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Ligand-gated purinergic receptors regulate HIV-1 Tat and morphine related neurotoxicity in primary mouse striatal neuron-glia co-cultures

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Abstract

Emerging evidence suggests that opioid drugs, such as morphine and heroin, can exacerbate neuroAIDS. Microglia are the principal neuroimmune effectors thought to be responsible for neuron damage in HIV-infected individuals, and evidence suggests that opioid drugs acting via μ opioid receptors in microglia aggravate the neuropathophysiological effects of HIV. Key aspects of microglial function are regulated by the P2X family of ATP activated ligand-gated ion channels. In addition, opioid-dependent microglial activation has been reported to be mediated through P2X₄ signaling, which prompted us to investigate whether the cation-permeable P2X receptors contribute to the neurotoxic effects of HIV and morphine. To address this question, neuron survival, as well as other endpoints including changes in dendritic length, extracellular ATP levels, and intracellular calcium levels, was assayed in primary neuron-glia co-cultures from mouse striatum. Treatment with TNP-ATP, a non-selective P2X antagonist, prevented the neurotoxic effects of exposure to morphine and/or the HIV Tat, or ATP alone, suggesting P2X receptors mediate the neurotoxic effects of these insults in striatal neurons. Although P2X₇, and perhaps P2X₁, receptor activation decreases neuron survival, neither P2X₁, P2X₃, nor P2X₇ selective receptor antagonists prevented Tat and/or morphine-induced neurotoxicity. These and other experiments indicate the P2X receptor family contributes to Tat-and morphine- related neuronal injury, and provide circumstantial evidence implicating P2X₄ receptors in particular. Our findings reveal that members of the P2X receptor family, especially P2X₄, may be novel therapeutic targets for restricting the synaptodendritic injury and neurodegeneration that accompanies neuroAIDS and opiate abuse.

Keywords

HIV-1; NeuroAIDS; Tat; opioid drug abuse; ionotropic purinergic (P2X) receptors; striatal neurons; astroglia; microglia; dendritic injury; neurodegeneration

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Conflicts of Interest

The authors declare no competing financial interests.

Introduction

HIV-associated neurocognitive disorders (HAND) encompass a broad spectrum of neurological conditions that can range from mild to severe symptoms (Antinori et al., 2007; McArthur et al., 2010). Despite the widespread use of combined antiretroviral therapy (cART), the prevalence of HAND is still approximately half of HIV positive individuals (Kraft-Terry et al., 2010; McArthur et al., 2010; Harezlak et al., 2011). Opiate abuse and HIV are interrelated epidemics. Almost 25% of HIV cases in the USA in 2011 were directly related to injection drug use (Centers for Disease Control 2011), and opiate exposure may promote the progression of HIV infection to AIDS (Donahoe and Vlahov, 1998; Bell et al., 2002; Kumar et al., 2004; Byrd et al., 2011; Meyer et al., 2013), and can intrinsically alter neuropathogenesis by directly affecting neuronal, astrocyte and microglial function (Hauser et al., 2012).

In the central nervous system (CNS), microglia are productively infected by HIV-1 and in response release neuroinflammatory and excitotoxic products (Persidsky and Gendelman, 2003; González-Scarano and Martín-García, 2005). Both microglia and astroglia are important sites where opiates converge and exacerbate the pathobiology of HIV-1 Tat (Suzuki et al., 2011; Zou et al., 2011). Activation of μ opioid receptors can compound the neurotoxic effects of HIV by dysregulating intracellular calcium ($[Ca^{2+}]_i$), elevating reactive oxygen species (El-Hage et al., 2005; Zou et al., 2011), and glutamate uptake (Zou et al., 2011) in astrocytes, as well as exacerbating cytokine and chemokine release (El-Hage et al., 2005; Hauser et al., 2006). Despite evidence for pronounced opioid and HIV interactions in the CNS, the mechanisms involved are incompletely understood (Hauser et al., 2012).

The P2X receptors are activated by binding ATP (for review of purinergic system see (Burnstock and Kennedy, 2011)). They are expressed by neural cells and have been implicated in microglial activation and neuroinflammation (Ohsawa et al., 2007; Horvath and DeLeo, 2009). There are currently seven known members of this family, P2X₁₋₇, which are ligand gated ion channels, mostly permeable to Ca^{2+} but that can also flux Na^+ and K^+ . P2X₄ is a likely candidate for involvement in HIV and opioid signaling since it has been shown to be upregulated on microglia and involved in other neuroinflammatory signaling (Ulmann et al., 2008). Previous work also suggests that the effects of morphine on microglia are mediated through P2X₄ and μ opioid receptor interactions (Horvath and DeLeo, 2009). They found that morphine-dependent microglial migration was inhibited by TNP-ATP (P2X₁₋₇ antagonist), but not by PPADS (pyridoxal-phosphate-6-azophenyl-2',4'-disulfonic acid; a somewhat preferential P2X₁₋₃ & 5-7 antagonist), suggesting P2X₄ mediation. Here we provide evidence that the P2X system is involved in HIV and opioid neuropathogenesis, and provide circumstantial evidence that the P2X₄ receptor subtype may be preferentially mediating many of these events.

