INTERACTIONS OF FENTANYL WITH GP120 IN THE CENTRAL NERVOUS SYSTEM

By

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ABSTRACT
Meagan Elizabeth Buchanan: Interactions of Fentanyl with gp120 in the Central Nervous System
(Under the direction of Dr. Jason Paris)

The Human Immunodeficiency Virus (HIV) has affected more than 75 million individuals worldwide. HIV not only affects the peripheral system through the targeting of T cells, but also causes adverse effects in the central nervous system. Individuals suffering from HIV often experience neuropathic pain and are prescribed opioids to combat the chronic pain. Along with the increase of opioids prescribed to HIV patients, the number of deaths related to the synthetic opioid, fentanyl, has increased drastically. Drug abuse and HIV are interlinked epidemics; individuals who are HIV positive and referred to as “NeuroAIDS.” The HIV envelope protein, gp120, has been shown to have a wide variety of neurotoxic effects on the CNS through increasing cell death of astrocytes through stress-mediated apoptosis, increasing the permeability of the Blood Brain Barrier (BBB), increasing the expression of proinflammatory cytokines and chemokines, and altering and damaging mitochondria. The purpose of these experiments was to determine whether gp120 showed direct neurotoxic effects and/or indirect neurotoxic effects and whether fentanyl would act synergistically with gp120. Through conducting a Live/Dead Assay, Immunocytochemistry for GFAP/IBA1, and a Cytokine Array, it was determined that gp120 shows neither direct nor indirect neurotoxic effects, and fentanyl does not exacerbate the effects of gp120.
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<table>
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<tr>
<td>AIDS</td>
<td>Acquired Immunodeficiency Syndrome</td>
</tr>
<tr>
<td>ANI</td>
<td>Asymptomatic Neurocognitive Impairment</td>
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<tr>
<td>BBB</td>
<td>Blood Brain Barrier</td>
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<td>BDNF</td>
<td>Brain-Derived Neurotrophic Factor</td>
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<td>cART</td>
<td>Combined Antiretroviral Therapy</td>
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<tr>
<td>CD4</td>
<td>Cluster of Differentiation 4</td>
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<tr>
<td>CDC</td>
<td>Centers for Disease Control and Prevention</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
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<tr>
<td>CXCR4</td>
<td>C-X-C Motif Chemokine Receptor 4</td>
</tr>
<tr>
<td>CCL5</td>
<td>C-C Motif Chemokine Receptor 5</td>
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<tr>
<td>DAPI</td>
<td>4',6-Diamidino-2-Phenylindole</td>
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<tr>
<td>GFAP</td>
<td>Glial Fibrillary Acidic Protein</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-Protein Coupled Receptor</td>
</tr>
<tr>
<td>HAD</td>
<td>HIV Associated Dementia</td>
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<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
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<tr>
<td>IBA1</td>
<td>Ionized Calcium Binding Adaptor Molecule</td>
</tr>
<tr>
<td>IDU</td>
<td>Injecting Drug Users</td>
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<tr>
<td>MND</td>
<td>Mild Neurocognitive Disorder</td>
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<td>NIDA</td>
<td>National Institute on Drug Abuse</td>
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1. Introduction:

a. HIV worldwide and in the U.S.

The human immunodeficiency virus (HIV) epidemic has infected more than 75 million people worldwide since the first reported cases in 1981, and an estimated 35 million people have died due to complications associated with HIV (“HIV/AIDS,” 2018). Currently, there are approximately 37 million individuals living with the infection (Deeks, et al., 2015); roughly 26 million of these individuals live in Sub-Saharan Africa, (Kharsany & Karim, 2016) and approximately 1.1 million people live in the United States, accounting for an estimated 162,500 who are unaware of their condition (“Drug Use and Viral Infections,” 2018). In Sub-Saharan Africa, HIV is most prevalent among women aged 15-24 due to cultural interactions (CDC). In the United States, the populations most affected by HIV are homosexual African American men and homosexual Hispanic/Latino men; both populations have experienced an increase in HIV diagnoses in recent years, whereas all other populations have experienced a decrease in diagnoses (“Estimated HIV Incidence and Prevalence in the United States,” 2018).

