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Potential Interactions Between GPER1 and HIV Tat-mediated Neurotoxicity

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POTENTIAL INTERACTIONS BETWEEN GPER1 AND HIV TAT-MEDIATED NEUROTOXICITY

By

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ABSTRACT

SHIWANI THAPA: Potential interactions between GPER1 and HIV Tat-mediated neurotoxicity

(Under the direction of Dr. Jason Paris)

Great advances have been made in the treatment of HIV, however, new infections remain consistent each year with no effective cure. The early entry of HIV virus into the central nervous system is thought to contribute to the development of HIV-associated neurocognitive disorders. One of the mechanisms of neurological impairment may involve actions of the neurotoxic HIV viral protein, trans-activator of transcription (Tat). Tat can be secreted from infected cells and acts as an excitotoxin, increasing the intracellular flux of Ca^{2+}, promoting mitochondrial dysfunction, and neural cell damage/death. Previous experiments have shown that steroid hormones such as estrogen can exert protective effects against Tat-mediated neurotoxicity; but, the site(s) of this protection action are unclear. The primary purpose of this thesis was to begin to assess the role of the non-traditional estrogen receptor, G-protein coupled receptor 1 (GPER1), in Tat-mediated neurotoxicity. The capacity of a GPER1 agonist and antagonist were assessed for their influence on Tat-induced production of reactive oxygen species (ROS) and subsequent cell death using SH-SY5Y human neuroblastoma cells. The results revealed that GPER1 agonism attenuated Tat induced ROS production and prevented Tat-mediated cell death. Conversely, antagonizing GPER1 modestly reduced ROS production; albeit, did not completely attenuate Tat-mediated increases, and exert no effects on cell death.

Further investigation of non-traditional sites of endocrine action may reveal novel therapeutic targets for the treatment of neurological diseases, within and beyond the field of neuroHIV.

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LIST OF ABBREVIATIONS

AIDS	Acquired Immunodeficiency Syndrome
Allop	Allopregnanolone
ANI	Asymptomatic Neurocognitive Impairment
BBB	Blood Brain Barrier
BDNF	Brain-Derived Neurotrophic Factor
cART	Combined Antiretroviral Therapy
CM-H_2 DCFDA	5-6- chloromethyl-2',7'-dichlorodihydrofluorescein diacetate
CNS	Central Nervous System
CREB	cAMP-Response Element Binding Protein
DCF	Dichloroflorescein
DMEM	Dulbecco's Modified Eagle Medium
ERs	Estrogen Receptors
GPER1	G-protein Coupled Estrogen Receptor 1
HAND	HIV-Associated Neurocognitive Disorders
HIV	Human Immunodeficiency Virus
MND	Mild Neurocognitive Disorder
NDs	Neurological Disorders
NMDA	N-methyl-D-aspartate Receptor
PKC	Protein Kinase C
P13K	Phosphoinositide 3-Kinase
PMCA	Plasma Membrane Ca^{2+} ATPase
ROS	Reactive Oxygen Species

Tat	Trans-Activator of Transcription
VPR	Viral Protein R

1. INTRODUCTION:

a. Prevalence of HIV worldwide and in the U.S.:

The human immunodeficiency virus (HIV) remains an ongoing concern worldwide and in the United States with no effective cure. Since the start of the HIV epidemic, 75.7 million people have been infected and approximately 32.7 million people have died from acquired immunodeficiency syndrome (AIDS) or related illnesses (UNAIDS, 2020). Worldwide, there are currently about 38 million people globally living with HIV in 2019 (36.2 million adults and 1.8 million children; UNAIDS, 2020). Although progress has been achieved in preventing and treating HIV, there were an estimated 1.2 million people living with HIV in the United States at the end of 2018 (CDC, 2021). HIV can be transmitted via contact with bodily fluids (blood, semen, rectal fluids, vaginal fluids and breast milk) from an infected person. Transmission occurs when these fluids come in contact with a mucous membrane or a damaged tissue or are directly injected (from needle or syringe; CDC, 2020). The pathogenesis and transmission dynamics concerning HIV have evolved in the post-combined antiretroviral therapeutic (cART) era.Treatment options are now available, however vaccine and cure strategies remain to be achieved (Simon, et al., 2006). The development of cART has greatly reduced medical morbidity and mortality, but still has not decreased the prevalence of HIV-associated neurocognitive disorders (HAND; Heaton, et al., 2011) wherein infection manifests as neurological disturbances in approximately 50% of HIV-infected patients (Saylor, et al., 2016).

b. HIV-associated neurocognitive disorders (HAND)

HAND is characterized by impaired short term memory along with neurological disorders ranging from mild difficulty with concentration, impaired decision-making, and lack of coordination, to progressive dementia (Ghosh, et al., 2017). Notably, in the post-cART era, the most severe form of cognitive impairment (HIV-associated dementia) has been reduced (~2%). But the prevalence of additional cognitive impairment remains. In the modern day, HAND is characterized by asymptomatic neurocognitive impairment (ANI), mild neurocognitive disorder (MND), and to a lesser extent HIV-associated dementia (Ghosh, et al., 2017). The neuropathogenesis of HAND is generally considered to be initiated and driven by HIV invasion and replication within the brain parenchyma. This occurs via productive infection of brain perivascular macrophages and endogenous microglia, and perhaps to some degree by restricted infection of astrocytes. This invasion of the central nervous system (CNS) is believed to occur early in HIV infection (Heaton, et al., 2011). The CNS can subsequently serve as a reservoir for ongoing HIV replication, thereby limiting the opportunity for treatment (Heaton, et al., 2011). Importantly, cART is poorly retained within the CNS and cannot target latent HIV reservoirs (Fields, et al., 2019). As such, cART cannot presently eradicate HIV from the CNS compartment. HAND combined with a unique spectrum of opportunistic infections and malignancy, comprise neuroHIV (Clifford, et al., 2013).