Materials and Methods

Experiments were conducted in accordance with procedures reviewed and approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee and conform to Association for Assessment and Accreditation of Laboratory Animal Care guidelines.

Cell Culture: Mixed glial bed layer preparation

Striatal mixed glial cultures were prepared from P0–P2 ICR (CD-1®; Harlan Laboratories, Indianapolis, IN) mouse pups. Striata were dissected, minced, and incubated with trypsin (2.5 mg/ml; Sigma, St. Louis, MO) and DNase (0.015 mg/ml; Sigma) in 10 mL of Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen, Carlsbad, CA) with 25 μ M

glutamate (30 min, 37 °C). Tissue was then triturated, resuspended in 10 ml glial maintenance medium made of DMEM with 10% defined fetal bovine serum (Hyclone, Logan, UT), and cells were filtered twice through 70 µm pore nylon mesh. Cells were plated onto 24-well plates at a density of 3×10^5 per well and maintained in media that contained 10% serum. Medium was changed 24 h after being plated and then every 2–3 d afterwards. Glia were allowed to reach confluence, which took approximately 10 d.

Co-culture with neurons

Striatal neurons were prepared from E15–E16 ICR (CD-1[®]; Harlan Laboratories) mouse embryos. Striata were dissected, minced, and incubated with trypsin (2.5 mg/ml) and DNase (0.015 mg/ml) in 10 mL of Neurobasal medium supplemented with B27 (Invitrogen) and 0.5 mM L-glutamine (Invitrogen) (30 min, 37 °C). Tissue was then triturated, resuspended in 10 ml of supplemented Neurobasal medium and cells were filtered twice through 70 µm pore nylon mesh. Neurons were placed on top of a confluent mixed glial bed layer at a density of $0.8\text{--}1.0 \times 10^5$ cells per well. Neuron-glia co-cultures were maintained in complete Neurobasal medium and allowed to mature for about 5 d prior to start of the experiments, with media exchanged 24 h after neurons were plated and then every 2–3 d. Our mixed-glia cultures have been previously characterized to contain approximately 91% astrocytes and 8% microglia (Zou et al., 2011).

Immunocytochemistry

Cell cultures were fixed for 15 min in 4% paraformaldehyde in phosphate buffer (pH 7.2 at 4 °C), permeabilized in 0.1% Triton-X 100, and rinsed 3×20 min in PBS, pH 7.2. Cultures were incubated in diluted primary antiserum overnight at 4°C in PBS, pH 7.2 with 1% crystalline grade BSA (Calbiochem/EMD Millipore, Billerica, MA) and 0.1% Triton-X 100. Rabbit anti-P2X₄ receptor (1:500; Alomone Labs, Jerusalem, Israel) was colocalized with either mouse anti-microtubule-associated protein 2 (MAP2) antibodies (1:500; EMD Millipore) for identification of neurons, or with goat anti-ionized calcium binding adapter molecule 1 (Iba1) (1:500; Abcam, Cambridge, MA) antibodies for the identification of microglial cells. Appropriate secondary antibodies conjugated to Alexa 488 or Alexa 596 (1:500 dilution; Molecular Probes/Invitrogen) were used to identify primary antibody targets. Nuclei were counterstained with Hoechst 33342 (1 µg/ml). Cultures were rinsed and mounted in Prolong Gold Antifade Reagent (Molecular Probes/Invitrogen). Samples were imaged using a Zeiss LSM 700 laser scanning confocal microscope equipped with a 63× oil immersion objective. Images were collected using ZEN 2009 Light Edition software (Carl Zeiss, Inc.; Thornwood, NY) and edited using Adobe Photoshop CS3.

Drug and HIV-1 Tat treatments

Recombinant Tat_{1–86} (100 nM) was obtained from HIV-1_{III_B}, ImmunoDiagnostics, Woburn, MA). Morphine sulfate (500 nM) and naloxone (500 nM) were used to target the µ opioid receptor (Sigma). NF499 (300 nM; Tocris Bioscience, Bristol, UK), while TNP-ATP (2',3'-O-(2,4,6-trinitrophenyl) adenosine-5'-triphosphate; 300 nM), PPADS (300 nM), BBG (Brilliant Blue-G) (1 µM), and A-317491 (1 µM), BzATP (3'-O-(4-benzoyl)benzoyl adenosine 5'-triphosphate) (300 µM) (Ma et al., 2009; Valdez-Morales et al., 2011), αβMe-ATP (αβ-methylene-ATP) (1 µM), and ATP (multiple concentrations) were all obtained from Sigma. Concentrations of NF499, A317491, PPADS, BBG, BzATP, and αβMe-ATP were chosen based on values shown to be effective elsewhere in the literature (Jarvis et al., 2002; Rettinger and Schmalzing, 2004; Raouf et al., 2007; Young et al., 2007; El-Ajouz et al., 2011).

