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HIV-1 Tat and Opioids: Implications for Stress-related Psychopathology

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HIV-1 TAT AND OPIOIDS: IMPLICATIONS FOR STRESS-RELATED PSYCHOPATHOLOGY

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ABSTRACT

The overarching goal of this dissertation was to assess the interaction of HIV-1 Tat protein with opioids like oxycodone and the hypothalamic-pituitary-adrenal (HPA) axis and develop novel therapeutic strategy to restore HPA dysfunction in adult HIV-1 Tat transgenic mice. In the past 3 decades, combined antiretroviral therapeutics (cART) has increased the life expectancy of HIV infected individuals, with a significant decline in the number of deaths. However, due the inability of cART to sufficiently target latent CNS viral reservoirs (predominantly microglia and astrocytes to a lesser extent), approximately 50% of the HIV^{+} infected population contends with neurological, neuropsychiatric and neuroendocrine complications, but the mechanisms are unknown. The central hypothesis of my dissertation is that neurosteroids like allopregnanolone can restore HIV-1 Tat mediated HPA axis dysregulation and underlying neurological and psychiatric complications, which are exacerbated by clinically prescribed opioids. Chapter 1 demonstrates the ability of HIV-1 Tat to promote HPA dysfunction by elevating basal circulating corticosterone and paradoxical adrenal insufficiency in response to a natural stressor in transgenic Tat-expressing mice, recapitulating the clinical endophenotype. Blocking receptor targets namely corticotropin-releasing factor (CRF) and glucocorticoid receptor (GR) in males partially-reinstated the HPA response in Tat-expressing mice implicating GR in these effects. Chapter 2 revealed HIV-1 Tat expression produced neuroHIV symptomatology like anxiety, depression, behavior disinhibition, cognitive impairment and potentiated oxycodone psychomotor effects in various behavioral tasks in adult transgenic mice. Pharmacological blockade of corticotropin-releasing factor receptor (CRF-R) and/or GR via systemic administration of

antalarmin and RU-486 attenuated psychomotor and anxiety-like behavior. Moreover, gonadal steroids like estradiol and progesterone ameliorated Tat-mediated neurotoxicity in cell cultures. Chapter 3 demonstrated the neuroprotective capacity of neurosteroids like allopregnanolone and 18 kDa translocator protein (TSPO) ligands to restore HPA dysfunction and concomitantly Tat-mediated behavioral deficits. Given the inability of cART to target Tat, novel adjunctive compounds such as allopregnanolone or TSPO ligands may provide further therapeutic recourse to curtail HIV-1 mediated HPA dysfunction and underlying neurological complications.

DEDICATION

This work is dedicated to my parents, Aziz and Roohi, as well as my brother, Mohammed Aziz, for their unwavering support. Without them, this would not have been possible. I am also very appreciative of my wife, Aleem, who has instilled in me a ravenous appetite for knowledge and a passion for studying. Additionally, I had wanted to dedicate this dissertation to my former and present mentors, who have not only taught me science, but have also enriched my life experience.

LIST OF ABBREVIATIONS OR SYMBOLS

AIDS	Acquired immunodeficiency syndrome
ANI	Asymptomatic neurocognitive impairment
BLT	Bone marrow-liver-thymus
BSNT	Bed nucleus of stria terminalis
cART	Combinative anti-retroviral therapeutics
CB	Cannabinoid
CeA	Central Amygdala
CMI	Cell mediated immunity
CNS	Central nervous system
CPP	Conditioned place preference paradigm
CRF	Corticotropin releasing factor
CRF-R	Corticotropin releasing factor receptor
CSF	Cerebrospinal fluid
D1	Dopamine receptor subtype 1
D2	Dopamine receptor subtype 2
DAT	Dopamine transporter
dCA	Didehydro-Cortistatin A
ETC	Electron transport chain
GABA	Gamma-aminobutyric acid

gp120	glycoprotein 120
gp41	glycoprotein 41
GR	Glucocorticoid receptor
GRE	Glucocorticoid response elements
HAD	HIV associated dementia
HAND	HIV-associated neurocognitive disorders
HDACi	Histone deacetylase inhibitors
HIV	Human immunodeficiency virus
HIVE	HIV encephalopathy
IDUs	Injection drug users
IV	Intravenous
KCC2	K+ Cl- cotransporter 2
LRA	Latency-reversing therapeutics/agents
MND	Mild neurocognitive disorder
mPFC	Medial prefrontal cortex
MSMs	Men who have intercourse with men
MSNs	Medium spiny neurons
Nef	Negative factor
PLWH	People living with HIV
PQC	Protein quality control
PTSD	Post traumatic stress disorder

RNA	ribonucleic acid
SE	S-Equol
SIV	Simian immunodeficiency virus
STAT	Signal transducer and activator of transcription
Tat	Transactivator of transcription
TNF-α	Tumor Necrosis Factor Alpha
TSPO	Translocator protein
VPR	Viral protein R

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Introduction

HIV-1 Patients and Neurological Dysfunction

Despite the recent advancements of improved patient care and clinical outcomes, human immunodeficiency virus (HIV) continues to be a global epidemic. In the United States, an estimated 1.2 million people are presently living with HIV (CDC, 2020), with approximately 49% actively seeking combinative antiretroviral therapeutics (cART) to treat HIV infection (NIAID, 2015). In contrast to early 1990s monotherapy, this fixed combination regimen uses three or more medications to reduce virus-induced resistance to these therapies (Richman et al., 1994). These therapeutics act by inhibiting various enzymes at different stages of the HIV life cycle. In particular, they inhibit reverse transcriptase, integrase, and protease enzymes which are crucial for HIV replication (Rhee et al., 2016). Specifically, the regimen includes nucleoside and non-nucleoside reverse transcriptase inhibitors, protease inhibitors, fusion/entry inhibitors (Jean et al., 2019). This combination therapy is critical for increasing $CD4^+$ T-cell counts, decreasing plasma viral loads, and increasing HIV-infected persons' survival rates (Jean et al., 2019). Indeed, in the past 3 decades, the advent of cART has extended the life expectancy of people living with HIV (PLWH) with a significant decline in the number of deaths (Antiretroviral therapeutic cohort collaboration, 2008). However, due to the inability of cART to accumulate in the CNS compartment and target latently infected $CD4^+$ T-cells harboring proviruses, HIV proteins continue to persist in the latent state in these reservoirs (Buzon et al., 2014; Chomont et al., 2009; Chun et al., 1997; Finzi et al., 1999; Mbonye and Karn, 2014; Ruelas and Greene, 2013; Siliciano et al., 2003). As such, approximately half of the HIV^+ infected population contend with a constellation of neurological symptoms, also known as neuroHIV (Heaton et al., 2010). Although

the prevalence of HIV-associated dementia (~2%), a severe type of dementia as defined by the Frascati criteria, remains low in the post-cART period, milder neurocognitive disorders (MND) and asymptomatic neurocognitive impairment (ANI) continue to prevail (McArthur et al., 2004; Simioni et al., 2010). These neurological symptoms encompass deficits in attention, concentration, motor control, and cognition (Mothobi and Brew, 2012; Tozzi et al., 2007; Winston and Spudich, 2020). Additional neuropsychiatric illnesses such as anxiety, depression, post-traumatic stress disorder, suicidal thoughts, and increased sensitivity to substance use disorders are also common in HIV^+ infected persons (Bing et al., 2001; Gaynes et al., 2008; Gielen et al., 2005; Hartzler et al., 2017; Neigh et al., 2016; Remien et al., 2019; Sherr et al., 2011). Hence, in order to overcome the limitations of cART, it is critical to identify the underlying mechanisms of neuroHIV, develop innovative treatments, and provide a functional cure for this disease.

Modes of HIV Transmission and Infection

HIV is transmitted by unprotected intercourse and blood transfusions from infected individuals, with the exchange of vaginal, seminal, and rectal fluids being of particular concern. Following the AIDS epidemic, a few high-risk categories for contracting HIV emerged: i) men who have intercourse with men (MSMs), ii) blood transfusion recipients, and iii) intravenous (IV) drug users (Do et al., 2017; Hill et al., 2018; Neaigus et al., 2016). HIV transmission is not restricted to MSMs; contact of body fluids with mucosal membranes or open wounds is sufficient to promote transmission in a HIV-negative person (Harris et al., 2019; van der Graaf and Diepersloot, 1986). Additional mechanisms of transmission were observed, including the following: i) medical personnel may be at risk due to needlestick accidents; ii) vertical transmission of HIV from mother to child (perinatally by body fluid exchange at birth, and occasionally via

breastfeeding or placenta; van der Graaf and Diepersloot, 1986). Although virus particles have been detected in other body fluids, transmission is very unlikely unless blood is present in those bodily fluids and fluid to blood contact occurs (CDC, 1987; van der Graaf and Diepersloot, 1986). Although cerebrospinal fluid (CSF) may contain detectable amounts of HIV particles in the CNS compartment, the likelihood of actual transmission by CSF contact is limited outside of occupational exposure (Bell et al, 1998; CDC, 1987; Nath, 2015).

Acquired Immunodeficiency syndrome (AIDS)

Upon interaction with infected virus, the virus infects peripheral $CD4^+$ T cells and CD8 macrophages, predominantly through CCR5 or CXCR4, depending on viral tropism (Berger et al., 1998; Clifford and Ances, 2013; Coakley et al, 2005; Smail and Brew., 2018; Zhou and Saksena, 2013). Following the establishment of the virus in the CD4 host cell, the virus confers $CD4^+$ T cell necrosis via mechanisms of apoptosis and pyroptosis (Doitsch et al., 2014; Doitsch and Greene, 2016; Laurent-Crawford et al., 1991; Terai et al., 1991). Some of the symptoms seen during this stage are influenza-like viral disease; fever, laziness, albeit these symptoms may vary from patient to patient (UNAIDS, 2016). The immune system becomes activated and creates antibodies against the viral infection, bringing the CD4 count back to pre-infection levels. This is the symptom-free stage of the latent state (Shah et al., 2010). This latent stage of HIV infection lasts for ~8 years, and during this time, the viral genome has been firmly entrenched inside the host genome, allowing the virus to continue reproducing successfully within infected cells (Harris and Bolus, 2008). Over this long-term period, $CD4^+$ T cells consistently drop. When the CD4 cell count goes below 200 cells/μL, the patient's clinical-stage transitions from HIV to AIDS (Crum-Cianflone et al., 2009). The patient's immune system is thus compromised, which increases the

susceptibility to various opportunistic infections which are usually well-tolerated by healthy individuals. Additionally, cell growth disruption has also been reported, increasing the vulnerability to Kaposi's sarcoma (Bohlius et al, 2014; Gilmore et al, 1983). Thus, a low CD4 count is one of the hallmarks of an impaired immune system (Jansen et al., 2005).

The relationship between immunological failure and the development of autoimmune disorders in people with human immunodeficiency virus (HIV) infection and AIDS is noteworthy. While AIDS is another sort of immune system dysfunction, a person's immune system is weakened or rendered ineffective in this circumstance, categorized into 4 stages; Stage 1: An acute HIV infection and an intact immune system; Stage 2: No visible AIDS symptoms, CD4 count is decreasing, indicating immunosuppression and no autoimmune diseases detected; Stage 3: Immunosuppression, low $CD4^+$ T-cell counts, and AIDS development [psoriasis or diffuse immune lymphocytic syndrome (similar to Sjogren's syndrome)], no autoimmune disorders are diagnosed at this stage; Stage 4: Restoration of immunological competence and resurgence of autoimmune diseases (including rheumatological syndromes; systemic lupus erythematosus, vasculitis, idiopathic thrombocytopenic purpura; Grave’s disease; (Zandman-Goddard and Shoenfeld, 2002).

Table 1: WHO Clinical Stages of HIV-1 infection.

Adapted and modified from Weinberg and Kovarik, 2010

WHO Clinical Stage	CD4 count	Symptoms
Stage –1	≥ 500 cells/μL	Asymptomatic Persistent generalized lymphadenopathy
Stage – 2	200 – 499 cells/μL	Moderate unexplained weight loss, Herpes zoster, Recurrent oral ulceration, Seborrhoeic dermatitis Fungal nail infections
Stage – 3 & 4	≤ 200 cells/μL	Persistent oral candidiasis Oral hairy leukoplakia Pulmonary tuberculosis, Acute necrotizing ulcerative stomatitis, gingivitis or periodontitis, Unexplained anemia, neutropenia, chronic thrombocytopenia, Central nervous system toxoplasmosis (after one month of life) Extrapulmonary cryptococcosis (including meningitis) HIV encephalopathy, Progressive multifocal leukoencephalopathy Symptomatic HIV-associated nephropathy or HIV-associated cardiomyopathy

Another caveat is the neurological manifestations of HIV infection. In the pre-cART era, the neurological problems were less well-documented given that HIV patients transitioned more quickly to AIDS and died. However, the post-cART era has brought relief with a greater number of people living with HIV on account of antiretroviral therapeutics. However, neurological

complications have now become more apparent. Thus, patient care in the post-cART era transitioned from managing acute HIV infection to chronic management of the infected population. As such, more systematic studies are needed to identify and treat the opportunistic infections and neurological sequelae in the HIV^+ population (Gelman et al, 2013).

Mechanism of viral entry into CD4 cell

The mature HIV virion is approximately 100 nm in diameter. The HIV virion harbors envelope proteins gp120/gp41 which have an affinity for the CD4 receptor of the host cell (Dalglesih et al., 1984; Klatzmann et al., 1984). Envelope proteins like gp120 are vital for their interaction with host CD4 receptors (Dalglesih et al., 1984; Klatzmann et al., 1984). This interaction is further followed by additional binding of chemokine coreceptors (CXCR4 or CCR5) (Alkhatib et al., 1996; Deng et al., 1996; Doranz et al., 1996; Feng et al., 1996). Hence, the preference of the virus for these receptors on the host cell lymphocyte or macrophage confers viral tropism (Arts and Hazuda, 2012). These interactions produce a conformational change in the viral envelope and expose the hydrophobic portion of gp41 protein for viral entry into the cell (Arts and Hazuda, 2012). However, it needs to be emphasized that other HIV envelope proteins like Env (Ladinsky et al., 2020) may likely contribute to virus entry into the host cell to mediate neurological manifestations and related psychopathology.

Infection of Central Nervous System

The HIV infection gets transmitted into the CNS rapidly after infection (Masliah et al, 2000). Various theories have been proposed to demonstrate HIV entry into the brain to establish an active infection. The most widely accepted is the "trojan horse theory," in which infected macrophages and perhaps infected $CD4^+$ T-lymphocytes from the periphery cross the blood-brain

barrier, permitting the infection to establish reservoirs in the brain, a compartment that the virion would normally not able to access on its own (Haase, 1986; Meltzer et al., 1990; Meltzer and Gendelman, 1992; Peluso et al., 1985; Zink et al., 1999; Zhou and Saksena, 2013). These infected macrophages can produce neuronal damage via various mechanisms (Zink et al., 1999). While most evidence supports the Trojan horse theory, other theories for viral entry into the CNS have also been proposed (Harouse et al., 1989; Kaul et al., 2001, 2005; Zhou et al., 2008; Zhou & Saksena, 2013). For instance, the phenomenon of transcytosis may be involved, wherein infected virions in the blood are incorporated into vacuoles with the aid of endothelial cells, and then transported to the brain (Argyris et al., 2003; Bobardt et al., 2004; Kaul et al., 2001; Mankowski et al., 1994). Further infection of the choroid plexus is then spread by cerebrospinal fluid (Burkala et al., 2005; Chen et al., 2000; Harouse et al., 1989). Irrespective of the viral entry mechanism, HIV will infect the microglial cells (immune cells of the CNS), astrocytes (glial cells that provide synaptic support to the neuron and carry out a myriad of additional functions, including immune-related support). While neurons are not directly infected with HIV, they are indirectly vulnerable to its effects due to the production of proinflammatory chemokines and cytokines by infected or uninfected microglia and astrocytes (Ajasin and Eugenin, 2020; Gelman and Nguyen, 2010; Kovalevic and Langford, 2012; Soulas et al., 2009).

Hence neurological disorders in the HIV^+ population are likely produced by 3 mechanisms. Primarily by direct effects of viral proteins and HIV-promoted excitotoxins to damage or kill neurons, secondly by indirect effects of glial proinflammatory cytokines to promote neuroinflammation, damaging or killing neurons, and finally via opportunistic infections caused by increased T-cell destruction and compromised cell-mediated immunity (Lucas & Nelson, 2015).

Thus, microglial cells and perivascular macrophages are one of the main reservoirs which harbor the latent virus in the CNS (Abreu et al., 2019; Wallet et al., 2019). These reservoirs may contribute to neurological problems, given their ability to produce inflammatory cytokines and neurotoxins, thereby affecting astrocytes and neurons to produce neuronal apoptosis (Adle-Biassette et al., 1995). Given that microglial cells are latent viral reservoirs, they are likely to contribute in the emergence of drug resistance by different mechanisms like 1) mutations of HIV-1 in response to protease and reverse-transcriptase inhibitors (Hecht et al., 1998) and 2) transfer of resistance genes to peripheral tissues, thereby exacerbating the challenges for a functional HIV cure (Wallet et al., 2019). Thus, HIV's capacity to cause neurological infection is mediated mostly by direct cytotoxic effects and indirectly through activation of central reservoirs via pro-inflammatory cytokines to cause neuroinflammation. Thus, direct excitotoxicity and indirect neuroinflammation are the primary mechanisms for neuronal damage, thereby increasing the vulnerability to neurocognitive impairment in HIV^+ individuals. Hence, a better understanding of the mechanism of neuronal damage and progression of neurological sequelae will aid in the improvement of treatment strategies to combat neuroHIV symptomatology.

HIV associated neurocognitive disorders (HAND)

Following the discovery of AIDS, in addition to the opportunistic infection of multiple peripheral organ systems, neurological abnormalities were noted in a significant proportion of the HIV^+ population (Britton and Miller, 1984; Fenelon et al., 1986; Helweg-Larsen et al., 1986; Levy et al., 1985; Snider et al., 1983). This constellation of neurological symptoms was collectively referred to as HIV-associated neurocognitive disorders (HAND). Broadly, HAND were classified into three forms; asymptomatic neurocognitive impairment (ANI), mild neurocognitive disorder

(MND), and HIV associated dementia (HAD), arranged in order of increasing severity (Antinori et al., 2007; McArthur et al., 2010), with the most extreme cases likely displaying HIV encephalopathy (HIVE) (Everall et al., 2009). The most severe form of HAND, namely HAD, needs early diagnosis and treatment as it is not reversible by combined antiretroviral treatment (cART) (Sacktor et al., 2002; Tozzi et al., 2005). In the post-cART era, there has been a decreased prevalence of HAD and HIVE cases, although milder and asymptomatic forms still exist (Ellis et al., 2007; Everall et al., 2009; Heaton et al., 2011; McArthur, 2004; Sacktor, 2002). Neuroinflammation is viewed as the primary driver for the advancement of HAND (Everall et al., 2009; Glass et al., 1995; Levine et al., 2016; Persidsky & Gendelman, 2003). Although clinical parameters like viral load, $CD4^+$ cell count, and co-morbidities are better in HIV^+ patients now (post-cART era) than in the pre-cART era, neurocognitive dysfunction still is a challenge (Field & Ellis, 2019). In support, studies show the presence of different HIV quasispecies in the CSF despite suppressive cART, brain imaging studies of HIV^+ patients on cART shows persistent HIV-related neurodegeneration (Li et al., 2019), postmortem brains of HAND diagnosed HIV^+ patients revealed diminished neuronal integrity, astrogliosis, microgliosis, increased production of pro-inflammatory cytokines, and changes in mitochondrial architecture and integrity (reviewed in Field & Ellis, 2019). The diagnosis of HAND symptoms varies among patients, however generalized symptomatology is common, which encompass deficits in memory, learning and concentration, verbal capacity, mood disorders, processing activities of daily living, motor deficits, and challenges in processing visual data or performing spatial operations (Antinori et al., 2007; Dawes et al, 2008; Levine et al, 2016). The implications of HAND if untreated may progress into severe forms, which may impair the quality of life of the infected individuals (Antinori et al., 2007).

HIV proteins contribute to mitochondrial dysfunction in CNS and HAND

Although cART is now available, many neurological complications associated with HIV infection are likely to be caused by HIV replication and low-level production of HIV proteins in the brain and peripheral tissues (Ko et al., 2019; Levine et al., 2016; Tso et al., 2018). While cART has lowered peripheral viral load; antibodies against HIV proteins and HIV genomic DNA have been detected in the CSF and brains of HIV^+ patients on cART, suggesting that low-level viral replication and HIV protein expression in the brain may contribute to HAND (reviewed in Fields and Ellis, 2019). In support, HIV proteins including the transactivator of transcription (Tat), glycoprotein 120 (gp120), viral protein R (VPR), and negative factor (Nef) have been linked to immune activation, oxidative stress, altered calcium signaling, dysregulated mitochondrial function, autophagy, translocation of apoptotic factors like CytC and Bax to promote apoptosis and neurotoxicity (Reviewed in Fields & Ellis, 2019). Several studies, including one conducted in a rat model of HIV-induced neurotoxicity, have demonstrated that HIV proteins are involved in the development of mitochondrial dysfunction in the brain (Villeneuve et al., 2016). These investigations identified changes in the electron transport chain (ETC), glycolytic pathways, mitochondrial trafficking proteins, and proteins involved in a variety of energy pathways (Villeneuve et al., 2016).

Human Immunodeficiency Virus Structure and Proteins

HIV is a single-stranded RNA virion that encompasses 3 main regional elements; gag, pol, and env. These regions in the HIV genome are vital to produce various proteins by combinative approaches like differential splicing of open reading frames and post-translational modifications (Mailler et al., 2016). The single-stranded RNA genome is enclosed in the lipid membrane capsid

which comprises several proteins like p17/MA-matrix, p24/CA-capsid, p6, p7/NC-nucleocapsid which are products of the *gag* gene (Mailler et al., 2016). Their main functional role is to promote formation of the capsid shell and binding of the viral genome with host cell and interaction of the viral and host cell proteins (Mailler et al., 2016). The env gene produces gp120 and gp41 glycoproteins, critical for binding and infecting cells (Finzi et al., 2010). The pol gene is the proenzyme, necessary to drive the rate-limiting step of viral integration, and replication with the help of various enzymes like reverse transcriptase, integrase, and protease (Kaplan, 1994; King, 1994). Other proteins like Nef, Vif, Rev, Vpu, Vpr, and Tat are encoded by different genes (Kaplan, 1994; King, 1994; Langer and Sauter, 2017).

HIV phylogenetic classification

HIV-1 is further classified by substantial genomic variability due to a number of reasons, including the reverse transcriptase's (RT) lack of proofreading capabilities (Op de Coul et al., 2001; Roberts et al., 1988), the virus's fast turnover rate *in vivo* (Ho et al., 1995), host-selective immunological forces (Michael, 1999), and recombination events during replication (Temim, 1993). As a result, diverse HIV variants have been characterized into different phylogenetic groups, namely group M (main), group N (outlier), and group O (neither M or N; Ayouba et al., 2000; Gürtler et al., 1994; Simon et al., 1998). There are 10 identified phylogenetic subgroups or clades (A to K) within group M, which is responsible for the bulk of infections in the global HIV-1 pandemic (Buonaguro et al., 2007). The most prevalent ones are clade B and clade C (Hemelaar et al., 2006). We will examine clade B, given it is geographically distributed in the United States and Europe (Campbell et al., 2011; Tyor et al., 2013).

HIV functional domain and neuropathogenesis

Tat protein sequence is broken down into six distinct functional domains. The first five domains, which are coded by the first exon, are adequate for trans-activation of viral transcription and modulation of most Tat cellular functions (Clark et al., 2017). The arginine-rich basic domain serves as an RNA binding domain (RBD), a protein transduction domain (PTD), and a signal for nuclear localization (NLS) (Clark et al., 2017). The second exon codes for the C-terminal domain, which contains a tripeptide RGD motif and is not necessary for Tat activities in cell culture, but may have a role in viral pathogenicity *in vivo* (Clark et al., 2017; Li et el., 2008).

