neurons and astroglia. However, unlike Tat, opioids can have neuroprotective effects, preventing programmed cell death or allaying toxicity in some experimental systems.[24,48,49,62] The purpose of this study was to determine whether opioid drugs (morphine) might interact with HIV Tat protein to modify the viability of striatal neurons. Morphine is the active metabolite (deacetylated) of heroin that functions in brain making it an appropriate prototypic opioid drug of abuse.

EXPERIMENTAL PROCEDURES

Mixed-striatal cultures

Striata were isolated from unsexed newborn or 1-day-old ICR mice (Harlen Sprague-Dawley, Indianapolis, IN) as previously described;[73] however, using culture conditions that favored a balanced mixture of neurons and glia. The striata from two mice were pooled and considered as an independent sample (n=1). Growth medium contained Neurobasal™ medium (Gibco/Life Technologies, Grand Island, NY) supplemented with B-27 (2% v/v; Gibco/Life Technologies), L-glutamine (0.5 mM), and gentamicin (10 μg/ml). Briefly, striata were digested with 0.25% (w/v) trypsin in growth medium, centrifuged, and triturated in growth medium supplemented with 2.5% (v/v) donor horse serum. Cells were plated at a

mean density of 100,000 cells/coverslip onto poly-L-lysine (0.1 mg/ml) coated-15 mm-diameter glass coverslips and incubated at 35°C in 5% CO_2/95% air and high humidity. In addition, cells were maintained in serum-free growth medium transiently supplemented with 25 μM L-glutamate but then transferred into glutamate-free medium.[24] Animal procedures were approved by the University of Kentucky Animal Care and Use Committee and were in accordance with the National Institute of Health guide for the care and use of laboratory animals.

Immunocytochemistry

Cultures were evaluated for the presence of opioid receptor containing neurons at 6 days *in vitro*. Immunodetection of μ and κ opioid receptors was performed as previously described.[73] Rabbit anti-μ (MOR1; 1:2000), δ (DOR1; 1:2500), and κ (KOR1; 1:2000) affinity purified, polyclonal antisera (gift from Dr. Robert P. Elde) were used to detect μ, δ, and κ opioid receptors, respectively.[2-4] Secondary biotinylated goat anti-rabbit antibodies (1:250) were conjugated to avidin-peroxidase (Vectastain ABC kit, Vector Laboratories, Burlingame, CA) or Cy2-avidin (1:250; Amersham Life Science, Pittsburgh, PA). Nickel-intensified diaminobenzidine (DAB) was used as a substrate for peroxidase.[24]

For triple label studies, neurons were detected using rabbit anti-human protein gene product (PGP) 9.5 antisera (1:1200 dilution; Ultraclone, Cambridge, UK) followed by goat anti-rabbit antibodies conjugated to Alexa 488 (Molecular Probes). PGP 9.5 is a neuronal ubiquitin carboxyl- terminal hydrolase.[78] Astrocytes were detected using mouse anti-glial fibrillary acidic protein (GFAP) antibodies (1:300, Boehringer Mannheim, Indianapolis) followed by anti-mouse antibodies conjugated to Alexa 546 (Molecular Probes). Microglia were detected using biotinylated isolectin from *Griffonia simplicifolia* (IB_4) (Sigma)[12,17,61] (50 μg/ml for 16 h at 4°C in PBS) followed by incubation in streptavidin conjugated Alexa 350 (10 μg/ml in PBS, 1 h at room temperature; Molecular Probes). Once differential counts were performed on fluorescent neurons, astrocytes, and microglia, immature oligodendrocytes were identified in the same cultures using anti-O4 rat IgM monoclonal antibodies (1:1 dilution from ascites fluid; gift from Dr. M. Schachner, Zurich[6,71]). Primary O4 antibodies were detected with anti-rat IgM antibodies conjugated to peroxidase (Jackson ImmunoResearch, West Grove, PA) and visualized using nickel-intensified diaminobenzidine.[33] For studies on astrocyte viability, rabbit anti-GFAP antisera (1:600 dilution; Chemicon, Temecula, CA), followed by goat anti-rabbit IgG conjugated to Cy3 (Jackson) were used to detect astroglia in combination with ethidium monoazide and Hoechst dyes.