Assessment of neuronal survival

As previously described (Suzuki et al., 2011), a microscope (Zeiss Axio Observer Z.1) with environmental chamber (PeCon GmbH, Erbach, Germany) and a computer-controlled, x,y axis stage encoder was used to track individual neurons over time (Zeiss AxioVision 4.6 software; Mark&Find, and Time Lapse modules). Cultured neurons within multiple microscopic fields were randomly selected prior to the addition of drug and HIV-1 Tat treatments. Time-lapse images were taken every 30 min for 72 h. Media was not changed during the experiment. Approximately 30 healthy neurons with well-defined dendritic arbors and single axons were followed for individual treatment groups in each experiment using phase-contrast microscopy (40× magnification). Neuron death was recorded upon collapse and fragmentation of the cell body, and has been verified previously with ethidium homodimer staining (Buch et al., 2007). Data are presented as the percentage of surviving neurons relative to cells present at the onset of the experiment (0 h); experiments were repeated at least $n = 4$ times.

Measuring intracellular calcium concentration ($[Ca^{2+}]_i$)

Tat and morphine $\pm$ TNP-ATP-dependent changes in $[Ca^{2+}]_i$ were measured by ratiometric imaging of fura-2. Cells were then loaded with 1 μ M fura-2/AM (Molecular Probes/Invitrogen; 45 min, 37 °C) in Hank's balanced salt solution with 10 mM HEPES buffer (pH 7.2) and then incubated for an additional 30 min at 37 °C to allow for complete hydrolysis of the acetoxymethyl ester group. Data are reported as percentage change in fura-2 ratio at 340/380-nm excitation relative to values recorded at $T = 0$ for each neuron and proportional to $[Ca^{2+}]_i$. Due to the heterogeneous nature of purinergic receptor expression by striatal neurons (Amadio et al., 2007), we separated the cells into two groups: responsive (neurons displaying a robust response) and non-responsive (neurons lacking any response) neurons. Each response curve represents the mean $\pm$ SEM from at least $n = 3$ separate experiments—each consisting of the average percent control ratios of 5–6 responsive neurons. This allowed us to see effects that otherwise may have been undetectable due to high variability. Responsive neurons were included in graphed data and used for statistical analysis. The same numbers of neurons were observed across all treatment groups.

ATP assay

Neuron mixed-glial co-cultures were exposed to morphine (100 nM), HIV-1 Tat, and/or ATP (non-selective P2X agonist; Sigma) $\pm$ purinergic antagonists. Cells were pretreated for 15 min with ARL67156 (Tocris Bioscience), an ecto-ATPase inhibitor, to block the rapid breakdown of extracellular ATP, which allowed us to measure more accurately the amount of ATP being released. At 30 min, 1 h and 2 h following continuous exposure, 100 μ L aliquots of media were removed and placed into a 96 well plate. ATP was measured by luminescence reaction using an ATPLite™ Kit (Perkin Elmer, Waltham, MA) according to the manufacturer's instructions, except cell lysis solution was not added in order to preserve integrity of the cell and ensure measurement of only extracellular ATP levels. The aliquoted media was placed on a microplate reader (PHERAstar FS, BMG Labtech, Cary, NC) and ATP concentrations were calculated based on a standard curve.

Dendrite length measurements

After 72 h of treatment, cultures were fixed for 5 min with 4% paraformaldehyde, washed in PBS (3×5 min), and incubated with rabbit anti-microtubule associated protein-2 (MAP2) antibodies overnight at 4 °C (1:500 dilution; Chemicon/EMD Millipore, Billerica, MA). After washing of the primary antibody in PBS (3×5 min), the secondary antibody conjugated to Alexa 488 (mouse anti-rabbit; 1:500 dilution; Molecular Probes/Invitrogen) was incubated on the cells for 2 h. The secondary antibody was rinsed off (3×5 min) and

cells were counterstained with Hoechst 33342 (1 $\mu\text{g/ml}$) for 5 min and then washed off with PBS (3 $\times$ 5 min). Neurons were left in PBS at 4°C until images were taken. All images were acquired at 20 $\times$ magnification using a computer controlled microscope (Zeiss Axio Observer Z.1). Dendritic length was estimated by the number of intersections with calibrated concentric circles using a modified Sholl procedure (Sholl, 1953; Hauser et al., 1989), with slight modifications for *in vitro* measurements (Singh et al., 2005; Suzuki et al., 2011). All neurons that had clearly defined cell bodies located completely within the viewing field were included. Hoechst 33342 staining was used to label cell nuclei. MAP2-positive neurons that lacked dendritic processes but had clear cell bodies were counted as having 1 point of intersection. Neurons lacking dendrites may have died recently or were about to die. Including these neurons in our analysis allowed us to better represent groups in which additional cell death had occurred. Approximately 40–60 neurons were sampled per treatment group in each experiment. Data are presented as the mean dendritic length per neuron $\pm$ SEM from at least 4 independent experiments.