Tat-induced neurotoxicity is regulated by unique protein signatures found in Tat-B clade (Williams et al., 2020). In particular, presence of the dicysteine motif (C_{30} C_{31}) inside the Tat-B protein is critical for binding to NMDA receptor, thereby conferring neurotoxicity (Li et al., 2008; Williams et al., 2020). In support, Tat-B causes an increase in oxidative stress, kynurenine pathway metabolites, amlyloid beta, and glutamate, all of which have a detrimental effect on neuronal integrity than Tat-C clade (Bertrand et al., 2013; Rempel and Pulliam, 2005; Samikkannu et al., 2009, 2014a, 2014b; Williams et al., 2020). Tat-B also elicits a more robust inflammatory response than Tat-C, involving increased CCL2 levels, increased BBB degradation, and ultimately increased monocyte transmigration across the BBB (Ranga et al., 2004). As a result, Tat-B may be more efficient in enhancing infected monocyte transmigration into the brain, as well as inflammatory markers, promoting neurotoxicity, and viral products, which could be a rationale for HIV-B's increased neurovirulence compared to HIV-C clade (Campbell et al., 2011; Mishra et al., 2008; Ortega et al., 2013; Rao et al., 2008; Williams et al., 2020). Hence we will be evaluating the HIV-1 Tat acquired from clade B for our future studies.

Glycoprotein 120

The tripartite spike of the viral envelope is made up of glycoprotein 120 (gp120). gp120 is noncovalently coupled to gp41 on viral surfaces. After binding to CD4 and a chemokine co-receptor (CCR5 or CXCR4) on the host cell, gp120 enables membrane fusion and viral capsid deposition into the cytoplasm (Deng et al., 1996; Wu and Yoder, 2009). Thus, blockade of membrane fusion may be a viable strategy to combat the HIV infection progression (Eggink et al., 2019; Scarlatti et al., 1997). *In vitro,* gp120 causes neurotoxicity in the picomolar range via binding to either CCR5 and CXCR4 receptors on neurons (Bachis et al., 2003; Lipton, et al., 1991; Meucci & Miller, 1996), and/or via gp120 internalization and axonal transport in peripheral neurons (Berth et al., 2015). A transgenic mouse model that expresses gp120 in astrocytes exhibited signs of neurotoxicity and neuropathological characteristics similar to those found in HAND patient brains (Toggas et al., 1994). Recent subcellular neuropathological, functional, and imaging studies showed gp120 mice to exhibit hypersensitive neuroinflammatory profile, and promote apoptosis, mitophagy, and influence mitochondrial dynamics (reviewed in Field and Ellis, 2019; Young et al., 2022).

Viral Protein R and Nef

Unlike gp120 and Tat, Vpr and Nef are less explored HIV proteins, but they have substantial impacts on CNS mitochondria (Reviewed in Field and Ellis, 2019). Virion-bound Vpr is required for initial $CD4^+$ T cell and macrophage infection (Kogan & Rappaport, 2011). Nef promotes the survival of infected cells by downregulating cell-surface receptors in the immunological synapse (Chaudhry et al., 2005; Das & Jameel, 2005). Vpr and Nef alter mitochondrial activity in uninfected CNS cells, implicating their role in HIV-induced brain damage

(reviewed in Field & Ellis, 2019).

The Trans-activator of Transcription (Tat)

HIV-1 transactivator of transcription (Tat) is a regulatory protein vital for HIV replication (Das et al., 2011). Tat binds to the viral TAR genomic component, complexing with elongation factor (P-TEFb) to drive viral replication. Development of this complex further promotes phosphorylation and initiation of RNA Polymerase II, important for expression and maturation of the viral genome (Debaisieux et al., 2012; Kim & Sharp, 2001; Sobhian et al., 2010). Tat is synthesized at a low level by the reverse transcriptase enzyme during viral infection, and is critical for faster viral replication (Kim & Sharp, 2001). The length of the Tat protein varies according to the specific viral strain, which ranges from 80 to 103 amino acids (Debaisieux et al., 2012; Kamori and Ueno, 2017). The most essential aspect of Tat protein is that it contains conserved domains and sequences. Outstanding domains include a Zn^{2+} binding basic domain and a highly acidic N-terminal region (Bayer et al., 1995; Debaisieux et al., 2012). The internal interactions within the Tat protein's many domains are extremely dynamic and are regulated by a variety of external variables. As a result, the secondary structure of Tat is highly varied, with the crucial Zn^{2+} binding basic domain being critical for binding and stabilizing Tat's interactions with other regions (Debaisieux et al., 2012; Pantano et al., 2002, 2004; Rayne et al., 2010; Zhang et al., 2000). Additionally, the basic domain functions as a target sequence for plasma membrane penetration, allowing Tat to be secreted intact into extracellular space via vesicular transport without the necessity for cell lysis (Debaisieux et al., 2012; Frankel & Pabo, 1988; Green & Loewenstein, 1988; Rayne et al., 2010). Additionally, AIDS and neuroAIDS pathological states can be induced

even in the absence of dynamic viral replication due to the flexible structure and lack of spatial constraints of shortened Tat fragments, which can act at cell surface receptors and cross cell membrane to interact with intracellular segments other than TAR (Soulas et al., 2009). Indeed, all of these properties amplify HIV pathogenicity, making Tat a viable target for therapeutic intervention (Bayer et al., 1995; Cohen Avrahami et al., 2014; Debaisieux et al., 2012; Magnuson et al., 1995; Nath et al., 1996). Hence, the present dissertation will explore the capacity of HIV-1 Tat to produce behavioral, neurological and neuroendocrine manifestations.

Excitotoxicity mediated by HIV-1 Tat Protein

Excitotoxicity is one of the key processes through which HIV and other neurodegenerative illnesses such as traumatic brain damage and seizures produce neuronal injury (Avignone et al., 2005; Haughey et al., 2001; Li et al., 2008; Mattson et al., 2005; Mehta et al., 2013). Excitotoxicity occurs when a neuron becomes over excited as a consequence of excessive cation signaling, which may occur as a result of excessive cation input into the synaptic cleft, insufficient cation clearance, or inappropriate cation inhibitory feedback (Dong et al., 2009). Excitotoxicity does not occur as a single event, but rather refers to a cascade of pathological events which drive changes at the cellular and molecular level (Bouilleret et al., 2000; Mehta et al., 2013). While excitotoxic injury to neurons *in vivo* is not immediately deleterious, the accumulation of neuronal damage over time may result in the loss of functional capacity of neurons due to the caspase-mediated apoptosis event (Garden et al., 2002). Thus, these cascades of events determine the lag time between the emergence of neurological symptoms in HIV^+ patients and primary HIV diagnosis (Coleman et al., 2004; Levy and Bredesen, 1998).

In the post cART era, HIV^+ patients demonstrated neural dysfunction despite the lower

peripheral viral load, opening a new avenue for researchers on elucidating mechanisms underlying CNS neuronal damage. In search of mechanisms, we and others find Tat to be one of the most important neurotoxic proteins present in the HIV genome. Tat promotes neurotoxicity via direct (excitotoxic) and indirect (neuroinflammatory) actions (Ajasin and Eugenin, 2020; Marino et al., 2020; Paris et al., 2020; Tang et al., 2020; Wallace, 2021). HIV Tat is one of the first viral genes produced by infected cells when proviral DNA is integrated into the host cell's genome (reviewed in Field & Ellis, 2019). Tat is released from infected lymphocytes, monocytes and glial cells including astroglia (Ensoli et al., 1993; Fan and He, 2016; reviewed in Field & Ellis, 2019; Jin et al., 2012) Tat was found in pre-cART HIVE brains assessed via immunohistochemical techniques (Cowley et al., 2011; Del Valle et al., 2000; Hudson et al., 2000; Henderson et al., 2019; Johnson et al., 2013). A sensitive ELISA has also found Tat-specific antibodies in the CSF of individuals on cART (Bachani et al., 2013). Tat persists in some HIV^+ people despite viral suppression by cART regimens (Bachani et al., 2013). Tat binds to the LRP receptor of neurons (functional relevance to NMDA receptor) (Liu et al., 2000), promoting association with the PSD-95 and nNOS to the NMDA/LRP complex (Eugenin et al., 2007). Concurrently with an increase in nitric oxide signaling, these changes may induce death in neurons (Eugenin et al., 2007; King et al., 2010). Additional mechanisms of Tat-mediated neurotoxicity are a) dysregulated calcium homeostasis (Haughey et al., 1999; Haughey and Mattson, 2002; Hu, 2016); b) hyperactivation of glutamate receptor to mediate excitotoxicity (Gras et al., 2003); c) elevation of free radical oxygen species and oxidative stress in the cell (due to cysteine uptake inhibition, leading to glutathione depletion) (Gras et al., 2003; Kruman et al., 1998; Perl and Banki, 2000) d) Tat-mediated neuroinflammation resulting in increased amyloid-β production and Tau hyperphosphorylation (Canet et al., 2018; Wallace, 2021). Furthermore, the cysteine-rich region of Tat causes synaptodendritic damage and

may account for synaptic loss (Bertrand et al., 2013; Nath and Steiner, 2014), which may explain the cognitive abnormalities seen in this population. Tat is also demonstrated to mediate inflammatory gene expression and activation of microglia and astroglia (Rozzi et al., 2018; Teodorof-Diedrich & Spector, 2018; Thangaraj et al., 2018). As a result, HIV Tat not only impacts mitochondrial and neuronal structural integrity, but also promotes inflammation, oxidative stress, and mitochondrial dysfunction in glia, forming the basis for long-term neuroinflammation in a reservoir of low-level HIV infection. Hence, Tat-mediated neuronal dysfunction occurs not only through direct excitotoxicity mechanisms but also through bystander processes, resulting in an upregulation of inflammatory genes and cytotoxicity found in comorbid disorders such as HAND (Ajasin and Eugenin, 2020).

Comorbidity of HIV infection and opioid epidemic

A growing concern worldwide is the interaction of HIV infection with opioids (Bruce and Altice, 2007). HIV infection occurs by sharing needles and syringes among injection drug users (IDUs), which is an important mode of transmission in those who do not transmit HIV via sexual contact (CDC, 2017; Friedland, 1985; van der Graaf & Diepersloot, 1986). Moreover, IDUs have been shown to be significantly associated with risky sexual behaviors, thereby increasing the probability of transmission of HIV infection to others (Bruce and Altice, 2007). In support, a study revealed up to 45% of IDUs were HIV positive (Francis, 2003). Indeed, substance use makes treatment of HIV infection overly complicated, given substance abusers need to be primarily treated for substance use disorders and additional comorbidities (Durvasula and Miller, 2014; Francis, 2003). Moreover, while opiates are not by any means the only medications infused intravenously, they contribute to a significant extent to injection drug use (Bruce & Altice, 2007).

Additionally, opioids are increasingly prescribed to HIV^+ patients for neuropathic pain (Becker et al., 2016; Dowell et al., 2016; Edelman et al., 2013; Jeevanjee et al., 2014; Koeppe et al., 2013; Merlin et al., 2015, 2016; Silverberg et al., 2013; Swica and Breitbart, 2002; Tsao et al., 2012) raising concerns beyond illicit drug users. As such, the present dissertation proposes to characterize the interaction of opioids like oxycodone and HIV-1 Tat (neurotoxic HIV protein) as well as to provide a viable treatment option for HIV^+ opioid addicts.

Opioid use and HIV interactions

The link between opioids and HIV does not end with infection. Once the virus is detected, HIV proteins spread and may interact with opioids, resulting in neurological dysfunction (reviewed in Chilunda et al., 2019; Dutta and Roy, 2012; Hauser et al., 2012; Mahajan et al., 2008; Olin et al., 2012). Opioids prescriptions and illicit use is rising in the HIV^+ population (Becker et al., 2016; Edelman et al., 2013; Jeevanjee et al., 2014; Koeppe et al., 2013; Merlin et al., 2015, 2016; Silverberg et al., 2013; Swica and Breitbart, 2002; Tsao et al., 2012; Williams and Bisaga, 2016), thus making it more important to delineate the interactions between opioids and HIV and enable the development of targeted medicines aimed at slowing the trajectory of neurocognitive impairment in HIV-infected patients who use opioids (Dutta and Roy, 2012). Clinical reports of increased neuropathology and neurocognitive impairment in HIV^+ opioid addicts have been observed both before and during the availability of combination antiretroviral therapy (Bell et al., 1998; Byrd et al., 2011). Mounting evidence from Jeanne Bell's lab in the pre-cART era show a higher prevalence of cognitive impairment (~3.5-fold increase) among injection drug users (Bell et al., 1998). In the post cART era, clinical, neuroimaging, and neuropathological studies (Anthony et al., 2008; Bell et al., 1998, 2006; Byrd et al., 2011) paired with cell culture and animal model

studies revealed opioids to exacerbate the neuroHIV symptoms (Byrd et al., 2011; Bokhari et al., 2009; Fitting et al., 2010a, 2014; Gonek et al., 2018; Meyer et al., 2013; Nath et al., 2000, 2002; Noel et al., 2008; Silverstein et al., 2012; Turchan-Cholewo et al., 2006). Some of the underlying mechanisms are, opioids induce neuroinflammation in the central nervous system, which results in increased viral entry and replication, hence contributing to affective dysregulation and other types of neurocognitive problems (Cahill and Taylor, 2017; Norman et al., 2009; Roy et al., 2011; Smith et al., 2014; see Fig. 1). In models of the simian immunodeficiency virus (SIV) macaque model, opioids like morphine interacted with SIV to potentiate neuropathogenesis and mortality compared to the virus group alone (Bokhari et al., 2011). Other lines of evidence show morphine exposure to augment viral replication (Kumar et al., 2006). Some contrasting evidence shows morphine to reduce the progression to AIDS in the simian AIDS progression in the rhesus macaque model (Donahoe et al., 2009). Moreover, recent clinical evidence suggests the use of opioids like oxycodone in HIV^+ patients for neuropathic pain phenotype (Merlin et al., 2018; Silverberg et al., 2012; Swica and Breitbart, 2002). However, oxycodone interactions with HIV proteins are largely unexplored. Given the heterogeneity of human populations, it is challenging to get a representative sample and maintain experimental control in a clinical setting. Additionally, some reports indicate that there is no obvious correlation between substance abuse and HIV status. Thus, we and others isolated specific HIV proteins to investigate their interactions with illicit drugs in laboratory conditions in order to identify potential treatment targets. As such, my present dissertation will delineate the interactions of clinically prescribed opioids like oxycodone with HIV-1 Tat protein in both *in vitro* culture systems and *in vivo* transgenic mouse models.

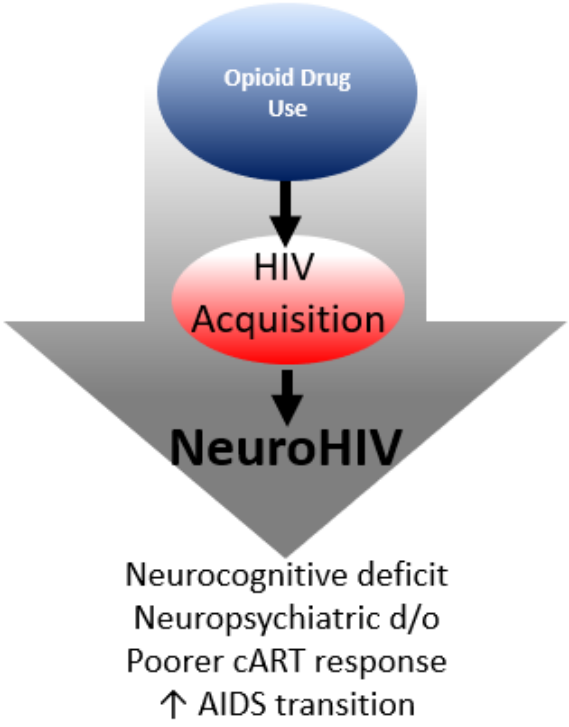

Figure 1: Schematic flow chart of opioid use to produce HIV acquisition and associated neuroHIV symptomatology.
Opioid abuse can further accelerate the course of HIV infection and its associated complications, particularly in the brain. Clinical research indicates that substance use might raise viral load, accelerate disease progression, increase HIV-related neurocognitive disorders diagnosis and exacerbate AIDS-related death, even in patients on antiretroviral therapy (ART). Additionally, individuals with substance use problems are less likely to take life-saving HIV treatment on a consistent basis, which accelerates the transition to AIDS.

Effects of HIV-1 Tat and Combined Substance Use on the Brain

A preponderance of the evidence demonstrates HIV-1 infection and combined substance use increases the severity of neuroHIV symptomatology among drug users (Atluri, 2016; Bell et al., 2006). Indeed, illicit drug use is a significant risk factor for HIV transmission (Bruce and Altice, 2007). HIV targets microglia in the CNS compartment as well as perivascular macrophages early in the infection (Wallet et al., 2019). HIV infection, on the other hand, does not start actively

infecting neural cells until the immune system has been severely compromised, which can lead to an increase in activated microglial cells, neuronal death, and cognitive deficits (Bell et al., 2006). Drugs of abuse exacerbate neuroHIV symptoms illustrated by the deposition of phosphorylated Tau proteins, often seen with neurodegeneration (Bell et al., 2006). HIV also infiltrates and modulates the dopaminergic system, which is an important mediator for drug reward and addiction (Kalivas and Volkow, 2005; Koob and Volkow, 2016) and increases the propensity for the development of HAND in drug abusers (Bell et al., 2006; Gaskill et al., 2013). Intriguingly, HIV-1 proteins like Tat and gp120 have promoted adaptations in the dopaminergic transmission (Fitting et al., 2015) which may underlie HIV-associated cognitive and motor dysfunctions (Berger and Arendt, 2000; Nath et al., 2000). In particular, HIV-1 Tat is a potent blocker of dopamine transporter (DAT) thereby producing dysregulation of dopamine homeostasis in the synaptic cleft (Bucci, 2015; Midde et al., 2012), via altering dopaminergic system recycling (Ferris et al., 2009), uptake kinetics (Zhu et al., 2009), and rapid DAT dysfunction (Wallace et al., 2006; Zhu et al., 2011). On the other hand, psychostimulants like cocaine and amphetamine act at DAT (site distinct from Tat; Zhu et al., 2009) and increase extracellular dopamine in the synaptic cleft (Kahlig and Galli, 2003). Together HIV-1 Tat and cocaine boost dopamine synaptic levels, which may contribute to HAND and addictive behaviors in HIV^+ users of psychostimulants (Sun et al., 2017).

Additional morphological and electrophysiological studies show HIV-1 Tat to differentially influence the striatal D1 and D2 receptor expressing medium spiny neurons (MSNs). HIV-1 Tat reduced dendritic spine density, increased dendritic damage (swelling or varicosities), and impaired neuronal excitability in D2 MSNs (Schier et al., 2017). These findings suggest that D2 MSNs are more vulnerable to HIV-1 Tat than D1 MSNs, thereby enhancing reward valence

(Schier et al., 2017). Moreover, the combination of Tat and cocaine changed the number of dendritic synapses (Bertrand et al., 2015). The enhanced neuronal damage caused by Tat protein and cocaine is due to altered L-type calcium channel expression and function (Napier et al., 2014; reviewed in Wayman et al., 2015a). Hence, L-type calcium channels may be a viable target for combined Tat and cocaine-associated neuropathology. Tat also causes depolarization of mitochondrial membrane potential in human and murine neuronal cells, including SH-SY5Y neuroblastoma cells (Malik et al., 2011), apoptosis, and increased reactive oxygen species (Malik et al., 2011; Suzuki et al., 2011), effects that were exacerbated by opioids like morphine (Fitting et al., 2014a; Malik et al., 2011; Maubert et al., 2016; Suzuki et al., 2011) and methamphetamine (Huang et al., 2021). When Tat and morphine were combined, proinflammatory cytokines like TNF-α, IL-1β, and IL-6 are elevated in primary murine astrocytes or microglia (Bokhari et al., 2009; El-Hage et al., 2005, 2006). As a result, HIV-1 Tat interactions with drugs of abuse including opioids and psychostimulants may result in more severe neuropathological cellular and molecular changes.

Furthermore, drug reward and addiction studies demonstrate the ability of Tat to interact with drugs of abuse and potentiate their effects (Cirino and McLaughlin, 2021). In the drug self-administration paradigm, a model of positive reinforcement tenet of the addiction cycle, effects of psychostimulants like cocaine was enhanced by Tat (Wayman et al., 2015b; 2016). In conditioned place preference paradigm (CPP), a model to assess drug reward, HIV-1 Tat expression potentiated cocaine's psychostimulant and rewarding effects (Paris et al., 2014a) and oxycodone rewarding effects (Salahuddin et al 2022a Unpublished*) and ethanol rewarding effects in the Tat Gt-tg bigenic mice model (McLaughlin et al., 2014). In the behavioral sensitization paradigm, HIV-1

Tat potentiated a) morphine psychomotor effects in male mice (Paris et al., 2020); b) methamphetamine mediated psychomotor effects in HIV-tg rats (Liu et al., 2009). Tat also increases reward deficits, a hallmark of depression-like behavior, and increased sensitivity to methamphetamine reward behavior in transgenic mice (Kesby et al., 2016). Other drugs of abuse substances potentially interact with Tat to modulate the dopaminergic transmission and influence structural and functional outcomes (Gaskill et al., 2017). Together, these pieces of evidence suggest the ability of Tat to directly or indirectly alter dopaminergic neurotransmission and potentiate the behavioral effects of psychostimulants and other drugs of abuse.

Sex Differences in HIV-1 and Substance Abuse-Related Neurological Sequelae

Epidemiological studies accounting for sex as a biological variable, demonstrated sex/gender differences in the vulnerability to neurological symptoms in the HIV infected individuals (Burlacu et al., 2019). Generally, women tend to be increasingly diagnosed with affective disorders like anxiety and depression compared to men (Albert, 2015; Kuehner et al., 2017; McLean et al., 2011). Intriguingly, contrasting evidence is reported in the HIV literature, wherein women with HIV elicited a decrease in the propensity to HAND (Bing et al., 2001; Lopes et al., 2012) improved clinical and immunological outcomes (Cabrerñoz et al., 2012; Collazos et al., 2007; Finkel et al., 2003) and lower viral load (Farzadegan et al., 1998; Grinsztejn et al., 2011) during early stages of HIV infection (Sterling et al., 1999); and slower disease progression post-HIV infection (Jarrin et al., 2008). Although these findings are not always observed as some reports suggested greater cognitive impairment among females when compared to males (Maki et al., 2015, 2018; Manly et al., 2011; Rubin et al., 2019) and some found no gender differences (Evans et al., 1997; Sewell et al., 2000; Tsao et al., 2004). Clinical studies investigating HIV pathological

outcomes are often not stratified by gender, sociodemographic, clinical, and behavioral characteristics (Rubin et al., 2021) and many are not controlled to assess the exogenous/endogenous steroid milieu in females that may confer neuroprotection. Animal models thus, offer appropriate means to evaluate the potential hormonal mediators that may confer protection/vulnerability to HIV-1 proteins. We have found "female-typical" steroids (i.e. those that fluctuate to a greater degree in females), such as progesterone and its metabolites, to confer protection against Tat-induced neurotoxicity, mitotoxicity, and the affective behavioral consequences of CNS Tat exposure (Paris et al., 2014b, 2016, 2020). Moreover, HIV-1 Tat expressing female mice demonstrated reduced neuronal dysfunctional states and improved behavior outcomes when compared to their male counterparts (Hahn et al., 2015). In particular, female mice revealed a) decrease in cellular deficits in the striatum, b) lower astrocyte activation and microglial 3-NT (a nitrosative cellular stress marker) c) higher dendritic spines d) lower disruption in the levels of excitatory and inhibitory synaptic proteins e) higher forelimb grip strength and reduced anxiety-like behavior (in light-dark box) (Hahn et al., 2015).