c. NeuroHIV

Within hours of viral acquisition (An, et al., 1999), HIV enters the CNS. Although the entry mechanism remains debated, there is strong support for what has become known as the "Trojan horse" theory. This theory postulates that soon after seroconversion, infected monocytes can traverse the brain microvascular endothelium. Monocyte-derived cells become easily infected. Both the immature monocytes or mature macrophages do not divide. In contrast,

because of their highly proliferative nature, infected CD4+ T-lymphocytes produce large levels of virus in the periphery. Thus monocytes, macrophages, and/or lymphocytes may carry the virus into the brain during disease. Free progeny virus may also cross the blood-brain barrier (BBB) before infecting monocyte lineage cells in the perivascular space (Zink et al., 1999). The infected macrophages can cause neuronal damage and destruction through multiple mechanisms (Zink et al., 1999).

Neurological disorders (NDs) in persons infected with HIV are largely caused by three main mechanisms, direct effects of HIV infection and toxic protein production, indirect neuroinflammation, and opportunistic processes as a result of immune challenge (Lucas, et al., 2015). Microglial cells, the CNS resident macrophages, are one of the major cellular reservoirs of latent HIV. This reservoir contributes to cognitive deterioration in the different forms of HAND that involve an increase in proinflammatory cytokines and increased neurotoxins that affect both astrocytes and neurons, promoting apoptosis (Adle-Biassette, et al., 1995). These cells are believed to be involved in the emergence of drug resistance and potentially re-seeding peripheral tissues following BBB disruption (Wallet, et al., 2019). The BBB is a selective barrier composed of microvascular endothelial cells lining the brain microvessels. It has an active interface between the circulation and the CNS, restricts free movement of substances between blood and CNS, and is critical in maintaining CNS homeostasis (Wilhelm, et al., 2011). HIV-derived cellular and viral toxins are known to alter the integrity of the BBB. Exposure to HIV alters tight junction expression (Dallasta et al. 1999; Boven et al. 2000; Persidsky et al. 2006; Chaudhuri et al. 2008b, a; Eugenin et al. 2011) and also increases transmigration of cells across the barrier (McRae, 2016). The mechanisms of HIV's capacity for neurological damage are largely thought to be driven by production of cytotoxic and/or pro-inflammatory HIV

proteins. Some proteins exert direct effects to promote neurotoxicity while others activate central reservoirs that mediate neuroinflammation.

d. HIV neurotoxic proteins: gp120, VPR, Tat

Several neurotoxins are produced by HIV that can promote neurotoxicity. The gp160 glycoprotein complex comprises the outer coat of the HIV virion. It presents itself as viral membrane spikes consisting of gp120 linked together and anchored to the membrane by gp41 protein (Yoon et al., 2010). The HIV envelope protein, gp120, initiates virus entry into T cells or macrophages through attachment to the CD4 molecule and subsequent binding to a chemokine coreceptor, CXCR4 or CCR5, depending on viral tropism (Bluel CC et al., 1996; Schmitz J E et al., 1999). Direct interaction of gp120 with either CCR5 and CXCR4, as well as N-methyl-D-aspartate (NMDA) receptors on neuronal surfaces promotes intracellular calcium influx, the generation of reactive oxygen species (ROS), and the activation of signaling pathways that lead to cellular apoptosis (Smith, et al., 2018). HIV viral protein R (Vpr) plays a unique role in neuropathogenesis with its ability to induce G2/M arrest along with its capacity to increase viral gene transcription (James, et al., 2016). Vpr can be released from HIV-infected cells and taken up by uninfected neuronal cells. Once Vpr enters the cells, it can act to increase intracellular calcium, driving mitochondrial dysfunction and ROS production. Although Vpr increases the permeability of the cytosolic membrane, it was found that Vpr inhibits calcium release from the cells by affecting endogenous levels of the plasma membrane Ca^{2+} ATPase (PMCA). Thus, it is likely that Vpr induces an array of biological events downstream of its capacity to dysregulate neuronal calcium homeostasis (Rom, et al., 2009). Other effects of Vpr include changes in cell cycle progression, dysregulation of cellular metabolism and signaling, and loss of cell viability (James, et al., 2016). However, one of the most well-studied neurotoxic

HIV proteins is the transactivator of transcription (Tat), which was the subject of the present thesis.