b. HIV transmission

HIV is transmitted from infected individuals through bodily fluids: blood, semen, pre-seminal fluid, anogenital fluids, and breast milk. Transmission occurs via viral contact with mucus membranes, tissue lesions, or via direct transmission into the blood
stream, such as occurs with injection drug use (“HIV/AIDS,” 2018). Individuals who inject drugs, hormones, steroids, or silicone can acquire HIV by sharing needles, syringes, or other injection equipment due to residual blood from an infected person. Additionally, the virus can survive within used injection equipment for up to 42 days, depending on environmental conditions (“HIV/AIDS,” 2018). The contribution of increased engagement in risky sexual behavior associated with impaired judgment associated with drug use is also a consideration (“HIV/AIDS,” 2018).

c. **HIV effects on the body**

HIV affects all organ systems in the body through its interactions with the immune system. HIV targets T cells and reduces cell-mediated immunity which leads to severe opportunistic infections and cancers. HIV infects and eradicates CD4+ T-cells, or helper T-cells. CD4+ T-cells mature in the thymus of the human body and aid B cells in the production of antibodies, induce macrophage activity, recruit neutrophils, eosinophils, and basophils to infection and inflammation sites, and produce cytokines and chemokines (Zhu & Paul, 2008). Cytokines and chemokines are secreted, small cell-signaling protein molecules, whose receptors are expressed on immune cells. These factors play a critical role in immune cell differentiation, migration, and polarization into functional subtypes and in directing their biological functions (Shachar & Karin, 2013).

d. **HIV and injecting drug users (IDU)**

While Sub-Saharan Africa contains 70% of the world’s population of HIV positive individuals, the main route in that region is heterosexual transmission (Aceijas et al.,
2004). However, in China, Indonesia, Vietnam, several Asian republics, the Baltic States, and North Africa the main route of transmission is primarily unsafe drug-injecting practices with additional HIV spread occurring through commercial sex work (Aceijas et al., 2004). Additionally, it is estimated that in many countries in Europe, Asia, the Middle East, and the Southern Latin America, the sharing of injecting equipment is the primary mode of transmission, accounting for 30–90% of all reported infections (Aceijas et al., 2004).

In the United States, injection drug use is associated with approximately one-third of all HIV infections, and approximately 10% of all new HIV diagnoses are attributed to injection drug use or male-to-male sexual contact and injection drug use (“HIV/AIDS,” 2018). Research has estimated that HIV positive IDU might significantly contribute to higher rates of transmission even when compared to those with ineffective or inconsistent treatment (Escudero, et al., 2017).

Even though awareness of transmission of HIV is at an all-time high in the United States, the prevalence of illegal opioid drug use also continues to climb. As a result, multi-person sharing of needles continues to be a common occurrence. To combat the transmission of HIV and other diseases through infected needles, the United States established the Syringe Service Programs (SSPs). SSPs allow individuals to exchange used syringes for sterile syringes; this has shown to decrease the spread of HIV, while not increasing the prevalence of illegal drug use (“Syringe Services Programs,” 2018). Additionally, even with the establishment of a treatment protocol for HIV, called
cART, HIV positive individuals with transmission via IDU continue to experience shorter lifespans (Marcus, et al., 2016).

e. Opioid toxicity

The most commonly injected drugs are opioids; the most abused opioid is heroin (Baciewicz, 2012). Chronic opioid use leads to multiple impairments in the central nervous system: increased microglial activation, increased axonal damage, increased neuronal loss, increased vasculopathy, and decreased astrocytes. Additionally, chronic opioid use has been shown to cause dizziness, sedation, hyperalgesia associated with increased sensitivity to pain, and increased rates of clinical depression and other mental illnesses (Arts & Hazuda, 2012).

