2014

The Role of P2X Receptors in HIV and Opiate-Related Neurotoxicity

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The Role of P2X Receptors in HIV and Opiate-Related Neurotoxicity

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

by

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May 2014
Acknowledgements

To begin with, I would like to say this is really a list of people and things that I would not be here without. So beyond an acknowledgment I would like to thank each and every one of you for being and a part of my Ph.D. experience and supporting me until the very end.

I would like to thank my Ph.D. advisor, Dr. Kurt Hauser. I appreciate the opportunity you have given me to be a member in your lab. I have learned so much from the lab environment and from you in particular. Although we may not have agreed on every single issue over the 6 years, your input has continually pushed me to be a better person and scientist.

I would also like to thank my committee members: Dr. Pamela Knapp, Dr. Steve Negus, Dr. Michael Fox, and last (but certainly not least!) Dr. Bill Dewey. You have all been there for me at different times and for various reasons over the years. A special thanks to Dr. Negus for his expertise in pharmacology principles and also to Dr. Dewey for not only his expertise but also his never-ending support in my timely and successful completion of the program. I also would like to thank Dr. Dewey for not only being a great committee member but a wonderful chair of our department. You do a great job at cultivating a positive and productive environment for everyone to work in.
I would like to thank all of the members in Dr. Knapp and Dr. Hauser labs, as well Dr. Nazira El-Hage. All of you have been there for both the good and bad over the years, to listen to whatever crazy things may be going on with me and share in the laughter as well as the tears. I could not have possibly made it through this experience without your support. Nazira: you have not just been a good co-worker but have also become a great friend. I appreciate your support and honesty in conversation over the years. Elizabeth Podhaizer (a.k.a. Zippy): beyond being great to work with in lab, you have been a better friend than I could have ever imagined or asked for. I can only hope that I have been able to be as good of a friend to you. Yun Kyung Hahn: Most of all, thanks for not killing me, I know it has been a struggle (laughing out loud to myself now)! Also, thanks for introducing to me to SpaWorld, along with other cool Korean things like karaoke and Gangnam style dance! Despite any past differences, I appreciate the daily excitement you bring to the lab and your friendship. Other lab members, I would like to give a special thanks to: Patrick Zou and Gigi Ji (love you guys!), Sylvia Fitting, Seth Dever, Joyce Balinang, and Will Marks. Also I would like to thank Kim Samano, a past lab member, who during her stay was also a great friend and co-worker.

I would like to gratefully thank the Rassendren and De Konick laboratories for helping to provide me with initial breeding pairs of P2X4 KO mice. I would also like to thank funding sources: RO1 DA019398 (KFH), T32 DA007027 (WLD)

I would like to thank the Department of Pharmacology and Toxicology at VCU, including my fellow graduate students, faculty members and great staff that make the department what it is. I would also like to thank MaryPeace McRae, while not a member in the department has also been a great friend. I would like to thank Subway and diet cokes as well. For those that know me, you
have probably seen me in line at Subway or in the act of eating a Subway sandwich. I know, especially my lab mates have observed my addiction to diet coke and would agree that acknowledging its role in my graduate work is not unjustified. They have both been a staple throughout my graduate career and I am not sure I could have made it without them.

I would like to give huge thanks my entire family. To my mom, thank you for your continued unwavering support throughout this crazy experience, and for being a positive and uplifting force in my life. To my dad, thank you for pushing me as child and helping to instill a strong work ethic in me, which has been a critical skill for graduate school. To my Aunt Mary Jeanne and Uncle Bill, who have really been like a second set of parents than an aunt and uncle, thanks for inspiring me to get my Ph.D. and for always encouraging me to do more than I thought I could do in the first place. I would like to thank my Uncle Tom, Aunt Tina, and Cousin Amanda for their support as well. I would also like to thank my grandparents Ira and Carol Davis, who unfortunately both passed away in December 2010, while I was in graduate school, but were the best grandparents I could have asked for and always supported me in every endeavor and I know would be so proud of me right now.

Finally I would like to thank my ex-fiancé Jason Ventrella who, over the past 9.5 years, has been the best friend I’ve ever had and my strongest supporter. Thanks for putting up with me for so long and being like a rock that I could count on more than my next breath. Oh and I can’t forget our dog Mojo Ventrella, thanks for your unconditional love and the joy that you have given and created over the years.
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Clarification of Contributions

Chapter 2: This work has been previously published elsewhere (Sorrell and Hauser, 2013)

Chapter 5:

Heat maps representing gene array data were generated by Blair Costin and Megan O’Brien (Figure 30)

RT-PCR data was generated by Seth Dever (Figure 31)
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List of Abbreviations

3NT: 3 Nitrotyrosine
ACh: Acetylcholine
AIDS: Acquired immunodeficiency syndrome
ATP: Adenosine triphosphate
asODN: Antisense oligonucleotide
BBB: Blood to brain barrier
bps: Base pairs
CNS: Central nervous system
DNA: Deoxyribonucleic acid
Env: Envelope
GABA: Gamma-aminobutyric acid
Gag: Group specific antigen
GIRKs: G-protein-activated inwardly rectifying K⁺ channels
GPCR: G-protein coupled receptors
HAART: Highly active antiretroviral therapy
HAND: HIV associated neurocognitive disorders
HIV: Human Immunodeficiency Virus
Iba1: Ionized calcium binding adapter molecule 1
KO: Knock-out
MAP2: Microtubule-associated protein 2
Nef: Negative factor
NMDA: N-methyl-D-aspartate
NMDG: N-methyl-D-glucamine
NNTC: National NeuroAIDS Tissue Consortium
P2X: Purinergic receptor family
PLCβ: Phospholipase Cβ
Pol: Polymerase
PPADS: Pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid
Rev: Regulatory for expression of viral proteins
RNA: Ribonucleic acid
ROS: Reactive oxygen species
TAT: Trans-activator of transcription
TNP-ATP: 2’,3’-O-(2,4,6-Trinitrophenyl) adenosine 5’-triphosphate
Vif: Viral infectivity factor
Vpr: Viral protein r
Vpu: Viral protein u
WT: Wild type
ABSTRACT

THE ROLE OF P2X RECEPTORS IN HIV AND OPIATE-RELATED NEUROTOXICITY

By: Mary E. Sorrell Ph.D.

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

Virginia Commonwealth University, 2014
Major Director: Kurt F. Hauser, Professor, Pharmacology and Toxicology

Emerging evidence suggests that opioid drugs can exacerbate neuroAIDS. Microglia are the principal neuroimmune effectors thought to be responsible for neuron damage in HIV-infected individuals, and evidence suggests that drugs acting via opioid receptors in microglia aggravate the neuropathophysiological effects of HIV. The P2X family of ATP activated ligand-gated ion channels regulates key aspects of microglial function. In addition, opioid-dependent microglial activation has been reported to be mediated through P2X₄ signaling, prompting us to investigate P2X receptors contribution to the neurotoxic effects of HIV and morphine. In vitro experiments showed treatment with TNP-ATP prevented the neurotoxic effects of morphine and/or HIV Tat, or ATP alone in a concentration dependent manner. This evidence suggests P2X receptors mediate the neurotoxic effects of these insults in striatal neurons. P2X₁, P2X₃, and P2X₇ selective receptor antagonists did not prevent Tat- and/or morphine-induced
neurotoxicity, implying cellular pathways activated may not involve these subtypes. Cells from P2X4KO mice show that activation of the P2X4 receptor on glia are necessary to cause Tat and/or morphine toxicity. However, data implied that baseline neuronal function may be altered due to lack of P2X4 receptor expression, and also gave evidence for altered Tat and morphine cellular signaling when the two are given in combination versus alone. Surgeries were performed on P2X4 KO and WT mice, which received intrastriatal Tat injections and morphine and/or naltrexone pellets. WT mice showed significant increases in inflammatory markers when treated with Tat and/or morphine. Increases in inflammatory markers were not seen in P2X4 KO mice, implying P2X4 receptors play a role in neuroinflammation resulting from Tat and/or morphine. Finally, human tissue samples from the National NeuroAIDS Tissue Consortium were analyzed. Changes in P2X5 and P2X7 mRNA were found in microarray data, but only changes in P2X7 mRNA levels were confirmed by RT-PCR. No changes in P2X4 mRNA levels were detected. Our experiments indicate the P2X receptor family contributes to Tat- and morphine- related neuronal injury, and reveal that members of the P2X receptor family, especially P2X4, may be novel therapeutic targets for restricting the synaptodendritic injury and neurodegeneration that accompany neuroAIDS and opiate abuse.
CHAPTER 1: Introduction

HIV

Human immunodeficiency virus or HIV is a member of the lentivirus genus, which is in the retroviridae family. It is also the virus that is known to be the causative agent of acquired immunodeficiency syndrome or AIDS. HIV-1 is the most common subtype of HIV around the globe except in some specific regions of Africa, which have a higher prevalence of HIV-2. As such, I will only be referring to the HIV-1 strain throughout the document. HIV-1 variants are classified into four groups: Group M for major, Group O for outlier, and two new groups, Group N and Group P. Group M also consists of at least nine genetically distinct subtypes, or clades, referred to as A, B, C, D, F, G, H, J and K (Hemelaar et al., 2011). It is important to remember that genetically different hybrid viruses can be made from two different subtypes meeting in an infected individual. This new hybrid virus can then infect other individuals providing a mechanism for new strains of virus to evolve, thus the number of groups and clades can change often (Spira et al., 2003; Taylor et al., 2008).

The HIV virion consists of a circular lipid bilayer that contains viral RNA and necessary enzymes (Fig. 1). Retroviruses are equipped with a unique enzyme, reverse transcriptase, which has the ability to change viral RNA into DNA once the virus has entered the host cell. Viral DNA is then translocated to the nucleus of the cell and can be integrated into the cellular genome. At this point viral and cellular proteins work together in order to express viral RNA that can either be made into viral proteins or new genomic RNA that can be assembled to make new viral particles (Fig. 2) (Barré-Sinoussi, 1996).
Figure 1. The HIV virion. The viral envelope consists of a lipid bilayer membrane (derived from the host cell) and envelope protein complexes (gp120 + gp41) that will help facilitate viral entry. Inside the viral envelope is the viral capsid that contains two single stranded RNA genomes and three viral enzymes (protease, reverse transcriptase, and integrase) (Barré-Sinoussi, 1996).
Figure 2. The HIV life cycle. HIV virions first enter the host cell. Envelope proteins mediate fusion of the viral lipid envelope with the cellular membrane. After entering the cytoplasm, reverse transcription occurs. Viral DNA can now be integrated into the host genome. Production of new virus particles is initiated by the transcription of new viral RNAs. The envelope glycoproteins are synthesized as a precursor protein in the endoplasmic reticulum/Golgi compartments and processed by a cellular protease. Assembly of new virions occurs at the plasma membrane immediately before release of new virions (Barré-Sinoussi, 1996).
**Figure 3. A simplified HIV gene map.** The HIV provirus is known to encode for at least 9 different proteins. These proteins can be divided into three classes based on function: structural (Gag, Pol and Env), regulatory (Tat and Rev), and accessory (Vpu, Vpr, Vif and Nef) (Ellis et al., 2007).
HIV in the CNS

Although HIV does not directly infect neurons in the central nervous system (CNS), the virus can infect other types of CNS cells that then indirectly lead to neuronal dysfunction. The main cell types infected by HIV in the CNS are microglia and perivascular macrophages. Microglia and perivascular macrophages are the immune cells of the brain, and are believed to originate from monocytes. Monocytes can enter the brain during embryogenesis, and then differentiate based on cues in the surrounding microenvironment (Jordan and Thomas, 1988; Guillemin and Brew, 2004).

Perivascular macrophages are located close to endothelial cells and the peripheral circulation. These cells are thought to be involved in initial CNS infection that can then infect resident microglia. Microglia can exist in one of two states: ramified or resting, and ameboid or activated. Ameboid microglia often have a spherical morphology and lack processes. They can travel long distances, and are capable of replication and phagocytosis. Ramified microglia have extended processes that constantly survey their surroundings. Microglia can also exist in other states, but these usually only occur in response to a disease state. One example is the multinucleated giant cell, which can occur in the CNS due to HIV infection (Boche et al., 2013).

HIV infection can cause microglia to transform from resting to activated states. Once in an activated state, microglia can have phenotypic differences based on the specific stimulus in which they are responding. This is similar to the different activation states in peripheral macrophages, M1 and M2, where response also differs based on what stimulus is present. However, regulation of active states in microglia has not been as well characterized (Boche et al., 2013; Schwartz et al., 2013). In the case of HIV infection, microglial activation leads to
cytokine and chemokine release that then activates molecular pathways in neighboring microglia as well as in nearby astrocytes and neurons, which can lead to neuronal injury and death. Also, HIV proteins from infected microglia can be released into the intracellular space and directly activate neurotoxic pathways.

HIV can also infect astrocytes, although only about 5% have been shown to exhibit infection (Eugenin and Berman, 2007). Astrocytes make up about 70% of the brain and contribute to maintaining neuron homeostasis by buffering extracellular glutamate levels, regulating neurotransmitter concentrations, releasing proinflammatory molecules, contribute to the integrity of BBB, and perform other supportive functions. As such, astrocyte dysfunction in even a small population may lead to abnormal neuron signaling and function (Eugenin and Berman, 2007; Eugenin et al., 2011). Astrocytes usually only have productive infection that occurs for a brief period after which they become latently infected (Brack-Werner et al., 1992; Nath et al., 1995; Eugenin and Berman, 2007; Eugenin et al., 2011). However, the ability of astrocytes to host productive viral infection may be restored by the presence of inflammatory molecules (Carroll-Anzinger and Al-Harthi, 2006). Latent viral infection is where the cell is still infected but new virus is no longer being made. This allows for astrocytes to behave as HIV reservoirs and may partially explain why CNS complications still exist even after the advent of highly active antiretroviral therapies (HAART).