Statistics

For all time-lapse and calcium imaging experiments a multi-way repeated measures ANOVA was used. For all other experiments a one-way ANOVA was performed. If significant overall differences were detected by ANOVA, Duncan's post hoc test was performed to assess intergroup differences. A *p*-value less than 0.05 was considered significant (Statistica; StatSoft, Tulsa, OK).

Results

Cellular localization of P2X₄ receptors in mouse striatal co-cultures

Although others have characterized P2X₄ receptor immunoreactivity in neurons and glia within the CNS (Garcia-Guzman et al., 1997; Lê et al., 1998), including the striatum (Amadio et al., 2007), most of this work has been done in tissue sections from rats and humans. Accordingly, we wanted to verify the presence of P2X₄ receptors and determine the cellular patterns of expression in our mouse primary mixed-glia and neuron culture system. P2X₄ receptor presence was confirmed in our primary striatal co-cultures (Fig. 1). P2X₄ receptor immunofluorescence was present in $87.7 \pm 5.3\%$ Iba-1 immunoreactive microglia in control cultures (Fig. 1a–f). P2X₄ receptor immunoreactivity appeared to be more intense in activated, amoeboid microglia (Fig. 1a–c), whereas a more dispersed expression pattern in quiescent microglia (Fig. 1d–f). Upregulation of the P2X₄ receptor at the cell surface of activated microglia has been shown to occur by others (Tsuda et al., 2003; Ulmann et al., 2008). P2X₄ receptor positive cells that lacked neuronal morphology and lacked Iba-1 immunoreactivity that appeared to be astrocytes were also observed (Fig. 1d–f). Moreover, a majority ($57.9 \pm 9.8\%$) of MAP2-immunoreactive neurons in control cultures also possessed P2X₄ immunoreactivity (Fig. 1g–i). Neurons typically displayed a slightly more stippled pattern of P2X₄ immunofluorescence than in ramified (quiescent) microglia (Fig. 1g–i). P2X₄ expression by neurons has been extensively described in the CNS (Lê et al., 1998; Ashour and Deuchars, 2004), including heterogeneous patterns of expression by striatal neurons (Amadio et al., 2007).

Evaluation of P2X receptor involvement in Tat and opioid related neuronal toxicity: Time-lapse experiments

Either morphine or Tat treatment alone caused a decrease in neuronal survival compared to control levels, while neuronal losses were significantly greater when morphine and Tat were combined compared to either group alone (Fig. 2a–b). The enhancement of the Tat toxicity in the presence of morphine was prevented by pretreatment with the opioid antagonist, naloxone. Pretreatment with TNP-ATP by itself had no effect on neuronal survival, but

reversed the neurotoxic effects of either morphine or Tat toxicity alone, or in combination (Fig. 2a–b). Lastly, PPADS by itself was intrinsically neurotoxic at the concentrations tested after 72 h (data not shown), invalidating its use in assessing a selective role of P2X_{1–3} & 5–7 receptors in morphine and/or Tat-induced neuronal losses.

TNP-ATP reversed Tat and morphine-related neuronal death in a concentration-dependent manner (Fig. 3a–c). The concentration-dependent protective effect was seen when the cells were treated with Tat or morphine alone, or Tat and morphine in combination. In all three cases, the highest concentration, 300 nM, completely prevented neuronal losses, further confirming results seen in Fig. 2. At intermediate concentrations, 100 nM and 50 nM, TNP-ATP partially blocked decreases in survival rates; these concentrations resulted in intermediary levels of protection that differed significantly different from both control levels, and Tat- and/or morphine- treated groups. At the lowest 10 nM concentration, TNP-ATP caused no change in the proportion of dying neurons caused by Tat and/or morphine at 72 h. Prior to 48 h, significantly more neuron losses were seen transiently with combined 10 nM TNP-ATP and Tat compared to Tat alone; however, this effect did not persist to 72 h (Fig. 3b). Though there was a trend toward increased neuronal death when 10 nM TNP-ATP was combined with morphine or morphine and Tat together (Fig 3a and 3c), the trend was not significant. It is possible that activating higher affinity P2X₁ and P2X₃ receptors is protective in this system.

Although Tat and morphine significantly increase neuronal death, a large number of cells (about 65–70%) survive, even after 72 h of continuous exposure. To further investigate the health of the surviving cells at this time and to evaluate whether one or more members of the P2X receptor subfamily might additionally mediate sublethal neuronal injury, potential changes in dendritic length were assessed in MAP2-immunoreactive dendrites using a modified Sholl analysis (Singh et al., 2005; Suzuki et al., 2011). Average dendritic length was significantly reduced in the population of the surviving neurons at 72 h following either Tat and/or morphine treatment. Similar reductions were seen in dendritic length irrespective of whether Tat and morphine were given separately or in combination. ATP, the positive control, significantly decreased dendritic length as anticipated; the decreases across all groups containing Tat or morphine, as well as ATP, were prevented with TNP-ATP (Fig. 4 a–b).