e. Tat

Tat is a viral HIV protein which is secreted from infected monocyte-derived cells (predominantly microglia and perivascular macrophages) within the CNS (Re M C et al., 1995; Hudson et al., 2000). It is present in the brain of HIV-infected individuals with and without cART treatment (Hudson et al., 2000). Tat is an essential viral protein for HIV replication and is critical for the stimulation of latently infected cells (Frankel et al., 1988). It promotes indirect neurotoxicity via the activation of glial cells, such as the microglia or astrocytes, that produce proinflammatory cytokines (Ensoli et al., 1993). It plays a critical role in viral rebound caused by interruptions in cART (Jin et al., 2020). Tat also exerts direct neurotoxic effects on neurons, independently or in concert with other viral proteins and inflammatory toxins to promote excitotoxic neuronal injury and/or death (Mattson et al., 2005). Tat activates NMDA receptors (Dreyer et al., 1990; Eugenin et al., 2007; Li et al., 2008), interrupts mitochondrial function (Brooke et al., 1998; Perry et al., 2005) and ATP production (Brooke et al., 1998; Norman et al., 2007; Turchan-Cholewo et al., 2006), and disrupts ion homeostasis (Ca^{2+}, Na^{+}, and perhaps K^{+}; Fitting et al., 2014; Greenwood and Connolly, 2007; Lee et al., 2003; Perry et al., 2005) resulting in synapto-dendritic injury (Greenwood et al., 2007; Park et al., 1996). As Tat can be released by infected monocytes and macrophages, it can accumulate at the BBB (Rayne, et al., 2010) and induce changes either via receptor-mediated pathways or through a direct uptake of the protein via active endocytosis (Rayne et al., 2010; De Marco et al., 2010). Tat also degrades tight junctions of brain endothelial cells via inhibiting occludin and promoting matrix metalloproteinase 9 cleavage (Xu et al., 2012). Shortly after exposure to Tat, rapid release of

ROS suggests impaired mitochondria membrane potential leading to alterations in mitochondrial size and subcellular localization in a calcineurin-dependent manner. Hence, Tat impairs mitochondrial dynamics in neurons, contributing to cell death (Rozzi et al., 2018).

f. Gender Differences in HIV acquisition:

Tat-mediated insults on the CNS are ameliorated by exogenous administration of steroid hormones (Kendall et al., 2005). In a study, long-term HIV Tat expression in the brain led to impaired short- and long-term memory but with more impairment in male mice (Marks et al., 2016; Qrareya et al., 2020). Moreover, aging-associated menopause, accompanied by estrogen decline may be related to the worse memory in females (Diaz and Roberta, 2012), which may further exacerbate the impact of Tat on female mice (Zhao et al., 2020; Qraryea et al., 2020). In a transgenic mouse model, HIV Tat_{1-86} is conditionally expressed in a CNS-targeted manner to demonstrate Tat-driven microgliosis within the striatum of male and female mice (Hahn et al., 2015). In a similar transgenic model, conditional Tat exposure was observed to increase microglial activation throughout limbic and extra-limbic brain regions of male mice (Paris et al., 2015). However, in an examination of sex differences, reactive nitrogen species were co-localized with microglia to a lesser extent in females, compared to males (Hahn et al., 2015). This coincided with reduced neuronal cell death, astrogliosis, and reduced motor/anxiety-like pathology among females exposed to central HIV Tat (Hahn et al., 2015). As such, gender may confer protection to some of Tat's neuroinflammatory and neurotoxic effects, but the mechanisms are not known. Classic steroid hormones and their neuroprotective metabolites may improve outcomes following exposure to HIV Tat (Paris et al., 2016). In support, classic estrogen actions reduce neurotoxicity in human cell cultures (Adams et al., 2010). In people, estrogen applied directly to the genital tract of post-menopausal women provides both enhanced barrier

function and reduced proinflammatory cytokine production in local epithelial cells when challenged with HIV (Dizzell et al., 2019). More recent studies reveal that estrogens may exert rapid signaling via actions at G protein-coupled receptors (Rodenas et al., 2017).

g. GPER1:

Traditionally, estrogens were thought to exclusively act via classical nuclear estrogen receptors (ERs), namely ERα. A second isoform of the classic nuclear receptor was later identified as ERβ (MacGregor et al., 1998). However, it has since been observed that some estrogens induce biological effects in only minutes after their application. This rapid effect of estrogens is thought to occur too quickly for canonical nuclear gene transcription and is rather thought to be mediated by novel receptors that exert transcriptional regulation via rapid signal transduction (i.e. “non-genomic”). One such estrogen-sensitive protein that has been identified is the G protein-coupled estrogen receptor 1 (GPER1; Rodenas et al., 2017). GPER1 (formerly known as GPR30) is thought to be localized at the cell membrane in its inactive state and is shown to activate kinase cascades and calcium (Ca^{2+}) flux within cells rapidly following activation (Maria and Nandini, 2018). This notion is conceptually consistent with predictions of a membrane-ER (mER) that can mediate non-genomic signaling by estrogens. GPER1 expression is widespread in the central nervous system (CNS) and contributes to spatial memory, anxiety, social memory, and lordosis behavior in mice (Hadjimarkou, Maria M, and Nandini Vasudevan, 2017). These data indicate that estrogens regulate normal function in the nervous, immune, skeletal, and cardiovascular systems, adipocytes, liver, pancreas, and kidney by activating GPER1 (Prossnitz, Eric R, and Helen J Hathaway, 2015). In the brain, GPER1 signaling, in response to estrogen, facilitates neuroprotection, social behaviors and cognition (Vajaria, et al., 2018). The GPER1 is Gs-coupled and inhibits Erk-1/-2 activity (Filardo, et al.,