The HIV virus is not thought to infect neurons or oligodendrocytes. While, the infection of oligodendrocytes by the HIV virus is somewhat controversial, there is no clear evidence to support that it occurs (Kramer-Hämmerle et al., 2005). Therefore, the main cells that are thought to be responsible for neuronal toxicity are the infected microglia cells that release both viral and cellular toxins, which then affect the function of nearby astrocytes and neurons.
Initial HIV infiltration into the CNS is thought to occur soon after infection (Annunziata, 2003). The most accepted theory of HIV infiltration into the CNS is the Trojan horse hypothesis (Georgsson, 1994; Liu et al., 2000), where infected perivascular macrophages are able to cross the blood to brain barrier and then infect microglia in the CNS (Figure 4). At this point, infected microglia may release inflammatory molecules affecting nearby cells or produce more virus that can then infect other microglial cells thereby spreading the virus and creating bystander damage. Inflammatory molecules can affect neighboring microglia as well as astrocytes. Astrocytes can also become activated in response to these inflammatory compounds and produce their own inflammatory products (El-Hage et al., 2006), as well as have a decreased ability to provide metabolic and trophic support to the neuron. Between indirect toxicity mediated by other neural cell types and direct insults to the neuron via release of proinflammatory molecules and viral proteins, neuronal damage and loss often occur due to HIV infection in the CNS (Fig. 4).
Figure 4. HIV Interactions in the CNS (the Trojan Horse hypothesis). HIV infected macrophages are able to carry the virus across the blood to brain barrier. These cells can then infect resident microglia in the CNS. Infected macrophages and microglia will then release virus as well other cytotoxic molecules including both viral and cellular proteins. The cytotoxic proteins can then affect neighboring neural cells. Cytotoxic insult to neighboring astrocytes leads to astrogliosis that can cause further release of proinflammatory molecules as well as cause a decrease in the ability of the astrocyte to give metabolic and trophic support to the neuron. Finally the cytotoxic proteins released from the microglia or astrocytes can directly interact with the neuron to activate cellular pathways. Overall this inflammatory environment leads to neuronal dysfunction and death (modified from Hauser et al., 2005).
HIV Associated Neurocognitive Disorders (HAND)

The above cellular mechanisms eventually lead to HIV associated neurocognitive disorders, or HAND. Presently, about 50% of HIV infected individuals suffer from HAND (Kraft-Terry et al., 2010; McArthur et al., 2010; Harezlak et al., 2011). HAND was first recognized in 1981 and has evolved to be characterized by three major categories: 1) asymptomatic neurocognitive impairment (ANI) 2) HIV-associated mild neurocognitive disorder (MND) and 3) HIV associated dementia (HAD). Respectively, these disorders range from mild to severe neurocognitive abnormalities that are exhibited by the infected individual (Fig. 5). HAD, the most severe form is a subcortical dementia that causes a marked impact on daily activities including distinct changes in cognition (e.g., learning, memory, attention, and executive function), motor coordination (e.g., psychomotor slowing and hypertonia), and behavior (e.g., apathy, irritability, emotional lability, and affective blunting). MND is a mild cognitive dysfunction that interferes with daily living to a limited extent, MND is characterized by mild but significant cognitive dysfunction, pronounced motor impairment and is associated with decreased adherence to treatment regimens, greater levels of unemployment. Finally, ANI is a subclinical cognitive dysfunction that does not interfere with daily function (Heaton et al., 1994; McArthur et al., 2005; Crossley and Brew, 2013).

Currently there is no agreement on the exact battery of tests used to evaluate these disorders. However, it is recommended that the following areas of neurocognition be tested: verbal/language, attention/working memory, abstraction/executive function, learning/recall, speed of information processing, and motor skills (Clifford and Ances, 2013; Mind Exchange Working Group, 2013). Besides psychological testing, other endpoints associated with inflammation (e.g. microglia activation, cytokine levels) and cellular morphology changes (e.g.
decreased synaptic and dendritic densities) can be used as biomarkers and signs of HAND. These tend to correlate better with neurocognitive dysfunction than viral load (Wiley and Achim, 1994; Masliah et al., 1997; Sá et al., 2004; Lyons et al., 2011; Kamat et al., 2012; Burdo et al., 2013; Yuan et al., 2013).
Figure 5. Diseases encompassed by HAND. HAND is comprised of several diseases that exhibit a range of symptoms from mild (ANI) to severe (HAD), with ANI characterized by moderate symptoms (Antinori et al., 2007).
NeuroAIDS in the Post-HAART Era

Although the incidence of neurocognitive impairment did not decrease after the advent of HAART, the proportion of individuals suffering from more severe symptoms decreased, while the number of patients experiencing minor symptoms has increased. Although many severe neurological complications that were once associated with HIV associated dementia (HAD) and evident as HIV encephalitis (HIVE) post mortem occur less frequently, neuroinflammation and microglial activation are still evident but to a lesser degree (Anthony and Bell, 2008; Zhou and Saksena, 2013). Another factor that may increase the incidence of HAND is that HIV infected individuals are living longer with HAART intervention.

HAART therapy consists of three main classes of antiretroviral therapies: nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), and protease inhibitors (PIs). Early entry inhibitors and integrase inhibitors may also be used (McGee et al., 2006). There is some evidence for HAART to decrease neurological complications caused by CNS infection (Kaul, 2009), however it does not provide complete protection against neurological symptoms (McArthur et al., 2005; Boissé et al., 2008; Brew et al., 2009). Although peripheral viral loads are well controlled by HAART therapy, the virus may exist in higher levels in CNS due to viral reservoirs and poor HAART penetrability of the BBB (Jevtović et al., 2009). NRTIs tend to penetrate the CNS better than NNRTIs or PIs, however they are quickly transported out of the CNS via efflux transport mechanisms. NNRTIs have varying abilities to penetrate the BBB, and PIs exhibit poor CNS penetration due to high levels of protein binding in the plasma (McGee et al., 2006). The early entry inhibitor, Maraviroc, and the integrase inhibitor, Raltegravir, both have moderate abilities
to cross the BBB (Tan and McArthur, 2012). CNS viral reservoirs may also give rise to viral mutations that enhance viral drug resistance (Boissé et al., 2008).

**HIV Viral Proteins**

As shown in Figure 3, the HIV genome encodes for 9 different proteins, of these gp120, Tat, Nef, and Vpr can be shed and directly lead to neurotoxicity. Tat and gp120 have been primarily focused on in the literature (Jones and Power, 2006). Tat is the HIV protein that will be investigated in the following studies. Once a cell becomes infected and provirus is present, only PIs are of use to combat HIV infection and these drugs act downstream of Tat production (Johnson et al., 2013). Hence, Tat may have a greater potential than gp120 to participate in inflammatory interactions that lead to neuronal complications.

**HIV-1 TAT**

Tat, standing for ‘trans-activator of transcription’, is one of the first proteins produced after HIV integration. Tat is encoded by 2 exons. The first exon encodes amino acid 1-72, including a proline-rich domain, cysteine-rich domain and basic domain. Exon 2 is the RGD-containing C-terminal domain, which can vary in length depending on the viral isolate. The full protein can range anywhere from 80 to 103 amino acids (Debaisieux et al., 2012). Tat is a potent transactivator of HIV-1. The first 72 amino acids (Tat1-72) have full transactivating abilities. This cysteine-rich region is necessary for Tat to form metal-linked dimers to resist proteolytic digestion and is essential for Tat function (Frankel et al., 1988; Garcia et al., 1988). The basic domain is important for nuclear translocation (Endo et al., 1989). We used Tat1-86, in our studies.
It can be secreted by infected cells at significant levels into the extracellular space and remain intact and functional (Westendorp et al., 1995; Hudson et al., 2000).

Other cells that are not infected by virus can still have direct contact with Tat. It can either be taken up into the cell (Chang et al., 1997; Liu et al., 2000) or interact with receptors on the cell surface (Albini et al., 1996; Ghezzi et al., 2000; Liu et al., 2000). In neurons, Tat has been proposed to undergo receptor mediated endocytosis that may be mediated by direct binding to several proteins, including CD26 (Gutheil et al., 1994), LDR (low density lipoprotein receptor) (Liu et al., 2000), extracellular matrix associated heparan sulfate proteoglycans (HSPG) (Chang et al., 1997), and surface integrins (Barillari et al., 1993). Tat also has the ability to activate the NMDA receptor (Magnuson et al., 1995; Eugenin et al., 2003; King et al., 2006a; Li et al., 2008). Overactivation of NMDA receptors leads to high Ca\textsuperscript{2+} levels, which can lead to cell death (Cheng and Reynolds, 1998; Haughey et al., 1999). The mechanism of NMDA receptor activation by Tat is unknown. Directly toxic pathways activated by Tat are thought to result in dendritic loss and cell death (Kruman et al., 1998; Bonavia et al., 2001; Haughey et al., 2001; Eugenin et al., 2003; Kim et al., 2008) via varying mechanisms and pathways (Singh et al., 2004; Kim et al., 2008).

Tat induced changes in intracellular calcium lead to endoplasmic reticulum (ER) and mitochondrial stress, which then can mediate cell death pathways (Kruman et al., 1998; Haughey and Mattson, 2002; Caporello et al., 2006). Mitochondria will release factors, such as cytochrome c, that activate caspase-mediated apoptosis. Apoptosis is programmed cell death, characterized by decreases in cell volume, membrane blebbing, chromatin condensation, and DNA fragmentation (Wyllie et al., 1980). It has also been shown that endonuclease G (endoG)
can also be released from mitochondria and activate apoptosis via caspase independent pathways (Li et al., 2001; Singh et al., 2004; Hauser et al., 2006).

Another mechanism for Tat induced neuroinflammation and cell death is its ability to inhibit autophagy pathways (Zhou and Spector, 2008; Li et al., 2011; Hui et al., 2012). Autophagy is essential for cell homeostasis and adaptation to environmental stresses. It plays a vital role in innate and adaptive immune mechanisms, including resistance to pathogen infection. Inhibition of autophagy can lead to increased necrosis, which would normally not occur. This type of cell death is associated with cell lysis and leakage of cell contents into the extracellular space, which leads to local inflammation and damage to the surrounding tissue (Ryter et al., 2014).

Indirect neuronal toxicity is caused through the actions of Tat on astrocytes and microglia that in turn release cellular toxins into the environment that then lead to neuronal toxicity. Astrocytes with increased levels of Tat have been shown to have a decreased ability to buffer glutamate levels (Eugenin et al., 2003; Zhou et al., 2004) and to excrete higher levels of NO, which can also initiate apoptotic events (Liu et al., 2002). Astrocytes treated with Tat have also been shown to release CCL2 (also known as MCP-1). CCL2 is important for the recruitment of monocytes to sites of inflammation and has been shown to be elevated in individuals with HAD (Conant et al., 1998; King et al., 2006a).

Treatment of microglia with Tat has also been shown to cause an increase in the amount of NO synthesis (Polazzi et al., 1999) as well as the release of proinflammatory cytokines and chemokines like CCL2, CXCL8, CXCL10, CCL3, CCL4 and CCL5, IL-1β and TNF-α. Activation of ERK1/2 MAPK, PI3K and p38 MAPK pathways have been shown to be involved
(Chen et al., 1997; Mayne et al., 2000; Sheng et al., 2000; D'Aversa et al., 2004). Tat can also cause pertussis toxin sensitive Ca\(^{2+}\) fluxes in microglia which implies activation of G\(_i\) coupled GPCRs is occurring. This is important because chemokine receptors are G\(_i\) coupled GPCRs, and activation of chemokine receptors can serve as chemoattractants to recruit other microglia (Albini et al., 1998).

**Opioid Signaling in NeuroAIDS**

Intravenous drug use 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). Opiates can also cause an increase the frequency and severity of NeuroAIDS (Arango et al., 2004; Anthony et al., 2005; Meyer et al., 2013; Smith et al., 2014). Heroin is one the most used drugs by injection drug users, with morphine being its major bioactive substrate. When taken, heroin quickly undergoes deacetylation to morphine via first pass metabolism. In the following studies we have chosen to use morphine to investigate HIV and opiate interactions. Please note that opiates are exogenous compounds that activate receptors, and opioids reference the family of receptors that are activated.

Opiate exposure can intrinsically alter neuropathogenesis by directly affecting neuronal, astrocytic and microglial function (Hauser et al., 2012) and 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). Morphine has been shown to increase the rate of viral replication in fetal brain cells co-cultured with a chronically infected monocytic cell line (Peterson et al., 1994).
This effect may be due to increased levels of CXCR4 and CCR5, which are known to be HIV co-receptors. HIV co-receptors enhance viral binding and subsequent entry into the cell. Greater numbers of cells being infected can then lead to increased viral replication as well as increased neuropathogenesis (Steele et al., 2003).

Aside from any neuroinflammation that may be caused directly by HIV infection, chronic morphine exposure on its own affects microglia function (Song and Zhao, 2001; Watkins et al., 2005). Chronic morphine treatment can activate μ opioid receptors on glia, leading to increased glial activation, which can be blocked by treatment with minocycline. Minocycline is a tetracycline derivative that is mainly used as a broad acting antibiotic. However, it has been shown to block microglial activation via mechanisms unrelated to its antimicrobial actions (Tikka et al., 2001; Cui et al., 2008; Huang et al., 2014). This glial activation can then lead to subsequent increases in the release of nitric oxide and proinflammatory cytokines (Chao et al., 1994; Peterson et al., 1998). Our own lab has also shown that morphine may alter microglial activity by altering ROS (reactive oxygen species) production levels; and can increase microglial motility by increased chemokine and cytokine release from astrocytes. (El-Hage et al., 2006; Turchan-Cholewo et al., 2009), This can then lead to further exacerbation of the symptoms seen in HIV neurodegeneration. Another possibility is that morphine may lead to aberrant neuron-glia communication (Johnston et al., 2004). Regardless of the scenario, glia are key contributors to HIV-1 and morphine-induced neurotoxicity. In fact, studies show that neuronal toxicity associated with HIV-1 and morphine is greatly reduced in the absence of glia, as well as in the presence of glia taken from μ opioid receptor knockout mice (Zou et al., 2011). This provides in vitro evidence that morphine, via glial μ opioid receptors, imparts significant neurotoxicity.
There are many cellular outcome measures that HIV-1 Tat and morphine can affect, including the ability of astrocytes to buffer glutamate, destabilize \([\text{Ca}^{2+}]_i\), and increase the release of ROS and cytokines. Despite the evidence, the underlying signaling pathways have not yet been identified. Moreover, different cellular targets and cell types can be differentially affected. The effects of opiates and HIV differ in a cell type, age dependent, and a regionally variable manner. A greater understanding of cell specific pathways is needed to find more effective treatments for HIV and opioid-related problems with neurocognition.

**Opioid Receptors**

Opioid receptors are classified as GPCRs (G-protein coupled receptors), which are predominately coupled to pertussis toxin-sensitive \(G_i/G_o\) proteins. Upon activation, these large, 7 transmembrane spanning receptors transduce their signal by second messenger signaling inside the cell. Depending on a variety of factors including the particular G-protein involved, RGS proteins, and other factors, GPCRs can act via multiple cellular pathways. Common events downstream of opioid receptors include the inhibition of adenylyl cyclases and voltage gated \(\text{Ca}^{2+}\) channels, and the activation of GIRKs (G protein-activated inwardly rectifying \(K^+\) channels) and PLC\(\beta\). There are three classical opioid receptors, \(\mu\) (MOR), \(\kappa\) (KOR), and \(\delta\) (DOR). More recently a fourth member, nociception/orphanin FQ (NOP), has been added. Endogenously these receptors are activated by peptides that fall into three main families, enkephalins, dynorphins, and endorphins, which derive from four precursors: proenkephalin, prodynorphin, pro-opiomelanocortin, and nociceptin/orphanin FQ. These peptides are released from neurons upon
stimulation in order to activate opioid receptors on nearby cells (Clapham and Neer, 1997; Waldhoer et al., 2004; Koppert, 2007; Dreborg et al., 2008; Sundström et al., 2010).

Aside from the endogenous peptides that naturally activate opioid receptors, there are many exogenous ligands that can activate this receptor system as well. In general agonists that selectively activate MOR or DOR are considered to be analgesic and rewarding, and those that are selective for the KOR tend to be dysphoric. Opiates are drugs of choice for the treatment of chronic pain. However, tolerance is caused by chronic opiate use. Classically, tolerance is thought to occur through desensitization of the receptor mediated by GRK (G protein coupled receptor kinases) receptor phosphorylation and recruitment of β arrestin, which will then bind to the phosphorylated receptor and block GPCR signaling. β arrestin can initiate subsequent endocytosis, leading to an overall reduction in the level of opioid receptors present at the cell membrane (DeWire et al., 2007). However, even though morphine induces tolerance, down regulation of MOR levels does not occur. Thus receptor desensitization and uncoupling to downstream signaling pathways may also play a significant role in tolerance. Over time, tolerance often leads to dependence and addiction (Waldhoer et al., 2004).