Recombinant Tat protein

Recombinant Tat was prepared as described previously[43] with minor modifications. 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. Previous studies demonstrating neurotoxicity have used Tat derived from the HIV BRU strain and this facilitates comparisons among studies.[47]

Furthermore, we have previously shown that the neurotoxic domain of Tat is well conserved across various strains of HIV.[47] The amplified *tat* gene was inserted into an *Escherichia coli* vector PinPoint Xa-2 (Promega). A deletion mutant from this plasmid was also prepared by deleting the sequence encoding amino acids 31-61 of Tat previously shown to contain the neurotoxic epitope.[55] This construct allowed the expression of the Tat proteins as a fusion protein naturally biotinylated at the N-terminus. The biotinylated Tat proteins were purified on a column of soft release avidin resin and cleaved from the fusion protein using factor Xa and eluded from the column followed by desalting with a PD10 column. All purification steps contained dithiothreitol to prevent oxidation of the proteins. Tat proteins were >95% pure as determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis followed by silver staining, and analysis by HPLC using a C4 column showed a single symmetrical peak. Western blot analysis showed that these preparations contained both monomeric and dimeric forms of Tat1-72, but the monomeric form only for TatΔ31-61. The functional activity of Tat1-72 was confirmed using a transactivation assay in HL3T1 cells containing an HIV-1 LTR-CAT construct.[43] The Tat preparation contained <1 pg/ml endotoxin as determined using a Pyrochrome Chromogenic test kit (Associates of Cape Cod, Inc., Falmouth, MA). The Tat protein was stored in a lyophilized form at −80°C in endotoxin-free siliconized microfuge tubes until taken for experimentation. Tat was immunoneutralized as described previously.[55]

Determination of toxicity

Viability assays were performed in striatal neuron cultures following exposure to opioids, HIV-1 Tat (1-72), mutant HIV-1 Tat (Δ31-61), and immunoneutralized HIV-1 Tat (1-72). Cells were continuously exposed to growth medium alone (untreated controls), morphine (10 nM, 100 nM, or 1 μM) (Sigma Chemical Co., St. Louis, MO), Tat (100 or 200 nM), mutant Tat (200 nM), immunoneutralized Tat (200 nM), morphine plus Tat, morphine plus mutant Tat, morphine plus immunoneutralized Tat, or morphine plus Tat and naloxone (3 μM; Research Biochemicals International, Natick, MA). To assess viability, the same neurons were photographed before and after treatment, and the proportion of dead cells was counted.[24] A neuron was considered dead/dying if there was vacuolization in the cell body, nuclear fragmentation, and/or disrupted cell processes. In other experiments, viability was determined by double-labeling cells with ethidium monoazide bromide (Molecular Probes, Eugene, OR) and Hoechst 33342-trihydrochloride trihydrate (Molecular Probes). Neurons were incubated with ethidium monoazide bromide (0.5 μg/ml; w/v) in Dulbecco's phosphate buffered saline (PBS) (Gibco/Life Technologies). Dead cells that failed to exclude ethidium monoazide became permanently labeled after the ethidium was bound by photo-affinity to DNA using a 45 W fluorescent light (15 cm distance) for 30 minutes at room temperature.[63] Cells were then fixed with Zamboni's fixative containing 3% paraformaldehyde and counterstained with Hoechst 33342 (15 μg/ml, Molecular Probes) in PBS for 15 minutes at room temperature. Hoechst labels DNA in all cells and permits assessment of nuclear morphology. The number of dead neurons (ethidium labeled nuclei) and total neuron numbers (Hoechst labeled nuclei) were counted and reported for individual cultures using a Nikon fluorescence microscope. Neurons were differentiated from other cell types based on morphologic criteria using Hoechst staining[75] and by using cell-type-specific markers.