Substance use comorbidities accentuate the neurological outcomes in HIV infected population (Altice et al., 2010). Numerous reports indicate that there are gender differences in substance use (Becker and Koob, 2016), but these distinctions are poorly defined in the HIV literature, with the majority of research focusing on male animal models (McLaurin et al., 2017). In general, men are more likely than women to engage in substance abuse (Becker et al., 2017); However, women who become addicted develop drug dependence more rapidly than men (Greenfield et al., 2007; Hernandez-Avila et al., 2004; Ridenour et al., 2005) and are even more susceptible to relapse following abstinence (Becker and Hu, 2008; Lynch et al., 2002; Robbins et

al., 1999). The variables that contribute to substance misuse differ, with peer influence and experimentation being the primary causes that motivate males to begin using substances. On the contrary, women's substance usage is driven by sexual or interpersonal relationships (Frajzyngier et al., 2007; Hser et al., 1987). This trend was recapitulated in the animal model of self-administration, such that female rats tend to escalate cocaine self-administration behavior faster and in greater amounts than males (Hu et al., 2004; Lynch et al., 2006) and also developed a preference for cocaine over food, a hallmark of addictive-behavior (Perry et al., 2013). Various clinical studies reveal circulating ovarian steroids may account for these subjective responses to psychostimulants (Sofuoglu et al., 1999, 2004), nicotine (Franklin et al., 2004), alcohol (Logue et al., 1981), and opiates (Back et al., 2011). Similarly, preclinical studies implicate gonadal hormones' role in gender variability in cocaine-induced behavioral responses (Carroll and Anker, 2010; Festa and Quinones-Jenab, 2004).

To this end, in female rats, estradiol increases and progesterone or its metabolite, allopregnanolone attenuates cocaine mediated drug-seeking behavior across three stages of addiction, namely acquisition (Becker and Hu, 2008; Jackson et al., 2006), escalation (Larson et al., 2007), and reinstatement (Anker et al., 2007, 2009). Estradiol and progesterone also demonstrated changes in the drug-induced behavioral sensitization, drug-self administration, and drug rewarding behavior as assessed by conditioned place preference (Bobzean et al., 2014; Cummings et al., 2014; Hu et al., 2004; Hu and Becker, 2003; Larson et al., 2007). When female rats were ovariectomized, by removing the source of their ovarian hormones, estradiol treatment enhanced sensitization to psychostimulants like cocaine and amphetamine (Hu and Becker, 2003; Peris et al., 1991; Souza et al., 2014) whereas progesterone reduced the cocaine sensitization and

stereotypy behavior (Souza et al., 2014). In ovariectomized female rats, estrogen receptor beta (ERβ) and not estrogen receptor alpha (ERα) enhanced cocaine-seeking behavior (Larson and Carroll 2007). Most of the effects mediated by estradiol and progesterone are via modulation of the dopaminergic signaling in a time-dependent manner within the reward pathway encompassing the dorsal striatum and nucleus accumbens (Yoest et al., 2018). In support, acute estradiol administration increases dopaminergic transmission, upregulating D2 receptor activation and dopamine transporter function in adult females, but not males (Bazzett and Becker, 1994; Becker, 1990; Calipari et al., 2017; Cummings et al., 2014; Yoest et al., 2018). Estradiol also enhanced the dopamine release and associated behavioral sensitization response in ovariectomized female and not castrated male rats (Cummings et al., 2014).

As stated earlier, women are more vulnerable to acquiring HIV-1 infection than men, however, post-infection women tend to maintain lower viral loads and demonstrated better clinical and immunological outcomes than men (Addo and Altfield, 2014; Griesbeck et al., 2016; Rechtien and Altfield, 2019; Ziegler and Altfield, 2016). Estrogen receptor-mediated signaling may contribute to these sex differences (Scully, 2018). Given that women tend to elicit elevated immune responses to HIV insult, faster progression of viral infection may be observed (Griesbeck et al., 2016). Moreover, women also tend to demonstrate increased vulnerability to affective dysregulation (Bing et al., 2001) which is also recapitulated in Tat expressing animal models (Makhathini et al., 2018a, 2018b; Paris et al., 2014a, 2014b; Schier et al., 2017). Other studies demonstrate men to be increasingly susceptible to affective dysregulation (Goggin et al., 1998; Lopes et al., 2012) and some studies did not stratify the outcomes based on gender (Sordo del Castillo et al., 2010). As such, controlling for other factors that may moderate or mediate the

pathological outcomes, estradiol displayed neuroprotective properties in animal models of HIV-1 Tat (Adams et al., 2010; Kendall et al., 2005; Lee et al., 2004; Turchan et al., 2001; Wallace et al., 2006) and HIV-1 gp120 (Corasaniti et al., 2005; Howard et al., 2001; Russo et al., 2005). Future studies need to be conducted on determining the role of estradiol on opioid-mediated reward and reinforcement-related behavior outcomes in Tat expressing mouse models.

Novel adjunct therapeutics for HIV suppression

Antiretroviral therapy has significantly improved the life expectancy of PLWH, owing to its capacity to efficiently inhibit viral replication, decrease drug resistance, and improve PLWH's quality of life (CDC, 2016). However, these medications may not be completely effective, as a small percentage of latent viral reservoirs (Siliciano et al., 2003) remain in the central nervous system (Henderson et al., 2019), raising concerns that HIV may cause additional neurological and neuropsychiatric disorders in HIV^+ infected individuals.

Novel strategies have been attempted to find a functional cure in the HIV^+ infected population. One of the strategies widely explored was the “shock-and-kill” approach. This strategy attempted to reactivate the virus using latency-reversing therapeutics/agents (LRA) (Ait Ammar et al., 2020; Battivelli et al., 2018). The expectation is to allow the infected cells to eliminate the actively replicating virus by the host immune system and cART. This strategy thus far has not been successful, because of heterogeneity of latent viral reservoirs with distinct phenotypic variation and metabolic properties and differences in patient’s compromised immune defense mechanisms (Ait Ammar et al., 2020). Only PKC agonists, which cause robust T cell activation, showed reproducible results among latency models (Spina et al., 2013). Bryostatin-1, a protein kinase C (PKC) agonist, was the only efficacious LRA, demonstrated via *ex vivo* manipulations from patient

cells (Bullen et al., 2014; Laird et al., 2015). In light of these findings, numerous researchers have reevaluated whether or not T cell activation is required for successful viral reactivation; or those who use polyclonal T cells, as well as those who use alternative pathways, have been forced to reevaluate their findings (no-T-cell activation property; Spivak and Planelles, 2016). HDAC inhibitors (HDACi) are chemotherapeutic drugs that can activate latent proviruses in resting $CD4^+$ T cells (Spivak and Planelles, 2016). Presently, HDACi is the most commonly tested latency-reversing agent class. Four HDACi are now in exploratory clinical trials, with limited efficacy (Spivak and Planelles, 2016)). None of the tested LRAs changed the latent reservoir size *in vivo* to date. Also, CRISPR/Cas9 gene therapy technique illustrated reductions in proviral DNA in plasma and tissue reservoirs of non-human primates (Mancuso et al., 2020), HIV-1 infected humanized mice (Dash et al., 2019), and proviral quasispecies (Dampier et al., 2014) as a potential curative therapy and may soon enter clinical trials.

Additional therapies were investigated, including those that target the various stages of viral replication and reduce the presence of latent viral reservoirs. Cortistatins are steroid-like alkaloids isolated from marine sponges that have been extensively explored as potential anti-Tat therapies by Dr. Valente's lab (Mediouni et al., 2019a). These compounds employ a "Block-and-Lock" strategy, preventing the virus from reactivating in cells even during treatment interruptions, while also locking the virus in a dormant state to inhibit active replication (Mediouni et al., 2019b). These compounds are potent blockers of Tat protein, which is an important protein in the HIV genome to drive HIV replication. The most promising cortistatin compound was didehydro-cortistatin A (dCA) which binds to the RNA binding site of Tat and blocks HIV-Tat replication without producing cellular toxicity (Mediouni et al., 2019b). The combination of dCA and cART

suppressed active HIV-1 viral replication, reactivation, and viral rebound of the latent viral reservoir in $CD4^+$ T cells isolated from aviremic individuals and bone marrow-liver-thymus (BLT) mouse model of HIV latency and persistence (Kessing et al., 2017). Additional chemical derivatives of dCA were sought to rationalize their ability to dock at specific binding sites of Tat protein (Mediouni et al., 2019b). Given dCA ability to inhibit Tat's expression early during the viral replication, capacity to penetrate latent viral reservoirs in the brain, good bioavailability, and additive potential with other antiretrovirals (Kessing et al., 2017; Mousseau et al., 2012, 2019ab), dCA and its novel steroidal based analogs holds potential as future anti-Tat therapeutics.

Sex steroidal based therapeutics, especially estrogen has gained prominence recently, given their ability to slow HIV transmission (Smith et al., 2000), ameliorate the neurotoxicity against synergy of HIV-1 Tat, gp120 viral proteins (Turchan et al., 2001) with cocaine (Kendall et al., 2005), confer protection against Tat-mediated inflammation of vascular endothelial cells (Lee et al., 2004), reduce neurotoxicity against HIV-1 protease-mediated apoptosis of neuroblastoma cells (Hawkins et al., 1999), decrease gp120 neurotoxicity and reinstating calcium homeostasis (Brooke et al., 1997; Brooke and Sapolsky, 2000). Indeed, estrogen promotes neuroprotection against glutamate-mediated excitotoxicity (Goodman and Mattson, 1996; Singer et al., 1996), beta-amyloid toxicity (Green et al., 1996), calcium insults (Mermelstein et al., 1996; Nakajima et al., 1995). Some of the estradiol neuroprotective and neuronal survival attributes include reduced apoptosis via modulation of anti-apoptotic Bcl or apoptotic Bax mechanism and interleukin-1β levels in the neocortex of rats (Corasaniti et al., 2005; Zhou et al., 2004), anti-oxidant properties to scavenge the oxygen free radicals (Behl et al., 1997; Keaney et al., 1994; Lacort et al., 1995; Mooradian, 1993), promotion of dendritic growth of neocortical neurons and increase in

neurotrophic factors (Brinton et al., 1997; Chowen et al., 1992; McEwen and Woolley, 1994).

Additional estrogen-based adjunctive treatments for HIV-associated neurocognitive disorders (HAND) were studied by Drs. Booze and Mactutus group. They have identified selective estrogen receptor β agonists, namely S-Equol (SE) and Phytoestrogens like daidzein and liquiritigenin to improve the HIV-associated neurological outcomes (McLaurin et al., 2020). S-Equol (SE) improved pre-attentive processes and stimulus-response learning in HIV transgenic rats (McLaurin et al., 2020); promoted reduction of combined cocaine and HIV-1 mediated synaptopathy (Bertrand et al., 2015). Phytoestrogens like daidzein and liquiritigenin repaired HIV-1 Tat-mediated synaptodendritic damage (Bertrand et al., 2014). Estrogens like 17 beta-estradiol have been effective in reducing HIV-1 Tat/gp120-mediated peroxynitrile-induced oxidative stress and loss of dopamine transporter function (Wallace et al., 2006). Some of the mechanistic studies highlighted the estrogen's anti-apoptotic effects to reverse HIV-1 Tat-mediated neuronal dysfunction (Adams et al., 2010). As a result, estrogen-based treatments offer therapeutic options for preventing the neurodegenerative and neurotoxic effects of HIV proteins like Tat and gp120 (Wallace, 2006).

HIV/AIDS and Neuroendocrine Dysfunction

HIV patients contend with additional comorbidities like endocrine dysfunction (Kalra et al., 2011). Some of the organs largely affected are adrenals, gonads, pituitary, thyroid, and metabolic and bone abnormalities (Kibirige and Ssekitoleko, 2013; Mirza et al., 2018; Zaid and Greenman, 2019). Chronic metabolic issues due to cART therapy in these patients included insulin resistance, hyperlipidemia, lipodystrophy, lipohypertrophy, and diabetes mellitus (Mirza et al., 2018). Bone abnormalities included osteoporosis and osteopenia (Mirza et al., 2018). Despite the

ability of gonadotrophin-releasing hormone (GnRH) to secrete sufficient gonadotropins (like luteinizing and follicle-stimulating hormone), hypogonadotropic hypogonadism was also increasingly reported in HIV^+ patients (Gomes et al., 2016; Poretsky et al., 1995; Rochira and Guaraldi, 2014; Wunder et al., 2007). Hypogonadism was defined as low testosterone levels in men and menstrual abnormalities including premature ovarian insufficiency levels in women (Dutta et al., 2017; Gomes et al., 2016; Rochira and Guaraldi, 2014; Poretsky et al., 1995; Wunder et al., 2007). In the post cART era, HIV therapy differentially modulated estradiol levels such that lopinavir/ritonavir combinative regimen increased estradiol levels and efavirenz decreased estradiol levels (McDonalad et al., 2018). Adrenal disorders include hypothalamic-pituitary-adrenal stress axis (HPA) dysregulation which encompasses elevated basal cortisol and adrenal insufficiency in HIV^+ patients (Mirza et al., 2018; Zaid and Greenman, 2019). In the pre-cART era, most of the endocrine abnormalities occur as a result of direct effects of HIV proteins, neoplasms, and opportunistic infections on various endocrine glands which may significantly worsen the quality of life and contribute to additional comorbidities and mortality (Membreno et al., 1987; Raffi et al., 1991; Schlienger and Lang, 1989; Zaid and Greenman, 2019). However, during the post-cART era, a decline in the incidence of opportunistic infections and associated endocrinopathies was observed (Zaid and Greenman, 2019). Overall, the clinical features of neuroendocrine dysfunction in HIV/AIDS patients may be masked with various infectious, non-infectious, and iatrogenic causes. Hence making it challenging for the clinicians for early diagnosis. Thus, animal models may be fundamental for systematic identification of the contribution of each of these factors and the development of adjunct therapeutics with improved efficacy against these manifestations.

HIV/AIDS and HPA Axis

A preponderance of data provided evidence for altered HPA axis in HIV-infected patients (Reviewed in Nicolaides et al., 2000). Indeed, in the pre-cART era, the pathogenesis of HPA dysfunction was mediated by opportunistic infections ranging from adrenalitis (Chrousos, 1995; Glasgow et al., 1985; Nassoro et al., 2019) to adrenal dysfunction seen in the late stages of AIDS (Gonzalez-Gonzalez et al., 2001; Lortholary et al., 1996; Stolarczyk et al., 1998; Wolff et al., 2001). Cytomegalovirus adrenalitis was the most prominent form of adrenalitis reported in up to 60% of the HIV patients (Hoshino et al., 1997; Oelkers, 1996; Pulakhandam and Dincsoy, 1990; Rotterdam and Dembitzer, 1993; Tomita et al., 1990). Autopsies of adrenals showed intra-adrenal inflammatory lesions with or without necrosis due to immunodeficiency in the HIV^+ population (Glasgow et al., 1985; Guarda et al., 1984; Nassoro et al., 2019; Niedt and Schinella, 1985; Welch et al., 1984). Potential mechanisms of adrenal dysfunction (insufficiency) include a) HIV infection or co-infection with *cytomegalovirus, mycobacteria, histoplasma, pneumocystis carinii* species (Freda et al., 1994) b) adrenal gland destruction by a tumor (sarcoma or lymphoma) (Jinno and Goshima, 2008) c) Hemorrhage of the adrenal cortices associated with coagulopathy (Jäättelä, et al., 1991; Natarajan et al., 1989) d) HIV mediated immune activation and release of cytokines like TNF-α leading to decreased adrenal secretion (Gaillard et al., 1990; Jäättelä, et al., 1990) e) Antifungals like ketoconazole mediated inhibition of 11-β hydroxylase enzyme, which is an important enzyme for steroidogenesis (Smith, 1994). Rifampicin mediated stimulation of cytochrome p450 enzyme activity leading to increased metabolism of antiretrovirals and cortisol (Burman et al., 1999; CDC, 2000; Dlodlo et al., 2005).

Given the decline of opportunistic infections and improved immune responsivity in the post

cART era, the incidence of adrenalitis has fairly dropped (Lo and Grinspoon, 2010). Estimates vary, but up to 46 % of HIV^+ patients in the post cART era, demonstrated HPA axis dysregulation (Afreen et al., 2017; Chrousos and Zapanti, 2014; González-González et al., 2001; Marik et al., 2002; Prasanthai et al., 2007; Sharma et al., 2018). Clinical diagnosis of the HPA axis in HIV/AIDS patients identified two important clinical endophenotypes namely, hypercortisolemia and secondary adrenal insufficiency (George and Bhangoo, 2013; Zapanti et al., 2008) based on whether subjects were in the early or late clinical-stage of AIDS respectively.

HPA Axis and Glucocorticoids

The hypothalamus-pituitary-adrenal (HPA) stress axis is essential for stress adaptation (Herman et al., 2016). The stress response is characterized by the release of corticotropin-releasing hormone (CRF) from the PVN nucleus of the hypothalamus on exposure to physiological, physical, immune, or drug abuse-related stressor challenges. The CRF then travels via the hypophyseal portal vein to the anterior pituitary gland to release adrenocorticotropic hormone (ACTH). The circulating ACTH then travels via systemic circulation to the adrenal cortex, to release (cortisol in humans; corticosterone in rodents), additional glucocorticoids, mineralocorticoids like aldosterone, sex steroids, and additional pregnane steroids like dehydroepiandrosterone (DHEA) (Sapolsky et al., 2000; Whitham et al., 2020; See Fig. 2). Glucocorticoids thus released, drive changes in the physiological system by mobilizing energy stores (promote glycogenolysis, gluconeogenesis, lipolysis) from liver, fat, and muscle stores (De Kloet et al., 1998). Once the stressor is subsided, glucocorticoids form a negative feedback loop and bind to the glucocorticoid receptor (GR) at the level of the hypothalamus and anterior pituitary to inhibit its own release via genomic (glucocorticoid response element and non-glucocorticoid response element) and non-

genomic molecular mechanisms (Croxtall et al., 2000; Gross and Cidlowski, 2008) and reinstate CNS homeostasis (Finsterwald and Alberini, 2014). Indeed, control of the excess release of glucocorticoids is modulated by negative feedback, which is critical for maintenance of HPA axis homeostasis as overshooting glucocorticoid response may lead to pathological states (Finsterwald and Alberini, 2014; Myers et al., 2012). Similarly, an individual gets habituated to repeated exposure to stressors, leading to sustained HPA axis activation (Grissom and Bhatnagar, 2009). Thus, chronic activation of HPA may manifest in various forms like a) chronic elevated basal cortisol (hypercortisolemia), b) sensitized stress response, and c) adrenal insufficiency (McEwen, 2006). Various factors related to stressors (chronicity, frequency, intensity) and individual (epigenome, early life adversity, sex, age) and environmental factors may interplay in the manifestation of HPA dysfunction (Herman et al., 2016). Furthermore, the engagement of limbic, brainstem, and hypothalamus circuits establishes the neurological basis for stress resistance (Herman et al., 2016; McEwen and Gianaros, 2010). These circuits regulate the physiological and behavioral stress response system, which could be adaptive in the short-term and maladaptive in the long term (McEwen and Gianaros, 2010). Thus, HPA axis dynamics entail a finely controlled physiological system's stress response (McEwen, 2007). Understanding the mechanisms underlying maladaptive response and restoration of the HPA axis to normal is a primary goal for integrative care and improved health outcomes.

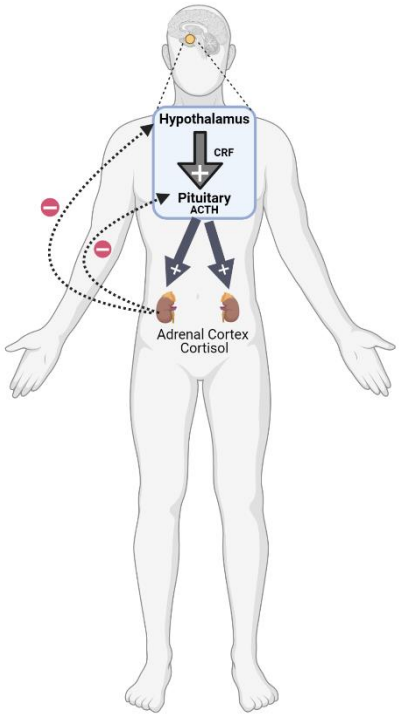

Figure 2: Schematic diagram of hypothalamus-pituitary-adrenal gland (HPA) axis.
The hormones involved in the HPA-axis are depicted. On exposure to a stressor, the hypothalamus produces and releases CRF, which stimulates the anterior pituitary to produce and release ACTH, which further stimulates the adrenal cortex to produce and release cortisol. Post-stress, cortisol forms negative feedback and binds to GR at the hypothalamus and pituitary to suppress its own release.

Glucocorticoid interaction with Glucocorticoid receptor

The HPA axis's final effectors are glucocorticoids, particularly cortisol. The released cortisol exists in two forms A) bound form or B) unbound form. Approximately 95% of the cortisol is bound to the corticosteroid-binding globulin (CBG) and transported in the blood. Less than 5% of cortisol is unbound and can be metabolized and excreted by enzyme transporters in the liver and kidney. Cortisol in its unbound state diffuses past the plasma membrane into the cytosol, where it is involved in signal transduction (Dittmar et al., 1997; Dittmar and Pratt, 1997; Pratt and Toft, 1997). The glucocorticoid receptor is a heterocomplex composed of a heat shock protein, a

stabilizing protein, at least one co-chaperone (FKBP52; FK506 binding protein), FKBP51, protein phosphatase 5, and cyclophilin 40. (Cheung and Smith, 2000; Dittmar et al., 1997; Dittmar and Pratt, 1997; Pratt and Toft, 1997). The binding of cortisol to the GR in the cytosol induces a conformational change in the GR heterocomplex, leading to nuclear translocation and homodimerization of the GR (Davies et al., 2002; Wochnik et al., 2005). The GR homodimer then elicits genomic effects via interaction with the glucocorticoid response elements (GRE) present in the regulatory zone of the glucocorticoid responsive genes (Drouin et al., 1989; Sakai et al., 1988). The GR co-activators or co-repressors are recruited and thus modulate the rate of gene transcription (Drouin et al., 1989; Sakai et al., 1988). The GR is also able to regulate the gene transcription independent of GRE mechanisms, via interaction with transcription factors, transducers, and activators like signal transducer and activator of transcription (STAT) (Ray and Prefontaine, 1994; Stöcklin et al., 1996). Additional evidence also points towards rapid non-genomic effects of glucocorticoids via interaction with various proteins (Croxtall et al., 2000). Thus, glucocorticoids produce their physiological and pathological responses via genomic (GRE and non-GRE) or non-genomic cytosolic mechanisms (Gross and Cidlowski, 2008).

The HPA axis and the immune-inflammatory response

The neuroendocrine-immune system interactions are regulated by the brain to elicit differential responses (Webster et al., 1997). Upon immune activation, several inflammatory cytokines like IL-1, IL-6, TNF-α, type 1 interferons (IFN α/β) are released (Chrousos, 1995). These cytokines confer protection from xenobiotics and also play a role in inducing autoimmune diseases (Coffman, 2006; Mosmann et al., 1986; Moudgil and Choubey, 2011). Additionally, the inflammatory cytokines can also independently activate the HPA axis at the level of CNS, pituitary,

and adrenals, albeit in a synergistic manner (Imura et al., 1991). Particularly, these cytokines can stimulate the pituitary and adrenal gland to release ACTH and cortisol respectively (Mastorakos et al., 1993, 1994). Additional inflammatory mediators like TGF-β, EGF, PAF may regulate the HPA axis by stimulating the inflammatory cytokines in a direct or indirect manner (Chrousos, 1995: Chrousos and Gold, 1992). Conversely, HPA axis activation is pivotal to combat the activated immune-inflammatory response during infection and protect the host cells from toxic inflammatory insult (Bellavance and Rivest, 2014). Glucocorticoids suppress the inflammatory cells and thereby the production of inflammatory cytokines like IL-1, IL-6, TNF-α, and other Th1 lymphocytes. Other cytokines like IL-2 and IL-4 may confer a glucocorticoid resistance state, by decreasing the affinity of the ligand to the glucocorticoid receptor. Hence, the bidirectional crosstalk between the HPA axis and cytokines during an infection or stressful stimuli is important in the regulation of stress response (Zapanti et al., 2008).