The Basal Ganglia: Where HIV-1 and Opiate Interactions Overlap

HIV preferentially targets the basal ganglia, and much of the neurotoxicity is mediated by HIV-infected or activated glia (Persidsky and Gendelman, 2003; Anthony et al., 2005; Kraft-Terry et al., 2010; McArthur et al., 2010; Harezlak et al., 2011; Steinbrink et al., 2013). The basal ganglia is also a site where many drugs of abuse have their effects and, as a result, may be a preferential area to look for HIV and opioid interactions (Nath et al., 2001; Nath, 2002; Nath et
al., 2002). The striatum, the largest component of the basal ganglia, has a very high density of \( \mu \) opioid receptors (Kuhar et al., 1973; Arvidsson et al., 1995; Mansour et al., 1995; Wang et al., 1999). Hence we have investigated HIV and opioid interactions in the striatum in all of our murine model studies (both \textit{in vitro} and \textit{in vivo}) (Sorrell and Hauser, 2014).

**Purinergic Receptors**

Purinergic receptors are characterized by the purine ligands that activate them, with the P1 receptor family being activated by adenosine and the P2 receptor family being activated by ATP. The P2 receptor family can be further subcategorized into P2X and P2Y receptor families, which are characterized according to receptor type: ligand-gated ion channels and GPCRs, respectively. The P2Y family consists of 8 members: P2Y\(_1\), P2Y\(_2\), P2Y\(_4\), P2Y\(_6\), P2Y\(_{11}\), P2Y\(_{12}\), P2Y\(_{13}\), P2Y\(_{14}\). Some numbers in the nomenclature appear to be absent (e.g. P2Y\(_3\), P2Y\(_5\), P2Y\(_7\), etc.) due to the fact that some receptors originally classified as P2Y later turned out to not be purinergic receptors and had been erroneously classified (Abbracchio et al., 2003). In the following work I will be focusing on P2X receptors. P2Y receptors have been reviewed in detail elsewhere (Weisman et al., 2012b).

There are currently seven known members of the P2X receptor family, P2X\(_{1-7}\). All of these are ligand gated ion channels having nearly equal permeability to Na\(^+\), K\(^+\), and significant permeability to Ca\(^{2+}\) (Ralevic and Burnstock, 1998; Khakh, 2001; North, 2002). Ca\(^{2+}\) permeability is similar to that of the NMDA receptor (Egan and Khakh, 2004). P2X ligand gated ion channels consist of 3 subunits that come together to form the ion channel. Each subunit consists of two trans-membrane domains and a large extracellular loop, with both the N and C
terminus being located in the cytosol (see upper left hand corner of Fig. 2) (Khakh and North, 2006). Three molecules of ATP are able to bind the receptor resulting in opening of the ion channel. There is evidence for the binding of ATP to occur in between two subunits and no evidence that less than 3 molecules of ATP can illicit partial channel activity (Browne et al., 2010; Coddou et al., 2011). The EC$_{50}$ of ATP to activate these receptors ranges from 1-10 µM, except in the case of P2X$_7$ receptors, which have a much higher EC$_{50}$ value of approximately 100 µM. Trace metals such as zinc and copper can also modulate the EC$_{50}$ values for P2X receptors, causing an increase or decrease depending on the metal and the receptor subtype (Acuña-Castillo et al., 2000; Khakh and North, 2006). Interestingly, upon prolonged ATP stimulation (several seconds), several of the P2X sub-members (P2X$_2$, P2X$_4$, and P2X$_7$), are able to undergo pore dilation. Larger cations, such as NMDG (N-methyl-D-glucamine) and even ATP itself, can also flux through the ion channel. At this time it is not clear how molecules that move through the dilated pore may affect cellular signaling pathways (Chessell et al., 1997; Yan et al., 2008; Samways et al., 2012).
Figure 6. Purinergic Receptor Family Outline and P2X Receptor Cartoon. Purinergic receptors are broken down into two subfamilies, P1 and P2, which are activated by adenosine and ATP respectively. The P2 subfamily is further broken down into P2X (ligand gated ion channels) and P2Y (GPCRs) receptors. Red highlighting denotes receptor subtypes that are most relevant to the work discussed in this document. Diagram in upper left hand corner depicts the structure of a P2X subunit and subunit confirmation in the membrane. A subunit consists of two trans-membrane domains, and a large extracellular loop, where 3 subunits come together to form functional ligand-gated ion channel (Khakh and North, 2006).
P2X₄ Receptors

The P2X₄ receptor undergoes slow desensitization and its receptor activity can be modulated by trace elements such as Zn²⁺ that decrease the EC₅₀ for ATP from approximately 7 µM to 2.5 µM. The zebrafish P2X₄ receptor was the first of the P2X receptors to be crystalized, where detailed atomic structure was revealed (Kawate et al., 2009). P2X₄ receptors have been shown to be readily involved in trafficking from lysosome vesicles to the cell surface. Probably due to their extensive N-linked glycans, these receptors are very stable in the lysosome and can traffic back to the cell surface where they are able to be functional ion channels (Qureshi et al., 2007). There are currently no selective agonists or antagonists that target the P2X₄ receptor over other P2X subtypes. Ivermectin is a selective allosteric modulator at P2X₄ receptors compared to the other P2X receptors; however, it does have actions at other non-purinergic receptor sites. Furthermore, ivermectin is hard to work with due to its lack of solubility in water (Khakh et al., 1999; Silberberg et al., 2007).

P2X₄ Receptors: Likely Candidates to Modulate HIV and Opioid Signaling

We hypothesize that the activation of the P2X₄ receptors on microglia may be a critical step in the synergistic toxicity of Tat and morphine to neuronal cells. Although the question of whether P2X₄ receptors play a role in HIV and opioid interactions has never been investigated before, several more recent studies suggest that this receptor subtype is involved in other inflammatory events, such as the pain pathways in the dorsal root ganglia and spinal cord (Tsuda et al., 2003; Ulmann et al., 2008; Tsuda et al., 2009). Furthermore, it has also been shown that
P2X₄ receptors that are specifically located on microglia are up-regulated after nerve injury are involved in certain types of pain (Tsuda et al., 2003; Ulmann et al., 2008). These studies are important because microglia play a major role in causing neurotoxicity due to HIV. Based on these findings, we hypothesize that P2X₄ receptors on microglia may also be a critical part of inflammation and neuronal damage seen in the basal ganglia of HIV patients, which is exacerbated with treatment of morphine.

We have further reason to hypothesize that the P2X₄ receptor is involved in morphine + Tat toxicity based on previous work that has shown that the effects of morphine on microglia are reportedly mediated through the P2X₄ receptor (Horvath and DeLeo, 2009). This paper observed that in cortical primary cell cultures when cells were treated with TNP-ATP (a P2X₁-7 inhibitor) but not when treated with PPADS (a P2X₁-3,5-7 inhibitor), morphine-dependent microglial migration was blocked. It also showed that after a 12 hr treatment with morphine, P2X₄ receptor levels were significantly increased on microglial cells in the cortex. A 2010 publication by the same group (Horvath et al., 2010) determined that injection of P2X₄ asODN (antisense oligonucleotide) into the lumbar region of the spine, as well as into the subarachnoid space, inhibited the development of chronic morphine tolerance. Since it has been further suggested that microglia and morphine interact via the P2X₄ receptor, we wished to investigate P2X₄ receptors in the basal ganglia and determine whether they might be involved in HIV and morphine’s interactive effects.
**Release and Breakdown of Extracellular ATP**

There are several possible sources for extracellular ATP, the first being dead or ruptured cells that release their contents, including ATP. Fairly high levels of extracellular ATP can be reached this way. For example, ischemia caused by stroke or a traumatic brain injury, can produce extracellular ATP levels as high as 1 mM at the site of the tissue trauma (del Puerto et al., 2013). ATP can also be released from cells that are alive. Release has been shown to occur through vesicular release in neurons, where it can be co-released with several other neurotransmitters, such as ACh, GABA and catecholamines (Burnstock, 2007). ATP can also be released from astrocytes (Burnstock, 2007). A final possibility is that P2X receptors that undergo pore dilation (upon prolonged ATP stimulation) can also flux ATP (Khakh and North, 2012). Although it has been shown that supernatant from HIV infected monocytes, as well HIV Tat or morphine treatment can lead to increases in extracellular ATP, no one has investigated the source of this ATP (Perry et al., 2005; Tovar-Y-Romo et al., 2013; Sorrell and Hauser, 2014). Once ATP is released into the extracellular space, its breakdown is rapid and occurs via a family of enzymes called ectonucleotidases that dephosphorylate or hydrolyze ATP back to adenosine, where it can then be recycled back into the cell and used to make more ATP (Goding, 2000).
Chapter 2: The use of pharmacological tools to investigate the role of the P2X receptor in Tat and/or morphine related neurotoxicity

Introduction

To date there have been only two other studies published that investigate purinergic receptors and how they may regulate HIV viral interactions. The first of these studies showed, through use of selective agonists and antagonists, that P2X₁, P2X₇, and P2Y₁ may all be involved in viral replication, however only the P2X₁ subtype was involved in viral entry (Hazleton et al., 2012). The second study looked at the ability of purinergic receptors to regulate neuronal injury in the hippocampus caused by the addition of supernatant from HIV infected microphages. The study showed ATP was present in HIV infected macrophage supernatant and by using several purinergic and NMDA/AMPA antagonists concluded that P2X receptors that are activated by this ATP are involved in neuronal injury and death due to HIV infection, at least partially due to their ability to modulate glutamatergic tone (Tovar-Y-Romo et al., 2013). These studies as well as studies mentioned in Chapter 1 led us to investigate whether P2X receptors, and P2X₄ receptors in particular, mediate HIV-1 Tat and morphine interactions.

We first characterized P2X₄ expression in our cell culture model. We wanted to both confirm their presence, as well verify our model exhibits receptor expression patterns consistent with what has already been shown in the literature. In order to study whether P2X receptors play a role in Tat and morphine related neurotoxicity, we first used and manipulated a primary mixed neuron and glia co-culture model with pharmacological tools. We initially used TNP-ATP (P2X₁-7 competitive antagonist) (Neelands et al., 2003) and PPADS (P2X₃&₅&₇ antagonist) (Gum et al., 2011) to investigate the P2X₄ receptor actions in particular. Although it initially seemed experiments done with PPADS helped point to P2X₄ modulation of HIV-1 Tat and morphine
mediated neuronal death (Fig. 3a), PPADS also caused neuronal toxicity when administered with alone (Fig. 3a-b), making it impossible to differentiate from PPADS verses Tat or morphine toxicity, and we could not conclude anything from cells treated with PPADS. After our initial studies only TNP-ATP was used to look at outcomes due to the activation of P2X receptors. Studies were conducted using TNP-ATP in a striatal murine primary cell culture model where neuronal death, changes in dendritic length, and increases in intracellular calcium levels were used as measures of neuronal instability. ATP is use to show results of direct P2X receptors activation, which can then be compared to outcomes induced by Tat and morphine treatment. Finally, selective agonists and antagonists were used to target P2X₁, P2X₃, and P2X₇ receptor subtypes in order to confirm any role they may play in Tat and morphine related neurotoxicity.
Figure 7. PPADS does not reverse Tat or morphine toxicity, but shows toxicity by itself (a-b). PPADS, which has a very low affinity to block the P2X<sub>4</sub> receptor, but does block several other P2X subtypes was not able to block morphine + Tat affiliated neuronal death. However, PPADS by itself was found to cause significant neuronal toxicity (a). This toxicity was shown to occur in a concentration dependent manner (b). The data represent the mean ± SEM from n=4 experiments. A one-way repeated measures ANOVA was performed followed by Duncans post hoc test. (*p <0.05 vs. control treated cells).
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 µM 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 $3 \times 10^5$ per well and maintained in media that contained 10 % serum. Medium was changed 24 h after being plated and then every 2–3 days afterwards. Glia were allowed to reach confluence, which took approximately 10 days.

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 trituated, 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 0.8–1.0×10^5 cells per well. Neuron-glia co-cultures were maintained in complete Neurobasal medium and allowed to mature for about 5 days prior to start of the experiments, with media exchanged 24 h after neurons were plated and then every 2–3 days. Our mixed-glia cultures have been previously characterized to contain approximately 90% astrocytes and 10% microglia (Zou et al., 2011).
**Figure 8. Characterization of glial cell layer.** All images are confocal; multiple z-stacks are compressed into a single image to show localization through entire cells. Left image is shown at lower magnification to give a representative view of glial cultures used for the co-culture system. Smaller panels to the right of each figure show color separation. Cells were immunostained for GFAP (green) and Iba-1 (red), and counterstained with Hoechst33342 nuclear marker (blue). Note that the majority of glia are GFAP+ astrocytes, with 10% Iba-1 microglia. Scale bar = 50 µM (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) (1:500; EMD Millipore) for identification of neurons, or with goat anti-ionized calcium binding adapter molecule (Iba1) (1:500; Abcam, Cambridge, MA) 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 HIV-1IIIB Tat1–86 (100 nM) (ImmunoDiagnostics, Woburn, MA) was the HIV protein used. 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) (multiple concentrations), 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) (See Table 1).
Table 1. IC$_{50}$/ EC$_{50}$ values (µM) for P2X antagonists and agonists

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<th>Antagonist</th>
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<th>P2X$_2$</th>
<th>P2X$_3$</th>
<th>P2X$_4$</th>
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<td>-----</td>
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<td>-----</td>
<td>&gt;100</td>
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<tr>
<td>NF499</td>
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<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>-----</td>
<td>-----</td>
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<tr>
<td>BBG</td>
<td>&gt;5</td>
<td>1.3</td>
<td>&gt;10</td>
<td>&gt;10</td>
<td>-----</td>
<td>-----</td>
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<td>1</td>
<td>&gt;500</td>
<td>3</td>
<td>&gt;100</td>
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<td>&gt;100</td>
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<td>BzATP</td>
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<td>0.08</td>
<td>7</td>
<td>&gt;500</td>
<td>-----</td>
<td>20</td>
</tr>
</tbody>
</table>

Notes: ----- indicates information is not yet available, Values listed are for either rat or human subunits (Bianchi et al., 1999; Jiang et al., 2000; Khakh and North, 2012). Also, values may be species specific in some cases (Jarvis and Khakh, 2009; Coddou et al., 2011)
**Assessment of neuron 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) (Fig. 9). 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. Medium 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) 2-3 fields of cells were used to count for all groups. Cells that drifted out of the field of view during experiments were not counted. 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.
Figure 9. Computer-aided, time-lapse tracking of neurodegenerative changes in the same cells. A within-subjects design is used to compare the survival of the same neuron before (0 h) and after treatment; data are the percentage of surviving neurons relative to pretreatment values. Neurons are digitally scanned and tracked individually at 30 min intervals for 72 h using a Zeiss Axio Observer Z.1 microscope, stage encoder, and environmental control.
Measuring intracellular calcium concentration ([Ca\(^{2+}\)]\(_i\))

Tat and morphine ± TNP-ATP-dependent changes in [Ca\(^{2+}\)]\(_i\) were measured by ratiometric imaging of fura-2. Cells were loaded with 1 µM 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 acetoxy-methyl 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 were 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 response) and non-responsive (neurons lacking any response) neurons. Each response curve represents the mean ± SEM from at least n =3 separate experiments—each consisting of the average percent control 340/380-nm excitation 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.