Tat and Morphine exposure increases extracellular ATP levels

ATP levels were measured in conditioned media to determine whether the endogenous ligand for the P2X receptors was elevated in response to opioid or HIV-1 protein treatment alone and in combination. Following 30 min incubation, Tat and morphine alone, or in combination, increased ATP levels when compared with controls (Fig. 5). While, at 1 h only Tat alone, and morphine and Tat in combination, continued to cause significant elevation in ATP. Finally, at the 2 h time point only combined Tat and morphine treatment resulted in significant increases in extracellular ATP levels (Fig. 5).

ATP and combined Tat and morphine-dependent increases in [Ca²⁺]_i are attenuated by a broad-acting P2X receptor antagonist

ATP, the natural ligand for P2X receptors, caused marked increases in intracellular calcium concentration ([Ca²⁺]_i) in neurons. Baseline [Ca²⁺]_i levels were established in neurons for 2 min prior to Tat/drug treatments. Neurons were exposed to increasing concentrations of ATP (10 nM, 100 nM, 1 μM, 10 μM, and 100 μM) at 2 min intervals. ATP caused concentration-dependent increases in the fura-2 340/380-nm excitation ratio indicative of rises in [Ca²⁺]_i (Fig. 6). Elevations were seen with exposure to 1 μM ATP, with significant increases occurring at both 10 and 100 μM concentrations.

We also found that treatment with morphine and Tat elevate the fura-2 340/380 ratio (Fig. 7). After establishing baseline levels, morphine was applied at 2 min and Tat was added at 5 min. The order of application was switched in a second group (data not shown). Similar results were obtained irrespective of whether morphine or Tat was given first, with the rise in $[Ca^{2+}]_i$ becoming significant at approximately 6 min (about 1 min after the last compound was administered). More interestingly, pretreatment with TNP-ATP prevented $[Ca^{2+}]_i$ increases caused by either ATP or Tat + morphine co-stimulation.

P2X₁, P2X₃, and P2X₇ receptor subtypes are not involved in HIV-1 Tat or morphine related neurotoxicity

To determine whether other P2X receptors are involved in the response to Tat and/or morphine, the potential role of P2X₁, P2X₃, and P2X₇ receptor subtypes in HIV and opioid-related neurotoxicity was explored using selective antagonists. As in the initial viability studies, morphine (with the exception of Fig. 8e where $p = 0.055$), Tat, or combined morphine and Tat $\pm$ naloxone, significantly decreased neuron survival as compared with control cells. In contrast to the initial experiments with TNP-ATP, none of the selective antagonists for P2X₁ (NF499) (Fig. 8a–b), P2X₃ (A-31749) (Fig. 8c–d), or P2X₇ (BBG) (Fig. 8e–f) receptor subtypes prevented morphine and/or Tat-induced neurotoxicity (Fig. 8).

Selective agonists for individual P2X receptor subtypes were used to further assess the potential neurotoxic role of these receptors and to confirm and extend the findings using selective P2X receptor antagonists when possible. The P2X receptor agonists tested were $\alpha\beta$ Me-ATP, which is selective for both P2X₁ and P2X₃ receptors, and Bz-ATP, which is selective for the P2X₇ receptor. P2X₁/P2X₃ receptor activation appeared to be marginally neurotoxic; causing significant neuronal losses by 72 h in some experiments (Fig 8a), but only partial reductions in neuronal survival that were not statistically significant when repeated in another set of experiments (Fig. 8c). To ascertain whether the marginal neurotoxicity might be due to opposing actions of $\alpha\beta$ Me-ATP at P2X₁ and P2X₃ receptors, the effects $\alpha\beta$ Me-ATP were challenged with P2X₁ and P2X₃ receptor antagonists (Fig. 8a,d). Interestingly, $\alpha\beta$ Me-ATP-induced neurotoxicity was not evident with P2X₁ receptor blockade (Fig. 8b), but caused significant reduction in survival despite P2X₃ receptor blockade (Fig. 8d), suggesting P2X₁ receptor activation might impart modest neurotoxicity. However, because the effects of $\alpha\beta$ Me-ATP were inconsistent and the P2X₁ antagonists only marginally decreased the neuronal vulnerability, these results are inconclusive. Unlike the selective agonist for P2X₁ and P2X₃ receptors, P2X₇ selective activation was highly neurotoxic (Fig. 8e) and the Bz-ATP-induced neuronal losses were completely blocked by selective P2X₇ blockade (Fig. 8f). Importantly, despite the suggestion that P2X₁ or P2X₇ receptor subtypes may affect striatal neuronal viability, as reported in the preceding paragraph, these receptor subtypes do not mediate the neurotoxic effects of HIV-1 Tat and/or morphine (Fig. 8b,d,f).