2002). GPER1 also activates cell-signaling cascades driven by phosphatidylinositol-3-OH kinase (PI3K; Revankar et al., 2005; Petrie et al., 2013), protein kinase C (PKC; Goswami et al., 2011), calcium mobilization (Revankar et al., 2005; Tica et al., 2011), and activation of other ion channels (Fraser et al., 2010; Goswami et al., 2011). Moreover, GPER1 is thought to dynamically modulate the PMCA which rectifies intracellular Ca^{2+} content (Tran, et al., 2015). GPER1 may thus contribute to estradiol's protective actions over HIV Tat, perhaps via its capacity to offset Tat's Ca^{2+}-mobilizing capacity thereby reducing mitochondrial dysfunction, ROS generation, and ultimately neuronal damage/death.

h. Hypothesis:

We anticipated that activation of GPER1 contributes to estradiol's protective effects on Tat-induced neurotoxicity. To test this, we conducted *in vitro* experiments using differentiated SH-SY5Y human neuroblastoma cells that were exposed (or not) to Tat protein and assessed for generation of ROS and subsequent cell death. We hypothesized that a GPER1 agonist, G1, would express potentially-protective effects over Tat-mediated cytotoxicity whereas the GPER1 antagonist, G36, would not.

2. MATERIALS and METHODS:

2.1 Cell Culture

Human SH-SY5Y neuroblastoma cells were obtained from ATCC (#CRL2266; Manassas, VA). These cells were seeded onto 96- or 24-well plates at a density of 0.5×10^4/well or 4×10^4/well, respectively, and maintained in growth medium: 89.5% DMEM/F12 (Life Technologies, Carlsbad, CA), 10% heat-inactivated fetal bovine serum (FBS; Thermo Scientific Hyclone, Logan, UT), and 0.5% antibiotic/antimycotic mixture (Life Technologies). Prior to

differentiation, these cells were incubated (37°C, 5% CO_2) and media was replaced every 2-3 days.

2.2 Differentiation:

The day after seeding, the growth medium was fully exchanged for differentiation medium #1. Differentiation medium #1 contained retinoic acid diluted 1:500 in growth medium (final retinoic acid concentration = 3.33 mM). One day later, media were fully exchanged for a serum-free differentiation medium #2. This medium consisted of BDNF diluted 1:200 in DMEM/F12 (supplemented only with the 0.5% antibiotic/antimycotic mixture; final BDNF concentration 1.85 μM). One day later, cells underwent experimental manipulations and were assayed after 20 h of incubation. Others find that these differentiation factors promote cell cycle arrest and the expression of mature neuron markers (i.e. a shift from nestin$^+$ to microtubule associated protein 2$^+$ expression and a polarized morphology; Constantinescu et al., 2007; Encinas et al., 2000).

2.3 Treatment:

Cells were treated with vehicle or HIV Tat (100 nM diluted in dH_2O; ImmunoDx, Woburn, MA), G1 (1 or 10 nM), or G36 (1 or 10 nM). G1 and G36 were dissolved in DMSO and diluted to concentration in media (diluted 1:10,000).

2.4 Measurement of overall ROS production

The indicator 5-(and-6)- chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA, Invitrogen, Carlsbad, CA), which is de-acetylated to dichlorofluorescein (DCF) was used to measure the levels of ROS production. Following the manufacturer's protocol, cells were loaded with 10 μM CM-H_2DCFDA in warm HBSS for 45 min and then washed twice before treatments were applied. Subsequently, two concentration-response

experimental designs were used. In one design, cells were treated with Tat (100 nM) and the GPER1 agonist, G1, ranging in concentration from 0.01 nM to 10 nM. In another design, the GPER1 antagonist, G36, ranging from 0.01 nM to 10 nM and Tat was held at 100 nM. Fluorescence was measured (ex/em = 485/520 nm) using a CLARIOstar (BMG Labtech, Inc., Cary, NC) microplate reader. ROS levels (quantified by arbitrary fluorescent units) were normalized to vehicle control cells and presented as the % change from control (n = 6-10 independent experiments).

2.5 Live/Dead Assay

After the treatment, a live/dead assay was conducted 20 h later to assess neuron viability/necrosis. Prior work utilizing time-lapsed microscopy (0–60 h) identified the 20 h time-point as the earliest time when pregnane steroid treated cells diverged from those that were Tat-treated on the measure of viability (Paris et al., 2016). Until ready for imaging, cells were incubated at 37 °C with 5% CO2 in the dark. 15 minutes prior to imaging, a working solution of propidium iodide (ex/em: 535/617 nm) and Hoechst 33342 (ex/em: 360/460 nm) was prepared by diluting stocks in Hank's Balanced Salt Solution (1:50 dilution) and then applied to the cells. Plates were imaged using a Ti2-E motorized, inverted microscope (Nikon Instruments Inc., Melville, NY). Using ImageJ Fiji software (Schindelin et al., 2012), the number of dead cells and total cells were quantified. The proportion of necrotic cells was calculated: [(propidium iodide + cell # / total cell #) ∗ 100].

2.6 Statistical analyses

Cell viability was assessed via two-way analyses of variance (ANOVA) with Tat condition (vehicle- or Tat-exposed) and GPER1 pharmacological treatment (vehicle, 1, or 10 nM G1 or G36) as factors. ROS was analyzed via repeated-measures ANOVA with Tat condition

(vehicle- or Tat-exposed) as the between-subjects factor and GPER1 pharmacological treatment (0-10 nM G1 or G36) as the within-subjects factor. To determine group differences main effects were followed by Fisher's Protected Least Significant Difference *post-hoc* tests (with all possible pairwise comparisons made). Interactions were delineated via simple main effects and main effect contrasts (corrected for family-wise error). For all the tests an alpha level of 0.05 was used to determine significance.