Extracellular ATP measurements

Neuron mixed-glial co-cultures were exposed to morphine (100 nM), HIV-1 Tat, and/or ATP (non-selective P2X agonist; Sigma) ± TNP-ATP. 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 µL 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 (PERAstar 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). The secondary antibody conjugated to Alexa 488 (mouse anti-rabbit; 1:500 dilution; Molecular Probes/Invitrogen) was incubated on the cells for 2 h. Cells were counterstained with Hoechst 33342 (1 µg/ml) for 5 min and then washed with PBS (3×5 min). Neurons were left in PBS at 4 °C until images were taken. All images were acquired at 20× 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 were counted in order to better represent
groups in which neurons may have been less healthy due to treatment. Approximately 40–60 neurons were sampled per treatment group in each experiment. Data are presented as the mean dendritic length per neuron ± SEM from at least 4 independent experiments.
Figure 10. Explanation of Sholl Analysis. A transparency sheet with concentric circles is placed on top of a neuron and centered at the cell body. Dendrite intersections with concentric circles are counted. Each circle corresponds to 10 μm in length. Total dendrite length was estimated for each individual neuron.
Statistics

For all time-lapse and calcium imaging experiments a one-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 P2X$_4$ receptors in mouse striatal co-cultures

Although others have characterized P2X$_4$ 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 P2X$_4$ receptors and determine the cellular patterns of expression in our mouse primary mixed-glia and neuron culture system. P2X$_4$ receptor presence was confirmed in our primary striatal co-cultures (Fig. 4). P2X$_4$ receptor immunofluorescence was co-localized in Iba-1 immunoreactive microglia (Fig. 4a-f). P2X$_4$ receptor immunoreactivity appeared to be more intense in activated, amoeboid microglia (Fig. 4a-c), whereas a more dispersed expression pattern in quiescent microglia (Fig. 4d-f). Up regulation of the P2X$_4$ receptor at the cell surface of activated microglia has been shown to occur by others (Tsuda et al., 2003; Ulmann et al., 2008). P2X$_4$ receptor positive cells that lacked neuronal morphology and lacked Iba-1 immunoreactivity that appeared to be astrocytes were also observed (Fig. 4d-f). Moreover, a majority of MAP2-immunoreactive neurons also possessed P2X$_4$ immunoreactivity (Fig. 4g-i). Neurons typically displayed a slightly more stippled pattern of P2X$_4$ immunofluorescence than in ramified (quiescent) microglia (Fig. 4g-i). P2X$_4$ 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).
Figure 11. Immunocytochemical colocalization 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 (arrow) (a–c), more quiescent microglia also display P2X₄ immunoreactivity but with less Iba1 overlap (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)
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. 12a-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. 12a-b).
Figure 12. 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 and 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 ± SEM from n=4 experiments. A one-way repeated measures ANOVA was performed followed by Duncans post hoc test. (*p <0.05 vs. control treated cells, δ 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.
TNP-ATP Acts Concentration-Dependently

TNP-ATP reversed Tat and morphine-related neuronal death in a concentration-dependent manner (Fig. 13a-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. 5. 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 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/morphine at 72 h. Prior to 48 h, there were significantly more neuron losses with 10 nm TNP-ATP when compared to Tat alone. However, this effect did not persist to 72 h (Fig. 13b). There was a trend toward increased neuronal death with 10 nM (Fig. 13a and 13c); however, the trend was not significant. It is possible that activating higher affinity P2X₁ and P2X₃ receptors is protective in this system. Finally, it should be noted that concentration curve experiments were performed independently of one another, so it may not be appropriate to compare levels of neuronal death induced by Tat and morphine alone versus Tat and morphine combined.

Next, we show that ATP, our positive, concentration-dependent causes neuron death. Importantly, like with Tat and morphine treatment, TNP-ATP was able to concentration-dependently block this effect (Fig. 14a-b). Treatment with 50 nM ATP was not different than controls. However, 100 nM, 500 nM, and 1 µM ATP all caused significant increases in cell death when compared to control cells (Fig. 14a). TNP-ATP was able to concentration...
dependently prevent neuron death caused by 1 μM ATP, where 300 nM TNP-ATP completely blocked cell death and is not different than control treated cells. 100 nM TNP-ATP partially blocked ATP caused neuron death, being both significantly different from control cells and ATP alone. 50 nM and 10nM ATP were not significantly different than ATP alone (Fig. 14b).
Figure 13. 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 ± SEM from n = 4 experiments. A one-way repeated measures ANOVA was performed followed by Duncans post hoc test (*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).
Figure 14. ATP leads to cell death in a concentration dependent manner, which can be prevented by TNP-ATP, also in a concentration dependent manner. Multiple ATP treatments, 100 nM, 500 nM, and 1 µM ATP, all caused significant increases in cell death when compared to control cells (a). 300 nM TNP-ATP prevented neuron death caused by 1 µM ATP. 100 nM TNP-ATP partially blocked neuronal death caused by ATP, as survival was significantly different from both control cells and 1 µM ATP. TNP-ATP treatment of 50 nM and 10nM were not significantly different than 1 µM ATP (b). The data represent the mean ± SEM from n = 4 experiments. A one-way repeated measures ANOVA was performed followed by Duncans post hoc test (*p < 0.05 vs. control treated cells, #p < 0.05 vs. 1 µM ATP treated cells).
P2X Receptors also Modulate Changes in Dendritic Length due to Tat and Morphine Treatment

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 MAP2 positive 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. 15 a-b). Potentiated decreases in dendrite length were not observed in combination Tat and morphine treatment versus Tat and morphine treatment alone as was seen in our neuronal survival assay. This may be due to differences in sensitivity of the assays (with in subject design versus population analysis). Accordingly, since we did not see any added affect of Tat and morphine in combination, there was no difference between Tat + morphine treatment versus Tat + morphine + naloxone treatment; as there was no added morphine toxicity for naloxone to block.
Figure 15. 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 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 in a and b are from the same experiment, but separated into two graphs for clarity. The data represent the mean ± SEM from n = 4 experiments. A one-way ANOVA was performed followed by Duncans post hoc test (*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).
**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. 16). At the next time-point (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. 16).
Figure 16. 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 ± 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 ± SEM from n = 6 experiments. A one-way ANOVA was performed followed by Duncans post hoc test (*p < 0.05 vs. controls at the same time).
ATP and combined Tat and morphine-dependent increases in 
\([\text{Ca}^{2+}]_i\) are attenuated by a broad-acting P2X receptor antagonist

ATP, the natural ligand for P2X receptors, caused marked increases in intracellular calcium concentration ([\(\text{Ca}^{2+}\)]_i) in neurons. Baseline [\(\text{Ca}^{2+}\)]_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 [\(\text{Ca}^{2+}\)]_i (Fig. 17). 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 elevates the fura-2 340/380 ratio (Fig. 18). 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 [\(\text{Ca}^{2+}\)]_i becoming significant at approximately 6 min (about 1 min after the last compound was administered). More interestingly, pretreatment with TNP-ATP prevented [\(\text{Ca}^{2+}\)]_i increases caused by either ATP or Tat + morphine co-stimulation.
Figure 17. ATP elicited concentration-dependent increases in [Ca\(^{2+}\)]. Neuron-glia cocultures were treated cumulatively with increasing concentrations of ATP (at 2 min intervals). Despite some tendency for [Ca\(^{2+}\)] to increase after applying 1 µM ATP, significant increases are not evident until 10 µM ATP is applied. Data represent the mean ± SEM from n =3 experiments. A one-way repeated measures ANOVA was performed followed by Duncans post hoc test (*p <0.05 vs. control and TNP-ATP pretreated cells).
Figure 18. TNP-ATP attenuated increases in neuronal \([\text{Ca}^{2+}]_i\) 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 \([\text{Ca}^{2+}]_i\) 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 ± SEM from n = 3 experiments. A one-way repeated measures ANOVA was performed followed by Duncans post hoc test (#p <0.05 vs. control and TNP-ATP pretreated cells).
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. 19e where \( p = 0.055 \)), Tat, or combined morphine and Tat ± 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. 19a-b), P2X₃ (A-31749) (Fig. 19c-d), or P2X₇ (BBG) (Fig. 19e-f) receptor subtypes prevented morphine and/or Tat-induced neurotoxicity (Fig. 19). 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 αβMe-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 19a), but only partial reductions in neuronal survival that were not statistically significant when repeated in another set of experiments (Fig. 19c). To ascertain whether the marginal neurotoxicity might be due to opposing actions of αβMe-ATP at P2X₁ and P2X₃ receptors, the effects αβMe-ATP were challenged with P2X₁ and P2X₃ receptor antagonists (Fig. 19a,d). Interestingly, αβMe-ATP induced neurotoxicity was not evident with P2X₁ receptor blockade (Fig. 19b), but caused significant reduction in survival despite P2X₃ receptor blockade (Fig. 19d), suggesting P2X₁ receptor activation might impart modest neurotoxicity. However, because the effects of αβMe-ATP were inconsistent and the P2X₁ antagonists only marginally
decreased the neuronal vulnerability, these results are inconclusive. Unlike P2X<sub>1</sub> receptors, P2X<sub>7</sub> activation was highly neurotoxic (Fig. 19e) and the Bz-ATP-induced neuronal losses were completely blocked by selective P2X<sub>7</sub> blockade (Fig. 19f). Importantly, despite the suggestion that P2X<sub>1</sub> or P2X<sub>7</sub> 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. 19b,d,f).
Figure 19. 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. A one-way repeated measures ANOVA was performed followed by Duncans post hoc test (*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).
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 neuronal death due to Tat or morphine treatment, increases in neuronal $[\text{Ca}^{2+}]$, 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 (P2X$_{1-7}$ antagonist), the selective blockade of P2X$_{1-3}$ & 5-7 by PPADS (Gum et al., 2011) was inherently neurotoxic—implying a differential involvement of the P2X$_4$ receptor subtype, or perhaps multiple P2X subtypes, in influencing striatal neuron viability. Finally, unlike TNP-ATP, the selective blockade of P2X$_1$, P2X$_3$, or P2X$_7$ receptors was not able to prevent Tat or morphine neurotoxicity, further supporting our hypothesis that the neurotoxicity is selectively occurring through the activation of P2X$_4$ receptors. Our findings strongly support P2X receptor mediation of these events, and circumstantially support a role for the activation of P2X$_4$ receptors in these events. Furthermore, we speculate that the data suggest 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 P2X$_4$ receptors (expressed by subpopulations of striatal neurons and glia) resulting in abnormally high levels of $[\text{Ca}^{2+}]$ and neuronal injury including decreased dendritic complexity and increased neuronal death. The time difference to get
increases in $[\text{Ca}^{2+}]_i$ by direct application of ATP versus Tat + morphine also supports that indirect mechanisms lead to this effect, which makes sense if ATP release has to occur first. Addition of apyrase (enzyme that causes hydrolysis of ATP) to groups treated with Tat and/or morphine could be useful in future experiments to more directly assess the role of ATP release.

As determined in the present study, P2X$_4$ 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 P2X$_4$ receptors, including increased levels of expression by astrocytes in striatal lesions caused by 6-hydroxydopamine (Amadio et al., 2007). Collectively, the findings suggest that P2X$_4$ 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 the 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. Yet, based on published information, we believe that the 300 nM TNP-ATP concentration used in most of our studies is likely to completely block P2X$_1$, P2X$_3$, P2X$_{2/3}$ and P2X$_4$ receptor populations and markedly attenuate P2X$_2$ receptor activity (Dunn et al., 2000; Ma et al., 2004), while failing to significantly antagonize P2X$_7$ receptors, although others have used
concentrations greater than 300 nM TNP-ATP to examine P2X actions (Guo et al., 2007; Solini et al., 2007). By contrast at 100 nM, TNP-ATP fails to block or only partially blocks P2X\textsubscript{2} and P2X\textsubscript{4} receptors, while continuing to preferentially block P2X\textsubscript{1}, P2X\textsubscript{3}, and P2X\textsubscript{2/3} receptor subtypes, which have a lower affinity for TNP-ATP (Virginio et al., 1998). Importantly, we found 300 nM, but not 100 nM, TNP-ATP sufficient to prevent ATP, and morphine and/or Tat neurotoxicity in the neuron survival assay. We also found 300 nM TNP-ATP was able to fully prevent the Ca\textsuperscript{2+} destabilizing or neurotoxic effects of Tat and/or morphine in our system. Lastly, it should be noted that the IC50 values for P2X\textsubscript{5} and P2X\textsubscript{6} receptors have not been investigated, perhaps due to their reported inability to form functional homomeric ion channels in human cells (Collo et al., 1996).

Measurement of extracellular ATP showed concentrations of ATP did not rise above 300 nM, while low µM ATP (< 10 µM, except for in the case of P2X\textsubscript{7}) (Lynch et al., 1999; Brône et al., 2007; Agboh et al., 2009) is needed to activate P2X receptors. However, the actual concentration of ATP at the cell surface may be greatly diluted by the comparatively large volume of media that is present in cell culture wells. For this reason, extrapolating actual ATP concentrations seen by cells from the ATP concentrations measured may not be appropriate in this assay.

The use of more selective antagonists showed that P2X\textsubscript{1}, P2X\textsubscript{3}, and P2X\textsubscript{7} receptor subtypes were not involved in HIV-1 or morphine related toxicity. While the absence of toxicity does not exclude the possibility of increased inflammation (Suzuki et al., 2011), heightened inflammation is generally associated with heightened neuronal injury in our cell culture model.
Despite some evidence that P2X<sub>1</sub> and P2X<sub>3</sub> 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 P2X<sub>1</sub> and P2X<sub>3</sub> receptors, the P2X<sub>7</sub> 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-1β signaling, and NF-κB signaling (Bai and Li, 2013). For the above reasons, we were surprised to find that P2X<sub>7</sub> receptors were not involved in the neurotoxic effects of Tat or morphine. One possible explanation is, with an EC50 > 30 µM (Jacobson et al., 2002; Hervás et al., 2005), P2X<sub>7</sub> 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 1-10 µM (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 in our primary cell culture model.

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 Tat-induced 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 [Ca<sup>2+</sup>]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 $[Ca^{2+}]_i$, through both direct and indirect effect. 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 P2X$_4$ 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 P2X$_4$ 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 P2X$_4$ 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 P2X$_4$ receptor in particular, in HIV-1 Tat or morphine related neurotoxicity in primary neuron mixed glial co-cultures from mouse striata. Our work also concurs with findings from another laboratory demonstrating that P2X receptors mediate increases in neuronal $[\text{Ca}^{2+}]_i$ 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.
Chapter 3: Evidence that P2X₄ receptor activation is necessary in Tat and/or morphine related neurotoxicity via cells from P2X₄⁻/⁻ mice

Introduction

Since there are no selective agonists or antagonists for the P2X₄ receptor, genetic manipulation of the receptor was an attractive strategy to complement pharmacological experiments in Chapter 2. The laboratory of Dr. Yves DeKonick graciously provided initial P2X₄⁻/⁻ mice breeding pairs. The generation of these mice has been previously described elsewhere (Sim et al., 2006a), and are described in more detail in the next chapter (Chapter 4) (Fig. 23). Primary cell cultures from P2X₄⁻/⁻ mice were used to confirm a role for the P2X₄ in Tat and morphine associated neurotoxicity. Manipulations in combinations of WT or P2X₄⁻/⁻ glia and/or neurons allowed us to investigate what role the P2X₄ receptor may be playing through these different cell types as well.
**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 C57BL/6J (Jackson Laboratories, Bar Harbor, ME) or P2X₄KO 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 µM 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 days afterwards. Glia were allowed to reach confluence, which took approximately 10 days.