To arbitrarily sample cells, the x-axis microscope stage controller was moved along an equatorial line through the center of the coverslip and all cell clusters entering the field of vision were sampled. Between 500-1000 neurons were analyzed per culture and at least four cultures were assessed per treatment condition. Each culture consisted of cells derived from separate mice. Neuronal losses were reported as the percentage of total neurons relative to untreated control cultures. Astroglial identity was assessed by morphologic criteria confirmed by GFAP immunocytochemistry. Approximately 200-300 astrocytes were analyzed per culture.

Statistics

Statistical analyses were performed using analysis of variance (ANOVA) and Newman-Keuls *post hoc* test (if significant treatment effects were noted by ANOVA) (Statistica, StatSoft, Tulsa, OK). Treatment effects were considered significant if $P < 0.05$. Photomicrographs were overlaid using Photoshop 5.0 (Adobe).

RESULTS

Characterization of phenotypes in mixed-striatal cultures

The main cell type in the striatal cultures was multipolar neurons, which were noted by 3 to 5 neurites and PGP 9.5 immunoreactivity (54.3 ± 1.6%; $n = 4$) (Fig. 1A,C-E). Astroglia were identified by GFAP immunoreactivity and comprised 41.8 ± 1.7% ($n = 4$) of total cells; of these most were the flat, polyhedral (type 1) astrocyte variant (Fig. 1B,F-G). Phenotypically, a majority of the neurons were immunopositive for both μ and κ opioid receptors (Fig. 1C-E). Fewer cells expressed δ receptor immunoreactivity (data not shown). Subsequent studies analyzed the effects of opioids and Tat on neurons. IB_4 isolectin bound-microglia comprised 0.7 ± 0.3% ($n = 4$) of the total population (Fig. 1F); 2.6 ± 0.6% ($n = 4$) were O4 immunoreactive oligodendrocytes at various stages of development (not shown).

Morphine and HIV-1 Tat (1-72) neurotoxicity

Toxicity between morphine and Tat was initially confirmed using repeated measures analysis (time lapse photomicrographs) of living neurons identified prior to treatment and at 16 h following treatment (data not shown). Marked synergistic toxicity between morphine (1 μM) and Tat (100 nM) was evident at 16 h that caused a 55.6% reduction in viable neurons compared to untreated groups or groups exposed to morphine alone ($P<0.01$). Neurotoxicity was denoted by shrunken, vacuolated cell bodies and retracted neurites. These initial observations prompted examination of the later 24-h time point using markers for cell viability. At 24 h, morphine and Tat together synergistically increased the proportion of dying cells, identified by their failure to exclude ethidium monoazide, by nearly two-fold (Fig. 2C). The ethidium monoazide-labeled dying cells often-displayed condensed, pyknotic nuclei using Hoechst 33342 blue-fluorescent dye (Fig. 2B). The combined toxicity was seen over a range of morphine (10 nM, 100 nM, or 1 μM) and Tat (100 nM or 200 nM) concentrations, and the effects of 1 μM morphine were prevented by naloxone (3 μM) (Fig. 2C). Importantly, neither the highest concentration of morphine (1 μM) nor Tat (200 nM) alone affected neuronal viability compared to untreated controls (Fig. 2C). Although Tat treatment alone resulted in a slight, albeit insignificant 10.2% increase in toxicity at 24 h,

Tat alone can be neurotoxic with prolonged exposure (J.A. Gurwell and K.F. Hauser, unpublished).[23,39,44,58] The proportion of ethidium monoazide-positive dying cells was 6 ± 2% in untreated control cultures.