f. Opioids and HIV

While the mechanisms in which opioids contribute to the increased neurocognitive impairments are not completely understood, the regulation of immune cell functions is thought to play a role (Jaureguiberry-Bravo, et al., 2018). Studies have shown that opiate abuse may act at multiple glial-cell types to further compromise neuron function and survival (Hauser, et al., 2012). Opioids also allow easier access to HIV to enter the brain by weakening the blood brain barrier (BBB) and cause greater nerve cell injury (“Opioid Overdose Death Rates,” 2018). Additionally, opioids potentiate the effects of HIV by promoting the progression to Acquired Immunodeficiency Syndrome (AIDS), increasing the frequency and severity of HIV encephalitis, and
potentiating the symptoms of HIV Associated Neurocognitive Disorder (HAND) (Jaureguiberry-Bravo, et al., 2018).

g. The effects of fentanyl

From 2002-2017, there was a 4.1-fold increase in the total number of opioid overdose related deaths and a 900% increase in the number of individual seeking treatment for prescription opioid abuse (Kolodny A, et al., 2015). In 2016 alone, more than 42,000 individuals in the U.S. died from opioid overdoses – more than 115 per day – and synthetic opioids were involved in approximately 50% of overdoses (“Opioid Overdose Death Rates,” 2018). The most commonly used synthetic opioid was fentanyl; from 2002-2017, there was a 22-fold increase in the total number of deaths due to fentanyl (“Fentanyl and Other Synthetic Opioids Drug Overdose Deaths,” 2018). Fentanyl is approximately 50 times more potent than heroin and 100 times more potent than morphine and negates naloxone, an opioid antagonist that reverses and blocks the effects of opioids (“Opioid Overdose,” 2016). It is because of these reasons that fentanyl is driving the opioid epidemic.

h. HIV patients and pain

Due to the neuropathic properties of HIV, over 50% of HIV patients suffer from chronic pain and are often prescribed opioids to combat the pain (Liu, Bolong et al., 2016). Studies show that opioids exacerbate the negative effects of HIV on the nervous system by promoting damage of the neurons, activation of caspase dependent and independent pro-apoptotic pathways, and disruption of glial functions; opioids can also
activate glial cells, such as microglia and astrocytes (Liu, Bolong et al., 2016). The activation of glial cells may facilitate the expression of hyperalgesia by releasing cytokines, chemokines, and brain-derived neurotrophic factor (Liu, Bolong et al., 2016).

i. **NeuroAIDS**

Drug abuse and HIV are interlinked epidemics; individuals who are HIV positive and abuse drugs show more severe symptoms of neurocognitive impairments linked to HIV, sometimes collectively referred to as “NeuroAIDS.” Since the development of combined antiretroviral therapy (cART) in 1996, the number of deaths due to HIV has decreased and individuals are able to live longer with the infection (Saylor, et al., 2016). cART aids in counteracting progression of HIV in the peripheral nervous system but cannot accumulate in the central nervous system due to its inability to permeate the blood brain barrier (Kumar, et al., 2018). Due to the increase of life expectancy, HIV-infected patients now contend with more neurocognitive impairments: mood disorders, cognitive impairment, behavioral disinhibition, motor impairment, and peripheral neuropathies associated with HIV patients (Saylor, et al., 2016). These neurocognitive impairments, which affect approximately 50% of all HIV patients, have been deemed as HIV Associated Neurocognitive Disorder (HAND) (Arts & Hazuda, 2012). Three categories of HAND have been discovered by researchers: Asymptomatic Neurocognitive Impairment (ANI), Mild Neurocognitive Disorder (MND), and HIV Associated Dementia (HAD) (Arts & Hazuda, 2012). Individuals who suffer from ANI show no impairment in activities of daily living (Arts & Hazuda, 2012) but show HIV associated cognitive impairment of at least two cognitive areas, such as memory, processing speed,
attention, sensory perceptual and motor skills (Kumar, et al., 2018). Individuals who suffer from MND show impairment in activities of daily living (Arts & Hazuda, 2012) with a decline in brain function, movement skills, and shifts in behavior (Kumar, et al., 2018). Individuals who suffer from HAD show marked impairment in activities of daily living and include individuals who suffer from HIV associated dementia and HIV-encephalitis, a neuro-inflammatory condition characterized by the presence of activated and infected microglia, multinucleated giant cells, astrogliosis, and loss of myelin (Saylor, et al., 2016). cART has successfully decreased the severity of HAND, but not the prevalence; the majority of individuals that are HIV positive now live with HAND but suffer from the less severe types (Saylor, et al., 2016). Additionally, HIV positive individuals with transmission via IDU have shorter lifespans even with the addition of cART (Festa & Meucci, 2012).