3. RESULTS:

3.1 HIV Tat increased, and GPER1 agonism attenuated, ROS in SH-SY5Y cells

To ensure the quality of ROS assessment via DCF fluorescence, positive controls were first assessed (Fig. 1). Compared to vehicle control, hydrogen peroxide (H_2O_2) significantly increased DCF fluorescence whether applied 24 h or 1 h prior to assay [$F(3,28) = 10.35$, $p < 0.0001$ - 0.03] (Fig. 1). SH-SY5Y cells were treated with vehicle or Tat in combination with the GPER1 agonist, G1 (0, 0.01, 0.1, 1, or 10 nM). Tat exposure significantly interacted with G1 concentration [$F(4,56) = 2.73$, $p < 0.05$] (Fig. 2A). Exposure to Tat significantly increased ROS (indicated by an increase in the proportional DCF fluorescence) compared to control vehicle-treated wells ($p = 0.002$). Any concentration of G1 significantly attenuated Tat-mediated increases in ROS signal ($p = 0.0001$ - 0.04; Fig. 2A). Conversely, when cells were treated with the GPER1 antagonist, G36, only Tat significantly influenced ROS. There was a main effect for Tat exposure to significantly increase ROS compared to vehicle-treated control cells [$F(1,72) = 23.05$, $p < 0.05$] (Fig. 2B).

3.2 HIV Tat increased SH-SY5Y cell death, but not when GPER1 was agonized

SH-SY5Y cells were exposed to Tat protein and assessed for viability via live-dead assay against G1 (1 or 10 nM; Fig. 3A). Tat and G1 exposure significantly interacted [$F(2,54) = 3.50$, p

< 0.05] such that only Tat administration alone significantly increased cell death compared to vehicle-treated controls ($p = 0.03$; Fig. 3A). G1-treated control groups had notably greater cell death than did vehicle-treated controls, but these were not significantly different. When SH-SY5Y cells were exposed to G36 (1 or 10 nM), only a main effect for Tat to significantly increase cell death was observed [$F(1,42) = 5.11$, $p < 0.05$] (Fig. 3B). Seeding density (assessed by counting the number of live and dead nuclei via Hoechst stain) did not significantly differ across treatment groups in either experiment (170-227 cells/field in Experiment #1 and 141-193 cells/field in Experiment #2).

4. DISCUSSION:

The initial hypothesis that the GPER1 agonist, G1, would exert protective effects over Tat-mediated cytotoxicity, whereas the GPER1 antagonist, G36, would not, were partially upheld. Consistent with prior work, the addition of Tat increased cellular ROS (Fitting et al., 2014; Smith et al., 2018) and promoted cell death in SH-SY5Y cells (Salahuddin et al., 2020; Paris et al., 2020; Zhu et al., 2009). Activating GPER1 attenuated the Tat-mediated production of ROS at any concentration assessed. Notably, the greatest concentration of G1 (i.e. 10 nM) also reduced baseline ROS, irrespective of Tat treatment. These data support the notion that GPER1 activation may serve to modulate intracellular signaling via ROS. The activation of GPER1 promotes rapid mobilization of intracellular Ca^{2+} stores and thereby stimulates production of ROS (Lei et al., 2017). Tat did increase cell death as expected, but results for G1 agonism to protect SH-SY5Y cells were ambiguous. G1 did prevent Tat from significantly increasing cell death, but may have also exerted some toxic effects on its own; more observations are needed to disambiguate these results. Conversely, antagonizing GPER1 with G36, did not prevent Tat-mediated ROS production. However, a notable reduction was observed at any concentration.

While not significant, G36 did cause some reduction in ROS production on its own which may have contributed to this effect. Consistent with this, G36 did not alter Tat's capacity to promote cell death. These findings extend what was previously known.

While on cART, women typically present with a higher CD4+ T-cell count in comparison to men and CD4 count can be associated with mortality in HIV patients (Maskew et al., 2013). HIV-infected women present with greater immune activation than men and typically progress less quickly to NeuroHIV than men (Griesbeck et al., 2016). Some of the potential protective effects observed in women may be conferred by actions of endogenous steroids. In particular, pregnane steroids may ameliorate HIV protein-mediated neurotoxicity, partly via actions at non-traditional receptor targets. In support, the progestogen, allopregnanolone (AlloP), is a potent, positive allosteric modulator of GABA(A) receptors (Majewska et al., 1986; Paul and Purdy, 1992) which is produced in response to immune challenges (Billiards et al., 2002; Ghezzi et al., 2000). It is also an antagonist at L-type calcium channels (Earl and Tietz, 2011) and a negative allosteric modulator of NMDA receptors when sulfated (Johansson and Le Grevès 2005; Maurice et al., 2006). AlloP may possess potential protective effects over HIV induced neurological dysfunction (Paris et. al, 2020). In some studies, it was found that progesterone was minimally protective (Kendall et al., 2005; Wallace et al., 2006); while, estrogen with its antioxidant properties attenuated microglial activation in response to Tat or gp120 (Bruce-Keller et al., 2001; Corasaniti et al., 2005; Zemlyak et al., 2005). Hence, the novel targets of estrogens and progestogens should be investigated for their capacity to improve neurological dysfunction (Paris et al., 2016). Studies using a transgenic mouse model, find an initial underlying difference in male versus female vulnerability to Tat exposure; all the cases where severity differed between sexes found, Tat-exposed male mice to have poorer outcomes, while Tat-exposed females were

similar to controls. Males also showed an enhanced state of glial activation and synaptic damage, similar to cognitive and motor impairments in HIV patients (Hahn et al., 2015). Estrogen is generally considered neuroprotective and can attenuate or prevent Tat-induced neurotoxicity. The mechanism(s) involved are not known but GPER1 is a novel component to investigate.