The specificity of Tat toxicity was tested by treating cultures with morphine (1 μM) in combination with Tat (200 nM), TatΔ31-61 (200 nM), or immunoneutralized Tat (200 nM) (Fig. 2D). Previous studies have shown that Tat residues 31-61 have both neurotoxic and neuroexcitatory properties.[55] Morphine (1 μM) and Tat (200 nM) synergistically increased neuronal death compared to all other treatment groups ($P<0.03$) (Fig. 2 D). Toxicity was not evident with either mutant Tat or immunoneutralized Tat by themselves, or with morphine (Fig. 2D).

Astrocyte viability

Dying cells were identified as neurons based on their morphology and the presence of PGP 9.5 immunoreactivity. Nevertheless, because some of the dying cells had degenerated so severely that they neither displayed evidence of a neuronal morphology nor possessed cell type specific immunoreactive markers, we examined the effect of opioids and/or Tat on the survival of astrocytes, the other major cell type present in our cultures, at 24 h (Table 1). Opioids and/or Tat had no effect on astrocyte viability, providing additional confirmation that the synergistic toxicity was restricted to neurons at 24 h. The proportion of ethidium monoazide-positive cells with astrocytic morphology was 1.5 ± 0.4% in untreated control cultures.

DISCUSSION

Morphine and HIV-1 Tat (1-72) display a synergistic striatal neurotoxicity

The findings present novel evidence that opioids and HIV-1 Tat protein are synergistically toxic to striatal neurons through a direct action on neural cell targets. Importantly, the enhanced toxicity was clearly mediated through opioid receptors, since the neurodegenerative effects of morphine were prevented by concurrent naloxone administration. Because morphine is a preferential μ opioid receptor agonist and a large proportion of the neurons in our cultures possess μ opioid receptor immunoreactivity as occurs *in vivo*,[76] our results infer that μ receptors may be preferentially involved. Nevertheless, a definitive role for μ receptor involvement requires additional study using reagents that are more selective. Naloxone is a μ, δ, and κ opioid receptor antagonist. In addition, although subsets of the neurons in our cultures can possess μ, δ, and/or κ receptor immunoreactivity, it is uncertain whether opioids and Tat are directly affecting neurons or acting via glial intermediaries. Subpopulations of striatal astrocytes,[73] as well as oligodendroglia[33] and microglia[14] can express opioid receptors and potentially mediate the effects of morphine and/or Tat in neurons.

Morphine and HIV-1 Tat (1-72) concentrations

Therapeutic levels of morphine in plasma following oral or intravenous administration for acute pain can approach 100 nmol/L (the concentrations in the central nervous system are likely to be lower[79]).[41,42] In extreme instances, concentrations in human cerebrospinal fluid

can be greater than 400 nM following intracerebroventricular administration for severe cancer pain.[70] In contrast, opioid dependent individuals reportedly tolerate opioid blood levels that are 2.5 to 100-fold greater than typical therapeutic concentrations for acute pain.[32] In rats, morphine concentrations in the nervous system can transiently approach 1.5-μg/g tissue weight following a 10-mg/kg intravenous dose.[11] Thus, the morphine concentrations used that increase Tat toxicity in the present study are likely to mimic those seen with chronic drug abuse, but importantly are less likely to be realized at therapeutic dosages for pain management.

Tat protein is produced during HIV infection and can be released by infected cells.[13,18,50,64,65] Furthermore, extracellular Tat is functionally active.[19] The concentrations of Tat in our cultures are 1.5-3.0 μg/ml (100-200 nM, respectively). Concentrations of Tat are approximately 1 ng/ml in the serum of HIV infected patients and 4 ng/ml in the conditioned media of HIV infected cells.[1,77] The extracellular space within the central nervous system comprises a small proportion of total brain volume,[59] and may be dramatically reduced with pathophysiological changes.[74] For this reason, it is conceivable that neurotoxic concentrations of Tat such as those used in this study may be achieved within the confined extracellular space of the HIV infected striatum.