j. HIV envelope protein gp120 and its effect on NeuroAIDS

The HIV protein gp120 is a highly glycosylated envelope protein that assists with viral entry into the cell through a conformational change in CD4 and a g-protein coupled receptor (GPCR) (Rich et al., 2019). While CD4 is necessary for viral entry, it is not sufficient, and HIV requires an additional interaction with GPCR chemokine co-receptors. Through the binding of viral glycoproteins and cell surface glycoproteins the process of adsorptive endocytosis begins. Broadly, HIV often parsed into three primary strains X4, R5, and a dual X4 and R5 strain, albeit, additional strains exist, particularly in world regions with poor virulence control. These strains are determined by the selectivity of the gp120 molecule for a particular co-receptor, called a “tropism” (Rich et
al., 2019). In X4-tropic strains, gp120 is associated with the CXCR4 chemokine receptor, and in R5-tropic strains, gp120 is associated with the CCR5 chemokine receptor (Rich et al., 2019). The CXCR4 co-receptor is the C-X-C motif chemokine receptor 4 and is one of the chemokine receptors expressed primarily on T-cells (Charo et al., 2018). In the periphery, CXCR4 plays a role in cell migration and acts as a receptor for extracellular ubiquitin by increasing intracellular calcium ion levels (“C-X-C Chemokine Receptor Type 4,” 2018). The CCR5 co-receptor is the C-C motif chemokine receptor 5 and is one of the chemokine receptors expressed on multiple cell types, including macrophages and some types of T-cells (Woollard & Kanmogne, 2015). R5 strains predominate in early stages of HIV infection, and X4 strains emerge in later stages (Woollard & Kanmogne, 2015).

Gp120 has been shown to have a wide variety of neurotoxic effects on the CNS. Gp120 increases cell death of astrocytes through stress-mediated apoptosis (Shah et al., 2012). Additionally, gp120 alters the BBB by increasing the permeability; the BBB aids in proper functioning and homeostasis of the CNS by regulating the trafficking of molecules (Shah, Ankit et al.). Gp120 increases the expression of proinflammatory cytokines and chemokines which promotes leukocyte chemotaxis to inflammation sites (Charo et al., 2018). Although the mechanisms are not fully understood, gp120 enlarges and damages mitochondria and impairs the transport of mitochondria along microtubules (Avdoshina et al., 2016). Gp120 also inhibits microtubule polymerization and tubulin acetylation, which results in impaired axonal function (Avdoshina et al., 2017). These findings suggest that the mechanism of gp120 neurotoxicity is contributed to the detrimental effects on microtubules.
k. **Hypothesis**

To determine if gp120 was directly neurotoxic, we exposed differentiated human neuroblastoma cells to X4-gp120 and/or R5-gp120 (10 – 1000 pM). To assess the extent to which gp120 exerted indirect toxicity via neuroinflammation, in the absence or presence of an abused opioid, primary mixed glia were obtained from mice and exposed to X4- or R5 gp120 (500 pM) with or without co-exposure to fentanyl. We hypothesized that gp120 would show both direct and indirect neurotoxic effects and fentanyl would potentiate these effects.
2. Methods

2.1 Description of Cells and Cell Culture Processes

a. SH-SY5Y human neuroblastoma cells

An SH-SY5Y cell line was received from the American Type Culture Collection (ATCC; Manassas, VA). Cells were relocated from growth flasks into 24 well plates and allowed to grow in media (DMEM/F12 89.5%, Fetal Bovine Serum 10%, Antibiotic (PSF) 0.5%) in an incubator kept at 37°C and 5% CO₂. Once the cells were at 50% confluency, Retinoic Acid (50 μg retinoic acid, 33.3 mL 95% EtOH) was added to begin differentiation. After ~6 days, BDNF (10 μg BDNF, 1 mL DMEM/F12) was added to continue differentiation. Throughout the differentiation process, a full media exchange occurred every 48 hours and plates were kept in an incubator (Paris et al., 2019).