Cytokines' role in HIV progression to AIDS

The immunopathogenesis of HIV progression to AIDS is characterized by a decrease in $CD4^+$ T cell count and loss of T helper (Th) cell function (Shearer et al., 1995). The progression of HIV to AIDS is characterized by a decline in type 1 cytokines (IL-2, IL-12, and IFN γ) and an increase in type 2 cytokines (IL-4, IL-5, IL-6, and IL-10). Given the reciprocal relationship between type 1 cytokines mediated cell-mediated immunity (CMI) and type 2 cytokines mediated humoral immunity, a decrease in the type 1 cytokines leads to an exaggerated humoral immune response (Clerici, 1995). CMI is important in preventing the progression of HIV to AIDS and humoral immunity confers poor clinical prognosis (Clerici, 1995). Thus, the transition of type 1 to type 2 cytokines predicts the following outcomes a) decline in the CD4 cell count; b) progression

to AIDS. This hypothesis of HIV progression to AIDS was supported by a weak type 1 cytokine/strong type 2 cytokine profile of seropositive HIV patients and conversely, the decline in the progression was manifested by a strong type 1 cytokine/ weak type 2 cytokine profile (Clerici et al., 1997). Given, glucocorticoids are important in regulating the Th1/Th2 cytokine balance, the extent of HPA activation in the HIV patients may thus determine the host's progression to AIDS (George and Bhangoo, 2013).

Pathogenetic Mechanisms of HPA Axis Dysfunction- Hypercortisolemia & Glucocorticoid resistance in AIDS patients

In a substantial number of HIV^+ infected population, basal cortisol levels were markedly increased (~35-55%) when compared to normal subjects (Christeff et al., 1992) during the early, middle and late stage of HIV infection (Christeff et al., 1992; Grinspoon and Bilezikian, 1992; Verges et al., 1989). These levels though higher were in the physiological range (Norbiato et al., 1992). However, in AIDS patients, the diurnal cortisol levels at 0800h were significantly higher compared to normal subjects (Membreno et al., 1987). Some of the possible reasons for hypercortisolemia phenotype observed in HIV^+ subjects were a) shift in the steroid metabolism of cholesterol from DHEA, aldosterone, and 17-deoxysteroids to cortisol as a central adaptive response to stress (Brown et al., 1991; Grinspoon and Bilezikian, 1992; Hofbauer and Heufelder, 1996); b) increased plasma corticosteroid-binding globulin levels were reported in HIV^+ patients (Martin et al., 1992); c) surge in proinflammatory cytokines like (IL)-1β and IL-6 which may directly stimulate adrenal cortex thereby increasing systemic cortisol levels (Biglino et al., 1995; Tauveron et al., 1994) or by the direct effects of viral proteins like gp120 on hypothalamic CRF release (Costa et al., 2000; Raber et al., 1996) or Vpr protein capacity to act as a GR co-activator

(Kino et al., 1999) and combined Vpr and Tat ability to increase glucocorticoid hypersensitivity (Chrousos and Zapanti, 2014; see Fig. 3). The capacity of adrenal glands to mount cortisol response was restored in the majority of AIDS patients as assessed by ACTH stimulation test (Dobs et al., 1988) with some advanced AIDS patients demonstrating blunted adrenal responsivity to CRF infusion (Lortholary et al., 1996). Additionally, AIDS patients in late stages of infection, demonstrated, hypercortisolemia phenotype with clinical features of peripheral glucocorticoid resistance (Norbiato et al., 1992). Intriguingly, these patients also demonstrated adrenal insufficiency phenotype (weakness, fatigue, anorexia, hyperpigmentation, hypotension, electrolyte complications like hyponatremia) (Eledrisi and Verghese, 2001). This phenomenon of elevated basal cortisol levels with signs of adrenal insufficiency could be possibly explained by glucocorticoid resistance characterized by increased GR density and decreased GR affinity observed in mononuclear leukocytes of AIDS patients (Norbiato et al., 1992). Additional lines of evidence, reveal the cAMP-mediated release of CRF and ACTH from the rat anterior pituitary corticotrophs and murine AtT-20 cell line (Xie et al., 1999). Given, Tat acts on L-type calcium channels (Hu et al., 2016) and cAMP-mediated CRF and ACTH release are mediated by Ca^{+2} influx via L-type calcium channels (Fig. 5), hence selective targeting to block L-type calcium channels to offset Tat's excitotoxic insults by use of neurosteroids may be one of the potential strategies to reinstate HPA homeostasis.

Role of viral factors: Vpr, Tat, and gp120 on HPA

The HIV proteins may influence the HPA axis (Chrousus and Zapanti, 2014). HIV-1 accessory protein, Tat, transactivates the HIV-1 LTR promoter protein in the HIV genome to drive transcription (Das et al., 2011). Tat interacts with the coactivator molecules p300/CREB-binding

protein and p160 to enhance tissue glucocorticoid sensitivity via accumulation of the positive transcription elongation factor-b on glucocorticoid responsive promoters (Kino and Chrousos, 2004). HIV-1 Tat promoted glucocorticoid resistance in splenocytes of HIV-1 Tat male transgenic mice (Paris et al., 2020). Viral protein R (Vpr) is a 96-amino acid accessory protein, responsible for virus incorporation into the host cell, nuclear translocation of host-virion complex, transcription, and initiation of apoptosis (Andersen and Planelles, 2005; Sawaya et al., 2000). Vpr is a GR co-activator and promotes glucocorticoid hypersensitivity (Kino et al., 1999). Combined Vpr and Tat also promote increased viral proliferation rates via enhancing the glucocorticoid hypersensitivity of target tissues and suppressing the host immune system activity (Chrousus and Zapanti, 2014). The envelope protein gp120 enhance IL-1 synthesis of peripheral blood monocytes (Wahl et al., 1989); enhance plasma ACTH and corticosterone levels and pituitary ACTH content in gp120-transgenic mice (Raber et al., 1996); exogenous gp120 infusion to rats enhances expression of CRF mRNA and hypothalamic protein concurrent with elevated CRF release (Pozzoli et al., 2001) and stimulation of hypothalamic PVN to promote CRF and AVP release (Costa et al., 2000; Table 2)

Table 2: Effects of various viral proteins like HIV-1 Tat, Vpr and gp120 on the HPA axis.

Virus/Viral Product	Effect	Reference
HIV (Vpr)	Enhances GR activity (acts as GR co-activator) to induce glucocorticoid hypersensitivity and potentiate glucocorticoid receptor signaling	Kino et al. 1999, 2002; Sherman et al. 2000
Tat and Vpr	Contribute to viral proliferation by enhancing glucocorticoid hypersensitivity of target tissues	Kino and Chrousos, 2004
HIV (gp120)	Direct stimulating effects of hypothalamic CRH and AVP release in rats	Costa et al, 2000
	Elevated plasma corticosterone and adrenocorticotrophic hormone (ACTH) levels and pituitary ACTH content	Raber et al., 1999

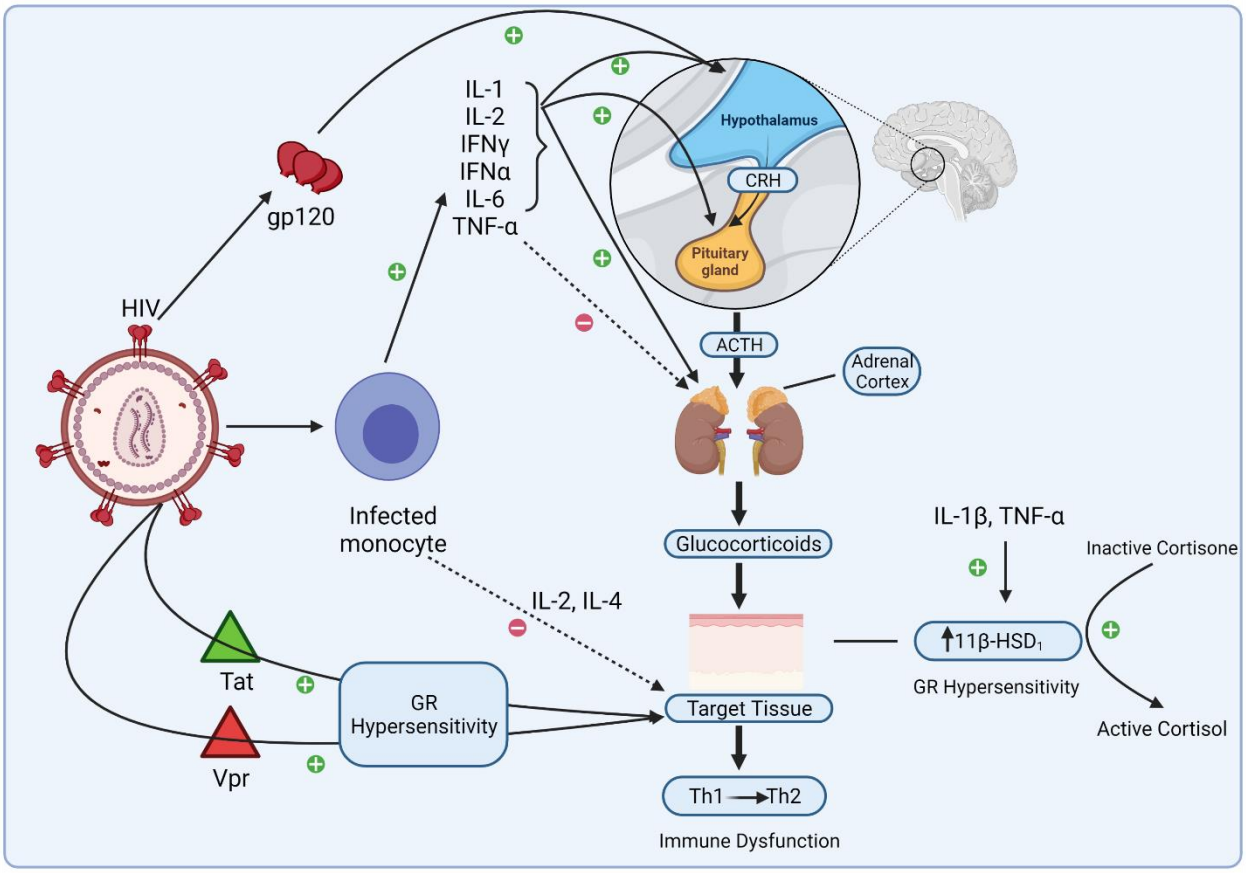

Figure 3: Schematic representation of HIV-1 protein effects on the hypothalamic-pituitary-adrenal (HPA) axis.
Adapted and modified from Chrousos and Zapanti, 2014

Glycoprotein (gp) 120 stimulates cortisol secretion directly through the HPA axis. Accessory proteins for HIV-1 Vpr and Tat promote glucocorticoid action (GR1) by enhancing target tissue glucocorticoid sensitivity. Inflammatory cytokines stimulate HPA activity at all three levels of the HPA axis, resulting in increased cortisol release. Hypersensitivity to glucocorticoids results in immunological dysfunction. Some HIV-infected individuals demonstrate glucocorticoid resistance, which is likely due to interleukin (IL)-2 and IL-4's suppressive effects on glucocorticoid activity in target tissues. The cytokines interferon (TNF)-α and IL-1β stimulate the activity of hydroxysteroid dehydrogenase type 1 (11β–HSD1), hence increasing glucocorticoid hypersensitivity.

Combinative antiretroviral therapeutics influence on HPA

Combinative antiretroviral therapeutics (cART) has markedly improved the life expectancy of HIV^+ patients (Collins et al., 2016). However, cART intervention could add new risk factors by its ability to transform an acute infection into a chronic inflammatory condition concurrent with an increase in proinflammatory cytokines like IL-1β and TNF-α (Chrousos and Zapanti, 2014). These cytokines may promote 11β-HSD overexpression, leading to conversion of inactive cortisone to active cortisol, thus shifting the cytokine profile from Th1 to Th2 (Chrousos and Zapanti, 2014; Norbiato, 2012, Fig. 3). Increased systemic cortisol levels lead to the development of metabolic complications like AIDS-related insulin resistance and lipodystrophy syndrome (ARIRLS) which is reminiscent of Cushing syndrome (Lo et al., 1998). Particularly protease inhibitors (PIs) inhibit CYP3A4, an enzyme necessary to metabolize glucocorticoids to an inactive form. Thus, the pharmacological activity is pronounced in the HIV^+ population owing to its reduced metabolism, thereby increasing the risks of the development of iatrogenic "Cushing" syndrome (Saberi et al., 2013). Given the ability of cART to influence HPA, HIV^+ patients need to be monitored regularly for any untoward complications.

Effects of Opiates on the HPA axis

With increasingly prescribed opioids to HIV patients for neuropathic pain (Merlin et al., 2016), it is evident to assess their effects on the HPA axis. The basis of opiates influence on the HPA axis is largely determined by the drug administration states viz steady-state (maintained by constant osmotic pump or drug implant) or on/off state (Kreek, 2007). As such, differential effects of opiates on the HPA axis have been reported (George et al., 2012; Kreek et al., 2002). Acute opioid exposure activates HPA, elevating CRF levels and downstream corticosterone (Koob and

Kreek, 2007). However, chronic administration suppresses HPA diurnal axis rhythmicity (Vuong et al., 2010) but withdrawal from opiates may activate the HPA axis (Culpepper-Morgan and Kreek, 1997; Koob and Kreek, 2007). Particularly, daily episodes of withdrawal seen in opioid-addicted subjects, may cause sustained HPA axis activation (Koob, 2020; Koob and Kreek, 2007;) and conversely immune suppression (Eisenstein, 2019), thereby increasing vulnerability to substance use disorders. Likewise, when opioids are misused, their initially pleasurable effects wear off and their constant usage is mostly driven by their desire to avoid the negative consequences of addiction (George et al., 2012; Koob, 2020; Koob and Kreek, 2007;). Furthermore, it is plausible to assume that given opioids are HPA activators, HIV proteins may interact with opioids to influence the HPA axis (Chrousus and Zapanti, 2014; George and Bhangoo, 2013; Zapanti et al., 2008). Thus, opiate use in the context of neuroHIV and HPA axis may need to be considered as it may influence the clinical outcomes.

Stress impact on the GABAergic signaling

Given that cART is poorly-retained within the CNS (a major reservoir for latent and active HIV-1), and the majority of the HIV^+ infected population contend with HPA dysfunction, novel adjunctive therapeutics are required for a functional cure. One of the mechanisms which underlie the HPA dysregulation phenomenon may be alterations in GABAergic transmission leading to reduced GABA levels, the lower density of GABAergic interneurons, and modification of $GABA_AR$ subunit expression (Boero et al., 2019; Luscher et al., 2011). Under normal conditions, GABAergic interneurons at the level of hypothalamic PVN and adjacent forebrain project inhibitory inputs to CRF neurons (Cullinan et al., 2008). However, this inhibitory regulatory mechanism is disrupted under stressful conditions, demonstrated by rapid and reversible

downregulation of GABAergic transmission (quantified by binding assays of GABA and its modulators), hence promoting increased CRF signaling in different brain regions of male rats as seen in preclinical paradigms of acute stress (forced swim, carbon dioxide inhalation, mild foot-shock, and handling) (Biggio et al., 2007; Drugan et al., 1989). Another plausible explanation for loss of GABAergic inhibition, following acute stress, involves downregulation and dephosphorylation of Ser940 residue of K^+/Cl^- co-transporter (KCC2) (Hewitt et al., 2009; Sarkar et al., 2011) prompting a change in GABAergic transmission on CRF neurons from inhibitory to excitatory, leading to increased CRF signaling and inefficient HPA inhibition (reviewed in Boero et al., 2019). Additionally, acute or chronic stress differentially modulates the expression of $GABA_A$ subunit types, especially the extrasynaptic α4/δ subunit which is pivotal for its tonic inhibition property in granule and pyramidal cell neurons of the hippocampus of male rodents (Maguire and Mody, 2007; Serra et al., 2006). Certain glial cells (especially astrocytes) and CNS neurons synthesize steroids *de novo* in the brain (neurosteroids) or from peripheral progesterone and act in a paracrine manner to influence GABAergic transmission (Lambert et al., 2003). In particular, neurosteroids bind with extrasynaptic α4/δ $GABA_A$ receptors and could be explored as a viable therapeutic to enhance the GABAergic inhibitory tone and restore HPA homeostasis (Fig. 4).

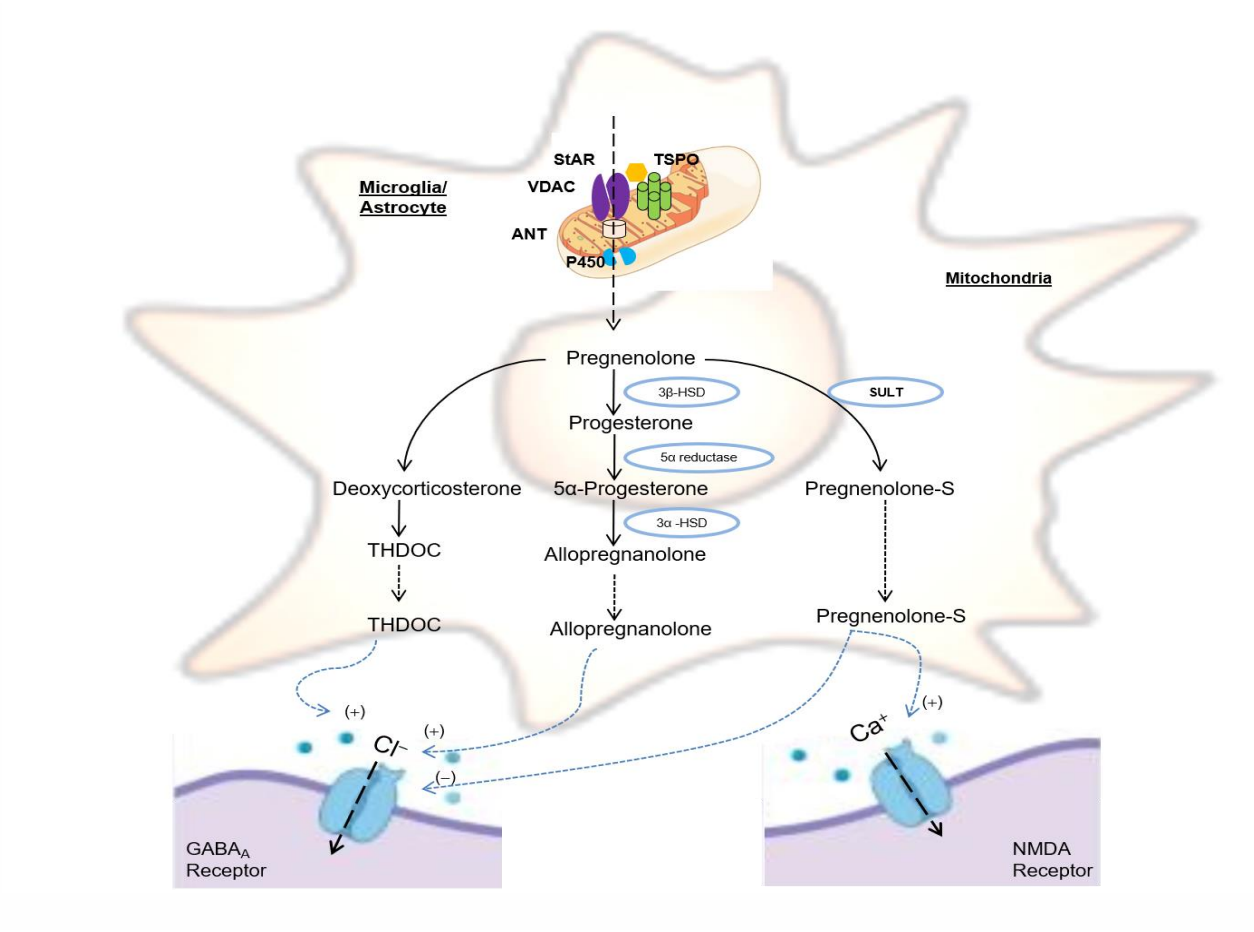

Figure 4: Schematic diagram of production of various neurosteroids from the precursor, Cholesterol.

Various neurosteroids including THDOC and Allopregnanolone are produced from cholesterol. Additionally, receptors and signaling mechanisms that may regulate steroidogenesis, are also shown.

Neurosteroidogenesis may ameliorate HIV-1 Tat-mediated HPA dysregulation, particularly excessive CRF signaling that may underlie substance use disorders

Upon acute drug exposure, extrahypothalamic CRF system of extended amygdala activation leads to binge/intoxication, withdrawal, and relapse/reinstatement of drug addiction (Koob, 2020; Zorrilla et al., 2014). CRF neurons are abundantly present in the PVN nucleus of the

hypothalamus, where its main role is to mediate HPA stress axis activation (Rivier and Vale, 1983). Additionally, quantitative analysis of whole-brain of male mice revealed CRF neurons presence in other extrahypothalamic sites like the amygdala particularly CeA and BSNT (bed nucleus of stria terminalis) (Peng et al., 2017). The extrahypothalamic sites mostly mediate the emotional and behavioral response to stress (Schreiber and Gilpin, 2018). CRF-Rs are composed of CRFR1 and CRFR2 (Dedic et al., 2018). CRFR1 is present in the brain and is mainly responsible for anxiogenic behavior. The role of CRFR2 is less well understood, but may involve maintenance of homeostasis (Dedic et al., 2018).

Following 30 minutes of acute stress, HPA axis activation causes elevation of corticosterone and allopregnanolone (Purdy et al., 1991), which represent a compensatory mechanism to restore the GABAergic inhibition on the CRF neurons in the PVN nucleus of the hypothalamus, thereby reinstating HPA homeostasis (Morrow et al., 2021). In support, allopregnanolone (AlloP) offsets anxiety-like behavior produced by CRF exposure, prevents the release of CRF from hypothalamic explants, and also downregulates the CRF gene expression following adrenalectomy (Patchev et al., 1994). Exogenous AlloP administration, prior to stress attenuated the stress-induced increase in ACTH and corticosterone (Owens et al., 1992; Patchev et al., 1996). Consistently, physiological AlloP (10-100nM), inhibited the stress-induced CRF release via GABAergic receptors modulation in neonatal male and female mice (Gunn et al., 2013, 2015). Intriguingly, systemic administration of AlloP to non-stressed adult male rats demonstrated an increase in hypothalamic CRF and circulating ACTH and corticosterone (Naert et al., 2007). This study shows that AlloP regulates the HPA axis by increasing circulating corticosterone levels in normal conditions but decreasing them in stressful situations (Morrow et al., 2020). Overall,

these lines of evidence reveal the importance of neurosteroidogenesis to restore homeostasis in the CRF signaling at both hypothalamic and extrahypothalamic sites, thereby reducing the vulnerability to substance use disorders.