Potential clinical significance of striatal neurotoxicity

Our basic experimental studies *in vitro* provide a possible explanation why opioid drug use causes increased dementia and motor dysfunction in a significant proportion of HIV-infected individuals. As recently noted by Dr. Alan Leshner, the Director of the National Institute on Drug Abuse, “Drug abuse and HIV are truly interlinked epidemics” (see[52]). “2.4 million Americans use heroin and as many as 30 % of injecting drug users are HIV positive[52] (see also[9])”. In a patient cohort in Scotland, 59% of HIV positive injecting drug users with an extensive (but not exclusive) history of opioid abuse have pathological changes associated with HIV encephalitis at autopsy.[8,9] Within this cohort (homosexuals and drug users), cognitive impairment often coincided with HIV encephalitis. Similarly, in injecting drug users sampled throughout the United Kingdom, individuals with HIV infection are more likely to develop encephalitis.[15]

Morphine and HIV-1 Tat (1-72) can induce apoptotic pathways

The loss of neuronal viability (inability to exclude ethidium) was accompanied by nuclear shrinkage and chromatin compaction (pyknosis) and/or fragmentation (apoptotic bodies). Tat has been shown to activate apoptotic cascades such as caspase and Par-4 in primary neurons in culture.[39] Interestingly, depending on the cell type and dosage, opioids can have paradoxical neuroprotective or neurodegenerative effects, which likely relates to the ability of opioid receptors to couple to cell death signaling pathways in different systems.[27] In some experimental paradigms, opioids are neuroprotective.[24,48,49,62] Interestingly, morphine may exaggerate HIV-envelope protein gp120-induced early proliferative increases in kidney fibroblasts[68] and in gp120 interactions with substances of abuse can at times be protective.[72] Clearly, however, HIV is a multisystem disease, and opioids themselves have diverse effects that are system specific.[27] More typically, when opioids affect cell viability, they exacerbate apoptotic events or induce cytotoxicity. Morphine can induce toxicity in cerebellar Purkinje

cells.[25] Fentanyl, a selective μ agonist drug, is neurotoxic to the limbic system of rats at high dosages[35] and can exacerbate the effects of ischemia-induced damage to the basal ganglia.[34] Morphiceptin, a μ agonist, enhances staurosporine or wortmannin-induced apoptosis in embryonic chick neurons.[21,51,66,67,69,80] Alternatively, in other studies, opioids have no intrinsic toxicity, but will synergistically enhance cell losses only if apoptosis is induced by other factors,[16,21,22,45] which is similar to the present findings. It is important to note that morphine alone is not toxic to our striatal neurons, even following prolonged exposure (7 days) to high concentrations (1 or 10 μM), whereas prolonged exposure to Tat (100 or 200 nM > 72 h) is intrinsically neurotoxic (K.F. Hauser, unpublished).[38,39] Thus, morphine is accelerating the toxic effects of Tat, and studies now in progress are addressing the nature of the interactions and the particular signaling pathways involved.

Conclusions

Our present results should be interpreted with some caution, because they represent an acute response of cells isolated *in vitro* to relatively high concentrations of morphine. Many of the detrimental effects of opioids result from high, fluctuating levels and a failure to accommodate to changes in opioid signal intensity.[37,57] Steady state or moderate therapeutic exposures have few side effects. Moreover, with chronic opioid exposure, tolerance develops at the molecular, cellular, and systems levels. It is yet uncertain whether Tat would be more toxic in opioid tolerant cells. In addition, non-opioid factors present *in vivo*, such as specific EGF-family ligand-erbB signaling events, may normally modify or negate the neuronal response to opioids.[60] The particular signaling pathways or temporal relatedness involved in opioid-Tat interactions remain speculative. Lastly, although we have assessed overt losses of neuronal populations, it is uncertain whether the effects are direct or indirect since the astroglia (~40%) or microglia (albeit <1%) may intervene and mediate aspects of the opioid-Tat synergism. Irrespective of these issues, our findings are an important first step and suggest that opioid drug abuse may contribute to the progression of HIV-associated dementia through a direct neurotoxic mechanism.