There are several mechanisms by which GPER1 may be beneficial for HIV-mediated neurotoxicity. Although not clearly understood, GPER1 has been observed in the hypothalamus (Xu et al., 2009), pituitary gland (Hazell et al., 2009), hippocampal formation, and amygdala (Tian et al., 2013) in both male and female rodents. This may indicate that GPER1 plays a role in the control of emotions and regulation of endocrine responses. Moreover, GPER1 also exhibits a role in lexical cognitive function as it is expressed in the cholinergic neurons of the basal forebrain. (Hammond and Gibbs, 2011). It has also been noted that GPER1 signals via G(s) proteins, stimulating cAMP production, and triggering cleavage of membrane-tethered heparin-bound epidermal growth factor (EGF). Hence, this results in transactivation of the EGF receptor, intracellular Ca^{2+} mobilization and ERK1/2 activation (Olde Björn, and L M Fredrik Leeb-Lundberg, 2009). In one study, G-1 increased the level of cAMP response element binding (CREB) protein and binding of NF-κB to the promoter region for glutamate transporters in rat primary astrocytes, thereby enhancing neuroprotection (Hadjimarkou, Maria M, and Nandini Vasudevan, 2018). Another study suggests that GPER1 promotes neuroprotection in cultured cortical neurons via its ability to signal death associated protein kinase 1 (DAPK1) and to downregulate subtype 2B containing NMDA receptors. Thus, there may be different signalling pathways downstream of GPER1 that exert neuroprotection (Vajaria, Ruby, and Nandini Vasudevan, 2018). GPER1 may also interact with traditional estrogen receptors to exert mitochondrial protection. When ROS is produced , intracellular Ca^{2+} increases, promoting

reduced ATP, acetyl CoA, and an imbalance in the NADH/NAD^+ ratios, thus disrupting NFκB signaling pathways. Signaling factors downstream of GPER1 can phosphorylate and activate ERalpha receptors. Subsequently this leads to changes in the expression and activity of nuclear respiratory factor 1 (NRF-1) and mitochondrial transcription factor A, ultimately stimulating mitochondrial fusion which may help offset Tat's mitotoxic capacity (Klinge et al., 2020).

The mechanisms by which GPER agonism influences ROS production are unclear. While conducting the ROS assay, it was anticipated that exposure to Tat would increase the production of ROS in the cells. When Tat is introduced into the cell, it activates Ca^{2+} excitatory ion channels, promoting disruption of the membrane potential (Fitting et al., 2014). Mitochondria help maintain homeostasis through storing the excess Ca^{2+} within their own membrane; this promotes the production of ROS and the mitochondrial membrane potential can be compromised (Fitting et al., 2014). The rapid production of ROS causes alterations in mitochondrial size and cellular dysfunction. Hence, Tat promotes cell death via impairment of mitochondrial dynamics in neurons. G1-decreases in ROS may occur following decreases in intracellular Ca^{2+}. G1 also appeared to decrease ROS on its own (irrespective of Tat) at the highest concentration suggesting it has some inherent capacity to decrease ROS. Surprisingly, G36 did appear to reduce ROS levels in response to Tat, although the attenuation was partial, not concentration-dependent, and only occurred when Tat was present. This partial attenuation did not reach statistical significance. Other GPER1 antagonists have been later found to have partial agonist activity (e.g. G15); albeit, this has not yet been reported for G36.

Targeting non-traditional endocrine receptors may promote therapeutic advancement in the field of neurological diseases including neuroHIV. Novel, steroid-based therapeutics have been increasingly explored and found to reduce Tat mediated neuropathology. The

didehydro-cortistatin A (dCA), a marine sponge glucocorticoid analogue, prevents Tat's capacity to transactivate the HIV LTR and improves Tat-induced inflammation; it also attenuates Tat's capacity to potentiate cocaine-mediated conditioned place preference (Mediouni et al., 2019, 2015; Mousseau et al., 2012). Also S-equol, an estrogen receptor β (ERβ)-acting isoflavone, inhibits Tat-mediated synaptic loss in an ER-β-dependent manner with or without influence by cocaine (Bertrand et al., 2015). Steroid mechanisms may also play a role in reducing interactions with drugs of abuse including opioids. One of the findings show that oxycodone, a clinical opioid, plays a role in down-regulating classic and novel estrogen receptor gene expression such as ERα and GPER1 in human neuroblastoma cells. Other findings have presented ER involvement in the desensitization of Mu-opioid receptor, partially explaining interactions between these systems (Micevych et al., 2009). Thus, for the opioid-using population (licit and illicit), adjunctive therapeutics that can maintain endogenous hormone milieu may be beneficial for neuroHIV (Paris et al., 2020).