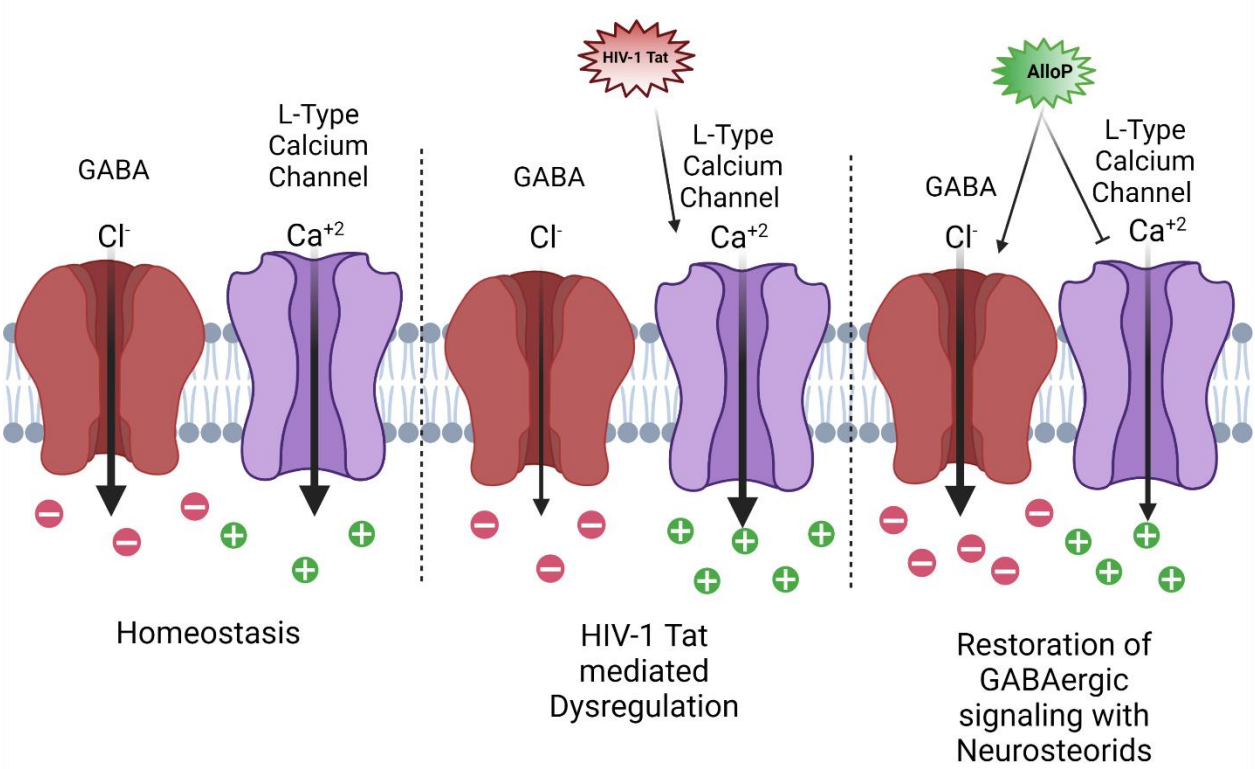

Figure 5:Schematic diagram of restoration of HIV-1 Tat-mediated GABA(A) receptor-mediated signaling by neurosteroids like Allopregnanolone.

HIV-1 Tat infection impairs GABA-mediated signaling by reducing $GABA_A$ receptor production and internalization and increasing the L-type calcium channel signaling. Neurosteroids, such as allopregnanolone (AlloP), restore homeostasis by promoting GABAergic signaling via its actions at synaptic and extrasynaptic receptors and its capacity to antagonize L-type calcium channels.

Neurosteroidogenesis as a potential defense mechanism for neuropsychiatric disorders

Neurosteroids are synthesized *de novo* in the brain from cholesterol (Baulieu, 1998) and may also reach the brain from peripheral sources like adrenals and gonads (Charlier et al., 2015).

The most potent neuroactive steroids are 3α,5α reduced metabolites of progesterone namely, allopregnanolone (3α,5α-THP) and deoxycorticosterone (3α,5α-THDOC). These steroid metabolites are positive allosteric GABAergic modulators that alter the neuronal excitability at $GABA_A$ synaptic and extrasynaptic receptors (Belelli et al., 2002; Puia et al., 1990; Reddy and Rogawski, 2002). At nanomolar concentrations, these neurosteroids greatly potentiate GABA neurotransmission on the GABAergic receptors to promote the Cl^- influx thereby producing an inhibitory tone (reviewed in Porcu et al., 2016). At micromolar concentrations, they directly activate the receptor channel (Belelli and Lambert, 2005; Carver and Reddy, 2013). 3α,5α-THP, and 3α,5α-THDOC can also bind to extrasynaptic receptors that express δ subunits at high concentrations (Belelli and Lambert, 2005; Carver and Reddy, 2013). Due to their ability to modulate the GABAergic neurotransmission, neuroactive steroids can produce antidepressant, anxiolytic, anticonvulsant, and antinociceptive effects. Moreover, neurosteroids also exert neurotrophic, neuroprotective, and anti-apoptotic effects in animal models of ischemic insults and traumatic brain injury (Frye and Sturgis, 1995; Guennoun et al., 2015; He et al., 2004; Rasmusson et al., 2017, 2018; Rossetti et al., 2016) and reversal of neurodegeneration in Parkinson's and Alzheimer's disease (Adeosun et al., 2012; Brinton, 2013). Impairment of neurosteroidogenesis may increase vulnerability to several neuropsychiatric and neurodegenerative disorders (Porcu et al., 2016).

Steroidogenesis dysregulation in HIV

In the post cART era, endocrine challenges related to HIV infection are beginning to be elucidated. The interaction between HIV and the endocrine system is dynamic and potential bidirectional crosstalk exists between steroids and HIV. Human endogenous steroids influence

HIV replication and neuropathology, whereas HIV virotoxins may alter the body's ability to make endogenous steroids.

HIV-1 proteins that contribute to neuroHIV also disrupt neurosteroidogenesis

With the advent of cART, HIV infection has become a chronic neurological problem (Gates and Cysique, 2016). As such, HIV^+ infected population contends with neuroHIV symptoms which include a constellation of neurological problems which encompass deficits in memory, concentration, attention, and motor skills (Heaton et al., 2011). Clinical evidence show post-mortem brains obtained from HIV^+ infected population, showed a reduction in the enzymes required in the neurosteroidogenesis, including cytochrome P450scc, 5α-reductase, and 3α-hydroxysteroid dehydrogenase (HSD) when compared to HIV negative controls (Maingat et al., 2013). Consistently in a similar research study, when human fetal astrocytes were exposed to HIV-infected supernatants, 5α -reductase and 3α-HSD protein expression were decreased (Maingat et al., 2013). Recent research in 99 HIV^+ patients revealed that at least eight neurosteroids were downregulated (which also predicted depressive symptoms), including pregnenolone sulfate, dehydroepiandrosterone-sulfate (DHEA-S), and 5-androstane 3β,17β -diol monosulfate (Mukerji et al., 2021).

Using animal models of HIV-1 Tat and gp120, we and others have recapitulated the clinical endophenotype of neuroHIV symptomatology, increasing anxiety, and depression-like behavior, behavior disinhibition, cognitive impairment, and deficits in sensorimotor gating (Hahn et al., 2016; Nass et al., 2020; Paris et al., 2014bc; 2016, 2020). Notably, these behavioral changes occurred concurrently with neurosteroid dysregulation at the level of the brain and periphery (Paris et al., 2020).

In light of the HIV virotoxins' mitotoxic properties (Tat, gp120/nef, viral protein R), HIV infection has been linked to changes in CNS steroid production. Similar to other models of neural injury, conditional expression of HIV-1 Tat protein in the mice model has been shown to impair pregnane neurosteroidogenesis (Paris et al., 2020). In particular, Tat elevated central pregnenolone and 3α,5α-THP and its 3β isomer (3β,5α-THP) and 20α-hydroxylated metabolite levels and decreased deoxycorticosterone concurrent with promoting Tat-mediated glucocorticoid resistance in primary splenocytes (Paris et al., 2020). Indeed, neurosteroidogenesis is a critical component of the adaptive response to stress. Disrupted steroidogenesis might be caused by Tat's ability to dysregulate cholesterol metabolism and disrupt central steroidogenesis (Bandaru et al., 2013). Furthermore, RNA-seq analysis revealed Tat's ability to dysregulate cholesterol and lipid gene expression in rat neurons, increasing free and total cholesterol and cholesteryl ester, consequently affecting the downstream synthesis of neurosteroid metabolites (Mohseni Ahooyi et al., 2018). Moreover, Tat also disrupted LXR signaling leading to dysregulation of cholesterol homeostasis and thereby implicating vulnerability to HAND (Cotto et al., 2018). Tat and gp120 have also been shown to elevate the levels of toxic sphingolipid, ceramide which may underlie impaired synthesis of steroidogenic enzymes, leading to dysregulated steroidogenesis and increased vulnerability to cellular dysfunction and death (Haughey et al., 2004, 2008). Additional line of evidence underlies, Tat's ability to compete with cholesterol for the carboxyl-terminus of peripheral-type benzodiazepine receptor (important for cholesterol binding and transport into mitochondria) and produce a conformational change, thereby decreasing cholesterol influx through the mitochondria and impairing steroidogenesis (Li et al., 2001). Furthermore, other HIV proteins also exert direct toxic effects on mitochondria, an important organelle for steroidogenesis. In general, HIV proteins (Tat, Nef, Viral Protein R, gp120) altered the mitochondrial dynamics, biogenesis and membrane

potential, glycolytic pathways, ATP production, oxidative stress, mitophagy, calcium signaling, apoptosis, and protein quality control (PQC) (De Simone et al., 2016; Duggan et al., 2021; Field and Ellis, 2019; Lecoeur et al., 2012; Rojas-Celis, 2019; Rozzi et al., 2017; reviewed in Salahuddin et al., 2021a; Teodorof-Diedrich and Spector, 2018, 2020; Thangaraj et al., 2018, 2021; Villeneuve et al., 2016; Fig. 6). Given, neurosteroids are non-traditional modulators of the HPA axis, fluctuations of these neurosteroid levels may implicate changes in HPA axis sensitivity to fight an external stressor and further predispose individuals to neurological and neuropsychiatric complications. Hence, Tat may be a potential therapeutic target for future exploration of neurosteroids and their influence on HPA function and related behaviors.

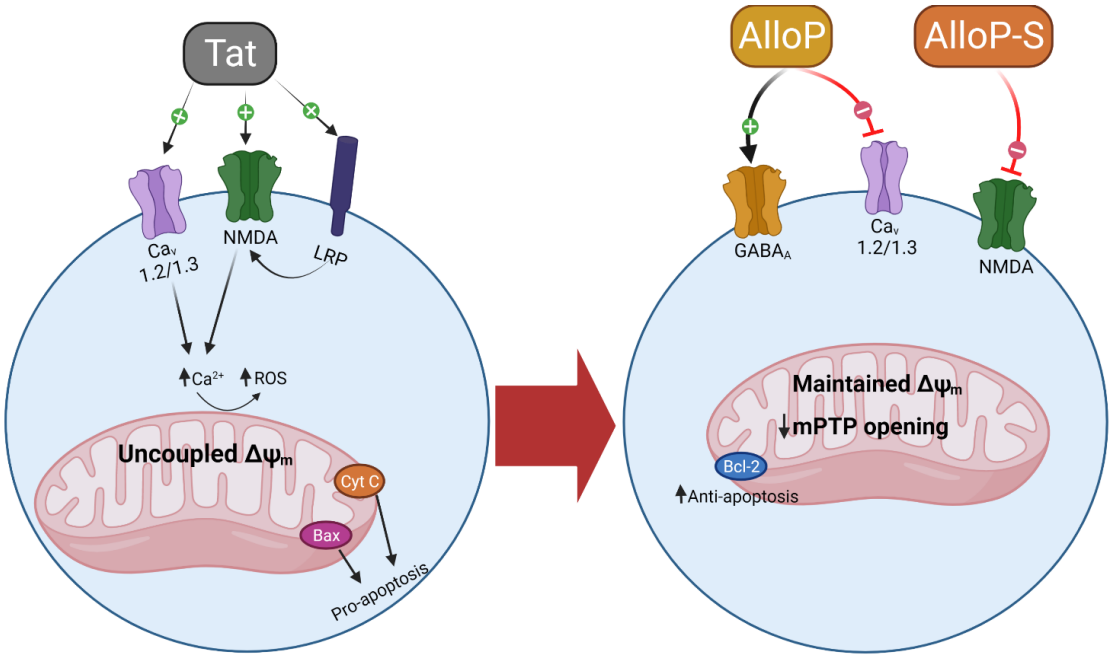

Figure 6: Schematic diagram of HIV-1 Tat effect on cation channels and downstream mitochondrial changes and potential of AlloP to mitigate those effects. Adapted and modified from Ref. Salahuddin et al., 2021a Copyright (2022) Mohammed F. Salahuddin, Fakhri Mahdi, Emaya Moss, Nicholas S. Akins, Jing Li, Hoang V. Le, Jason J. Paris; Allopregnanolone and neuroHIV: Potential benefits of neuroendocrine modulation in the era of antiretroviral therapy; Journal of Neuroendocrinology, John Wiley & Sons, © 2021 British Society for Neuroendocrinology. Volume34, Issue2 Special Issue: Special Issue of papers from the Virtual International Meeting STEROIDS and NERVOUS SYSTEM, TORINO, ITALY - February 2021 February 2022; e13047

HIV proteins activate calcium channels, thereby altering mitochondrial membrane potential, promoting the generation of reactive oxygen species, and contribute to cell damage and death (Left Pane). Allopregnanolone (AlloP) is a positive allosteric modulator of $GABA_A$ receptors; it inhibits L-type Ca^{2+} channels and restores mitochondrial bioenergetics equilibrium, hence possibly counteracting the excitotoxic effects of HIV proteins. Allopregnanolone-sulfate is an NMDA receptor antagonist (Right Pane).

Neuroendocrine modulators may influence HIV-related pathology

Anti-retroviral therapeutics have dramatically increased the life expectancy of PLWH, attributed mostly to its ability to effectively block viral replication, reduce drug resistance, and improve the quality of life of PLWH (CDC, 2008). However, these drugs are not able to eradicate

the virus completely, a small proportion of latent viral reservoirs are still prevalent in the central nervous system (Henderson et al., 2019) raising concerns of HIV to cause additional neurological and neuropsychiatric complications in the infected population. Given, neurosteroidogenesis is hypothesized as an adaptive mechanism to reinstate HPA axis homeostasis following a stressful episode (Morrow et al., 1995). Thus, exogenous neuroactive steroids may be sought as novel adjunct therapeutics for a functional cure.

Given the poor bioavailability, quick redistribution from CNS compartment, addictive potential, safety and efficacy, tolerability of neurosteroids, innovative techniques to modulate neurosteroidogenesis using translocator protein 18 kDa (TSPO) may illustrate a better therapeutic strategy (Porcu et al., 2016). TSPO is part of a large multiprotein complex responsible for the transport of cholesterol into the inner mitochondrial membrane of glial cells in CNS, cells of gonads, and adrenal cells (Papadopoulos and Lecanu, 2009; Rupprecht et al., 2010). Their main role is to promote neurosteroidogenesis (Frye, 2009; Papadopoulos et al., 2015; Papadopoulos and Lecanu, 2009). Indeed, neurosteroidogenesis plays an important role to curtail stress-induced HPA activation (Crowley and Girdler, 2014; Gunn et al., 2015). The TSPO specific ligands demonstrated an increase in circulating corticosteroid levels especially in hypophysectomized animals than controls (Cavallaro et al., 1992). Additionally, TSPO drug ligand-mediated neurosteroidogenesis may be beneficial in circumstances when neurosteroid levels have been depleted thereby increasing propensity to neurological behavioral deficits (Costa et al., 1994; Rupprecht et al., 2010). Several preclinical and clinical studies have shown a promising role of TSPO ligands in several disease states including brain damage, traumatic brain injury, anxiety and panic attacks, PTSD, Alzheimer's disease, and brain tumors (Rupprecht et al., 2010). TSPO ligands

like FGIN-1-27 were also demonstrated to reverse hypogonadism in young male rats by increasing testosterone production (Chen et al., 2019). Pretreatment with neurosteroids like TH-DOC demonstrated attenuation of stress-induced increase in plasma ACTH and cortisol (Owens et al., 1992). Three studies showed the promising role of endogenous neurosteroids in the context of HIV-1. One of the studies demonstrated a lower HPA- related neurosteroids to predict depression-like phenotype in HIV patients (Mukerji et al., 2021). Secondly, a clinical trial demonstrated the efficacy of DHEA in the improvement of depressive symptoms in HIV^+ infected population (Rabkin et al., 2006), and third (from my lab) demonstrated HIV-1 Tat to activate the HPA-related neurosteroids in the CNS (Paris et al., 2020).

Using Tat-transgenic mice, the functional effects of pregnane steroids on neuroHIV-like behavior have been shown. In support, a supraphysiological dose of progesterone (4 mg/kg daily for 7 days) or at a physiological dose (4 mg/kg once every 5 days for 15 days) alleviated Tat-mediated anxiety-like behavior in ovariectomized mice. However, the 5α-reductase inhibitor, finasteride (50 mg/kg), counteracted the protective effects of progesterone, indicating that metabolism to AlloP is responsible for these therapeutic effects (Paris et al., 2016). We subsequently discovered that AlloP dose-dependently attenuated the psychomotor effects of opioids (Paris et al., 2020). Estradiol did not improve the anxiety-like behavior, rather, counteracted the positive benefits of progesterone when co-administered with progesterone (Paris et al., 2014b). Likewise, AlloP improves neuroHIV-like behavior in mice (Paris et al., 2016; 2020), and may be a viable therapeutic for future assessment for its role in HPA modulation in transgenic mice (Fig. 6). Given neurosteroids play an important role in homeostatic control of the HPA stress axis, and HIV^+ patients exhibit dysregulated HPA, the

present dissertation will assess the capacity of TSPO ligands like FGIN-1-27 and allopregnanolone to increase neurosteroidogenesis and restore HPA dysfunction and associated neurological sequelae (Fig. 7).

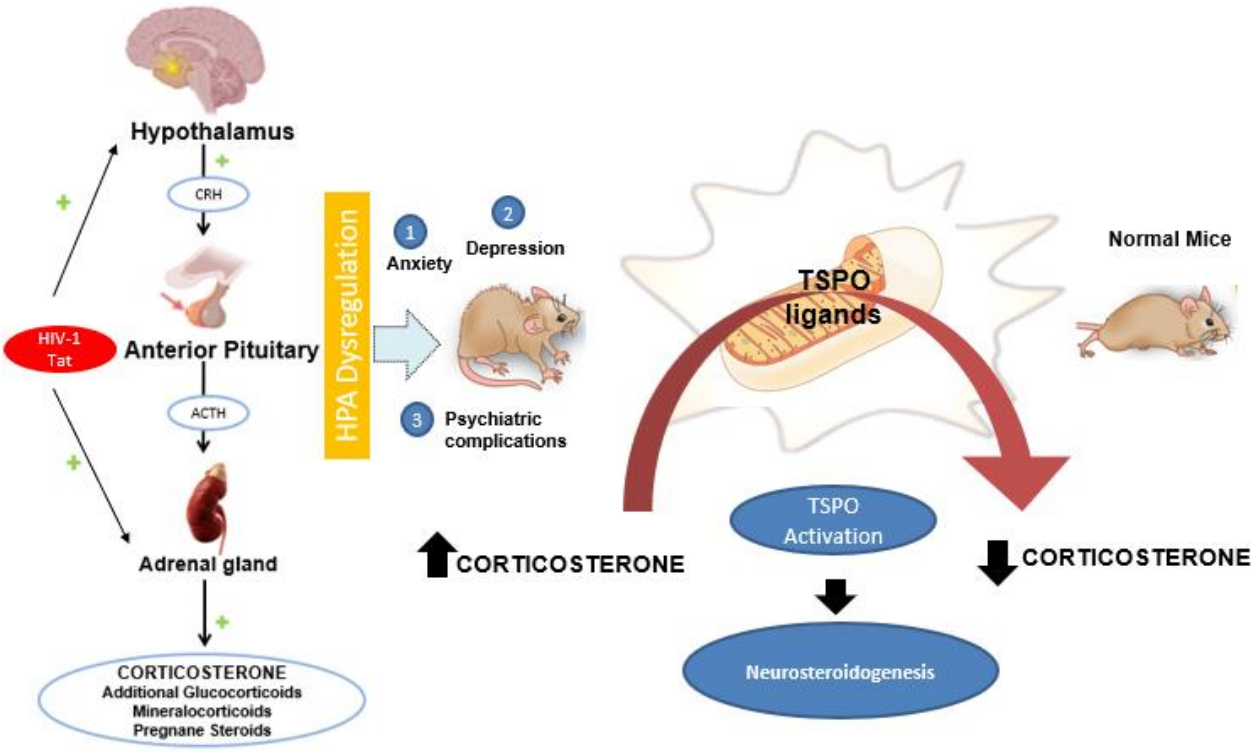

Figure 7: Schematic diagram of neurological sequelae associated with dysregulation of HPA axis.

Courtesy: Adapted from BrainStorm-Making sense of neuroscience research (Salahuddin, 2021b)

HIV-1 Tat, neurotoxic protein may confer HPA dysregulation (increased elevated circulating corticosterone) in mice and the hypothetical use of TSPO (a protein that transports the substrate for hormone synthesis, cholesterol, to the mitochondria) to initiate steroidogenesis, thereby correcting the corticosterone imbalance and alleviating anxiety, depression, and other psychiatric complications.

Research Goals

In humans, the HPA axis is the principal regulator of environmental stress. Stress causes HPA activation via a neuroendocrine cascade of events to produce cortisol, which is crucial to fight stressors and restore homeostasis. However, chronic stressor leads to dysregulated HPA axis producing atrophy of the hippocampus, cognitive impairments, and other psychiatric illnesses. In the post cART era, ~50% of patients present a constellation of neurological disorders including affective, cognitive, antinociceptive, and motor deficits (collectively termed as neuroHIV) concurrent with HPA axis dysfunction (Saylor et al., 2016; Zapanti et al., 2008). Some of the plausible underlying mechanisms for HPA axis dysfunction include chronic perceived stress, direct infiltration of viral proteins in CNS to mediate neurotoxicity, cytokines-mediated immune system activation, and cART side effects (Jacobs et al., 2018). Although evidences show opioids worsen neuroAIDS symptomatology, however it is increasingly prescribed to HIV^+ patients for chronic pain phenotype (involving avascular necrosis, and localized and widespread musculoskeletal pain), thus making the recipe for health misadventure (Bell et al., 1998; Merlin et al., 2016). The HPA axis dysregulation is commonly observed in many stress-related psychiatric disorders, especially in depressed patients and childhood trauma survivors (Carpenter et al., 2007; Varghese and Brown, 2001). Considering all these factors, HPA dysfunction in HIV^+ patients may contribute to increased vulnerability and exacerbation of neurocognitive, affective, and neuropsychiatric complications and we propose HPA modulators as novel adjunct therapeutics for a functional cure. In search of potential targets, we sought HIV neurotoxic proteins, namely trans-activator of transcription (Tat) for further investigation.

Overall Hypothesis:

We hypothesized that combined expression of HIV-1 Tat and opioids may produce HPA stress axis activation and associated neurocognitive and neuropsychiatric complications (see, Figure 8)

Thus, my dissertation is broadly divided into 3 aims/chapters.

Approach

Chapter 1: Assess the effects of HIV-1 Tat and/or clinical opioids (i.e. oxycodone) on the HPA and/HPG axes in an HIV-1 Tat transgenic mouse model.

Hypothesis: We hypothesized that HIV-1 Tat may promote HPA and/HPG dysfunction which is demonstrated by increased circulating basal corticosterone and changes in circulating estradiol and progesterone levels.

Approach: We will assess HPA activation by measuring circulating plasma corticosterone, estradiol, and progesterone and expression of corticotropin-releasing factor (CRF) at the levels of adrenal (circulation) and hypothalamus respectively. We will further assess the pharmacodynamic targets (glucocorticoid receptor or CRF receptor) via systemic administration of pharmacological antagonists.

Chapter 2: Assess the effects of HIV-1 Tat and/or clinical opioids (i.e. oxycodone) on psychomotor, cognitive, and HPA related behavior (depression and anxiety) in an HIV-1 Tat transgenic mouse model.

Hypothesis: We hypothesized that expression of HIV-1 Tat would potentiate oxycodone-mediated psychomotor responding, affective dysfunction, and cognitive impairment. We also anticipated

that pharmacological blockade of the HPA feedback loop with antalarmin and/or RU-486 may attenuate the behavior deficits.