**Co-culture with neurons**

Striatal neurons were prepared from E15-E16 C57BL/6J (Jackson Laboratories) or P2X₄ KO 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 tritured, 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 0.8–1.0×10^5 cells per well. Neuron-glia co-cultures were maintained in complete Neurobasal medium and allowed to mature for about 5 days prior to start of the experiments, with media exchanged 24 h after neurons were plated and then every 2–3 days. Our mixed-glia cultures have been previously characterized to contain approximately 91 % astrocytes and 8 % microglia (Zou et al., 2011).

Assessment of neuron 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. Medium 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.

**Statistics**

For all time-lapse experiments a two-way repeated measures ANOVA was used. 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

Presence of P2X$_4$ receptors on glia are necessary to cause Tat and morphine related neuron death

To examine whether activation of P2X$_4$ receptors on neurons or on glia is responsible for the Tat and morphine related neurotoxicity seen in our cell culture model, we treated WT neurons placed on top of P2X$_4^{-/-}$ glia and compared with the same treatments in co-cultures with both WT glia and neurons. As in previous experiments with groups containing both WT glia and neurons, Tat, morphine, and ATP treatment alone caused a significant decrease in neuron survival, where Tat + morphine was significantly more toxic than either treatment alone (Fig. 20a). In co-cultures where glia did not express the P2X$_4$ receptor, toxicity caused by Tat and/or morphine treatment was completely prevented, whether given alone or in combination. ATP toxicity was also prevented in these co-cultures (Fig. 20b).
Figure 20. Glia from P2X$_4^{-/-}$ mice confirm the receptors on this cell type are critical for Tat- or morphine-induced neuronal toxicity (a-b). Cells treated with Tat, morphine, Tat + morphine, and ATP were all significantly different than the control group cells. Tat + morphine treated cells were also significantly different than Tat or morphine alone (a). All KO groups were significantly different from their corresponding WT group except the KO control (b). Graphs represent N=4 experiments. A two-way repeated measures ANOVA was performed followed by Duncans post hoc test (*P < 0.05 vs. WT control, †P < 0.05 vs. WT Tat, §P < 0.05 vs. WT Morphine, ‡P < 0.05 vs. WT T+M, δP < 0.05 vs. corresponding WT group (except for KO control)).
P2X₄ KO on both glia and neurons prevents Tat and morphine toxicity, however toxicity is increased in KO controls verses WT controls

In order to investigate the role of the P2X₄ receptor in Tat and morphine mediated toxicity P2X₄KO co-cultures were compared with WT/C57BL6J primary co-cultures. Both groups were treated with Tat and morphine alone, as well as in combination, and with ATP. All treatments caused significant neuron death in WT cells in comparison with WT controls. As in WT cultures from ICR mice (Chapter 2) significantly more cell death was seen with Tat and morphine in combination versus when the two were given alone (Fig. 21a). Cells from P2X₄⁻/− mice that were treated with both Tat and morphine were no longer susceptible to neuronal death. ATP and morphine alone treatment also no longer caused significant neuronal death in cells from KO mice. However, untreated control KO cells exhibited significantly more death than WT controls. This same toxicity was seen in the KO Tat treated cells, but this group was not significantly different than KO control cells (Fig. 21b).
Figure 21. Cells from P2X$_4^{−/−}$ mice confirm receptors critical role in Tat or morphine neuronal toxicity (a-b). Cells treated with Tat, morphine, Tat + morphine, and ATP were all significantly different than the control group cells. Tat + morphine treated cells were also significantly different than Tat or morphine alone (a). All KO groups were significantly different from their corresponding WT group. KO Tat treated cells were also significantly different than WT control cells (but not different than KO control cells). Also note that KO T+M is significantly different than both KO control and KO Tat. (b). Graphs represent N=4 experiments. A two-way repeated measures ANOVA was performed followed by Duncans post hoc test (*P < 0.05 vs. WT control, # P < 0.05 vs. WT Tat, δ P < 0.05 vs. WT Morph, & P < 0.05 vs. WT T+M, KO control, KO Tat, § P < 0.05 vs. WT ATP).
P2X<sup>4</sup><sup>−/−</sup> on neurons prevented combined Tat + morphine toxicity but did not prevent toxicity when Tat or morphine were administered alone

To investigate what role P2X<sub>4</sub> receptors that are present on neurons play in Tat- and morphine-related neurotoxicity, we compared all WT co-cultures with co-cultures of glia from WT mice and neurons from P2X<sub>4</sub><sup>−/−</sup> mice (Fig. 22). As shown in previous experiments, Tat and morphine treatment alone caused significant neuronal death, with significantly greater cell death from combination treatment. ATP treatment, our positive control, also caused a significant increase in cell death (Fig. 22a). In co-cultures containing KO neurons with WT glia, we did not see prevention of the neuron death induced by Tat, morphine, or ATP as we saw in co-cultures containing P2X<sub>4</sub><sup>−/−</sup> glia and WT neurons. However, we were surprised to see that toxicity from combined Tat and morphine treatments was prevented (Fig. 22b). We also observed increased toxicity of KO control versus WT control neurons under these conditions, as was seen when the receptor was knocked out on both neurons and glia in Fig. 21. Finally, Tat treatment also caused significantly more neuronal death in groups that contained neurons from P2X<sub>4</sub><sup>−/−</sup> mice as compared to cultures with WT neurons and WT glia.
Figure 22. Neurons from P2X₄ KO are not protected from Tat or morphine treatment alone but are protected when Tat and morphine are given in combination (a-b). Cells treated with Tat, morphine, Tat + morphine, Tat + morphine + naloxone and ATP were all significantly different than the control group cells. Tat + morphine treated cells were also significantly different than Tat or morphine alone (a). KO groups, control, Tat, and Tat + morphine, were significantly different from their corresponding WT group. KO cells treated with Tat + morphine were not significantly different that WT Controls. Morphine and ATP treated cells were different from WT controls but not from WT cells that received the same treatment (b). Graphs represent N=4 experiments. A two-way repeated measures ANOVA was performed followed by Duncans post hoc test (*P < 0.05 vs. WT Control, # P < 0.05 vs. WT Tat, δ P < 0.05 vs. WT Morphine, § P < 0.05 vs. WT T+M, ¶ P < 0.05 vs. WT ATP, & P < 0.05 vs. KO Control).
Discussion

Time-lapse experiments with cells from knockout animals show that P2X₄ receptors on glia are necessary for Tat and morphine neurotoxicity in our cell culture model. However, the role for the receptor on neurons in Tat + morphine induced neurotoxicity is not as clear, mainly due to decreased neuronal survival under control conditions. Although more experiments investigating P2X₄ receptors on neurons are warranted, differences at baseline suggest they may play a neuroprotective role and participate in homeostatic cellular processes.

In order to better understand the role that P2X₄ receptors may be having on glia verses neurons, we mixed cells from WT and KO mice in our co-culture model and compared them to all WT cells. The first combination we tried was P2X₄⁻/⁻ glia co-cultured with WT neurons. Excitingly, cultures with P2X₄⁻/⁻ glia completely prevented combination Tat and morphine toxicity as well Tat and morphine toxicity separately. Implying the presence of P2X₄ receptors on glia in particular is necessary for Tat- and/or morphine-induced neurotoxicity. These results very closely resembled blockade of Tat and morphine neurotoxicity by TNP-ATP seen in Fig. 12 (Chapter 2), providing circumstantial evidence that TNP-ATP may be having its effects by inhibiting P2X₄ receptors on glia as well.

When the P2X₄ receptor is not present on both glia and neurons, results suggest a more complex role for P2X₄ receptors expressed on striatal neurons. Combined Tat + morphine neurotoxicity was blocked in P2X₄⁻/⁻ cells, further confirming the presence of these receptors on glia is critical for Tat and morphine related neurotoxicity but also not ruling out a role for the receptor on the neuron. However, in these same experiments control P2X₄⁻/⁻ cells underwent significantly more neuronal death than WT control cells, suggesting P2X₄ receptors normally
play an important and perhaps neuroprotective role. Data suggest that the neurotoxicity is coming from the P2X4\(^{-/-}\) neurons and not the P2X4\(^{-/-}\) glia, since we don’t see neurotoxicity under control conditions in experiments where only P2X4\(^{-/-}\) glia are used. P2X4\(^{-/-}\) cells treated with Tat also exhibited increased toxicity when compared to WT controls (but were not different than KO controls). However, we do not see this same effect in the P2X4 KO Tat + morphine group, suggesting that Tat may elicit activation of different signaling pathways when given by itself as compared to when given in the presence of morphine.

In our last condition, where P2X4\(^{-/-}\) neurons and WT glia were compared to WT neuron and glia cultures, further evidence is given that Tat, morphine, and Tat + morphine may activate distinct cellular pathways that are differentially mediated by P2X4 receptors on neurons. Surprisingly, Tat- and morphine-induced neurotoxicity was still prevented, even through WT glia were present. This is not what we expected after switching WT glia for P2X4\(^{-/-}\) glia and observing complete prevention of Tat- and/or morphine-induced neurotoxicity, which seemingly confirmed our hypothesis that P2X4 actions on glia in particular lead to Tat- and/or morphine-induced neurotoxicity. Although these data do not disprove our hypothesis, they do suggest that it is not the complete story. Since neurotoxicity was also prevented when P2X4\(^{-/-}\) neurons were switched for WT neurons, it appears that having the receptor present on both cell types is necessary for Tat and morphine combined neurotoxicity. Implying that neuron-glial interactions, which involve activation of P2X4 receptors on one or more cell types, play a complex role in the balance between neurotoxic and neuroprotective outcomes in response to Tat and morphine combined treatment.

However, in the case of Tat or morphine treatment alone neurotoxicity was not prevented. In this experiment we cannot determine the role neuronal P2X4 receptors play in
morphine-induced neurotoxicity. While there is no difference between morphine-induced neurotoxicity in all WT co-cultures versus co-cultures where P2X$_4^{-/-}$ neurons have been used instead of WT neurons, which would imply they play no role; there was also no difference between P2X$_4$ KO morphine and P2X$_4$ KO control groups. Neurotoxicity due to baseline differences and morphine treatment cannot be differentiated here.

Tat toxicity alone was also not prevented, but in this case the amount of neuron death was actually significantly more than in cultures with P2X$_4^{-/-}$ neurons as compared to those with WT neurons. The increased Tat toxicity may be partially explained due to decreased neuron survival rates to start with in control cells, as normal Tat toxicity may be combining with baseline levels. Data implies this additional Tat toxicity is due to lack of the P2X$_4$ receptor presence on the neuron since we don’t see this effect in co-cultures containing only P2X$_4^{-/-}$ glia. However, another interesting point is that when P2X$_4^{-/-}$ neurons are co-cultured with P2X$_4^{-/-}$ glia this additional Tat toxicity is also not present, giving evidence that glia may provide protection against Tat toxicity is in the later situation.

Overall, this experiment gave evidence for an unexpected yet important role for the P2X$_4$ receptor on neurons that may differ from one treatment condition to another and suggests that a signaling pathway shift may occur in response to combined Tat and morphine treatment, as opposed to the recruitment of multiple pathways at once. Finally, data from Tat treatment alone in these experiments suggest that neuronal death is regulated in a complex way, where multiple factors may affect the balance between neurotoxicity and neuroprotection.
In conclusion, our in vitro model confirms P2X4 receptors on glia are necessary for Tat- and/ or morphine-induced neuronal toxicity. However, we cannot rule out that P2X4 receptors on neurons may also modulate Tat and morphine related neurotoxic actions. Differences in baseline neurotoxicity in co-cultures containing P2X4−/− neurons interfered with our ability to assess the role of P2X4 receptors on neurons. One possible explanation for baseline differences is that these cells have been shown to exhibit differences in NMDA and AMPA subunit compositions (Wyatt et al., 2013); however, further characterization has not yet been performed and is warranted. At this time further experiments are necessary to better describe the role of neuronal P2X4 receptors in Tat- and/or morphine-induced neurotoxicity.
Chapter 4: Evidence that P2X$_4$ receptor presence modulates Tat and/or morphine related neurotoxicity via *in vivo* studies with P2X$_4$ KO mice

**Introduction**

Our next goal was to investigate the function of the P2X$_4$ receptor and its ability to modulate Tat and morphine interactions in a whole animal model. Primary cell culture experiments provide for quicker readouts when compared with *in vivo* experiments and the ability to focus on particular brain regions; however, these cells are de-afferented from other parts of the brain as well as from peripheral input. For these reasons it is important to validate the translatability of new findings in cell culture to *in vivo* models. Although the Tat transgenic mice have previously been a valuable tool to investigate Tat and morphine interactions in our lab, due to the lack of availability of selective P2X$_4$ antagonists and agonists, we have chosen to work with P2X$_4^{-/-}$ mice and perform intrastriatal injections of Tat instead.

As mentioned in the Chapter 1, the basal ganglia is an area of the brain that is targeted by HIV infection. The major input nucleus of the basal ganglia is the striatum (Smith et al., 1994; Kincaid et al., 1998; Bolam et al., 2000) a brain area made of 95% GABAergic neurons that can be divided into two classes, D1 and D2 (the type of dopamine receptors that are expressed on these neurons) (Kreitzer, 2009). These two populations are thought to promote the execution of motor programs, and control voluntary movement (Gerfen and Surmeier, 2011; Ralph J A Oude Ophuis, 2014). As such, we chose measurement of grip strength and locomotor activity to assess behaviors that may be impacted by changes in striatal function.