Discussion

Our findings provide evidence that the P2X receptor family mediates key aspects of Tat or morphine induced neurotoxicity. Pharmacological intervention with TNP-ATP indicated P2X receptor family involvement in Tat or morphine caused neuronal death, increases in neuronal $[Ca^{2+}]_i$, and reductions in dendritic length. Moreover, findings that Tat or morphine increase extracellular ATP levels, and that administration of exogenous ATP can mimic morphine's and/or Tat's effects provides further support that ATP acts as a critical intermediate in the neurotoxic actions of HIV-1, as well as those of morphine. Furthermore, recent studies demonstrate that levels of extracellular ATP are increased by HIV infection and are reportedly necessary for HIV replication (Hazleton et al., 2012). Interestingly, in

contrast with TNP-ATP (P2X₁₋₇ antagonist), the selective blockade of P2X₁₋₃ & 5-7 by PPADS was inherently neurotoxic—inferring a differential involvement of the P2X₄ receptor subtype, or perhaps multiple P2X subtypes, in influencing striatal neuron viability. Finally, unlike TNP-ATP, the selective blockade of P2X₁, P2X₃, or P2X₇ receptors were not able to prevent Tat or morphine neurotoxicity, further supporting our hypothesis that the neurotoxicity is selectively occurring through the activation of P2X₄ receptors. Our findings strongly support P2X receptor mediation of these events, and circumstantially support a role for the activation of P2X₄ receptors in these events. Furthermore, we speculate that the data suggests that one or more P2X receptors may be potential novel therapeutic targets for use in the prevention of HAND.

Our current mechanistic model proposes that morphine and/or Tat cause excess levels of extracellular ATP, which activates P2X₄ receptors (expressed by subpopulations of striatal neurons and glia) resulting in abnormally high levels of [Ca²⁺]_i and neuronal injury including decreased dendritic complexity and increased neuronal death. As determined in the present study, P2X₄ receptors appear to be widely expressed by neurons and microglia in the striatum. Moreover, prior studies indicate that astroglia (Kukley et al., 2001; Ashour and Deuchars, 2004) express P2X₄ receptors, including increased levels of expression by astrocytes in striatal lesions caused by 6-hydroxydopamine (Amadio et al., 2007). Collectively, the findings suggest that P2X₄ receptors may be pivotal in coordinating ATP-directed, bidirectional glial-to-neuronal communication among neurons, microglia, and astrocytes.

Our non-selective P2X antagonist, TNP-ATP, provides strong evidence that cation-permeable P2X subfamily of receptors mediates Tat and/or morphine neurotoxicity. While we cannot point to a specific P2X receptor from data obtained in these experiments, one thing to keep in mind is that TNP-ATP differs markedly in its affinity for different P2X receptor subtypes (Virginio et al., 1998; Coddou et al., 2011). Prior studies have evaluated human and rat P2X receptors; however, comparable studies have not been done in the mouse P2X receptor family. Although others have used concentrations greater than 300 nM TNP-ATP to examine P2X actions (Guo et al., 2007; Solini et al., 2007), we found 300 nM was sufficient to fully prevent the Ca²⁺ destabilizing or neurotoxic effects of Tat and/or morphine in our system. Based on published information, the 300 nM TNP-ATP concentration used in our study is likely to completely block P2X₁, P2X₃, and P2X_{2/3} receptor populations and markedly attenuate P2X₂ and P2X₄ receptor activity, while failing to significantly antagonize P2X₇ receptors. Though electrophysiological verification would help confirm this in our system, other investigators provide compelling evidence that 100 nM TNP-ATP preferentially blocks P2X₁, P2X₃, and P2X_{2/3} receptors (Dunn et al., 2000; Ma et al., 2004), while partially or failing to block P2X₂ and P2X₄ receptors, which have a lower affinity for TNP-ATP (Virginio et al., 1998). Also, it should be noted that the IC₅₀ values for P2X₅ and P2X₆ receptors have not been investigated, perhaps due to their reported inability to form functional homomeric ion channels in human cells (Collo et al., 1996).

The use of more selective antagonists showed that P2X₁, P2X₃, and P2X₇ receptor subtypes were not involved in HIV-1 or morphine related toxicity. While the absence of toxicity does not exclude the possibility that inflammation is not increased (Suzuki et al., 2011), heightened inflammation is generally associated with heightened neuronal injury in our cell culture model (Zou et al., 2011; Podhaizer et al., 2012). Despite some evidence that P2X₁ and P2X₃ receptor subtypes are involved in neuroinflammation and neurodegeneration (Apolloni et al., 2009), we did not see evidence for a role of these P2X receptor subtypes in the modulation of Tat or morphine neurotoxic interactions. In contrast to P2X₁ and P2X₃ receptors, the P2X₇ receptor is known to regulate microglial function, and has been shown

to modulate neuroinflammatory processes accompanying spinal cord injury, Alzheimer's disease, Parkinson's disease, inflammatory pain, neuropathic pain, trauma, multiple sclerosis, IL-1 β signaling, and NF- κ B signaling (Bai and Li, 2013). For the above reasons, we were surprised to find that P2X₇ receptors were not involved in the neurotoxic effects of Tat or morphine. One possible explanation is, with an EC₅₀ > 30 μ M (Jacobson et al., 2002; Hervás et al., 2005), P2X₇ receptors are not activated until ATP is present at very high concentrations. This is a much greater concentration than required to activate other P2X receptors, for which the EC₅₀ for ATP is 1–6 μ M (Lynch et al., 1999; Brône et al., 2007; Agboh et al., 2009). Accordingly, Tat and morphine may not release sufficient extracellular ATP to activate this subfamily of receptors.