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Abbreviations

AIDS	acquired immunodeficiency syndrome
ANOVA	analysis of variance
DAB	diaminobenzidine
GFAP	glial fibrillary acidic protein
HIV	human immunodeficiency virus
HIV-1	human immunodeficiency virus type 1
HPLC	high performance liquid chromatography

IB_4	isolectin from *Griffonia simplicifolia*
LTR	long terminal repeat
PBS	phosphate buffered saline
PGP 9.5	protein gene product 9.5

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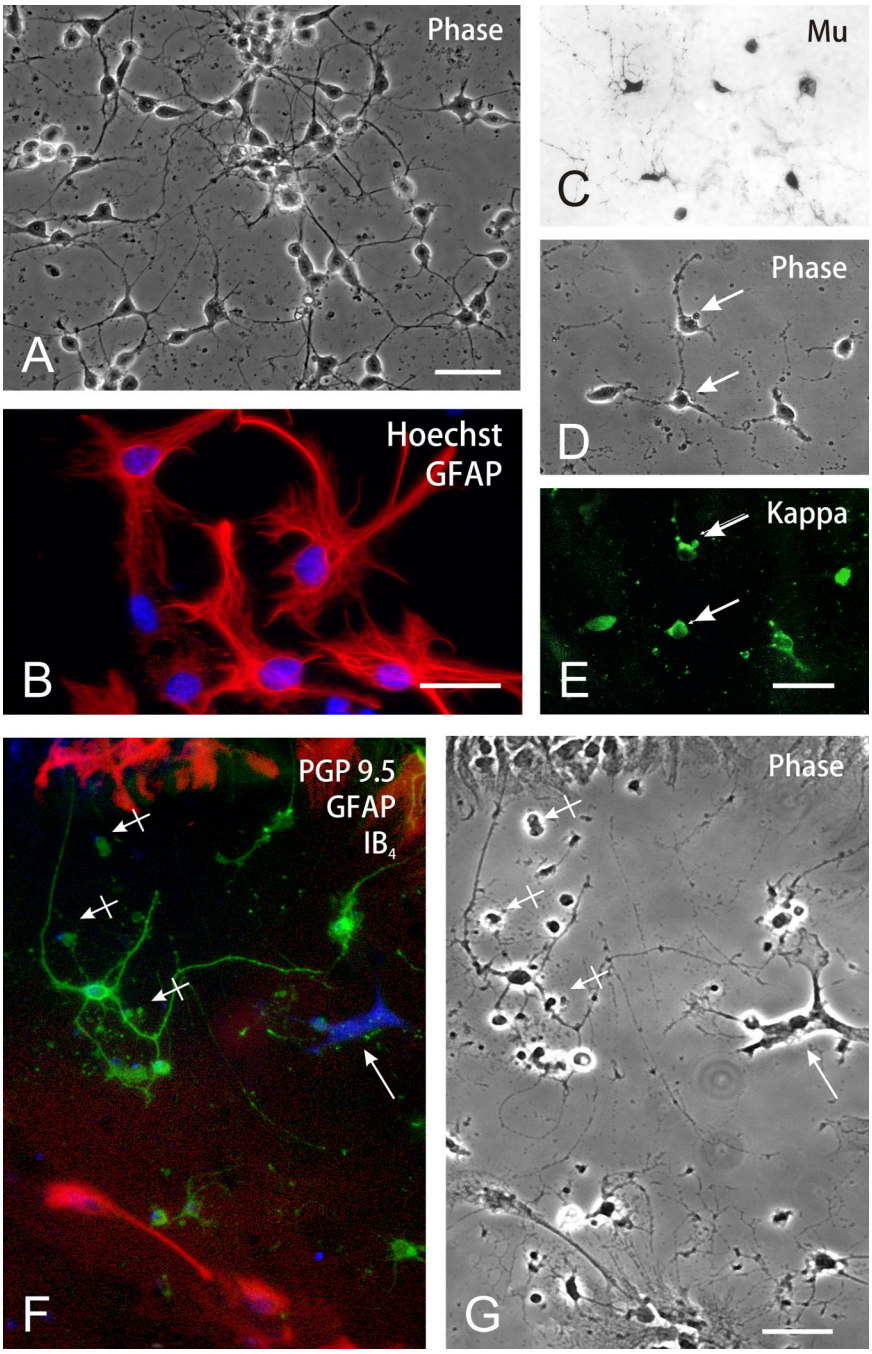

Fig. 1.