Due to the onset of SARS-CoV-2, the live/dead assay experiments associated with G1 were interrupted. The results presented contain preliminary data wherein there was high baseline variance, particularly for the G1 live/dead assays. This may have been caused in part by the large, non-optimal spread in seeding density that is noted for these assays. This thesis was also intended to include a behavioral component assessing the cognitive effects of GPER1 agonists/antagonists on Tat expression in transgenic mice; however, these experiments were interrupted by SARS-CoV-2 and are still ongoing in the lab.

In conclusion, exposure of SH-SY5Y neuroblastoma cells to HIV Tat resulted in increased production of ROS and subsequent cell death. GPER1 agonism attenuated Tat induced ROS production and attenuated cell death. The agonist G1 and antagonist G36 had some

perplexing common effects to both reduce ROS. As all doses of G1 were effective in blocking Tat-mediated ROS, future studies should focus at lower concentrations, and include a time course study to assess how long the protection may last. The mechanisms involving potential GPER1-mediated neuroprotection are not yet known. Further investigations regarding GPER1 and its mechanisms may reveal novel therapeutic targets for the treatment of neuroHIV and related neurological disorders.

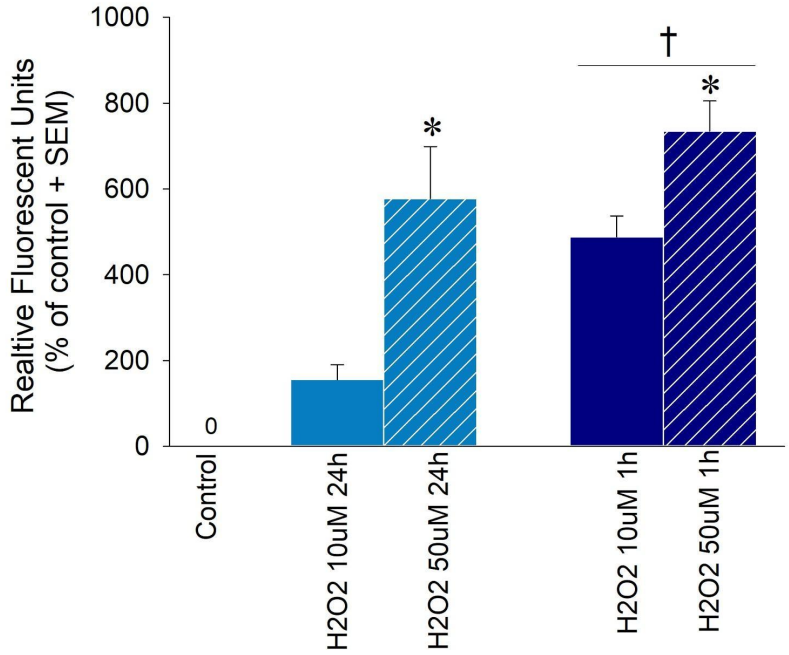

Figure 1: Quality of ROS assessment via DCF fluorescence with H_2O_2 whether applied 24 h or 1 h prior to assay.

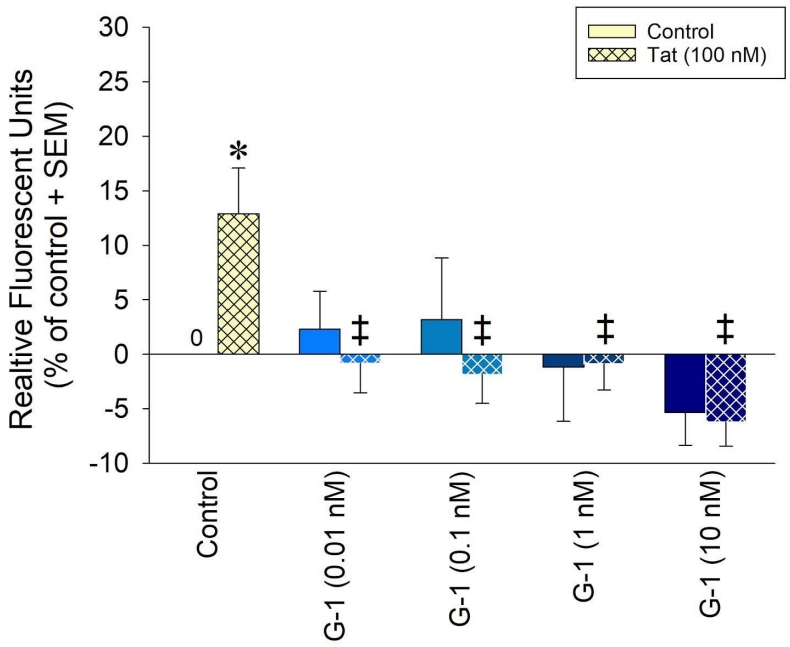

Figure 2A: Percent ROS production in DCF with addition of Tat (100 nM) and varying concentrations of G1.