HIV-1 Tat's neurotoxic actions have been authoritatively reviewed elsewhere (Mocchetti et al., 2012). Tat has been shown to interact with several membrane receptors, such as integrins, low-density lipoprotein receptor related protein (LPR), and the NMDA receptor (Liu et al., 2000; Li et al., 2008). While the literature points to NMDA receptors as major regulators of calcium levels during HIV-1 related neurotoxicity (Bonavia et al., 2001; Eugenin et al., 2007; Li et al., 2008; Buch et al., 2011), the present work suggests that P2X receptors may additionally contribute to the loss in neuronal [Ca²⁺]_i homeostasis caused by Tat. Furthermore, P2X receptors may affect AMPA and NMDA receptor levels and function (Gordon et al., 2005; Pankratov et al., 2009; Tai et al., 2010; Baxter et al., 2011), which suggests P2X receptors on neurons and glia may be exerting control over [Ca²⁺]_i through both direct and indirect effects. This concept has also recently been alluded to elsewhere in the context of HIV neuroinflammation (Tovar-y-Romo et al., 2013).

In contrast to HIV-1 Tat, morphine can be neuroprotective (Zou et al., 2011) or neurotoxic (Hu et al., 2002; Iglesias et al., 2003; Lim et al., 2004), depending on the cell type and context (Zou et al. 2011). At the concentration and times tested in our primary mixed-glial and neuronal cell culture system, continuous morphine exposure was typically neurotoxic. Importantly, P2X receptors have been shown to assist in regulating the actions of morphine (Tai et al., 2010; Zhou et al., 2010) with some evidence for the selective role of P2X₄ receptors in mediating morphine-mediated microglial migration, and in the development of morphine tolerance (Horvath and DeLeo, 2009; Horvath et al., 2010). Compelling evidence has recently been provided demonstrating that morphine-dependent expression of P2X₄ receptors by microglia is essential for the development of morphine-induced hyperalgesia (Ferrini et al., 2013). Our study adds to the growing list of examples where MOR and P2X₄ receptor systems may be functionally intertwined and is the first to show that P2X receptor subfamily plays a role in morphine-induced neuron injury. Although we have found that morphine can potentiate the neurotoxic effects of Tat (Zou et al., 2011), and postulated that P2X receptors might mediate the synergistic interaction, nothing can be inferred about a potential role of P2X receptors in the interaction *per se* since P2X blockade ameliorated the cytotoxic effects of both morphine and Tat alone.

Thus, the findings provide evidence implicating P2X receptors, and circumstantial evidence for the P2X₄ receptor in particular in HIV-1 Tat or morphine related neurotoxicity in primary neuron mixed glial co-cultured from mouse striata. Our work also concurs with findings from another laboratory demonstrating that P2X receptors mediate increases in neuronal [Ca²⁺]_i and the culling of dendritic spines in rat hippocampal neurons treated with supernatant from HIV infected monocytes (Tovar-y-Romo et al., 2013). Although further work is needed to expand the understanding of the underlying molecular pathways associated with cation-permeable, ligand-gated purinergic receptors, we propose that members of the P2X receptor subfamily may be valuable therapeutic targets for the treatment of neuroAIDS.

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Fig. 1. Immunocytochemical co-localization of P2X₄ receptors with striatal microglia and neurons from WT mice

(a–i) P2X₄ receptor immunofluorescence (green) is readily colocalized in microglia (red Iba1 immunofluorescence) (a–f). The neuronal marker, MAP2 (red fluorescence) in the bottom left panel and the P2X₄ receptor (green fluorescence); merged images (g–i). Microglia with an activated morphology display P2X₄ receptor immunofluorescence mostly at the surface of the cell (arrow) (a–c), while more quiescent microglia tend to display P2X₄ immunoreactivity within the cytoplasm and with less overlap with Iba1 (arrow) (d–f). Neurons (arrowheads) are also positive for the P2X₄ receptor, but the fluorescent product is less intense than in microglia (g–i). A cell possessing P2X₄ receptor, but not Iba-1,

immunoreactivity that is likely to be an astrocyte is denoted by an asterisk (**d–f**); all images (**a–h**) are at the same magnification as (**i**); cell nuclei were counterstained with Hoechst (*blue fluorescence*).

Fig. 2. Time-lapse studies show Tat + morphine-induced neurotoxicity is prevented by pretreatment with TNP-ATP

(a–b) Tat and/or morphine treatment caused significant neuron death compared to controls, while naloxone blocked the effects of morphine (a). Neurotoxicity was prevented in cells pretreated with TNP-ATP (300 nM). The TNP-ATP + Tat (T), TNP-ATP + morphine (M), TNP-ATP + T + M, TNP-ATP + T + M + naloxone (N), and TNP-ATP + ATP groups differed significantly from their corresponding groups that are lacking the antagonist (b). The data (a & b) are from the same experiment, but separated into two graphs for clarity (the control group in a and b is identical). The data represent the mean $\pm$ SEM from $n = 4$ experiments (* $p < 0.05$ vs. control treated cells, $\delta p < 0.05$ vs. Tat treated cells, $\#p < 0.05$ vs. morphine treated cells, $\$p < 0.05$ vs. corresponding groups lacking TNP-ATP; note, however, that the survival of neurons treated with TNP-ATP alone did not differ from control neurons).