Characterization of neural cells in mouse striatal cultures (A-G). (A) Phase contrast photomicrograph of striatal cells at 5-7 days *in vitro*. A majority of the cells are neurons; scale bar = 20 μm. (B) Astrocytes were characterized by large nuclei with multiple nucleoli and dispersed heterochromatin (Hoechst 33342 blue immunofluorescence) and glial fibrillary acidic (GFAP) immunofluorescence (Cy3 red fluorochrome); scale bar = 25 μm. (C-E) Subpopulations of striatal neurons possessed μ (C), κ (E), and to a lesser extent δ (not shown), opioid receptor immunoreactivity. D & E show phase contrast and κ opioid receptor

immunofluorescent images of the same cells; scale bar = 20 μm. (F-G) Triple-label-identification of neurons, astrocytes and microglia in striatal cultures. Neurons were identified using anti-PGP 9.5 indirect immunofluorescence (Alexa 488, green product), while astrocytes were labeled using anti-GFAP indirect immunofluorescence (Alexa 546, red product) and microglia were detected using IB_4 biotinylated isolectin-conjugated (via avidin) to Alexa 350 (blue fluorescent product) (arrow) (F). A majority of the cells were neurons; microglia comprised 0.7% of the total cells. (G) A phase-contrast photomicrograph of the same cells as in Fig. 1F, some of which were non-viable/degenerating (hatched arrows); PGP 9.5 immunoreactivity is retained by many degenerating neurons; scale bar = 20 μm.