In order to directly assess striatal inflammation we used the markers Iba1 (microglia
marker) and 3NT (marker associated with the generation of reactive oxygen species). This allowed us to investigate both changes in microglia number as well as whether or not microglia may be activated.
Methods

Animals

3-6 month old male mice were used for all surgeries. Animals weighed approximately 25-30g. C57BL/6J mice were used as WT controls to compare with P2X₄KO mice, which are on the same C57BL/6J background as the transgenic mice. WT C57BL/6J breeding pairs were obtained from Jackson Labs (Bar Harbor, Maine) and P2X₄ KO mice breeding pairs were obtained from the Dr. Yves de Koninck laboratory at Laval University (Quebec, Canada). The genotype of the original breeding pairs received was verified via PCR (Fig. 24). More specific details of the P2X₄ gene deletion in these mice has been previously described elsewhere (Sim et al., 2006a); however, in brief these mice were made by a targeting vector that was constructed via the insertion of a LacZ neomycin cassette, between a BglII site located 30 bases upstream of P2X₄-initiating methionine and a BamHI site 172 bases downstream of the first exon/intron boundary. This resulted in a 337 base deletion encompassing the first exon (including P2X₄-initiating methionine) and 172 bases of the downstream intron (See Fig. 23). Animal colonies were maintained in the vivarium facilities at Virginia Commonwealth University after initial breeding pairs were obtained. 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.
Figure 23. Generation of P2X₄ KO mice. Boxes represent open reading frames; LacZ gene insertion occurred upstream of the P2X₄-initiating methionine resulting in the deletion of the entire coding region of exon 1 and of the first exon–intron splice site. Note abbreviations: TK, Thymidine kinase; NEO, neomycin; Ex1, exon1; B, BamH1; Bg, BgIII; S, SacI; X, XbaI (Sim et al., 2006b).
PCR detection of P2X$_4$ mRNA in KO and WT mice

Reverse transcription PCR (RT-PCR) was performed to confirm expression of P2X$_4$ mRNA in WT C57BL/6J vs. P2X$_4$ KO mice. Tail clips were taken from animals and total RNA was isolated using DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA) as per the manufacturer’s protocol. The final elution volume was 200 µl. 2 µl of final product was using along with Taq Polymerase, dNTP were purchased from Bioline (Bioline, Taunton, MA). PCR was performed to detect P2X$_4$ mRNA expression using primers to recognize WT P2X$_4$ mRNA (both sequences directed against Exon1) (forward 5’-GGTGGTGCGCAGCGACAA and reverse – 5’CCAATGACGTAAGCCAGGAT) and primers to recognize KO P2X$_4$ mRNA (first sequence directed against small part of Exon 1 that remains and second sequence directed against LacZ gene) (forward – 5’CCAATGACGTAAGCCAGGAT and reverse 5’AAACGCCGAGTTAAGCCCAT). 2 µl of sample from WT C57BL/6J with no primers added was used as control. Amplified products were analyzed on 2% agarose gels and visualized with ethidium bromide.
Figure 24. PCR verifies genotype of P2X₄ KO mice. No band is seen in the control (no primers are present), a band at 200 bps (Exon 1) is seen from the WT mRNA, and a band at 450 bps (Exon 1 + LacZ gene) is seen from KO mRNA obtained from all 4 mice that were used to originally start the P2X₄ KO mouse colony at VCU.
**Animal Surgeries**

For all surgeries, mice were initially placed under anesthesia using isoflurane gas at 5% and then animals were placed on stereotaxic apparatus (Stoelting, Wood Dale, IL) where anesthesia was maintained with 3% isoflurane. Mice recovered from anesthesia under a warming lamp and were returned to their cages when they displayed normal activity. Mice were euthanized at 5 days after intrastriatal Tat and/or systemic opiate exposure. 8 animals were included in each group. Also it should be noted that mice that received combined Tat and opiate exposure where given additional warming time and received systemic sterile saline injections after surgery. These measures were taken due to decreased survival rates observed in this treatment group as compared to the others.

**Intrastriatal Injections**

1 µL injections of either saline (control) or 25 µg (2 nmol) rTAT$_{1-86}$ HIV-1 IIIB (ImmunoDX, Woburn, MA) were injected intrastriatally using a 30-gauge syringe (Hamilton, Reno, NV) under aseptic conditions. Tat was received lyophilized and reconstituted in saline before injection. Striatal injections were made at the coordinates AP = +0.7 mm, ML = 2.0 mm and DV = -4.0 mm from bregma (Hof, 2000). The needle was stereotactically placed at the above coordinate over a 2 min period. A saline or Tat injection then took place over 1 min followed by a 1 min wait period in which the needle was allowed to remain in place before withdrawal to minimize Tat backflow along the needle tract as the syringe was then withdrawn (over 2 min).
**Insertion of Time Release Pellets**

Continuous, time-release pelleted implants (NIDA, Rockville, MD) were used to administer vehicle (placebo implant), morphine (25 mg), and/or naltrexone (30 mg). Immediately after stereotaxic injection and while still under anesthesia pellets were implanted. Under aseptic conditions, the subscapular skin was lifted and a 3-mm incision made with a microscalpel. A 1.5 cm deep pocket is created with forceps, placebo or opiate drug pellets are inserted, and the pocket closed with 3-4 sutures.

**Behavioral Assays:**

Before being euthanized on day 5, mice were first tested for locomotor activity, which was immediately followed by grip strength testing. Animals were euthanized immediately after last behavioral test.

**Locomotor Activity**

To assess locomotor activity, mice were placed in clean plastic cages (28 x 16 cm) inside sound-attenuating chambers and total distance traveled was recorded for 10 min and analyzed by the ANY-maze (Stoelting, Wood Dale, IL) video tracking system. Subjects were randomly assigned to testing boxes on test day.
**Grip Strength**

Mice were tested on a computerized grip strength meter (Columbus Instruments, Columbus, OH), with a single digital force gauge. Mice were gently pulled across horizontal platform with grip bars, where computer software analyzed the force as mice released their grip. Trials were repeated 3 times during each test session and average grip strength was recorded. The instrument automatically records grams-force applied, which was then divided by the animal’s body weight to account for possible strength differences due to weight. Data are reported as force applied (g)/body weight (g).

**Tissue Handling and Immunohistochemistry**

Mice were deeply anesthetized with 5% isoflurane and euthanized by intracardiac perfusion with Zamboni’s modified phosphate buffered 4% paraformaldehyde. The brains were dissected and fixed for an additional 12 h before further processing. The forebrain, including the striatum, was serially sectioned (10 µm thick) in the coronal plane. To allow uniform penetration of the immunocytochemical reagents into 10 µm thick tissue sections, sections were permeabilized in 0.1 % Triton-X 100, 1% crystalline grade BSA (Calbiochem/EMD Millipore, Billerica, MA) in PBS, pH 7.2 for 1 hr and rinsed 3×20 min in PBS, pH 7.2. Tissue sections were then incubated in diluted primary antiserum for 1 hr in PBS, pH 7.2 with 1% crystalline grade BSA. Iba1 (ionized calcium binding adapter molecule 1) was detected by anti-Iba1 (1:200; Wako Chemicals, Richmond, VA) and 3NT was detected by using nitrotyrosine antibody raised against 3-Nitrotyrosine (1:100; Santa Cruz Biotechnology, Santa Cruz, CA). Primary antibodies were allowed to incubate overnight at 4°C and then washed 3 x 20 min in PBS, pH 7.2.
Immunoreactivity was visualized with appropriate secondary antibodies conjugated to Alexa 488 or Alexa 596 (1:500 dilution; Molecular Probes, Eugene, OR). Striatal tissue sections were counterstained with Hoechst 33342, which labels all cell nuclei.

**Quantitative Microscopy**

An up-right fluorescent microscope (Zeiss Axio Imager D1) was used to systematically sample cells near (300 ± 100 µm) the site of Tat injection within the striatum. The injection epicenter was identified by pronounced Iba1 and 3NT immunoreactivity caused by needle insertion (Fig. 25). Tissue sections were randomly assigned before analysis for cells to be sampled either to the left or right of the injection epicenter. Microglia (Iba1 positive cells) and cells producing 3-nitrotyrosine (3NT positive cells) were counted. 3NT is a byproduct in the production of peroxynitrate, a ROS (reactive oxygen species), so its presence in Iba1 positive cells implies that these microglia are also in an activated state. Stereological analysis was not applied, because the non-uniform distribution of injected Tat makes it unrealistic to define a reference volume for the gradient of Tat within the striatum. Instead, the relative changes in the proportion of Iba1/3NT positive cells were determined from the total Hoechst-labeled cells. Because the introduction of a sterile syringe needle alone caused some glial changes along the needle tract as noted below, and may induce subtle injury/inflammatory changes elsewhere, treatment groups were always compared with vehicle-injected control values at 300 ± 100 µm from the injection epicenter. Typically, 200–300 cells total were sampled and average values recorded for each animal. Data are reported as the mean ± SEM with 8 mice per group.
Figure 25. Needle track located in striatum of mouse. Needle track mark, located in striatal tissue, could be identified by pronounced Iba1 (green) and 3NT (red) immunostaining that outlined the shape of a needle. Cells were counterstained with Hoescht in order to label nuclei (blue).
Statistics

A two-way ANOVA was used in all behavioral and quantitative microscopy assays. 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

Locomotor activity shows differences between P2X$_4$ KO and WT mice when treated with both Tat and morphine

Mice were given 10 min to move freely in a box that they had never been placed in before in order to test their baseline activity in a novel environment. Tat treated mice did not differ from the control group animals. Both WT and KO animals that received morphine moved significantly less during the time period than control animals, but since both genotypes display the same behavior P2X$_4$ receptors do not play an important role in this outcome. Interestingly P2X$_4$ KO animals that received both morphine and Tat also moved significantly less than the WT control group, which was not exhibited by WT mice that received the same treatment. Although, WT Morphine +Tat mice did not perform significantly different when compared with WT Morphine mice (Fig. 26).

Grip strength reveals baseline differences between P2X$_4$ KO and WT mice, as well as between Tat treated groups

Next we measured grip strength, where P2X$_4$ KO control animals applied significantly more force than WT animals. Also WT Tat mice applied less force when compared with WT control mice, and there was no difference between WT controls and P2X$_4$ KO Tat treated mice (Fig. 27). No significant changes were observed between other groups.
Figure 26. Morphine treatment caused a significant decrease in locomotor activity in both WT and P2X₄ KO mice, while Tat and morphine combination treatment caused a decrease in activity in only P2X₄ KO mice. Morphine treated WT and KO mice are both significantly different than WT controls but not different from each other. KO mice treated with Morphine + Tat were both significantly different from WT control and WT Morphine + Tat treated animals. However, WT M+T was not different than WT Morphine. Each group represents N=8 mice. A two-way ANOVA was performed followed by Duncans post hoc test (*P < 0.05 vs. WT Control, δ P < 0.05 vs. WT Mor + Tat).
Figure 27. **P2X$_4$ KO control animals have increased grip strength compared to WT control animals, and WT Tat mice exhibit a decrease in grip strength.** Control P2X$_4$ KO mice had a significantly stronger grip than WT control mice. WT mice that received Tat had a decreased ability to grip when compared with WT control mice, P2X$_4$ KO Tat treated mice did not see this same decrease; however, WT and KO Tat treated mice were not significantly different than each other. Each group represents N=8 mice. A two-way ANOVA was performed followed by Duncans post hoc test (*P < 0.05 vs. WT Control).
Changes in proportions of Iba1 and 3NT positive cells in P2X4 verses WT mice gave evidence for P2X4 receptor involvement in striatal neuroinflammation from Tat and/or morphine

Immunohistochemistry was done in order to examine inflammation in striatal tissue in P2X4 KO versus WT mice (Fig. 28 - 29). WT animals treated with morphine and Tat alone, as well as in combination, showed increases in the percentage of Iba1 positive cells. These same increases were not seen in P2X4 KO mice that received the same treatments (Fig. 29a). Implying that increases in the number of microglia present due to Tat and morphine treatment is dependent on the presence of P2X4 receptors. The overall number of cells that were both 3NT and Iba1 positive also increased due to Tat and morphine treatment alone, as well as in combination, implying increased amounts of activated microglia. Increased 3NT immunoreactivity in P2X4 KO mice was not observed given the same treatments, implying increases seen in WT animals in response to Tat and/or morphine treatment may be P2X4 dependent (Fig. 29b). There were no baseline differences between P2X4 KO and WT animals in either Iba1 or 3NT positive cells.
Figure 28. P2X$_4$ KO mice treated with Tat and morphine did not exhibit higher levels of colocalized Iba1 and 3NT immunostaining as compared to WT mice that received the same treatment (a-d). All sections were incubated with the primary antibodies Iba1 (green) and 3NT (red) and counterstained with Hoescht in order to label nuclei (blue). Both tissues from WT and P2X$_4$ KO mice show low levels of either Iba1 or 3NT staining (a-b). In WT mice that were treated with both Tat and morphine noticeably more Iba1 and 3NT positive cells are present as compared to control mice (c). In tissue from P2X$_4$ KO mice that received both Tat and morphine, the amount of Iba1 staining looks similar to tissue from control mice. 3NT levels may be elevated (this was not quantified) but not on cells that are also Iba1 positive (d).
Figure 29. WT mice exhibit increases in Iba1 and 3NT positive cells due to Tat and/or morphine treatment, which is not seen in P2X4 KO mice (a-b). WT Tat and WT morphine treated animals, as well as WT animals that received both morphine and Tat exhibited significantly higher percentages of Iba1 positive cells when compared with the percentage of Iba1 positive cell WT control striatal tissue. Striatal tissue from P2X4 KO animals did not show increases in Iba1 positive cells when compared with WT controls due to any treatment (a). Increases in the percentage of 3NT positive (that were also Iba1 positive) cells were observed in striatal tissue sections from WT Tat, WT morphine, and WT morphine + Tat treated animals. KO mice did not show increases with the same treatments, which were significantly different from WT paired groups but not WT controls (b). Percentages are based off of total number of cells present in the field as identified by Hoescht nuclei staining. Graphs represent cells counted from N=8 mice. A two-way ANOVA was performed followed by Duncans post hoc test (*P < 0.05 vs. WT Control, **P < 0.001 vs. WT Control, ***P < 0.0001 vs. WT Control, #P < 0.05 vs. WT Tat, αP < 0.05 vs. WT Mor, δP < 0.05 vs. WT Mor + Tat).
Discussion

Overall, locomotor activity and grip strength assays showed little response to genotype differences. Animals that received time-released morphine pellets and saline intrastriatal injections exhibited less activity in the 10 minutes they had to explore a novel environment, but this effect was independent of genotype. While acute morphine exposure has been shown to have motor activating effects in mice on the C57BL/6J background (Castellano and Oliverio, 1975), since the testing occurred on day 5 after the surgery, the mice had most likely already undergone tolerance to this effect. Mice were only tested on day 5. When mice were observed on day 1 and day 2 after surgery the mice did appear to exhibit the typical increase in locomotor activity that has been seen by others (Castellano and Oliverio, 1975; Murphy et al., 2001); however, this effect was clearly not present on day 5. Decreases in activity were prevented when animals received the opioid antagonist naltrexone. Since there was no difference between P2X$_4$ KO mice and WT mice the P2X$_4$ receptor is not expected to play a role in the effect.

Surprisingly, the decrease in motor activity due to morphine was not seen in WT mice receiving combined morphine and intrastriatal Tat injections. Seemingly, Tat acts to negate the decrease in activity seen due to morphine, which is not what we would expect due to the fact HIV patients often suffer deficits in motor coordination (Woods et al., 2009). Although, Tat has the ability to travel between cells and may be able to travel to sites other than where injected, the entire brain is unlikely to receive the same exposure to Tat as in HIV infected individuals. Accordingly, motor deficits in patients may not be exclusively regulated by the striatum, and this would not be surprising as other areas of the brain contribute to motor activity and are variably affected due to HIV infection. Alternatively, much of the striatum may be spared, as we
did not inject bilaterally. We do not understand the exact mechanism behind this result; however, one possibility is that Tat may illicit astrocytic calcium signaling waves that may start in the striatum but have their effects elsewhere (Burnstock, 2007). Another interesting outcome from this data set is that we do see decreased motor activity in P2X$_4$ KO mice that are given morphine and Tat as compared with WT control animals and WT animals that also received Tat and morphine. However, WT M+T was not different that WT morphine, so differences due to genotype remain inconclusive.