Fig. 3. TNP-ATP pretreatment caused concentration-dependent reductions in Tat and/or morphine-induced neurotoxicity
 (a–c) Exposure to neurotoxic levels of morphine (500 nM) (M) (a), Tat (100 nM) (T) (b), and combined Tat and morphine (T+M) (c), were fully prevented by concurrent administration of 300 nM TNP-ATP, while lower TNP-ATP concentrations failed to or only partially blocked the neurotoxicity. The data represent the mean $\pm$ SEM from $n = 4$ experiments (* $p < 0.05$ vs. control treated cells, § $p < 0.05$ vs. morphine treated cells, \$ $p < 0.05$ vs. Tat treated cells, # $p < 0.05$ vs. Tat + morphine treated cells).

Fig. 4. TNP-ATP pretreatment negated Tat and/or morphine-mediated decreases in synaptodendritic injury and mean dendritic length

(a–f) Neurons in striatal neuron-glia co-cultures treated with medium alone (controls), ATP, or Tat (T) and/or morphine (M) showed significant decreases in mean dendritic length compared with controls (a). Treatment with TNP-ATP (300 nM) significantly increased the length of the average dendrite compared to corresponding treatments without TNP-ATP (b). The data (a & b) are from the same experiment, but separated into two graphs for clarity. The data represent the mean $\pm$ SEM from $n = 4$ experiments ($*p < 0.05$ vs. control treated cells, $\#p < 0.05$ vs. corresponding treatment group lacking a P2X receptor antagonist). Appearance of neurons exposed to Tat + morphine (c, e) and TNP-ATP + Tat + morphine

(d, f); TNP-ATP allays Tat and/or morphine-induced dendritic injury as denoted by reduced varicosities **(d, f)**. All images **(c–f)** are at the same magnification; MAP2 (*green immunofluorescence*) and Hoechst counterstained nuclei (*blue fluorescence*).

Fig. 5. Tat and/or morphine administration increased ATP levels in medium from neuron-glia co-cultures

Tat and/or morphine markedly elevated ATP levels at 30 min, while sustained increases, were seen at 1 h following Tat $\pm$ morphine exposure, but not with morphine alone. Although combined treatment with Tat and Morphine tended to increase ATP levels compared to Tat or morphine exposure alone, the effect was not significant. Tat alone significantly increases ATP levels at 2 h. Data represent the mean $\pm$ SEM from $n = 6$ experiments (* $p < 0.05$ vs. controls at the same time).

Fig. 6. ATP elicited concentration-dependent increases in $[Ca^{2+}]_i$

Neuron-glia co-cultures were treated cumulatively with increasing concentrations of ATP (at 2 min intervals). Despite some tendency for $[Ca^{2+}]_i$ to increase after applying 1 μ M ATP, significant increases are not evident until 10 μ M ATP is applied. Data represent the mean $\pm$ SEM from $n = 3$ experiments (* $p < 0.05$ vs. control and TNP-ATP pretreated cells).

Fig. 7. TNP-ATP attenuated increases in neuronal $[Ca^{2+}]_i$ caused by combined Tat and morphine exposure

Striatal neuron-glia co-cultures were treated with morphine at 2 min and Tat at 5 min. Images were taken at 1 s (0–3 min and 5–6 min) or at 30 s (3 to 5 min and 6–10 min) intervals. Significant rises in $[Ca^{2+}]_i$ were seen after 6 min of continuous exposure to combined morphine and Tat, and were significantly attenuated by pretreatment with TNP-ATP (300 nM). Data represent the mean $\pm$ SEM from $n = 3$ experiments ($^{\#}p < 0.05$ vs. control and TNP-ATP pretreated cells).

Fig. 8. Selective antagonists for P2X₁, P2X₃, and P2X₇ subtypes do not block Tat + morphine-induced neurotoxicity

(a–f) Striatal neuron-glia co-cultures treated with Tat, morphine, Tat + morphine, and Tat + morphine + naloxone exhibited significant neuronal death compared to controls (a, c, e). Tat and/or morphine-induced neurotoxicity was not prevented by administering P2X₁ (NF499) (300 nM), P2X₃ (A-31749) (1 μM), and P2X₇ (BBG) (1 μM) antagonists; while NF499, A-31749, and BBG alone had no effect on neuronal survival (b, d, f). All data represent the mean ± SEM from n = 4 experiments (*p < 0.05 vs. controls, #p < 0.05 vs. Tat, \$p < 0.05 vs. morphine, δp < 0.05 vs. Tat + morphine + naloxone, §p < 0.05 vs. BzATP).