Approach: We will assess psychomotor, depression- and anxiety-like, and cognitive behavior using behavioral tasks (e.g., open field/light-dark transition, tail suspension, and novel object recognition tests) in response to Tat or clinical opioid (e.g. oxycodone) exposure. Additionally, psychomotor and HPA related behavior endpoints will be also assessed following systemic antagonism of pharmacodynamic targets (glucocorticoid receptor or CRF receptor).

Chapter 3: Assess the protective effects of neuroendocrine modulators in an HIV-1 transgenic mice model.

Hypothesis: We hypothesized that neuroendocrine modulators like FGIN-1-27 and AlloP would rescue the Tat-mediated HPA activation/dysregulation and neurological sequelae.

Approach: We will infuse a steady-state concentration of AlloP to the brain as a potential HPA regulator and assess the HPA-related behavior. We will also assess an 18 kDa translocator protein (TSPO) activator, FGIN-1-27, to attempt to restore Tat-mediated HPA dysregulation and neurological behavior deficits.

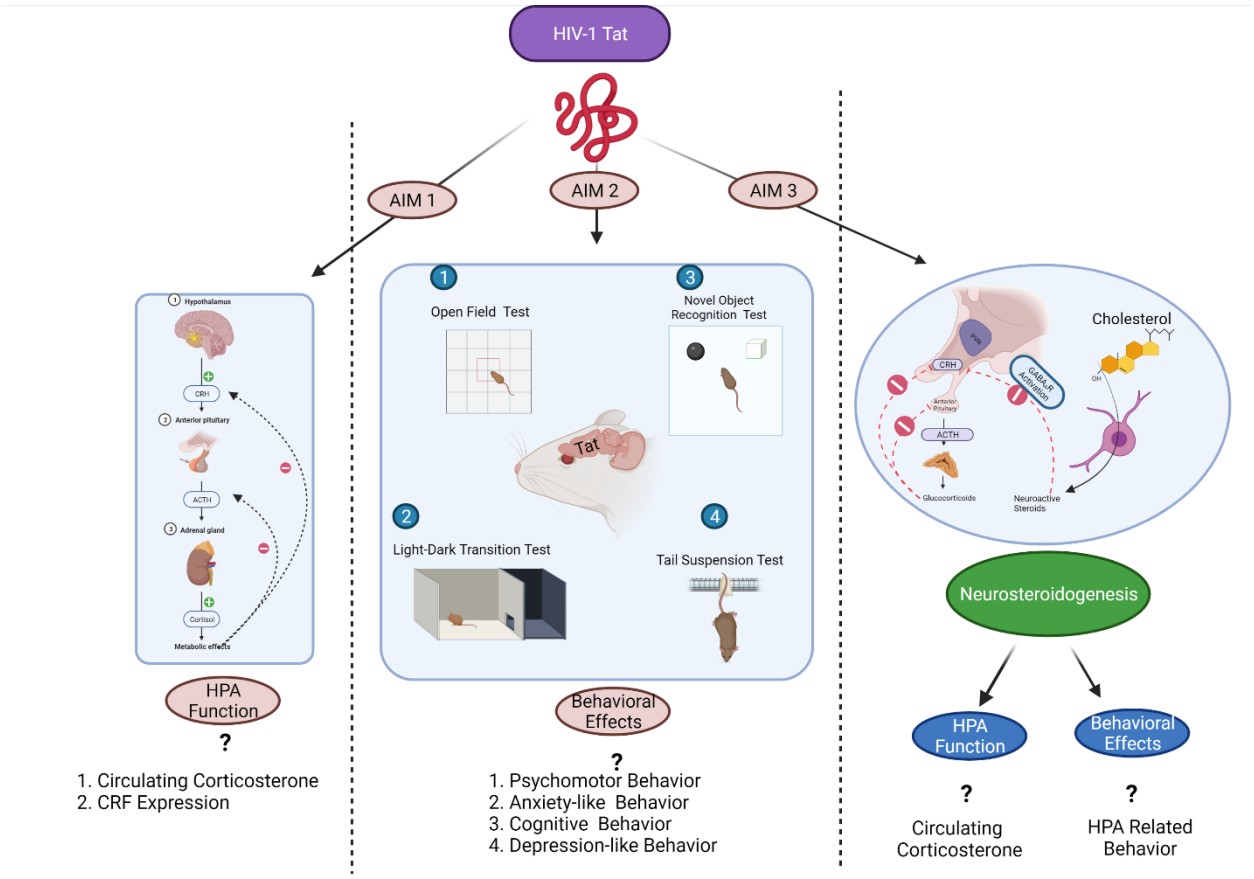

Figure 8: Schematic diagram of the experimental design.

In Aim 1, the effect of HIV-1 Tat on HPA function will be examined. In Aim 2, the effect of HIV-1 Tat on neuroHIV behavior (anxiety, depression-like, psychomotor, and cognitive behavior) will be examined. In Aim 3, we will examine the influence of neurosteroidogenesis on HPA function and HPA-related behavior.

Innovation

The innovation of this dissertation includes assessment of 1) the combined effect of HIV-1 Tat and clinically prescribed opioids like oxycodone on HPA and HPG function, 2) the combined effect of HIV-1 Tat and oxycodone on neuroHIV behavior, 3) the potential for neuroendocrine modulators like FGIN-1-27 and AlloP to restore HPA function and associated behavior deficits.

The neuroendocrine and behavioral mechanisms underlying the altered HPA dysregulation and its subsequent impact on neuroHIV symptomology are not well characterized. A greater understanding of the neuroendocrine factors associated with neuroHIV is essential and will catalyze interventions to promote health and improve the quality of life of HIV^+ patients.

MATERIALS and METHODS

Ethical Approval

All the protocols and procedures were pre-approved by the Institutional Animal Care and Use Committee (IACUC) (vide protocol # 18-004 & 21-2005) at the University of Mississippi. All the procedures were carried out as per the ethical guidelines illustrated by the National Institute of Health (vide NIH Publication No. 85-23).

Subjects & Housing

For the *in vivo* experiments, adult HIV-1 Tat transgenic male and female mice (age 2-6 months) were used. Transgenic HIV-1 Tat(+) mice constitute both the GFAP-rtTA transcription factor and TRE-Tat transgene and express the Tat_{1-86} protein via conditional, GFAP-relegated expression in a doxycycline-dependent manner (Bruce Keller et al., 2008; Gonek et al., 2018). Conversely, Tat(−) control mice expressed the transcription factor (GFAP-rtTA) necessary to activate transgene induction, but did not express the TRE-Tat transgene itself (Bruce Keller et al., 2008). Animals were bred in a vivarium at the University of Mississippi and housed (2-5/cage) in a temperature and humidity-controlled environment on a 12:12 reverse light cycle (lights off at 9 am) with *ad libitum* access to food and drinking water.

HIV-1 Tat induction

To induce HIV-1 Tat expression, daily doxycycline hyclate was administered (30mg/kg., i.p. QD for 5 days; Cayman Chemical, Ann Arbor, MI) which was freshly made in sterile saline 0.9% w/v, followed by 2 days of doxycycline washout to control for any non-specific effects

(Doxycycline half-life ($t_{1/2}$) = 5–6 h in mice; Lucchetti et al., 2019). We and others have shown expression of Tat mRNA was upregulated in the brain and spinal cord (Salahuddin et al., 2021; Fitting et al., 2012; Figure 9) and the effect of Tat-mediated impairments were stable for at least 21 days of doxycycline induction (Paris et al., 2014c). Hence, all the behavioral testing was conducted within 14 days of doxycycline administration (see behavioral timeline; Figure 14).

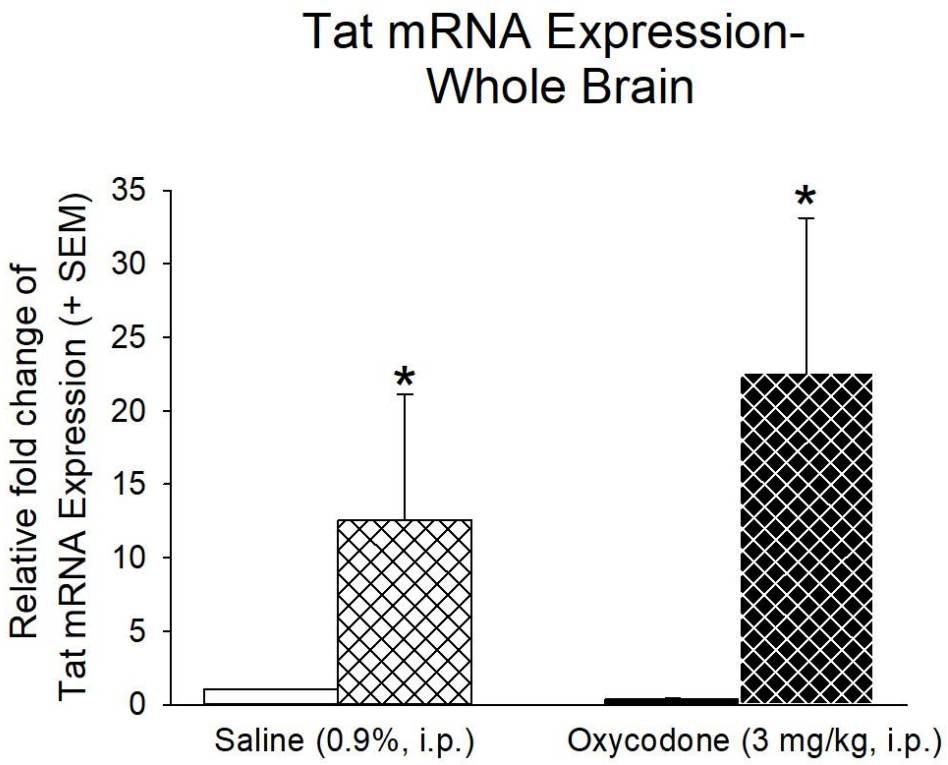

Figure 9: Fold changes of tat mRNA expression in the whole brains of female transgenic mice. Ref. © 2021 Salahuddin et al., 2021c, Licensee MDPI, Basel, Switzerland distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

Tat(−) (open bars) and Tat(+) (hatched bars) mice (n = 3/group) were administered acute saline or oxycodone and mRNA estimation was performed via qRT-PCR. * indicates a main effect of genotype wherein Tat(+) mice differ from Tat(−) controls, $p < 0.05$.

Estrous Cycle determination

Estrous cycle was determined in female mice by assessing the vaginal smear daily at 9:00 h. Briefly, female mice underwent daily vaginal lavage with cytology assessed for estrous cycle phase under light microscope holding x 50 magnification capacity (Fig. 10). The cell morphology assessed for the presence/absence of leukocytes was used to determine the estrous cycle stage. Proestrus was defined as (majority of nucleated epithelial-like cells), estrus (majority of cornified cells), metestrus (nucleated, cornified, and leukocytic cells), diestrus (majority of leukocytic cells; Paris et al., 2014b; Fig. 11). The vaginal smear was collected at the start of the dark cycle, as such, based on the sample collection time in my study, the proestrus phase was defined as stage when estrogen levels are declining and progestogens levels peaked (E_2:P_4 favors P_4; Scharfman and MacLusky, 2006) whereas diestrus phase was defined as stage when estrogens levels rising to peak and progestogens levels were nadir (E_2:P_4 favors E_2; Scharfman and MacLusky, 2006). These phase differences are important to control for hormonal variations that might influence behavioral outcomes and maintenance of reproductive capacity in female mice. When female mice entered a state of persistent estrus or diestrus for more than two cycles (typically 4-5 days) they were considered anovulatory as confirmed in prior work using rats (Paris et al., 2011; Walf et al., 2011). Estrous cycle was tracked daily for female mice followed by behavior assessment (either in proestrus or diestrus whichever came first) from the 8^{th} day to the 19^{th} day of the protocol (Salahuddin et al., 2020a, 2021c). The male mice were behaviorally assessed on the 8^{th} day of the protocol (Salahuddin et al., 2020b).

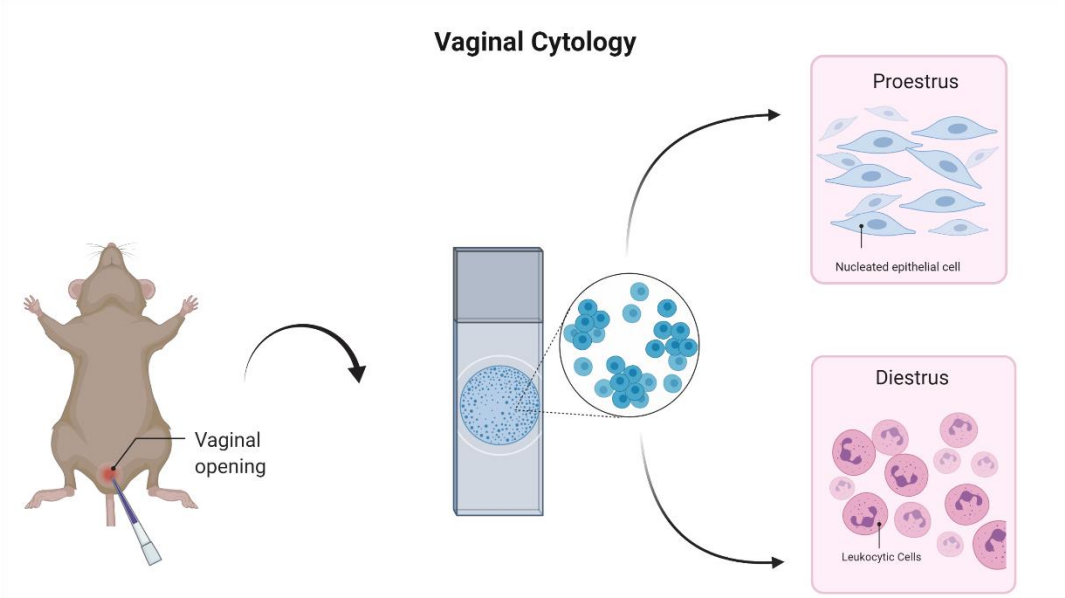

Figure 10: Schematic diagram of vaginal cytology.

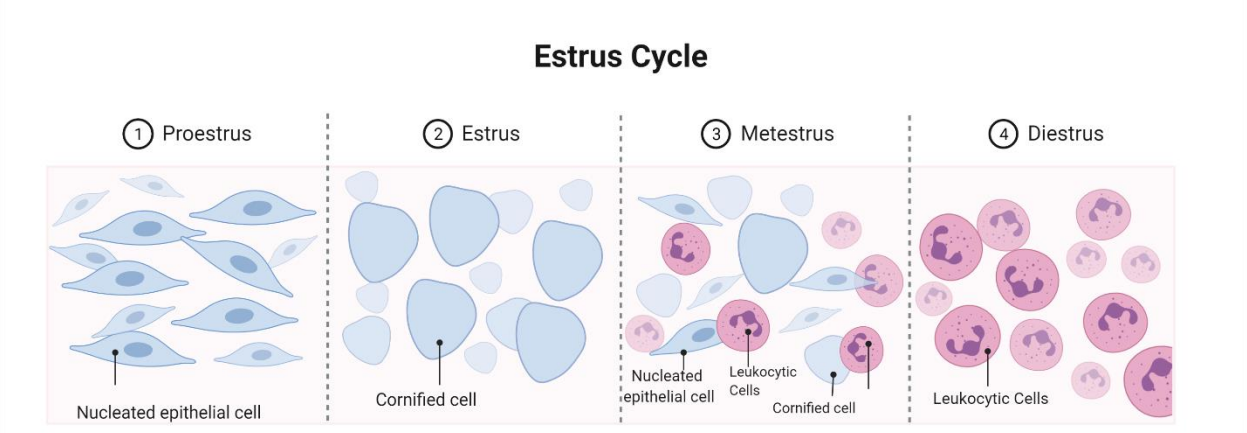

Figure 11: Schematic diagram of the estrus cycle.

Acknowledgements: Adapted and modified from Images Made with BioRender by Nina Kessler

Surgical manipulation

Ovariectomy

Some of the female mice were ovariectomized to remove the active source of gonadal hormones and then assessed for their behavioral outcomes. Female mice underwent bilateral ovariectomy under inhalational anesthesia using (2-4% isoflurane) based on our prior demonstrations (Paris et al., 2014b; Salahuddin et al., 2021c; Fig. 12). Post-surgical manipulation, mice were transferred to a clean cage with unlimited access to food, water, and acetaminophen (2mg/mL) for 96h period. Additionally, post-operative monitoring was carried out daily to account for their body weight, acetaminophen consumption, surgical incision site healing, and neurological signs. To limit any endogenous hormonal interference in the behavioral outcomes, 7 days of the hormone-washout period was carried out before any pharmacological manipulations.

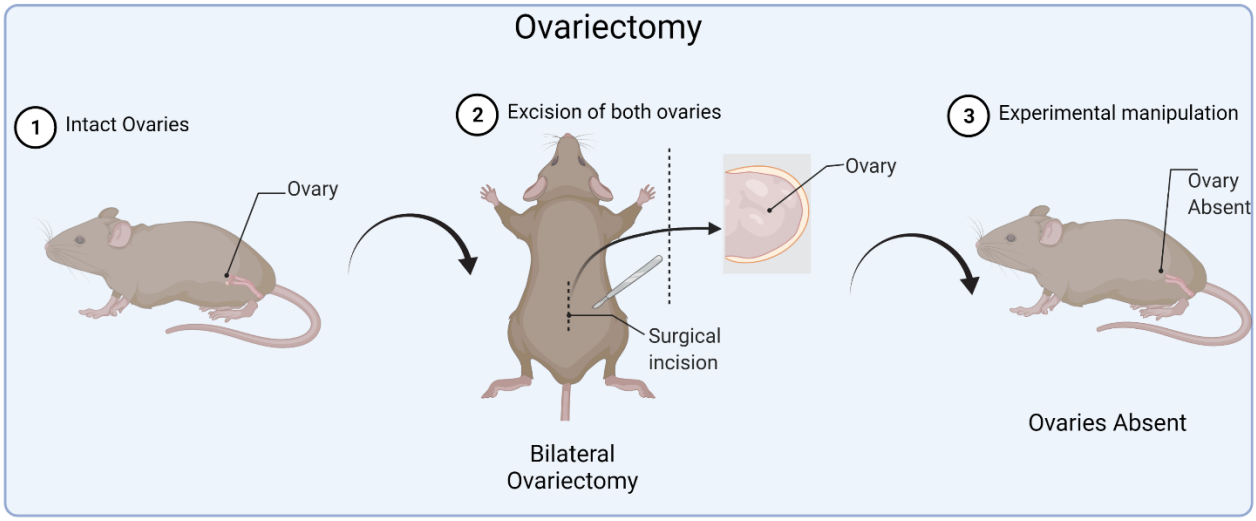

Figure 12: Schematic diagram of ovariectomy (removal of ovaries).

Stereotaxic osmotic infusion

Under isoflurane (2.5-4%) anesthesia, HIV-1 Tat transgenic mice that express Tat protein (or not) were stereotaxically implanted with ALZET osmotic pump calibrated to deliver drug [either vehicle (DMSO in saline (0.9%) in a ratio of 1:10,000) or FGIN-1-27 (5 µg) or allopregnanolone (100ng)] at a constant infusion rate of 1.0µL/hr (Model 2001) for 7 days. The osmotic pump was targeted to the lateral ventricle using the following coordinates from the mouse brain atlas (Bregma: AP: -0.5 mm, Lat: ±1.5 mm, DV: 2 mm; Leibrand et al., 2017; Paxinos et al., 1980). The pump was connected to the brain infusion kit (ALZET Brain Infusion Kit 3 #0008851) through polyethylene or vinyl catheter tubing (1.5cm long; provided with the kit). The penetration depth from the skull surface was adjusted with 2 spacers provided with the kit (Fig. 13). Following surgery, mice were transferred to fresh home cages and post-op monitoring was performed for 96h to observe neurological status, weight, and surgical site healing. Behavioral testing was performed after 7 days.

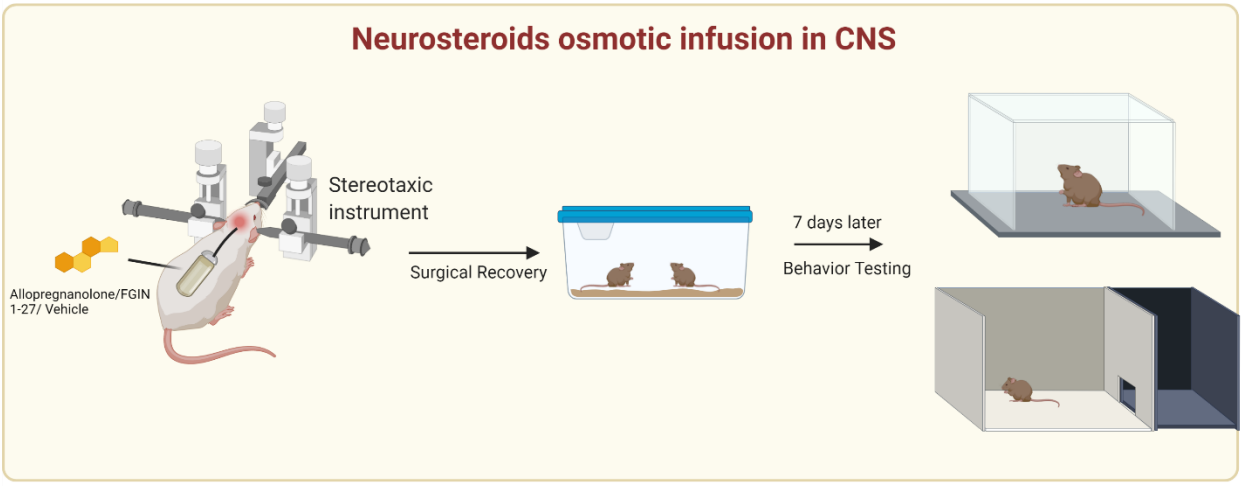

Figure 13: Schematic diagram of osmotic infusion of neurosteroids into the CNS.

Behavioral Assessment

Behavior testing of adult male (8th day) and female mice was carried out within (8th to 19th day) of their protocol. As stated, earlier, the estrous cycle was tracked among female mice and was subjected to behavior assessment when in proestrous or diestrous phase (whichever phase came first) of their estrous cycle. Previous studies found the affective behavior changes to be stable for at least 14 days from doxycycline induction, thus all the experimental mice were tested within 14 days of last doxycycline injection (Paris et al., 2014c; Fig. 14). Those mice which were irregular cyclers were excluded without testing. Prior demonstrations and present studies did not reveal either doxycycline administration or Tat expression to influence the estrous cycle length (Paris et al., 2014bd). All the mice were acclimated to the behavioral testing room environment 30 minutes prior to behavior testing and were assessed between 2-3h into the dark phase of the light cycle. The behavior was tracked and encoded by using ANY-Maze behavior tracking software (Stoelting Co., Wood Dale, IL).

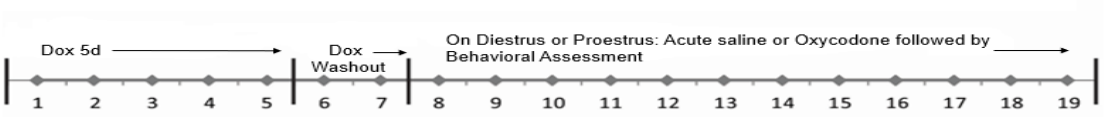

Figure 14: Behavioral timeline for female mice.

Behavioral Assays

Open Field

The open field test was used to assess motor and anxiety-like behavior (Hall and Ballachey, 1932). Briefly, mice were placed towards the lower-left corner of the transparent Plexiglas box (40 × 40 × 35 cm; Stoelting Co) with a brightly illuminated center (inner radius ~20cm) and allowed to explore the chamber for 5 min. The behavior was tracked and recorded using ANY Maze tracking software package. The total distance (m) and velocity of travel (m/s) was used as a proxy for the locomotor measure. Lesser time spent in brightly lit centers was used as a proxy for anxiety-like behavior (Figure 15, 16).