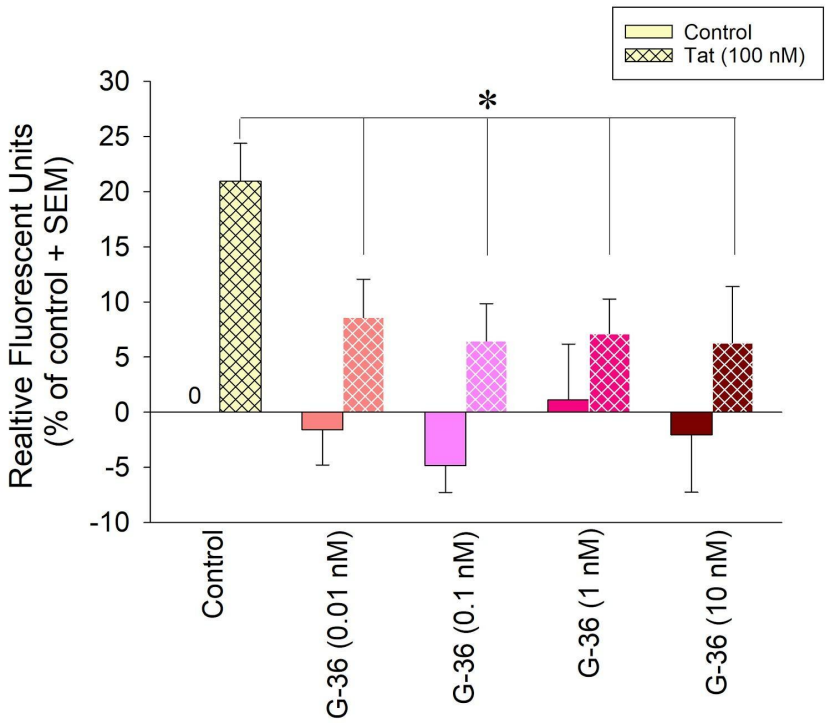

Figure 2B: Percent ROS production in DCF with addition of Tat (100 nM) and varying concentrations of G36.

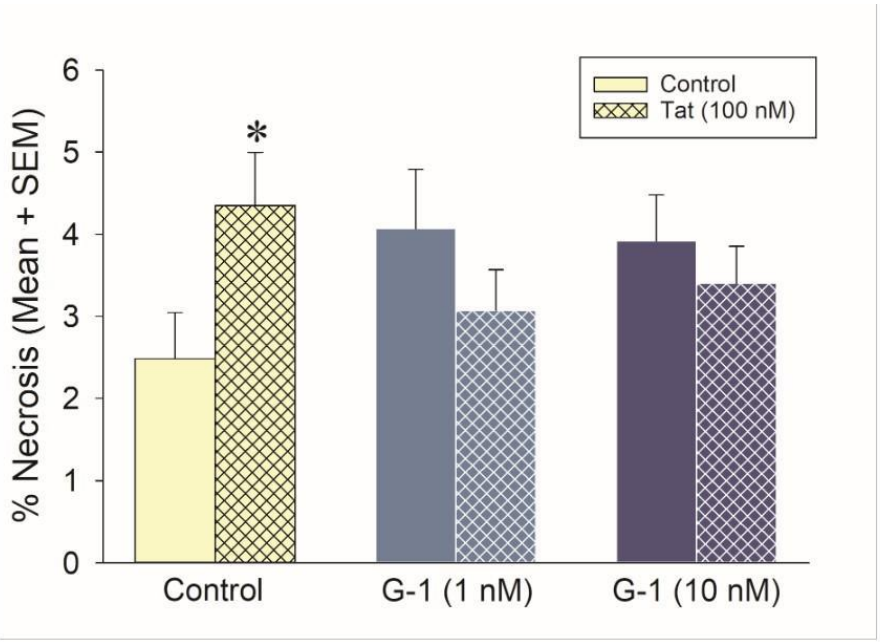

Figure 3A: Percent cell death of SH-SY5Y cells exposed to varying concentration of G1, with or without exposure to Tat (100 nM).

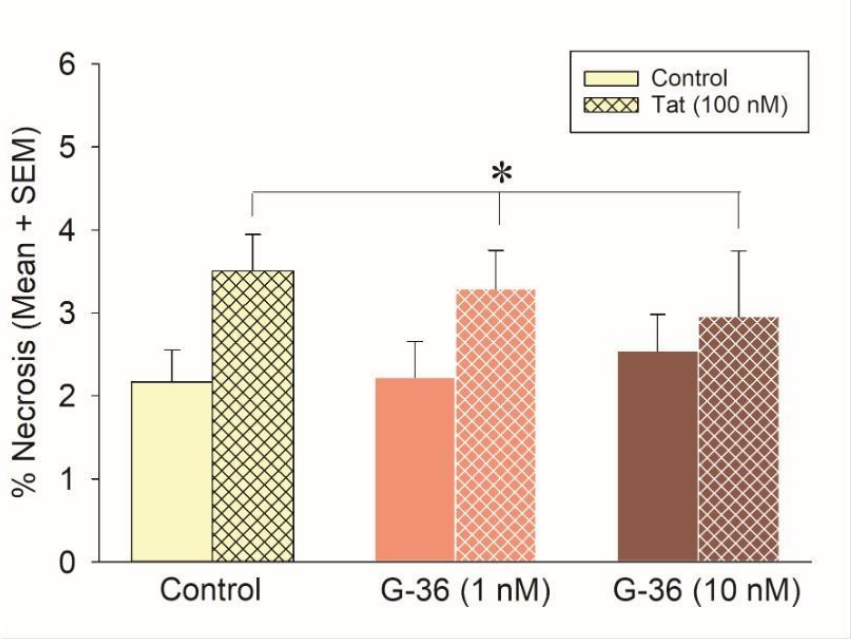

Figure 3B: Percent cell death of SH-SY5Y cells exposed to varying concentration of G36, with or without exposure to Tat (100 nM).

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