b. Primary mouse mixed glia cells

Primary mixed glial cultures were prepared as previously described (Paris et al., 2016). Briefly, mixed-glia were obtained from the whole-brain postnatal day 0–1 C57BL/6H Nsd. Brains were minced before being incubated (37°C, 5% CO₂) with trypsin (2.5 mg/ml) and DNase (0.015 mg/ml) in Dulbecco’s Modified Eagle’s Medium (DMEM; Life Technologies, Carlsbad, CA) for 30 min. Tissues were triturated and sequentially filtered through 100-μm and 40-μm diameter
pore cell strainers (Greiner Bio-One, Kremsmünster, Austria). Cells were plated at a density of 150,000 cells/well onto poly-L-lysine-coated (Sigma–Aldrich, St. Louis, MO) 24-well culture plates and maintained for 11-14 days in DMEM supplemented with 10% fetal bovine serum (Thermo Scientific Hyclone, Logan, UT). The mixed glial cells were harvested from 0-2 day old C57 BL/6HNsd pups and were seeded on 24-well plates at a density of 150,000 per well (Paris et al., 2016).

2.2 Experimental Procedures

a. Live/Dead Assay

After the SH-SY5Y cells were grown to 80% confluency and seeded onto a 24-well plate at a density of 50,000 cell/well, gp120 X4 and gp120 R5 received from ImmunoDX (Woburn, MA) were used to treat the cells. X4-gp120 and/or R5-gp120 were used at concentrations of 0, 10, 100, and 1000 pM. Fluorescents were added to the wells by mixing the fluorescents with the gp120 treatments or media per the controls. A master mix of propidium iodide/DAPI was created with gp120 media (one drop of propidium iodide/DAPI was added per mL of gp120 treatments/media) at time 0 and the number of red positive cells were counted at 0, 24, and 48 hours, but the cell death reached over 60% at 48 and no group differences were observed as such and only hours 0-24 were used for analyses. Propidium iodide is a membrane impermeable dye that only stains the DNA of a cell if the membrane is compromised. DAPI is a membrane permeable dye that
stains the DNA in all cells. By comparing those that were propidium iodide
stained and the total number of cells, the percent of dead cells was determined.

b. Immunocytochemistry for GFAP/IBA1

Wells were treated with gp120 X4 or gp120 R5 at a concentration of 500
pM, as well as fentanyl was obtained from: Sigma Aldrich (St. Louis, MO; #F-013)
diluted to final concentration in media at 1 µM. 24 hours later, cells were fixed in
4% Paraformaldehyde. Permeability solution (0.1% Triton X 100, 0.1% Bovine
Serum Albumin in PBS) was added to each well and incubated at room
temperature for 30 minutes. Then the permeability solution was removed and
blocking solution (1% Bovine Serum Albumin, 1% Normal Goat Serum in PBS) was
added to each well and incubated at room temperature for 30 minutes. Blocking
solution was removed and primary antibodies, (Wako (Richmond, VA) anti-Iba 1
Millipore (anti-gfap) mab360 Burlington, MA 1:250 dilution in Blocking Solution)
were added to each well and incubated overnight in a 4°C moisture chamber.
The next day, the primary antibodies were removed, and the wells were washed
with PBS 3 times for 10 minutes each. Secondary antibodies (Alexafluor 594 and
488 (Carlsbad, CA) 1:500 dilution in Blocking Solution) were added to the wells
and incubated in the dark for one hour at room temperature. After an hour, the
secondary antibodies were removed, and the wells were washed with PBS 3
times for 10 minutes each. Hoechst 33342 (1:10,000 dilution in PBS) was added
to each well and incubated at room temperature in the dark for 8 minutes. The
Hoechst was removed, and the wells were washed with PBS 3 times for 10 mins
each. The plates were kept in the dark until ready to be imaged by a Nikon Ti2 microscope. By using Image J software, the number of microglia were counted and integrated density of astroglia was calculated. Microglia were counted by selecting a random field with at least 200 cells per field and determining the number Hoechst positive cells and the number of Iba-1 positive cells. To determine the integrated density of astroglia, the images were uploaded to ImageJ and a threshold was developed to distinguish signal from the background. From the signal, Image J software was able to determine the intensity and the area. Integrated density was determined by calculating the mean intensity of signal multiplied by the total area of signal.