Assessment of grip strength showed a different outcome than the locomotor activity results. Regulation of these behaviors by different brain areas or brain circuitry could be a possible explanation for this. Locomotor activity can also involve modulation by the cortex, brainstem and spinal cord, whereas grip strength focuses on motor behaviors controlled by the extrapyramidal system (Misra et al., 2009). Here we saw decreases in grip strength due to Tat treatment which may mean that striatal regulation of this behavior is more important than in locomotor activity. Afferent connections from other areas of the brain may have less of an impact as well when compared with locomotor assessment. This same difference due to Tat treatment was not seen in the P2X$_4$ KO mice, but due to baseline differences between WT and KO control animals, we can not conclude these differences are solely based on changes in P2X$_4$ receptor function. Furthermore, there were no differences in either WT or P2X$_4$ KO mice that were treated with either morphine alone or morphine and Tat in combination. Although morphine has no effect on its own in this assay, it may counterbalance Tat effects when given in combination, as we no longer see decreased grip strength as with Tat alone.
It is difficult to draw clear conclusions based on the above behavioral outcomes, due to lack of effect (locomotor activity) and baseline differences (grip strength assay). Furthermore, these data do not support our hypothesis that P2X₄ receptor activation is linked to Tat- and/or morphine-induced neurotoxicity. As in any global knockout model, compensatory mechanisms developed due to changes in P2X₄ receptor levels may also be a factor, and differences in the grip strength assay between KO and WT control groups give evidence for this. Others have also shown evidence for baseline differences between P2X₄ KO and WT mice in several sensorimotor assays including, startle reflex (Bortolato et al., 2012), tactile sensitivity, and social interaction; however, differences were not observed in locomotor activity (as was also seen here), and anxiety-like behaviors (tested in elevated plus maze and light dark box) (Wyatt et al., 2013). Repeating of experiments or use of other behavioral assays may be useful to uncover the role of the P2X₄ receptor in this model. Another possibility would be to use a conditional P2X₄ KO mouse model in order to better avoid compensatory changes in the animal.

Immunohistochemistry with inflammatory markers, Iba1 and 3NT, gave more straightforward results. These data give evidence that P2X₄ receptors play an important role in Tat and/or morphine microglia mediated striatal inflammation. However, only 3NT cells that were also Iba1 positive were counted in 3NT immunostaining analysis. Accordingly we cannot rule out the possibility that other cells types may exhibit inflammation in P2X₄ KO mice that were treated with Tat and/or morphine. Results support conclusions made from in vitro data presented in Chapters 2 and 3 that support the hypothesis that Tat- and/or morphine-induced inflammatory outcome measures are mediated by striatal glia via P2X₄ receptors. Further analysis of neuron viability along with use of neuronal makers would be needed confirm Tat-
and/or morphine-induced effects in co-cultures containing P2X$_{4^{-}}$ neurons.
Chapter 5: P2X Receptor Expression in Brain Tissue from HIV Infected Individuals

Introduction

To address the potential role of P2XR family members in neuroAIDS, we used gene arrays to examine individual subtype differences among CNS tissues obtained from HIV-negative, HIV-positive, HIV-positive with neurocognitive impairment, and HIV-positive/impaired with encephalitis (HIVE) individuals. Recent findings demonstrate P2X7-R activation is directly neurotoxic (Sorrell and Hauser, 2014), and unpublished findings in striatal neural cells from P2X4-R-null mice (in preparation) show that P2X4-Rs mediate HIV-1 Tat-induced neurotoxicity in vitro. Together, this led us to explore the role of P2X4-R and P2X7-R, as well as other purinergic receptor subtypes, in the HIV-infected human brain. We further performed qRT-PCR validation and immunohistochemical localization using a subset of the same human tissue samples from the arrays.
Methods

**Human brain tissue**

Human brain tissue was obtained from the National NeuroAIDS Tissue Consortium (NNTC) Gene Array Project (Morgello et al., 2001; Gelman et al., 2012). Briefly, the array project consists of four groups of subjects (HIV-negative, \(n=6\); HIV-positive, \(n=6\); HIV-positive with neurocognitive impairment, \(n=7\); and HIV-positive with combined neurocognitive impairment and HIV encephalitis (HIVE), \(n=5\)) with samples taken from three brain regions (frontal lobe white matter, frontal cortex, and basal ganglia). Further details on the subject demographics, neurocognitive diagnoses, and impairment scores, as well as background information on the Gene Array Project itself can be found at http://www.nntc.org/gene-array-project. Details on the brain regions and numbers of individual samples analyzed for each HIV group in this study are summarized in Table 1 and have been previously described in published supplementary material (Dever et al., 2012).

**Microarray data analysis**

CEL files for arrays were retrieved from the NCBI Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/), GEO accession number GSE35864 (Gelman et al., 2012), and were reanalyzed as described previously (Dever et al., 2014). Briefly, robust multi-array average (RMA) analysis for probe intensity data normalization and multi-class linear models for microarray data (limma) analysis to access differential expression between subject groups were performed for each brain region (Dever et al., 2014). Heat maps were constructed from RMA

qRT-PCR

Total RNA was isolated using the miRNeasy Mini Kit (Qiagen, Inc.; Valencia, CA, USA) and used to generate cDNA templates by reverse transcription of 1 µg RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems; Carlsbad, CA, USA) according to the manufacturer's instructions. PCR reactions were performed in a total volume of 20 µL containing SensiMix SYBR qPCR reagents (Bioline USA, Inc.; Tauton, MA, USA) using a Corbett Rotor-Gene 6000 real-time PCR system (Qiagen, Inc.). PCR conditions consisted of an initial hold step at 95°C for 10 min followed by 40 amplification cycles of 95°C for 10 s, 58°C for 30 s, and 72°C for 30 s. Sequences of the primer sets used were forward: 5'-CTCACCATGAACCAGACACA -3' and reverse: 5'-GACAGACCCGGTTGAAAGCTA -3' for P2X4; forward: 5'-TTACAGCTGGAAACGGAGTG -3' and reverse: 5'-GTCCATCACATTGCTTTTG -3' for P2X5; forward: 5'-CTGTGAAGTCTCTGCCTGGT -3' and reverse: 5'-GGGACACTGTGGATTCTGAG -3' for P2X7; and forward: 5'-GCTGCGGTAAATCATGAGGATAAGA -3' and reverse: 5'-TGAGCACAAGGCCTTCAACCTTA -3' for TATA-binding protein (TBP). The specificity of the amplified products was verified by melting curve analysis and agarose gel electrophoresis. Melting curve analysis was performed after PCR reaction completes to help confirm there was no contamination from mispriming, primer dimers, etc. The melting temperature is dependent on the PCR product’s base composition. Accordingly, PCR products from a particular primer set
should have the same melting temperature. qRT-PCR data were calculated as relative expression levels by normalization to TBP mRNA using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Samples from different brain regions were analyzed together for each subject group as performed previously due to the limited availability of tissue (Dever et al., 2012).

**Immunohistochemistry**

Frozen white matter samples from the NNTC Gene Array Project subjects were sectioned and fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton X-100/0.1% bovine serum albumin (BSA), and blocked in 0.1% BSA/1% normal goat serum. Primary antibodies used were anti-P2X$_4$R (catalog number APR-002) at a 1:200 dilution and anti-P2X$_7$R (catalog number APR-004) at a 1:100 dilution from Alomone Labs (Jerusalem, Israel), and anti-Iba-1 (Abcam, Inc.; Cambridge, MA, USA; catalog number ab5076) at a 1:100 dilution. Immunoreactivity was visualized with appropriate secondary antibodies conjugated to Alexa Fluor 488 or 594 dyes (Molecular Probes; Eugene, OR, USA) used at a 1:200 dilution. Sections were counterstained with Hoechst 33342 to label cell nuclei. Samples were imaged using a Zeiss LSM 700 laser scanning confocal microscope equipped with a 63x oil immersion objective. Images were collected by use of the z-stack acquisition mode in ZEN 2009 Light Edition software (Carl Zeiss, Inc.; Thornwood, NY, USA), that was set to move through the z-plane in 5 µm increments covering a total of 20 µm. Z-stack images were later reconstructed and edited using Adobe Photoshop CS3 Extended 10.0 software (Adobe Systems, Inc.; San Jose, CA, USA).
Statistics

qRT-PCR data were analyzed by one-way ANOVA with Student Neuman-Keuls post-hoc test using GraphPad Prism 5 (GraphPad Software, Inc.; La Jolla, CA, USA) and are presented as the mean ± the standard error of the mean (SEM). A value of $p<0.05$ was considered significant.
Results

Though we were initially prompted by findings suggesting P2X₄R and P2X₇R can affect neuronal survival *in vitro* (Sorrell and Hauser, 2014), we also wanted to systematically explore the P2XR system more globally. To begin to examine P2X₄R and P2X₇R, as well as other P2X family members that might play a role in human HIV-1 neuropathogenesis, microarray data was retrieved and reanalyzed to examine P2X₁₋₇ subunit expression from subjects included in the National NeuroAIDS Tissue Consortium (NNTC) Gene Array Project (Gelman et al., 2012). Heat maps were generated displaying the expression pattern within individual P2X gene probesets included in the arrays for each subject in the four HIV groups across the three brain regions examined: the frontal lobe white matter, frontal cortex, and basal ganglia (Fig. 30a-c). Analysis of the microarray data showed a significant regulation of P2X₇ and P2X₅ subunit expression in the frontal cortex, as well as for P2X₇ subunit levels in the basal ganglia, when all the subject groups were compared, although this analysis did not reveal significant pair-wise differences between any two particular groups.

Accordingly, we further examined variations in P2X₅ and P2X₇ expression levels in the array data (Fig. 30d-f), and found a similar pattern of P2X₇ expression across the subject groups in the frontal cortex and basal ganglia. Lastly, it is also important to mention that our analysis does not allow for inter-brain region comparisons of expression levels for a particular gene as samples from each brain region were normalized and summarized separately.
Figure 30. P2X family member expression across HIV-infected subjects with varying levels of neurocognitive impairment (a-f). Heat maps are shown for P2X1-7 from the frontal lobe white matter (a), frontal cortex (b), and basal ganglia (c) of subjects that were HIV-negative (A1-A6), HIV-positive (B1-B6), HIV-positive with neurocognitive impairment (C1-C7), and HIV-positive with combined neurocognitive impairment and HIV encephalitis (HIVE) (D1-D5). Individual probesets included in the array for each P2X gene are given in parenthesis. Mean ± SEM of robust multi-array average (RMA) signal intensity values for P2X5 and P2X7 expression in the white matter (d), frontal cortex (e), and basal ganglia (f). Data generation by Megan O’Brien and Blair Costin, figure generated by Sylvia Fitting.
Based on the preliminary microarray data, there was obvious utility in validating changes in P2X$_5$ and P2X$_7$ mRNA expression levels. We also decided to further assess P2X$_4$ transcript levels based on our recent findings (Sorrell and Hauser, 2014), as well as evidence from the literature. P2X$_4$Rs have previously been shown to be involved in neuroinflammation and cytokine production (Donnelly-Roberts and Jarvis, 2007; Kawano et al., 2012b; Sakaki et al., 2013), as well as CNS disorders associated with excessive inflammation such as neuropathic pain, allodynia, and traumatic brain injury (Nasu-Tada et al., 2006; Ulmann et al., 2008; Beggs and Salter, 2013). As inflammation is a defining characteristic of HIVE, we reasoned that P2X$_4$Rs might also be involved in HIV-1 neuropathogenesis. To test this hypothesis, we evaluated the expression levels of P2X$_4$, along with P2X$_5$ and P2X$_7$, using qRT-PCR, and examined the pattern of P2X$_4$R and P2X$_7$R expression on cells by performing immunohistochemistry. Only a subset of the brain tissue samples used in the Gene Array Project were available for this purpose from the NNTC (Table 2) (Dever et al., 2012). We used the entire subset available in order to perform qRT-PCR and used representative samples to perform immunohistochemistry. Samples from different brain regions were pooled to perform qRT-PCR analysis.

Table 2. Regional distribution of brain tissue samples used for qRT-PCR.

<table>
<thead>
<tr>
<th>Brain region</th>
<th>HIV(-)</th>
<th>HIV(+)</th>
<th>HIV(+)-Impaired</th>
<th>HIV(+)-HIVE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frontal lobe white matter</td>
<td>3</td>
<td>2</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Combined frontal lobe white matter</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>frontal cortex</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal ganglia</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

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Although P2X$_4$ and P2X$_5$ mRNA expression levels were unaffected (Fig. 31a and b), we found that P2X$_7$ expression was significantly elevated in subjects with HIVE compared to uninfected subjects (Fig. 31c).
Figure 31. P2X7 but not P2X4 and P2X5 mRNA expression levels are elevated in HIV-infected subjects with combined neurocognitive impairment and HIVE (a-c). P2X4 (a), P2X5 (b), and P2X7 (c) expression was measured by qRT-PCR across the indicated groups of subjects. Error bars show the SEM. A one-way ANOVA was performed with Student Neuman-Keuls post-hoc test. P2X4: $F(3,24)=1.61$, $p=0.21$; P2X5: $F(3,24)=2.63$, $p=0.07$; and P2X7: $F(3,24)=3.05$, $p=0.048$; *$p<0.05$ when HIV-negative and HIVE groups were compared for P2X7. Data generated by Seth Dever.
Immunohistochemistry was used to determine the cellular location of P2X$_4$ and P2X$_7$ subunit expression in brain sections that were from representative samples of the white matter examined in the qRT-PCR analysis. P2X$_7$ subunit expression was found to co-localize on mostly Iba-1-positive microglial cells, whereas P2X$_4$ subunit expression was seen in Iba-1-positive cells, as well as other cell types in sections from both uninfected and HIV-1 subjects (Fig. 23a-d).

Confirmation of P2X subunit protein expression on Iba-1-positive cells supports the hypothesis that microglial P2XR activation may lead to neuroinflammatory signaling in neuroAIDS patients, although in the case of P2X$_4$R, inflammatory actions via activation on other cell types cannot be ruled out. Quantification of P2X protein levels in tissue samples from these groups of individuals will be useful for future studies to further confirm our qRT-PCR findings. However, we were not able to perform this analysis due to limited amount of brain tissue available to us.

Finally, greater numbers of Iba-1-immunoreactive, activated macrophages/monocytes and microglia appeared to be present in sections from HIV-1-positive individuals (Fig. 23b,d), which is in alignment with what has been shown in previous studies (Glass et al., 1993; Langford and Masliah, 2001).
Figure 32. P2X₄ and P2X₇ subunit antigenicity in uninfected and HIV-positive human brain tissue. P2X₄ (green) immunoreactivity in tissue sections from uninfected (a) and cognitively impaired/HIVE-positive (b) individuals; P2X₇ (green) immunofluorescent tissue from uninfected (c) and cognitively impaired/HIVE-positive (d) patients. Arrows denote microglia, which were labeled with the cell type-specific marker Iba-1 (red). Cell nuclei are counterstained with Hoechst dye (blue). Scale bar = 10 µm. Representative images are from frontal lobe white matter. All sections were cut 5 µm in thickness.
Discussion

Overall, human tissue studies give evidence that the P2X receptor family may play a role in NeuroAIDS. Unfortunately, we were unable to investigate the effects of opiates in HIV infected individuals. It is very hard to investigate the effects of a single drug in human studies due to the fact the most people are poly drug abusers and/or fail to accurately report drug usage. Also there is no group of patients from the NNTC that are substance abusers but HIV negative to serve as a control group. Aside from variable drug use, there are also several other factors to additionally take into account in human studies, such as differences in post mortem time intervals, different genetics associated with ethnicities and individuals, sex differences, as well differences in environment that may change protein expression and function at a regulatory level. Subjects were mostly male, middle-aged Caucasians who were enrolled at various locations across the country, with a wide variance in post mortem intervals (anywhere from 6-20 hours on average) (Gelman et al., 2012). The current study would greatly benefit from increased numbers of samples as well as more samples that better represent the populations as a whole. Furthermore, how the data is analyzed may also effect possible interpretations. For example, in contrast to the limma (parametric) analysis used in the present study, prior analysis of the same microarrays using local-pooled-error (nonparametric) tests for statistical comparisons (Gelman et al., 2012) did find a significant difference in P2X7 expression levels within the basal ganglia when uninfected (Group A) and HIVE (Group D) individuals were compared.