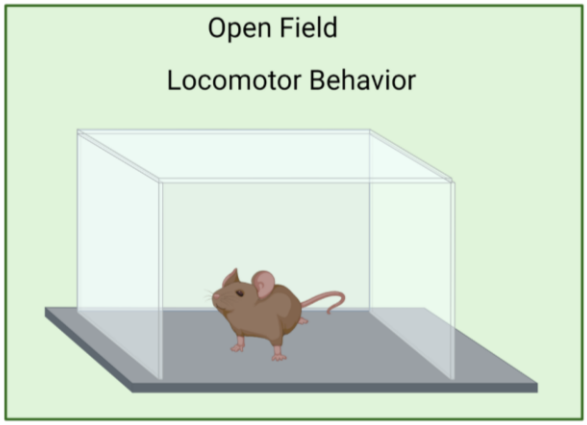

Figure 15: Schematic diagram of open field.

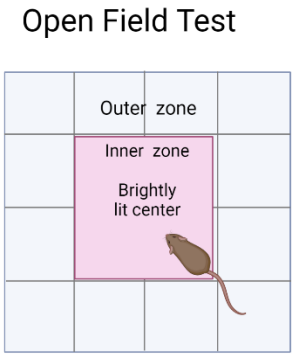

Figure 16: Schematic diagram of zones in an open field test.

Light-Dark transition test

The light-dark transition test was used to assess the anxiety-like behavior as demonstrated previously (Bourin & Hascoët, 2003). Briefly, mice were placed in the brightly-lit corner of a square Plexiglas box (40 × 40 × 35 cm; Stoelting Co., Wood Dale, IL, USA) that was uniformly partitioned into two compartments (one brilliantly lit side and one dark side) and permitted to explore for 5 min. The latency to enter the dark chamber and the time spent in the light zone were used as indices of anxiety-like behavior. The frequency of transitions between two chambers was utilized as an index of motor activity (Figure 17).

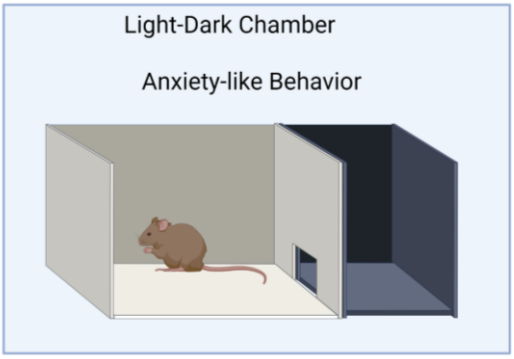

Figure 17: Schematic diagram of light-dark transition test.

Tail Suspension Test

Following the open-field behavior testing, the mice were subjected to a tail suspension test to assess for depression-like behavior (McLaughlin et al., 2017; Steru et al., 1985). Briefly, mice were suspended with a lab tape in a vertical position ~18inches above the ground. A plastic cup was used in order to prevent the mouse from tail-climbing. The behavior was recorded for 6 min (with the initial 2 min disposed of for acclimation). The time spent immobile (adoption of a complete static posture, with the exception of a body swinging from the earlier swing movement). The behavior was evaluated by 2 investigators who were blinded to the treatment groups. The greater time spent immobile was considered as an index of depression-like behavior (McLaughlin et al., 2017; Steru et al., 1985; Figure 18).

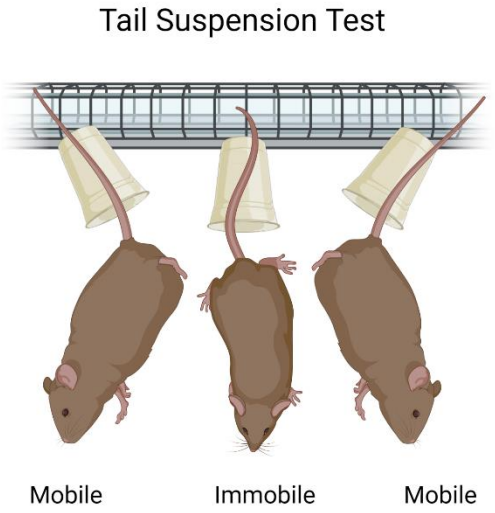

Figure 18: Schematic diagram of tail suspension test.
The time spent immobile is an index of depression-like behavior.

Forced Swim Test

The Porsolt forced swim test was used as a stimulus to activate the HPA stress axis as described previously (Porsolt et al., 1977). Briefly, mice were placed in a bucket of water ~22°C (room temperature) and permitted to swim for 15 minutes. Following the swim stressor test, mice were removed, dried with paper towels, and returned to their home cages (Salahuddin et al., 2020b, 2021c; Figure 19).

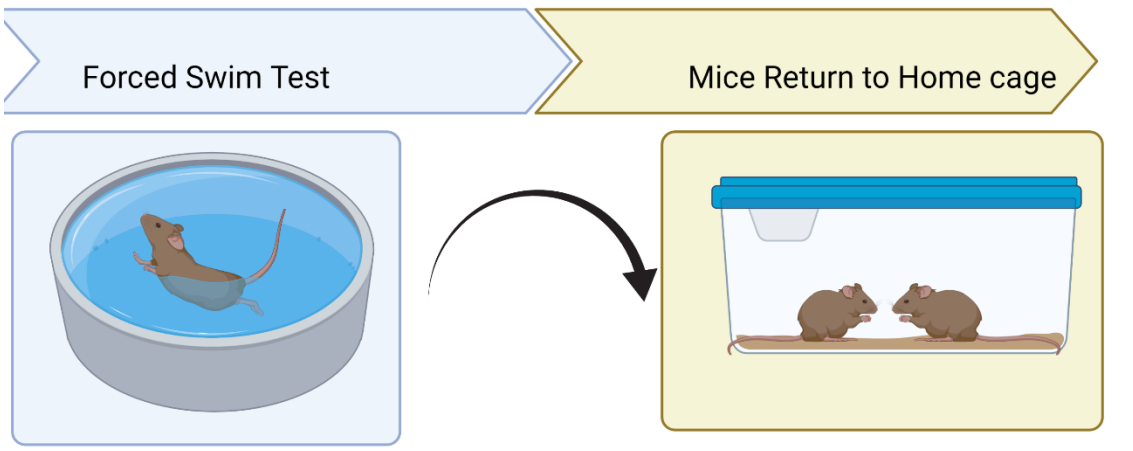

Figure 19: Schematic diagram of forced swim stress test.

The mice are placed in cold water (~22°C) for 15 minutes which is a natural stressor for mice to activate their HPA axis, followed by a return to home cage until behavior testing.

Novel Object Recognition Test

To assess the capacity of HIV-1 Tat to produce cognitive impairment, mice were assessed for short-term memory function in the novel object recognition task (Ennaceur and Delacour, 1988; Marks et al., 2016; Figure 20). Briefly, mice were acclimated to the testing room for 30 minutes with white noise. Following, mice were evaluated in a 10-min acquisition trial which comprised of investigating 2 objects (black spheres) set equidistant from one another and the northern side of a Plexiglas box (40 × 40 × 35 cm; Stoelting Co.). Post-acquisition trial, mice were transferred back to their home cage for a 4-hour inter-trial interval. Mice were then assessed in a 10-min testing trial which consisted of investigating two objects; a familiar object (black sphere) from the training phase and a novel object (white cube of similar size). The discrimination index was used as a measure to discern the recognition ability and determined by calculating the amount of time spent investigating the novel object by the equation: [(time spent

examining novel object/total time spent examining both the objects) × 100]. The novel object placement was counterbalanced between the left and right sides of the testing chamber to preclude any probable side preferences (Salahuddin et al., 2020a; Figure 20).

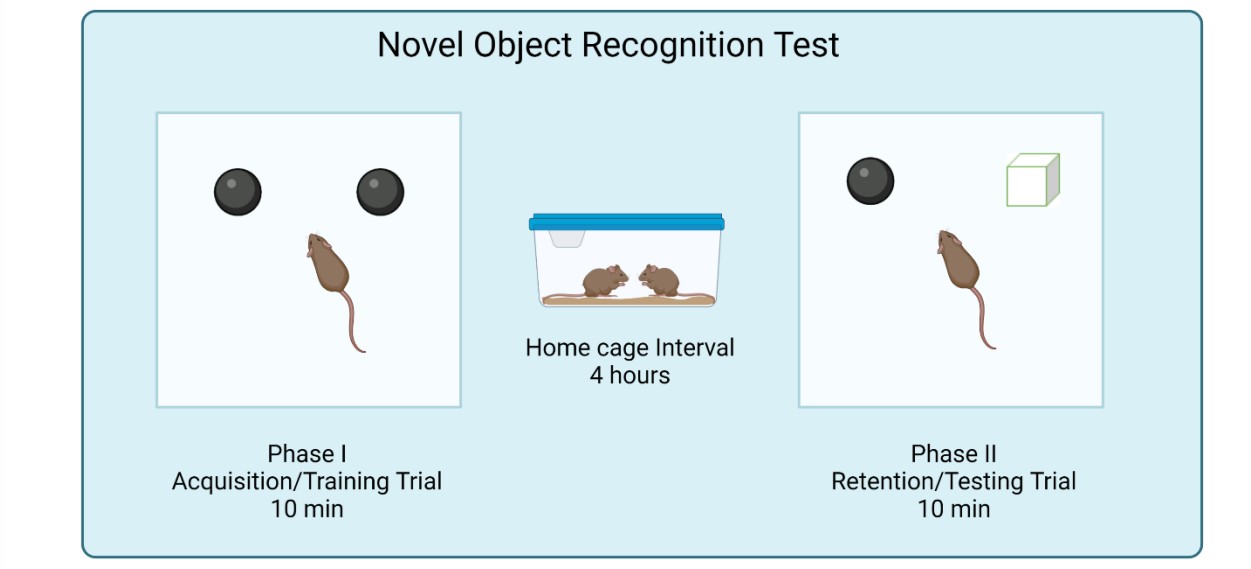

Figure 20: Schematic diagram of novel object recognition test.

The novel object recognition test is divided into 2 phases, the training phase (10min) & the testing phase (10min) which are four hours apart.

Steroidal Assay

Whole brain and trunk blood collection

Following behavior testing, mice were quickly sacrificed by cervical dislocation followed by rapid decapitation. Whole brains and trunk blood were collected, with the brains flash frozen and stored at -80° C until further use. Blood was allowed to clot and then centrifuged at 13,500 × *g* at 4°C for 20 min for separation of serum. The serum was strored at -80°C until further use.

Steroid Extraction

Circulating steroids like corticosterone, estradiol, and progesterone was isolated from serum utilizing ether-steroid extraction protocol as recently depicted (Paris et al., 2016). Briefly, the serum was incubated with 1mL of anhydrous ether, vortexed, and snap-frozen. The supernatant was collected and allowed to evaporate to dryness in a fume hood overnight (Figure 21). The dried tubes were then reconstituted to 5x (for Estradiol) or 25x (for Progesterone) or 50x (for corticosterone) to their initial volume with extraction buffer (Neogen Life Sciences, Lexington, KY; Salahuddin et al., 2020a)

Enzyme-Linked Immunosorbent Assay (ELISA)

Circulating steroids like estradiol, progesterone and corticosterone were assessed via ELISA based on manufacturer guidelines (Neogen Life Sciences; #402110, 402310, 402810) and as recently depicted (Paris et al., 2016; Salahuddin et al., 2020a). All the sample absorbance was read at 650 nm utilizing a CLARIOstar microplate reader (BMG Labtech Inc., Cary, NC; Figure 21). The antibodies cross-reactivities for the respective analyte of interest was 100%. However, corticosterone demonstrated other additional cross reactivity with other analytes like deoxycorticosterone (38%), 6-hydroxycorticosterone (19%), and progesterone (5.1%) and with some notable cross reactivities with other steroids ($\leq$ 2.7%).

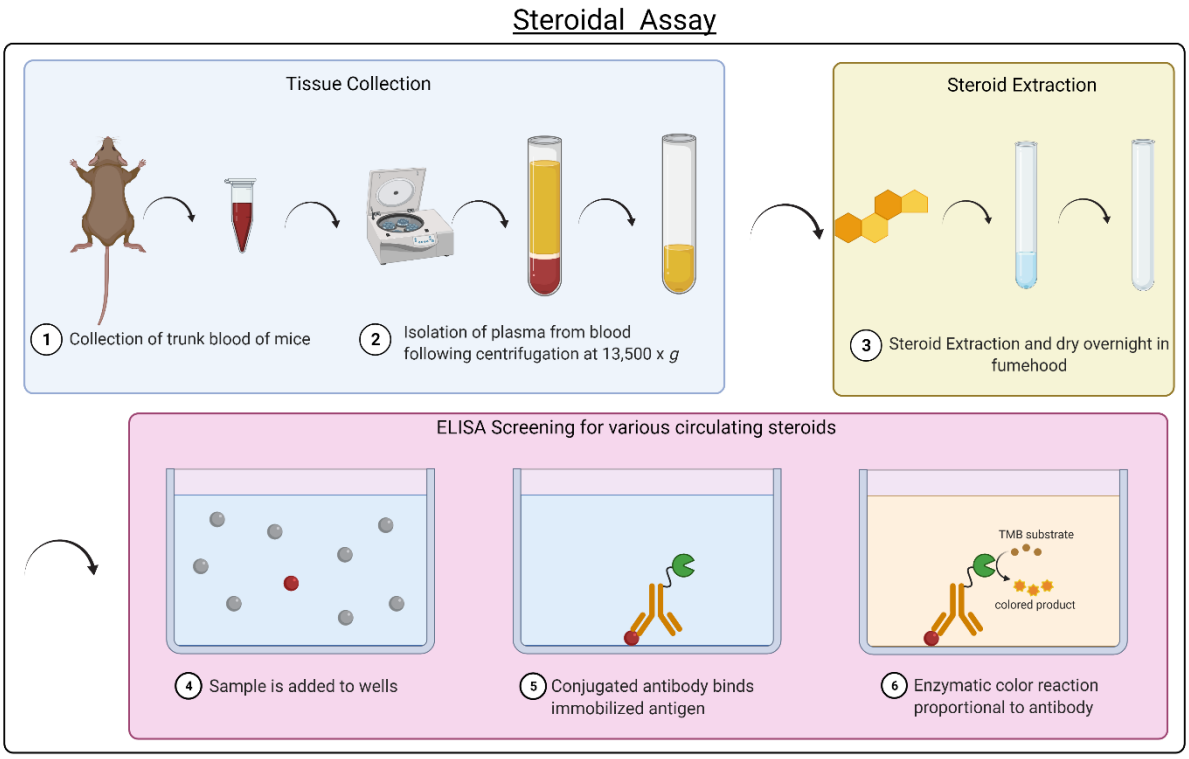

Figure 21: Schematic flow chart of steroidal enzyme estimation.

Western Blot Analysis

Western blot was used for detection and isolation of corticotropin-releasing factor (CRF). Briefly, the hypothalamus region of the brain was mid-sagittally dissected out and added to a cocktail of RIPA lysis buffer with a protease/phosphatase inhibitor (Halt Protease and Phosphatase Inhibitor Cocktail, Pierce, Rockford, IL) to reduce the extent of proteolysis, dephosphorylation, and denaturation to occur. The tissues were homogenized by passing ~20 times through a *27G* needle via syringe. The protein concentration of each lysate was determined via BCA (Bicinchoninic Acid) protein assay. Following protein quantification, brain lysates (20ug) were loaded on 4 – 20% Tris-HCl, TGX Stain-Free Gels (Bio-Rad Laboratories,

Hercules, CA). Electrophoresis was performed at 20mA for 1 hour to allow the migration of proteins complexes and their separation by size based on the ionic potential differences. The proteins from the gel were further transferred to a nitrocellulose membrane at 100 volts maintained at 4°C for 1 hour. Immunoblotting of the membrane was set up on a rocker with Odyssey® Blocking Buffer (LI-COR, Lincoln, NE) in TBS for 1 hour at room temperature. The protein of interest namely, CRF was detected using primary antibody [anti-CRF (IgG biotin-conjugated rabbit polyclonal, 1:1,000; Bioss Antibodies, Woburn, MA, #bs-0382R-Biotin)] and loading control anti-GAPDH [(mouse monoclonal, 1:2,000; BioLegend, San Diego, CA, #MMS-580S)] at 4°C overnight. The primary antibodies are incubated with fluorescent LICOR secondary antibodies conjugated against red and green fluoresceins (1:4,000) (IRDye® goat anti-rabbit 800CW streptavidin and goat anti-mouse 680RD; LI-COR) for 1 hour. The bands were read on CLX Licor Imager, and the intensities were evaluated using ImageJ programming software (National Institutes of Health, Bethesda, MD: Schindelin et al., 2012; Figure 22).

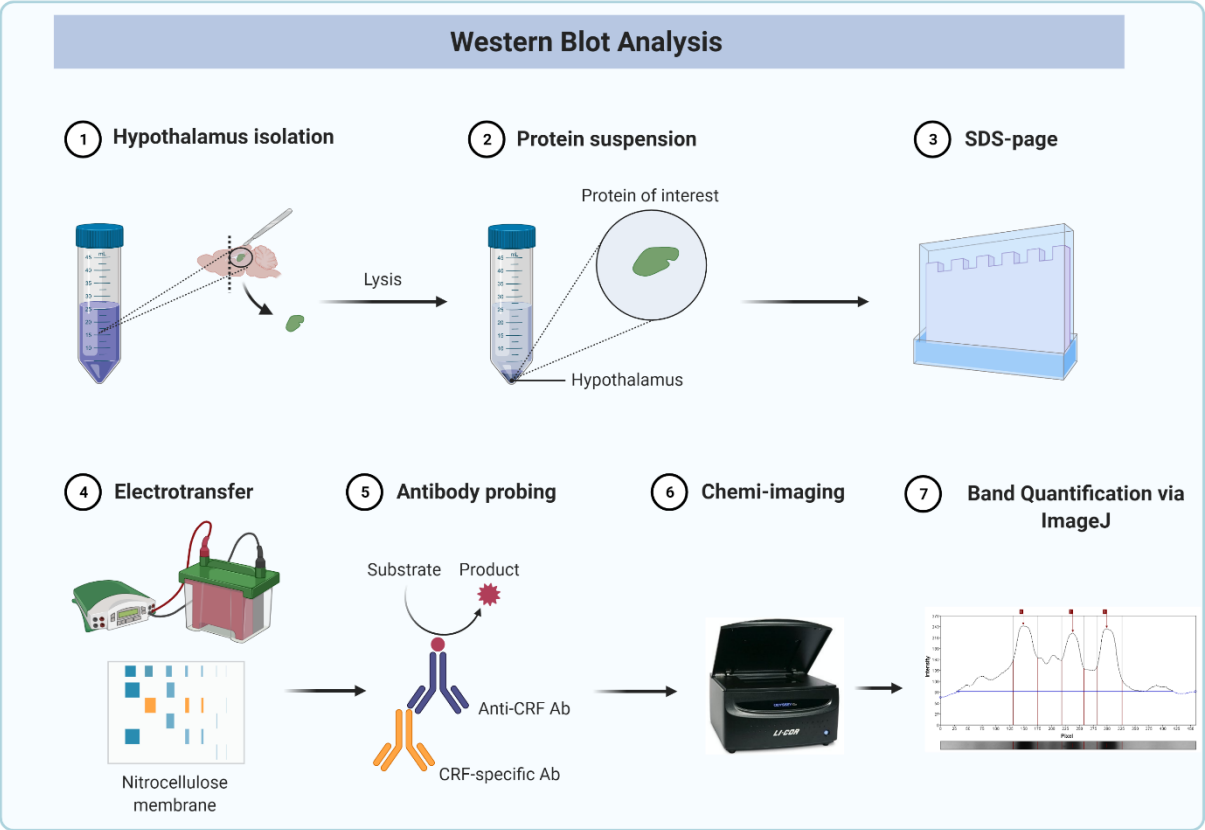

Figure 22: Schematic diagram of western blot analysis.

Acknowledgements: Adapted and Modified from Images Made with BioRender by Md Alamgir Kabir

Cell Culture

Human SH-SY5Y neuroblastoma cells (obtained from ATCC, #CRL-2266; Manassas, VA) were used to study the ability of gonadal steroids to confer neuronal protection. SH-SY5Y cells were seeded onto 24-well plates at a density of 2.5×10^4/well. Before differentiation, cells were incubated in media comprised of 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). On Day 1, cells were seeded onto the growth media. On day 2, retinoic acid (1:500) was added to the growth media to allow for differentiation characterized by the appearance of elongated neurites. On Day 3, the media was fully replaced with a serum-free differentiation medium (supplemented with antibiotic/antimycotic mixture) consisting of BDNF (1:200). On day 4, cells underwent experimental manipulations. On Day 5, twenty hours post-treatment, cell images were recorded on a scanning stage of Nikon Ti2-E motorized inverted microscope (Nikon Instruments Microscopy, Melville, NY; Figure 23). The differentiation with these factors promotes cell cycle arrest and articulation of neuronal markers (for example a shift from nestin+ to microtubule related protein 2+ expression and a diverged morphology) (Constantinescu et al., 2007; Encinas et al., 2000).

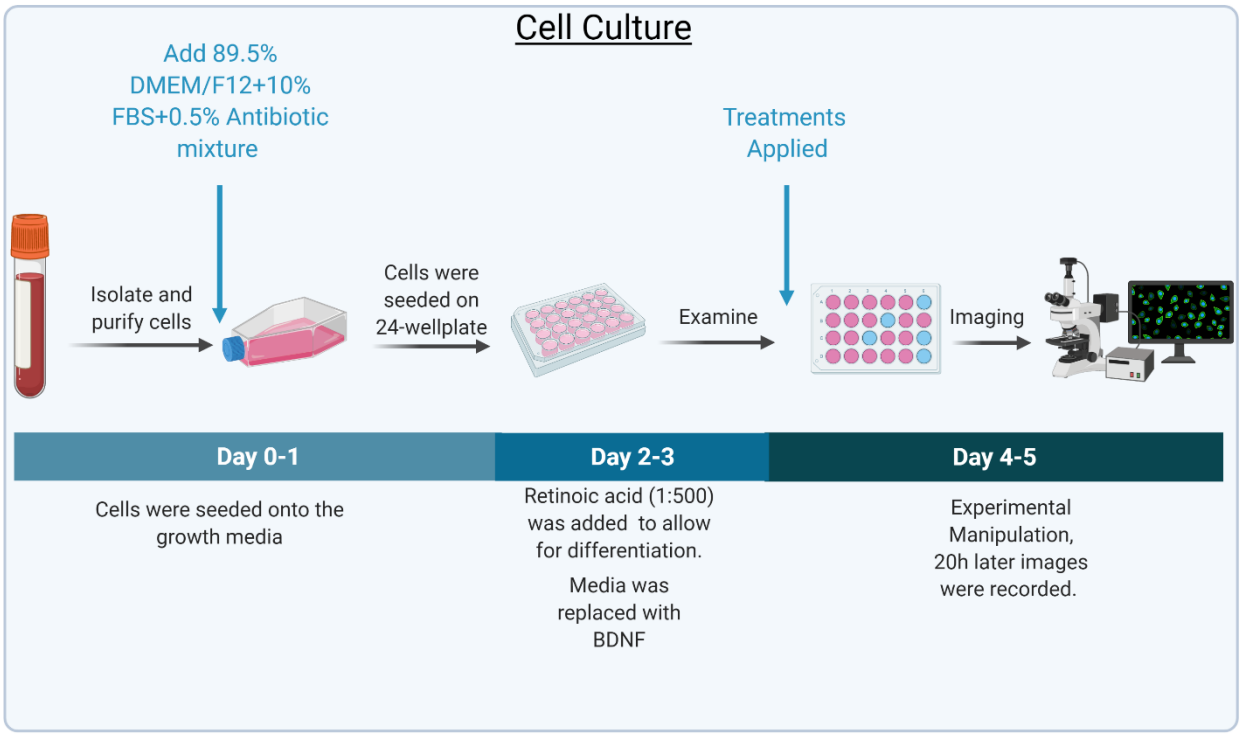

Figure 23: Schematic diagram of cell culture.