c. Cytokine array

Proteome Profiler Antibody Array: Mouse Cytokine Array Panel A ary-006 was purchased from R&D Systems, Inc. Minneapolis, MN and the array protocol for cell culture supernatants was performed according to manufacturing recommendations. To determine levels of mouse chemokines and cytokines, cell culture supernatants were placed on pre-coated membranes to determine the level of cytokine activity in cell supernatants for 38 cytokines. These cytokines included pro-inflammatory cytokines (TNFα, IFNγ, IL-1α, IL-1β, IL-2, IL-3, IL-6, IL-7, IL-12p70, IL-16, IL-17, IL-23, and IL-27), anti-inflammatory cytokines (IL-1ra, IL-4, IL-5, IL-10, and IL-13), and chemotactic/regulatory cytokines (C5, G-CSF, GM-CSF, TIMP-1, TREM-1, CD54, CCL1, CCL2, CCL3, CCL4, CCL5, CCL11, CCL12, CCL17,
CXCL1, CXCL2, CXCL9, CXCL10, CXCL11, and CXCL13). Images of the membranes were taken, and level of intensity was determined on a LI-COR imager.

d. Statistical analyses

Dependent measures for Live/Dead analyses were via separate, one-way Repeated Measures ANOVAs with gRp120 concentration as the between-subjects factor (0, 10, 100, and 1000 pM) and time as the within-subjects factor (0 or 24 h post-treatment). Dependent measures for immunocytochemical endpoints were assessed via separate, two-way ANOVAs with gp120 condition (control, X4, or R5) and fentanyl condition (vehicle or fentanyl) as between-subjects factors. Fisher’s Protected Least Significant Difference post hoc tests determined group differences following main effects. Interactions were delineated via simple main effects and main effect contrasts with alpha controlled for multiple comparisons. Analyses were considered significant when p < 0.05.
3. Results

3.1 Live/Dead Assay

To determine if direct neurotoxicity was present, SH-SY5Y human neuroblastoma cells treated with X4-gp120 and or R5-gp120 (0, 10, 100, or 1000 pM) were maintained in media containing DAPI and propidium iodide and imaged at 0, 24, and 48 hours. After 48 hours, there were no group difference between percent cell death (data not shown). There was a significant interaction [F(3,4)=3.59, p<.05)] from 0 to 24 hours such that X4-gp120 decreased cell death at 10 pM (Fig. 1A). Although cell death increased from 0 to 24 hours, there was no significant interaction between treatment conditions of R5-gp120 (Fig. 1B) and X4/R5-gp120 (Fig. 1C) (0, 10, 100, or 1000 pM) from 0 to 24 hours.

3.2 Immunocytochemistry for GFAP/IBA1

To determine if indirect neurotoxicity was present and if fentanyl exacerbated the effect, primary mouse mixed glial cells were treated with X4-gp120 or R5-gp120 (500 pM) with and without the presence of fentanyl. Although a decrease in frequency for microgliosis (Fig. 2A) and integrated density for astrogliosis (Fig. 2B) were both present, there was no significant interaction within groups.
3.3 Cytokine Array

To determine if neurotoxicity was present and if fentanyl exacerbates the effect through the influence of cytokines or chemokines, cell supernatant from the primary mouse mixed glial cells that were treated with X4-gp120 or R5-gp120 with and without the presence of fentanyl were taken and used on a cytokine/chemokine array. Although fentanyl caused an increase of the chemokines CXCL10 and CCL2 (Fig. 3A), the data did not reach significance. Additionally, there were no significant interactions concerning any cytokines (Fig. 3B) or individual interactions with either X4-gp120 or R5-gp120 (Fig. 3A/B).
4. Discussion

The expectations of these experiments were that gp120 would show both direct and indirect neurotoxic effects through increasing cell death, astrogliosis, microgliosis, and the presence of cytokines and chemokines. Additionally, it was expected that fentanyl would act synergistically with gp120 and exacerbate the effects. The live/dead assay showed a significant decrease in cell death of X4-gp120 at 10 pM from 0 to 24 hours; however, all other data were not significant. Due to the lack of direct neurotoxicity, indirect neurotoxicity was expected to occur through astrogliosis and microgliosis. Immunocytochemistry for GFAP and IBA1 did not reveal significant differences, but there was an apparent effect. To assess whether innate immune modulators were influenced, a cytokine array was performed. Fentanyl had an apparent effect with the chemokines CXCL10 and CCL2, but these data were exploratory (n=2/group) and groups did not significantly differ. Although the findings were intriguing, they did not reach significance and the original hypothesis was rejected.