RT-PCR results suggest that among the P2X1-7Rs, expression of the P2X7-R subtype may be differentially regulated during the HIV disease process in the CNS, and that increases in P2X7 subunit transcripts are associated more with encephalitis than with neurocognitive impairment. Greater sample sizes are necessary to fully confirm or deny these assertions. Moreover, the
P2X\textsubscript{7} mRNA increases may underestimate the actual changes since the number of white matter samples available from HIV(+)-HIVE individuals was lower than that from uninfected (HIV(-)) patients (Table 2). Samples from different brain regions were pooled in this analysis, so data does not account for differences based on brain region.

Despite the lack of changes in P2X\textsubscript{4} mRNA expression levels, P2X\textsubscript{4}R function may still be indirectly constrained by changes in P2X\textsubscript{7} transcripts, since P2X\textsubscript{4}R activation enhances P2X\textsubscript{7}R inflammatory activities and the co-expression of these receptors on microglia is required for full P2X\textsubscript{7}R function (Kawano et al., 2012a; 2012b). A possible role for P2X\textsubscript{4}R is less clear based on the current limited understanding of this receptor subtype.

Large amounts of ATP can be released extracellularly in situations involving neuronal injury, such as ischemia and spinal cord injury (Jurányi et al., 1999; Wang et al., 2004; Davalos et al., 2005; Melani et al., 2005). Extracellular ATP levels are markedly elevated in primary murine neuron and glia co-cultures following treatment with HIV-1 Tat (Sorrell and Hauser, 2014), and exposure to supernatants from HIV-1-infected human monocytes (Tovar-Y-Romo et al., 2013). Assuming excessive extracellular ATP occurs in HIVE or HAND, the elevated levels are likely to further activate P2XRs contributing to cellular inflammation and/or injury. Moreover, since both extracellular ATP and P2X levels appear to be affected, it is likely that purinergic signaling is widely disrupted in neuroAIDS. Since the response of individual P2X subtypes to HIV may differ with cognitive status and the presence of encephalitis, the disruptions to purinergic receptor signaling appear to be highly complex. Lastly, P2XRs reportedly can also mediate their effects by modulating the function of other receptors such as NMDA and GABA (Pankratov et al., 2002; Gordon et al., 2005; Pankratov et al., 2009; Tai et al., 2010; Baxter et al., 2011).
Previous results, shown in Chapter 2, found P2X$_7$R activation by the selective agonist BzATP to be intrinsically neurotoxic (Sorrell and Hauser, 2014), suggesting that the increases in P2X$_7$ expression levels during HIVE in the human CNS described here might contribute to these effects. Despite these findings, we are guarded in this interpretation, because in the same study HIV-1 Tat-induced neurotoxicity in neuron and glia co-cultures was unaffected by P2X$_7$R blockade (Sorrell and Hauser, 2014). However, HIV-associated neuroinflammation in humans occurs over many years and with multiple viral and cellular toxins. Thus, signaling pathways that are activated later during the disease process are not the same signaling pathways that respond to the initial infection and increases in HIV-1 proteins. The preliminary events following infection may be better modeled by primary murine culture systems. Emerging evidence in studies of spinal cord injury (Wang et al., 2004), ischemic injury (Arbeloa et al., 2012), and neuroinflammation (Weisman et al., 2012a) also suggest that P2X$_7$R blockade may be involved in anti-inflammatory and neuroprotective events. Nevertheless, there is hesitancy to conclude that P2X$_7$R signaling is causative rather than merely correlative in HIV-1 neuropathogenesis without additional studies in vivo, using infectious models, or following exposure to other HIV-1 proteins such as gp120.

Overall, our results suggest an as yet undefined role for P2X family members in HIV disease progression, and particularly for P2X$_7$R in patients with HIVE. Accordingly, pharmacotherapies directed against the P2X family, including potentially P2X$_7$R antagonists, may help to ameliorate HIV-associated neuroinflammation and/or neuronal injury in HIVE-positive patients.
Chapter 6: Conclusions and Future Directions

The role of P2X receptor involvement in HIV and opioid interactions has been investigated in a wide range of different in vitro and in vivo assays, with focus on the role of P2X₄ receptor involvement. Finally, we were able to obtain human tissue samples to probe for possible P2X and HIV interactions, although we could not investigate opioid interactions in this model. Data from using pharmacological tools in murine primary cell co-culture models gave strong evidence for the necessity of activation of P2X receptors in order for Tat or morphine associated neurotoxicity to occur, and circumstantial evidence for actions occurring at the P2X₄ receptor (Chapter 2). Using a similar primary cell co-culture model but using cells from P2X₄ KO mice instead of pharmacological tools, confirmed that activation of P2X₄ receptors on glia in particular are critical in Tat and morphine associated neurotoxicity. However, a yet undefined role for the receptor on neurons was implicated, where neuronal receptors may actually be neuroprotective (Chapter 3). Next we looked at P2X₄ actions in the whole animal, again via the use of P2X₄ KO animals. Behavior data showed little significance and did not support our hypothesis, however, signs of striatal inflammation due to the treatment of morphine and/or Tat were observed (Chapter 4). Finally, tissue from human subjects implies the P2X receptor system is involved in NeuroAIDS, however no evidence for the P2X₄ receptor involvement in particular was noted (Chapter 5). Overall the P2X receptor system seems to play an important yet complex role in HIV and opioid interactions, with murine model evidence implicating P2X₄ receptors on glia in particular.

Based on the results presented herein, our working hypothesis is that after initial insult due to HIV infection, which involves the release of toxic HIV proteins like Tat, ATP release
occurs. Although the exact source of extracellular ATP is not investigated here, ATP can be released by neurons and astrocytes, as well as by damaged or dead cells (see discussion in Chapter 1). Extracellular ATP then can activate P2X$_4$ receptors on glia in the striatum. This then leads to abnormal control of [Ca$^{2+}$]$_i$, decreases in dendrite length, and ultimately increased neuronal death.

Experiments using TNP-ATP in Chapter 2 confirm activation of the P2X receptor family is occurring due Tat and/or morphine treatment and blockade of P2X receptors prevent Tat and morphine associated neurotoxicity in multiple assays. Studies using selective antagonists for the P2X$_1$, P2X$_3$, and P2X$_7$ subunits plus evidence from the literature circumstantially strengthen the case for P2X$_4$ subunit involvement. Finally use of glia from P2X$_4$ KO animals in primary cell culture experiments and comparison of inflammatory markers in the striatum of WT verses KO animals that received intrastriatal Tat injections and/or morphine pellets point to the activation of P2X$_4$ receptors on glia leading to increases in inflammation in the striatum as a result of Tat and/or morphine. However, in vitro experiments that involved neurons from P2X$_4$ KO mice give rise to a more complex hypothesis involving possible baseline change in neuron function due to knockout of the P2X$_4$ receptor, and evidence for the activation of different signaling pathways depending on whether Tat or morphine are given alone as compared to when they are given in combination. Data from behavioral tasks also imply there may be differences in baseline function between KO and WT animals, as well as gave evidence that P2X$_4$ receptors in other areas of the brain and body may be involved in a more complex role of overall homeostasis in the animal. Behavior data supports the theory that Tat and morphine treatment in combination verses when compounds are given alone may activate different cellular signaling pathways. In general, it also supports themes that were suggested in Chapter 3 in response to data generated with neurons
from P2X4 KO mice, that specific pools of P2X4 receptors may in fact play a significant role in Tat and morphine related neurotoxicity, however when global P2X4 receptor function is assessed, it appears to affect multiple functions that are important to the overall function of the animal and how it may respond to Tat and morphine exposure.

Our work suggests selective antagonism of the P2X4 receptor may be a useful target in the clinic to treat symptoms associated with neuroAIDS. However, there are currently no selective agonists/antagonists for the P2X4 receptor, as such targeting proteins that participate in signaling downstream of receptor activation may be another option. Furthermore, since the receptor may elicit different signaling pathways based on cell location, it may be of benefit to target downstream targets that are cell type specific. The present work supports signaling associated with P2X4 receptors on microglia as the most promising target.

Further experiments are needed to investigate downstream signaling events associated with P2X4 receptor activation induced by Tat and/or morphine treatment. However, several pathways have been identified in microglia P2X4 signaling in response to other stimuli/conditions, including MAPK/ERK and PI3/Akt pathways (Fig. 33). Tat and morphine can also activate these same pathways, making them potential points of convergence (D'Aversa et al., 2004; Hauser et al., 2006; King et al., 2006b). Looking at other points of convergence such as changes in cytokine and chemokine level changes due to Tat and morphine treatment would also help to uncover P2X4 downstream signaling pathways.
Figure 33. Speculative molecular mechanisms in microglia. MOR activation can lead to up-regulation of P2X\(_4\) receptors on the cell surface (Horvath and DeLeo, 2009). These receptors can undergo trafficking from lysosomal vesicles due to multiple factors. After receptor undergoes activation, subsequent Ca\(^{2+}\) flux occurs, which leads to MAPK/ERK and PI3/Akt pathway activation. MAPK/ERK pathway activation has been linked to BDNF and chemokine/cytokine release. PI3K/Akt pathway activation has been linked to increases microglia migration (Horvath and DeLeo, 2009; Trang et al., 2009; Beggs and Salter, 2013). Ca\(^{2+}\) can lead to MAPK/ERK activation via activation of proline-rich tyrosine kinase 2 (PYK2), which then acts through the small GTPase Ras to induce the MAPK cascade (Lev et al., 1995; Berridge et al., 2003). Ca\(^{2+}\) can alter PI3K/Akt activity through activation of protein phosphatases (Nakazawa et al., 2005).
Unfortunately, we were unable to differentiate between the roles of P2X₄ receptors located on microglia versus astrocytes in our co-culture model. As mentioned in the methods section glia cultures contain about 10% microglia and 90% astrocytes. The removal of microglia from astrocyte cultures can be very time consuming and the process itself can be harmful to the cells. It is also hard to get pure microglial cultures without astrocytes present, as microglia do not survive well in isolation. This also complicates the ability of receptor silencing to be useful since it is hard to isolate either cell type. Ideally, a model that expressed or knocked out the receptor based off a cell type specific promoter would greatly facilitate the ability to differentiate between functions occurring through astrocytes versus microglia.

The use of conditional P2X₄ KO mice that could be induced by something like doxycycline would be useful in determining whether baseline differences seen between WT and KO groups are due to developmental compensation or if the same effect would still occur if P2X₄ receptors levels were altered later in development. Conditional KO animals that make use of the Cre/Lox technology would also be helpful to answer the question of what the lack of P2X₄ receptors in other areas of the brain aside from the striatum as well as in the periphery may be having on our Tat and morphine treatment paradigm. However, they are several subpopulations of neurons in the striatum, which makes finding a particular promoter to target that would encompass all striatal neurons very difficult. Additionally, targeting microglia or astrocytes in a particular brain region is also difficult, as their protein expression profile tends to be more homogeneous. Crossbreeding of P2X KO animals with inducible Tat transgenic mice, may also be an improved model, as chronic Tat presence that is more evenly distributed may better correlate with what occurs in humans.
Another thing to keep in mind for these experiments is that morphine released from implanted pellets is released systemically and likely able to activate receptors throughout the animal, not just in the striatum. Tat may also have effects elsewhere, but due to injection directly into the striatum it is probably having most of its direct effects there. One idea may be to also inject morphine into the striatum but daily dosing over a 5-day period provides a technical challenge, as having to perform multiple surgeries on the animal would likely have significant affects of its own. Insertion of an injection cannula may be able to minimize complications due to repeated injections. Even in this situation changes in striatal projections may alter the function of other areas of the brain that may exhibit a changed response due to lack of P2X₄ receptors being present.

Another aspect to keep in mind with both our in vitro and in vivo murine models, is that seeing one dose of Tat over several days, is not going to be the same as in a human where the brain is getting insulted by Tat and other HIV proteins for years and only over time do individuals start to show symptoms. However, it is impossible to study individual molecular pathways without looking at simpler systems. Our model does show good concordance with signs that are associated with neuronal injury that are seen in humans, such as increased intracellular calcium levels and decreased dendritic length. Also, the amount of neuronal death observed seems reasonable (around 30% with Tat and morphine treatment) to correlate with what may occur in humans. Dysfunction in increasing but initially, small proportions of neurons in targeted areas is what eventually adds up to cause alterations in brain function over time. Finding appropriate models to study HIV interactions in the CNS is a challenge to everyone in the field, but we believe that elucidating initial inflammatory events is important to not only
finding treatments aimed at prevention of neurocognitive deficits, but for better early biomarkers that may be associated with NeuroAIDS. However, we must also keep in mind the viral infection in humans over years or even decades may be much more complex than looking at a single HIV protein for a short duration due to single treatments in cell culture or whole animal models.

As with Tat, treating with single doses, or time-released treatments over several days as was done in our studies, is not going to exactly replicate what humans would see with opiate abuse that occurs over years or possibly decades. In both our in vitro and in vivo experiments, we use a higher dose of morphine, as opioid-dependent individuals can reportedly have very high opioid blood levels (Gurwell et al., 2001). However, opiate abuse patterns can vary greatly among different individuals and furthermore interactions with other drugs are also likely to be a factor, as most humans are poly drug users.

Another factor that affects Tat and morphine interactions in humans that are not addressed in our animal models is the genetic variability of an individual. The current work focuses on possible new targets and biomarkers to help in the treatment of neuroAIDS related symptoms. However, 50% of individuals will never see neurological complications, so it is apparent that these individual differences also play an important role. While, we do not fully understand these differences, polymorphisms of different proteins can cause differences in HIV disease progression, with perhaps that most notable being a mutation in the CCR5 protein, where individuals that are exposed to HIV do not get infected (Huang et al., 1996), however there are also many other polymorphisms that can affect HIV disease progression (Fellay et al., 2007;
Polymorphisms in the μ opioid receptor and differences in splice variants may also play a role in HIV and opioid interactions (Dever et al., 2012; 2014).

Another reason why some individuals may see neurocognitive symptoms and others do not is that an individual may respond differently based on genetic variation in the virus itself. Tat can differ in its amino acid structure with the different subtypes of the HIV virus, which can affect its transcriptional activity. We only used Tat made from HIV-1B subtype, so Tat from different HIV-1 subtypes may have yielded different results (Kurosu et al., 2002; Roof et al., 2002; Desfosses et al., 2005). Furthermore, differences in strain can also effect morphine’s interactions with the virus (El-Hage et al., 2011).

In conclusion, both in vitro and in vivo experiments using both pharmacological and genetic manipulation provide strong evidence in support of our initial hypothesis that activation of P2X4 receptors on microglia cells is necessary in Tat and morphine striatal toxicity. However, microglia and astrocyte function were not able to be assessed individually, and experiments involving neurons from P2X4 KO mice and assays assessing motor related behavior in P2X4 KO mice that were treated with Tat and/or morphine suggest a more complex model with the receptor having different effects depending on cell type and tissue location. Although data from human tissue did not show a correlation between P2X4 mRNA levels and HIV progression in the CNS of humans, this does not exclude normal levels of P2X4 mRNA and presumably protein function from having a significant effect on symptoms caused by neuroAIDS. Altogether, data suggests P2X4 receptors as promising new targets to prevent or treat neurocognitive symptoms associated with HIV, and that targeting a subpopulation of these receptors may provide the most
benefit.
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