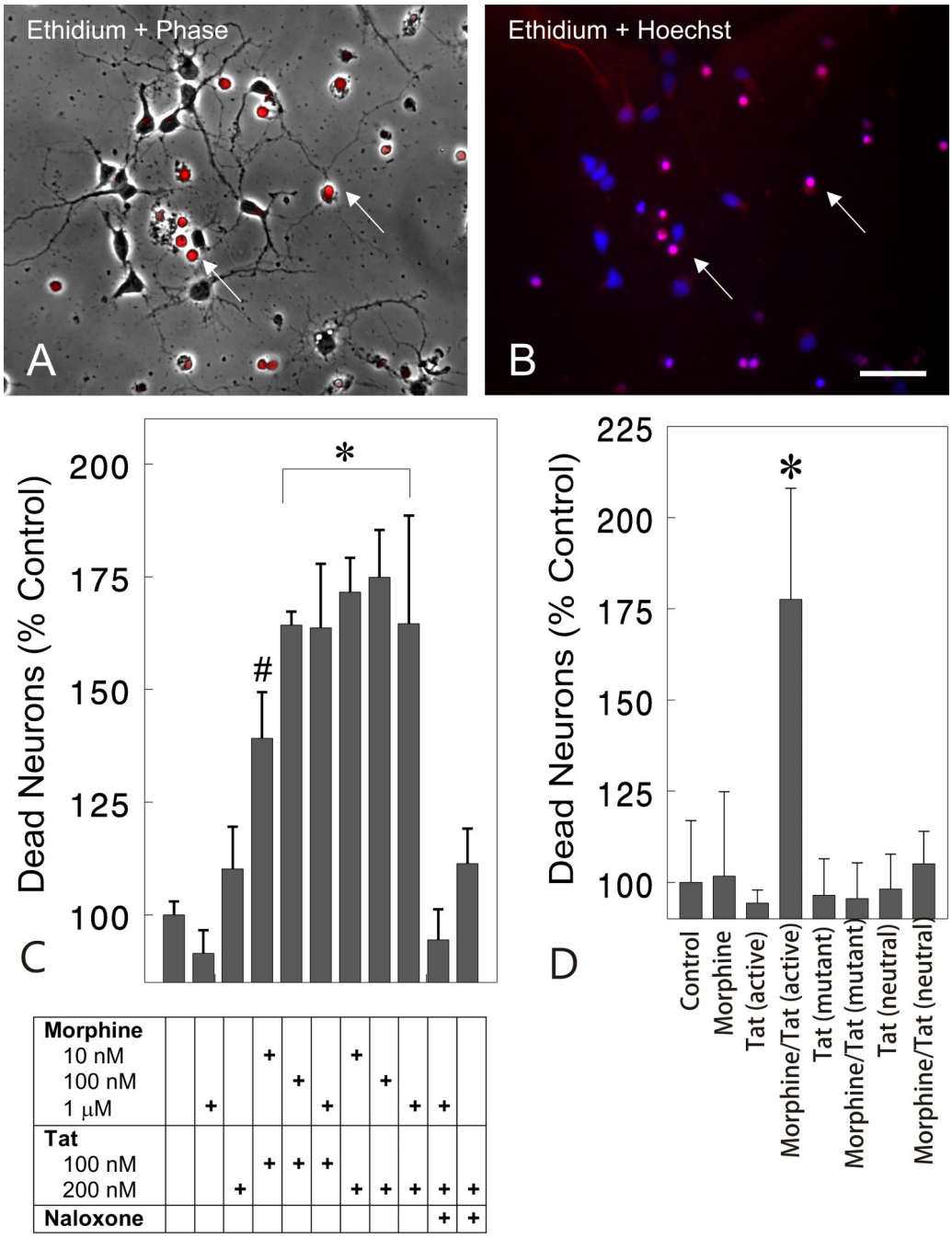

Fig. 2.

Effect of opioids and/or Tat on the survival of striatal neurons at 24 h. Phase contrast microscopy (A) with ethidium monoazide (red fluorescent overlay in A & B) and Hoechst 33342-labeling (blue fluorescence) of nuclear chromatin patterns (B) were examined in the same cells at 24 h following morphine plus Tat exposure. Dying cells, noted by the inability to exclude ethidiummonoazide, lacked neuritic processes (A-B). Nonviable cells additionally displayed dense, pyknotic heterochromatin with Hoechst dye suggestive of apoptosis (B). (C). The viability assay shows morphine and Tat had synergistic effects on

neuronal losses at 24 h. The effects of combined morphine (1 μM) and Tat (200 nM) toxicity was prevented by naloxone (3 μM) (C). The results are the mean ± SEM of 4-7 determinations on cells pooled from separate mice (*P < 0.02 versus untreated controls, treatment with Tat or morphine alone, or naloxone-exposed groups) ($^{\#}P$<0.05 versus untreated controls, Tat or morphine alone, or naloxone-exposed groups); morphine (Morph); naloxone (Nal); scale bar = 20 μm. (D). Tat Specificity. Synergistic toxicity of morphine (1 μM) and Tat (200 nM) (*P<0.03 versus untreated controls and all treatment groups) was attenuated in the presence of mutant Tat (31-61 deletion) (200 nM) and immunoneutralized Tat (200nM) (D). The results are the mean ± SEM of 5 determinations on cells pooled from separate mice.

Table 1

Effect of Opioids and/or Tat on Astrocyte Viability

Treatment	Non-Viable Astrocytes (%)[b]
Untreated	1.5 ± 0.4
Morphine	1.8 ± 0.3
Tat	0.9 ± 0.3
Morphine + Tat	1.6 ± 0.3
Morphine + Tat + Naloxone	1.3 ± 0.2

[a]Cell cultures were continuously exposed to opioids and/or Tat for 24 h in vitro. The results are the mean ± SEM of four cultures, each containing cells derived from separate mice.

[b]No significant effects on astrocyte survival were noted (ANOVA).