Previous literature has proposed direct neurotoxicity to neurons through gp120 initiating the release of a glutamatergic excitotoxin from infected microglia and macrophages (Howard et al., 1999). The agent reacts with NMDA receptor and calcium-dependent excitotoxic damage to neurons through calcium mobilization (Howard et al.,
Calcium mobilization causes a downstream reaction in which the generation of reactive oxygen species (ROS) and lipid peroxidation occur (Howard et al., 1999). The exact mechanism in which the gp120 mobilized calcium causes accumulation of ROS is unknown; however, accumulation has been shown to occur in both neurons and glia (Howard et al., 1999). Accumulation of ROS in neurons is consistent with the proposal of gp120 activating NMDA-dependent pathways, whereas ROS in glia indicate that gp120 mobilizes cytosolic calcium within astrocytes (Howard et al., 1999).

Additionally, gp120 has been shown to have direct neurotoxic affect through alterations in mitochondrial fusion/fission and mitochondria biogenesis (Fields et al., 2016). Mitochondrial fusion is dependent on MFN1, MFN2, and OPA1; mitochondrial fusion is dependent on DRP1 (Fields, et al.). Experiments have shown levels of MFN1, OPA1, and DRP1 were altered in the brains of HAND patients and in gp120 transgenic mice (Fields et al., 2016). Additionally, gp120 transgenic mice showed damaged mitochondria and increased markers of neuroinflammation (Fields et al., 2016).

Furthermore, the G protein coupled receptor CXCR4 and its endogenous ligand CXCL12 have been shown to play a role in neuronal proliferation, maturation, survival, and the regulation of dendritic spine density (Festa & Meucci, 2012). Both CXCR4 and CXCL12 are constitutently expressed in the human brain and CXCL12 has been shown to dictate neuronal survival, neuronal transmission, and alter excitotoxic pathways (Festa & Meucci, 2012). Disrupting the connection between CXCR4 and CXCL12 has negative impacts within the brain. Through the cleavage of CXCL12, the neuroprotective function
of CXCR4 is impaired and an alternate receptor, CXCR3, is activated and causes neuronal injury (Festa & Meucci, 2012).

As gp120 binds to CXCR4, MAP kinases are activated and CXCR4 internalization and dimerization is induced (Festa & Meucci, 2012). Gp120 then induces pathways associated with apoptosis, such as p38 and caspase-3, even in the absence of glia and CXCR4 internalization (Festa & Meucci, 2012). Additionally, gp120 has been shown to directly interact with neurons through experimentation that proved CXCL12, CXCR4 inhibitors, and Gi-signaling inhibitors all reduced or blocked gp120-induced neurotoxicity (Festa & Meucci, 2012).

Although these experiments did not match what was seen in previous literature, several features could have played a factor. For example, receiving gp120 from different manufacturers could have resulted in different results due to different amounts of glycosylation. After using the enzyme PNGase F to deglycosylate gp120, our lab has observed toxicity increased between 6-10% in a concentration-dependent manner. Additionally, the present experiment used SH-SY5Y cells that were differentiated in BDNF which may have been protective. Assessing different cell types could show different direct toxicity profiles as well as potential regional vulnerability via the assessment of primary cells from selected brain regions as opposed to those obtained from the whole brain as was done herein. As well, In lieu of time-lapse microscopy, propidium iodide was added at T0; due to unexpected toxic properties of propidium iodide, it caused an increase in cell death which may have masked our capacity to
see effects. In future experiments, the use of time-lapse microscopy or an end point analysis where cell death is measured at 24 or 48 would be beneficial. Lastly, cytokine arrays were informative, but revealed targets that were semi-quantitative; more observations are needed, and future studies will replicate the work and employ an ELISA on targets of interest, CCL2 and CXCL10.

Future avenues of research include determining the relationship of other HIV proteins, such as Tat and Vpr, that contribute to neurotoxicity and opioids. Tat, or the transactivator of transcription, has been linked to progressive neuronal deregulation that leads to the development of HAND and accelerated brain aging (Bagashev & Bassel, 2013). Tat increases the expression of chemokine receptors, induces cytokine release, induces oxidative stress, attracts monocytes, and promotes immune cell permeation across the BBB (McRae, 2016). Vpr plays an important role in the rate of viral replication and the effects of the viral on T cells; however, the most detrimental characteristic of Vpr is that it allows the replication of HIV within macrophages (Gonzalez, 2017).

While women account for 52% of all HIV infections worldwide and women aged 10-24 are twice as likely as men the same age to acquire an HIV infection (CDC), men are more likely to die due to an HIV infection (Maskew, et al., 2013). Women typically represent a higher CD4 count when compared to men while participating in cART (Maskew, et al., 2013). CD4 count is strongly associated with mortality in HIV patients; individuals with higher CD4 counts are less like to die (Lawn et al., 2008). While women are more susceptible to acquiring an HIV infection, there is higher immune activation in
women than men and women are less likely to progress as quickly to NeuroAIDS when compared with men (Griesbeck et al., 2016)

One of the hypotheses as to why women are less likely to progress to NeuroAIDS as quickly is the presence of female hormones. Recent studies have shown that both progesterone and allopregnanolone (AlloP) show neuroprotection when administered to animals with acute brain injury (Paris, et al., 2016). Researchers hypothesize that progesterone and AlloP protect neurons by suppressing the inflammatory response (Paris, et al., 2016). While progesterone and AlloP show protection, neither show evidence of enhanced proliferation or migration (Paris, et al., 2016).
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Figure 1A: Percent cell death of SH-SY5Y cell line treated with 0, 10, 100, and 1000 pM of X4-gp120 from 0 to 24 hours. There was a significant interaction [$F(3,4)=3.59$, $p<.05$] from 0 to 24 hours such that X4-gp120 decreased cell death at 10 pM.

Figure 1B: Percent cell death of SH-SY5Y cell line treated with 0, 10, 100, and 1000 pM of R5-gp120 from 0 to 24 hours. There were no significant interactions between treatment conditions.
**Figure 1C:** Percent cell death of SH-SY5Y cell line treated with 0, 10, 100, and 1000 pM of X4/R5-gp120 from 0 to 24 hours. There were no significant interactions between treatment conditions.

**Figure 1D:** Image of SH-SY5Y cell line stained with cell-permanent Hoechst and cell-impermanent Propidium Iodide stains.
Figure 2A: Frequency of microglia was determined by selecting a random field containing at least 200 cells and counting the number of Hoechst positive cells and Iba-1 positive cells. Although a decrease in frequency was present, there was no significant interaction within groups.

Figure 2B: Image of microglia (20X) stained with Ionized Calcium Binding Adaptor Molecule 1 (Iba-1) and the nucleus of the cell was stained with Hoechst.
**Figure 2C:** Integrated density of astroglia determined through ImageJ software by calculating the mean intensity of signal multiplied by the total area of signal. Although a decrease in integrated density was present, there was no significant interaction within groups.

**Figure 2D:** Image of astrocytes (40X) stained with Glial Fibrillary Acidic Protein and nucleus of the cell stained with Hoechst. Images were used to develop a threshold that would distinguish signal from the background. From the signal, Image J software was able to determine the intensity and the area.
Figure 3A: Cytokine activity was determined by measuring the levels of fluorescent units from the mixed glial cell supernatant on a cytokine array panel.

Figure 3B: Chemokine activity was determined by measuring the levels of fluorescent units from the mixed glial cell supernatant on a cytokine array panel.
**Figure 3C:** Cytokine Array Panels determine the level of mouse chemokines and cytokines produced by mixed glial cells. Intensity levels were determined by a LI-COR software.