Live Dead Assay

In order to assess the neuronal viability, differentiated SH-SY5Y neuroblastoma cells were treated with vehicle or HIV-1 Tat (100 nM diluted in dH2O, ImmunoDx, Woburn, MA), saturating concentration of oxycodone (500nM), estradiol (0, 1, or 10 nM) or progesterone (0, 1, or 10 nM). Steroids were dissolved in sterile DMSO and diluted to a concentration (1:10,000) in the media. Neuronal viability was assessed based on the principle of differential staining of live and dead cells. The live cells are indicated by a blue Hoechst stain and dead cells by a red-fluorescent ethidium homodimer-1 due to loss of membrane integrity. Neuronal viability was assessed utilizing the LIVE/DEAD® Viability/Cytotoxicity marker (Molecular

Probes, Eugene, OR) per company instructions. Briefly, the differentiated cells were incubated with experimental treatments, run in duplicate on a 24-well plate, and live/dead assay was performed 20h later. Our prior reports utilizing a 60h time-lapse microscopy revealed 20h as the earliest time point when the pregnane steroid-treated cells diverged from Tat-treated cells on a measure of cell viability (Paris et al., 2016). Dead RED (propidium iodide), a marker of cellular necrosis was used. The Dead red fluorescence was measured at excitation/emission wavelength: 535/617 nm) and nuclear Hoechst 33342 fluorescence was measured at (excitation/emission wavelength: 360/460 nm). A working solution of these fluorescence markers was prepared in Hank's Balanced Salt Solution (1:500 dilution) and replaced with growth media containing cells 15min prior to imaging (incubated at 37°C with 5% CO_2 in the dark). Plates were imaged using a Ti2-E motorized, inverted microscope (Nikon Instruments Inc., Melville, NY). The number of dead cells and total cells were quantified using ImageJ Fiji software (Salahuddin et al., 2020a; Schindelin et al., 2012; Figure 24) and the proportion of dead cells was calculated using the formula [(propidium iodide stained cell # / total cell #) * 100].

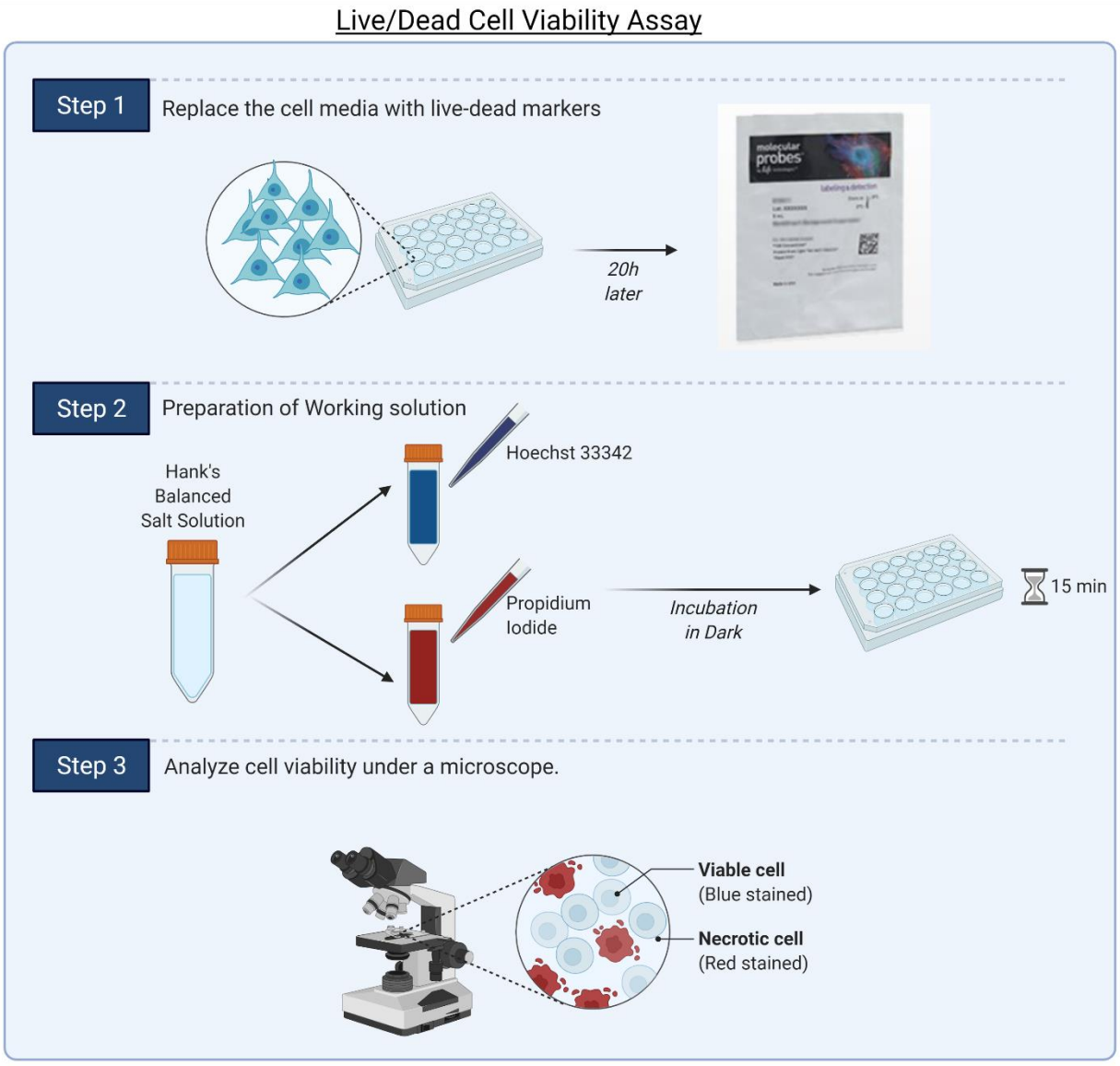

Figure 24: Schematic diagram of live/dead cell viability assay.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

In vitro

Differentiated SH-SY5Y cells were treated with either normal media or medium containing Oxycodone (500nM). TRIzol reagent and a Qiagen RNeasy Mini-Prep kit (Qiagen, Germany; #74104) were used to isolate RNA. A RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific; #K1651) was used to synthesize cDNA from 1 µg of isolated RNA. IDT supplied all of the primers (Coralville, IA; Table 3). qRT-PCR was carried out in a 25 µL final volume containing cDNA (1 µg), primers (400nM), and the PowerUp SYBR Green master mix (Thermo Fisher Scientific; #A25742). qRT-PCR was performed using a Bio-Rad CFX Connect Real-Time System, wherein the reactions were heated initially to 95 °C for 10 minutes, followed by 40 cycles of 95°C for 18 seconds/cycle; 60°C for 1 minute (Bio-Rad, Hercules, CA). The data are provided as the mean of three separate trials that were carried out in duplicate.

Ex vivo

The mice were euthanized, after behavior testing, and brains were flash frozen at -80° C until further use. On the day of RNA isolation and preparation, the hypothalamus was dissected out and tissue samples were homogenized in Trizol reagent and a Qiagen RNeasy Mini-Prep kit (Qiagen, Germany; #74104) were used to isolate RNA. Total RNA concentration was determined by using spectrophotometer optical density (260 and 280 nm). For each sample, the purity of the nucleic acid was estimated by accounting ratio (OD_{260}/OD_{280}) in the range of 1.7-2.0. A RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific; #K1651) was used to synthesize cDNA from 1 ug of isolated RNA. IDT supplied all of the primers (Coralville, IA). qRT-PCR was carried

out in a 25 µl final volume containing cDNA (1 µg), primers (400nM), and the PowerUp SYBR Green master mix (Thermo Fisher Scientific; #A25742). qRT-PCR was performed using a Bio-Rad CFX Connect Real-Time System, wherein the reactions were heated initially to 95 °C for 10 minutes, followed by 40 cycles of 95 °C for 18 seconds/cycle; 60 °C for 1 minute (Bio-Rad, Hercules, CA). Additionally, melt curve analysis [95°C, 1min, 70 cycles(1s)] was carried out immediately following amplification to identify any nonspecific product. As a negative control, we performed parallel reactions without template. The data are provided as the mean of three separate trials that were carried out in duplicate. All the resulting curves were sigmoidal and amplification efficiency was calculated to be 100%.

Table 3: Primers used for in-vitro Quantitative Real-Time-PCR. Ref. Salahuddin et al., 2020a

Receptor	Forward primer (5′–3′)	Reverse primer (5′–3′)
ERα	GCCAGGCTTGTCGTCTTAGG	TCCTTCACCACCGCCATTA
ERβ	AGATTCCCGGCTTTGTGG	GCTTCCGGCTGCTGTCA
GPER1	CCTCAACACTCACACACTCTGG	GATGTCTGGGCTGGTGCT
PR	ACCCGCCAGTGCCTCAGTCTCGTCT	GGCTTTCATTTGGAACGCCCACTGG
mPRα	GCTGTTCACTCACATCCC	TGGTGCAACCCCCAGA
mPRβ	GCGGCCCTGGTACTGCTGC	CACGGCCACCCCCACA
MOR	ATTGGTCTTCCTGTAATGTTCA	CAGGTTGGATGAGAGAATGTTAGTGT
KOR	CGTCTGCTACACCCTGATGATC	CTCTCGGGAGCCAGAAAGG

DOR	GCGGGAAAGCCAGTGACTC	TGCCCTGTTTAAGGACTCAGTTG
β-actin	GATCATTGCTCCTCCTGAGC	ACTCCTGCTTGCTGATCCAC
GAPDH	GGAAGCTCACTGGCATGGC	TAGACGGCAGGTCAGGTCCA
δ-subunit $GABA_AR$	GACTACGTGGGCTCCAACCTGGA	ACTGTGGAGGTGATGCGGATGCT
γ2-subunit $GABA_AR$	GGTGGAGTATGGCACCCTGCATT	AGGCGGTAGGGAAGAAGATCCGA
CYP11a1	CTGCCTCCAGACTTCTTTCG	TTCTTGAAGGGCAGCTTGTT
5α reductase1	CAGGAAGGGCAATGGGAGGGTGTT	TGTCTGGGGGTCAAAGGGGTCTGC
5α reductase 2	CATGCGGTTTAGCGTCGGTGTCT	CCAAAGCGTAGCCCATCCATTCAA
3α HSD	CACATTGGGAAGTTCACGAGACA	AAGCCAACTGGAATTCAAAAACCT

Ultra-Performance Liquid Chromatography (UPLC)-Mass Spectrometry (MS)

For UPLC-MS/MS, charcoal-stripped tissue (brain tissue derived from Tat-tg mice) was utilized to prepare both the calibration curve and quality control samples for analysis. A simple protein precipitation method was used for steroid extraction. Samples were homogenized (100 µL of PBS pH 7.4) and precipitated with 100 µL of acetonitrile followed by vortexing (2 min) and centrifugation (10 min at 14,000 rpm). After centrifugation, the supernatant solution was mixed with 50 µL of derivatizing solution (20 mg/mL of 2-hydrazinopyridine solution prepared in 0.5% trifluoroacetic acid ethanol solution) and incubated at 60°C for 1 h. Following incubation, 20 µL

of the internal standard solution (1 μg/mL) was added and vortex mixed. For sample analysis, aliquots of 2 μL were injected into the UPLC-MS/MS instrument (Salahuddin et al., 2021c).

Statistical Analyses

Behavioral, steroidal, RNA, and live/dead measures were analyzed via two-three-way analysis of variance (ANOVA) with genotype [Tat(–) and Tat(+)], drug condition (saline/vehicle or oxycodone), cycle phase (proestrous or diestrous), HPA/HPG blockade (vehicle or antalarmin or RU-486 or antalarmin+RU-486 or OVX) as between-subject factors. The dose-response and novel object recognition measures were evaluated using repeated-measures ANOVA with the same between-subjects variables and testing trial as the within-subjects factors (i.e. training or retention trial). For each treatment group, median effective doses (ED50; provided with 95 % confidence intervals) were calculated using non-linear regression (sigmoidal curvilinear modeling with variable slope) and a least-squares fit (bottom value constrained to 0). To delineate group differences, main effects were followed by Fisher's protected least significant difference post hoc tests. Interactions were defined using simple main effects and the main effect contrasts with family-wise error. Following omnibus inferential statistics and main effect contrasts, effect size measures (η2, Cohen's d) are provided. Data from qRT-PCR were computed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) and evaluated using the Student's t-test. All analyses were considered significant when $p \leq 0.05$.

CHAPTER 1

Interaction of Human Immunodeficiency Virus (HIV) and Opioids to promote HPA dysregulation

This chapter were previously published by

1. [Hormones and Behavior] [Salahuddin MF, Qrareya AN, Mahdi F, et al. Combined HIV-1 Tat and oxycodone activate the hypothalamic-pituitary-adrenal and -gonadal axes and promote psychomotor, affective, and cognitive dysfunction in female mice. Horm Behav. 2020;119:104649. doi:10.1016/j.yhbeh.2019.104649].

2. [International Journal of Molecular Sciences] [Salahuddin MF, Mahdi F, Paris JJ. HIV-1 Tat Dysregulates the Hypothalamic-Pituitary-Adrenal Stress Axis and Potentiates Oxycodone-Mediated Psychomotor and Anxiety-Like Behavior of Male Mice. Int J Mol Sci. 2020;21(21):8212. Published 2020 Nov 3. doi:10.3390/ijms21218212]

3. [Viruses] [Salahuddin MF, Mahdi F, Sulochana SP, Paris JJ. HIV-1 Tat Protein Promotes Neuroendocrine Dysfunction Concurrent with the Potentiation of Oxycodone's Psychomotor Effects in Female Mice. Viruses. 2021;13(5):813. Published 2021 Apr 30. doi:10.3390/v13050813]

Abstract

HIV infection is associated with co-morbid affective and stress-related neuropsychiatric disorders, which may be related to hypothalamic-pituitary-adrenal (HPA) stress axis dysfunction. The HPA axis is disrupted in up to 46% of HIV patients, however, the underlying mechanisms are unknown. Transactivator of transcription (Tat), a neurotoxic HIV-1 regulatory protein, may play a role. We hypothesized that disruption of the HPA axis may contribute to Tat-mediated interactions with oxycodone, a commonly prescribed opioid for HIV patients. Tat expression significantly increased circulating basal corticosterone levels both in transgenic male and female mice, recapitulating the clinical phenotype. Tat expression and acute oxycodone administration in female mice elevated corticotropin-releasing factor predominantly in the diestrous phase of the estrous cycle. Intriguingly males demonstrated paradoxical adrenal insufficiency in response to a natural stressor or pharmacological inhibition of HPA feedback. In Tat-expressing male mice, pharmacological inhibition of glucocorticoid receptors (GR) partially reinstated the stress response, implicating GR for these effects. Unlike male mice, female mice did not demonstrate adrenal insufficiency on exposure to a natural stressor or pharmacological blockade of HPA feedback. Rather OVX attenuated Tat/oxycodone interactions implicating gonadal hormones as drivers for neuroHIV behavior. Tat also elevated the E_2:P_4 ratio of circulating hormones in diestrus, and while acute oxycodone blunted this effect, repeated oxycodone exacerbated it. Taken together, these findings support the notion that Tat exposure can disrupt the HPA axis, increasing sensitivity to stress-related substance use and affective disorders.

1.1. Introduction

Human immunodeficiency virus type 1 (HIV-1) continues to be a significant public health concern in the United States, with over 1 million infected individuals (CDC, 2018). Antiretroviral combination therapy (cART) has significantly increased the life expectancy of HIV-positive individuals and decreased the incidence of HIV-associated dementia (Saylor et al., 2016). However, patients continue to experience a constellation of neurological symptoms (i.e., neuroHIV), most likely as a result of cART's incapacity to target neurotoxic HIV-1 proteins and latent CNS viral reservoirs like microglia/macrophages and astrocytes (Reviewed in Sanchez and Kaul, 2017). Additionally, opioid use may worsen neuroHIV symptomatology, a concern that extends to both illicit and licit opioid users, given that a potentially high proportion (8–52%) of HIV-1-infected individuals receive opioid prescriptions (Jeevanjee et al., 2014; Merlin et al., 2016; Silverberg et al., 2012).

The HIV infection disrupts multiple systems, including the hypothalamic-pituitary-adrenal (HPA) axis, a critical system responsible for orchestrating the resting state and adaptive response to stress. Numerous causes contribute to the dysfunction of the HPA axis. HIV directly affects the HPA axis by modulating host immune activity and altering cellular biological pathways via HIV-encoded proteins, as well as indirectly through immunodeficiency-associated opportunistic infections and various side effects of therapeutic compounds, including those used in combination antiretroviral therapy (Nicolaides et al., 2020). These modifications are further facilitated by the soluble factors or cytokines generated during viral infection and the chronic inflammatory state that ensues (Chrousos, 1995). These cytokines, which are generated during an immunological response, can both activate the HPA axis and induce glucocorticoid resistance

(Chrousos, 1995). Prior to the advent of cART, HPA dysfunction was predominantly "primary" (i.e., direct degeneration of the adrenals in greater than 50% of patients). In the post-cART era, HPA dysfunction is predominantly "secondary" (i.e. mediated at the CNS level), most likely due to cART's inability to curb CNS viremia. HIV- mediated secondary HPA dysfunction is characterized by high glucocorticoid levels (e.g. cortisol) at baseline and (adrenal insufficiency in response to a stressor, which appears counterintuitive) (Nicolaides et al., 2020). We and others believe that HPA dysfunction is related to a glucocorticoid insensitivity caused by HIV (Chrousos and Zapanti, 2014). The HPA axis is critical for an organism's resilience to physiological, psychological, and even immunological stressors. HPA factors have a wide range of pleiotropic effects on cytokine profiles, central excitation, and the peripheral stress response. Thus, increased basal glucocorticoids or adrenal insufficiency may increase vulnerability to neuroHIV symptoms like anxiety, depression, neurotoxicity, and cognitive impairment.

Although the mechanisms behind HIV-mediated dysregulation of HPA are unknown, they may involve neurotoxic HIV-1 proteins. The regulatory protein, transactivator of transcription (Tat), is a significant therapeutic target that may contribute to these outcomes. HIV-1 Tat causes excitotoxic damage directly to neurons and activates monocyte-derived cells (and, to a lesser extent, astrocytes) to induce neuroinflammation via cytokine production (Kaul et al., 2005). Preclinical animal models and cultured tissues have both shown that opioids can increase the severity of some of these effects (Bokhari et al., 2009; Fitting et al., 2014a, 2014b, 2010; Gonek et al., 2018; Nath et al., 2000, 2002; Noel, 2008). Henceforth, novel adjunct therapeutics to alleviate the neurotoxicity produced by the combination of opioids and Tat must be developed.

In the present dissertation chapter, we hypothesized that HIV-1 Tat and/or oxycodone would interact to cause HPA axis dysregulation, in a conditionally-inducible Tat transgenic mice model. In addition, we also anticipated that pharmacological inhibition of GR and/or CRF receptors would restore HPA function. Moreover, we also hypothesized that steroid hormones produced either peripherally or centrally would alter the HPA response.

In order to achieve the general objective, I have subdivided Chapter 1 into two aims.

Aim 1: Assess HPA activation by measurement of circulating corticosterone and corticotropin-releasing factor (CRF) at the levels of the adrenal (i.e. circulation) and hypothalamus, respectively.

Aim 2: Assess the pharmacodynamic targets (glucocorticoid receptor or CRF receptor) via systemic administration of pharmacological antagonists.

1.2. Materials and Methods

HIV-1 Tat Induction

To induce expression of the tat transgene (or not), Tat(−) and Tat(+) mice were administered doxycycline hylcate (prepared fresh daily and dissolved to 30 mg/kg, i.p., in 0.9% sterile saline; Cayman Chemical, Ann Arbor, MI) for 5 days, followed by 2 days without manipulation for doxycycline washout. Starting on day 8, the estrous cycle was assessed daily.

Determination of Estrous Cycle Phase

Estrous cycle was tracked by the daily collection of the vaginal epithelium as previously described (Paris et al., 2014b) with modification to the sample collection time. Samples were

collected at the start of the dark phase of the light cycle. At this time-point, the diestrous phase is characterized by E_2 levels that are rising to a peak and P_4 levels that are at nadir (E_2:P_4 ratio favors E_2; Scharfman and MacLusky, 2006). In the proestrus phase, E_2 levels are declining and P_4 levels are at their peak (E_2:P_4 ratio favors P_4; Scharfman and MacLusky, 2006). The cycle phase was determined by morphology as previously described (diestrus indicated by a majority presence of leukocytic cells and proestrus indicated by a majority presence of nucleated epithelial cells; Paris et al., 2014b).

Tissue Collection

Immediately following the completion of behavioral testing, mice were sacrificed via cervical dislocation followed by rapid decapitation. Whole brain and trunk blood were collected with brains flash frozen and maintained at −80° C. Blood was centrifuged at 13,500 × *g* at 4°C for 20 min and serum was stored at −80° C.

Steroid Extraction

Circulating steroids were isolated from serum using ether-steroid extraction as previously described (Paris et al., 2016). Briefly, serum samples were incubated with 1 mL of anhydrous ether and snap-frozen. The supernatant was collected, evaporated to dryness in a fume hood overnight, and reconstituted to 5x (for E_2) or 25x (for P_4 and corticosterone) the original volume in extraction buffer (Neogen Life Sciences, Lexington, KY).

Experiment 1: Assessment of Acute Oxycodone Exposure on Circulating Steroids

To assess the interaction between HIV-1 Tat expression and acute oxycodone exposure, mice were randomly assigned to receive vehicle (sterile saline, 0.9%, i.p.) or oxycodone hydrochloride (3 mg/kg, i.p.; Sigma-Aldrich, St. Louis, MO), either in their proestrous or diestrous phase of the estrus cycle, once 15 mins prior to open field and tail suspension behavioral testing. Mice were immediately euthanized (~30 min from saline or oxycodone injection) and ELISA was performed to estimate circulating corticosterone, estradiol, and progesterone levels in plasma, and Western blot was performed to estimate hypothalamic CRF protein expression in the brain.

Experiment 2: Assessment of Repeated Oxycodone Exposure on Circulating Steroids

To assess the interaction between HIV-1 Tat expression and repeated oxycodone exposure, mice were randomly assigned to receive vehicle (sterile saline, 0.9%, i.p.) or oxycodone hydrochloride (3 mg/kg, i.p.; Sigma-Aldrich, St. Louis, MO) once daily for 5 days, and assessed for novel object recognition behavioral testing either in their proestrus or diestrous phase of the estrous cycle. Post behavioral testing, mice were immediately euthanized and ELISA was performed to estimate circulating corticosterone, estradiol, and progesterone (in females) levels in plasma.

Experiment 3: Assessment of Acute Oxycodone Exposure on Circulating Steroids in Non-Stressed and Stressed mice

To begin to determine the HPA-axis interactions involved in exposure to HIV-1 Tat and acute oxycodone, mice were randomly assigned to undergo 15-min swim stress (or not) followed

by administration of vehicle (saline, 0.9%, i.p.) or oxycodone (3 mg/kg, i.p.) only once prior to behavioral testing. Fifteen minutes after drug administration, mice were assessed in an open field to determine their psychomotor response followed immediately by assessment in a light-dark transition test to determine anxiety-like behavior (Fig. 34A). Post behavioral testing, mice were immediately euthanized (~120 min from saline or oxycodone injection) and ELISA was performed to estimate circulating corticosterone, estradiol, and progesterone (in females) levels in plasma.

Experiment 4: Assessment of Repeated Oxycodone Exposure on Circulating Steroids in Non-Stressed and Stressed Mice

Given that most patients are exposed to opioids on a repeated dosing schedule, some mice were administered sterile saline (0.9%) or oxycodone (3 mg/kg) daily throughout the 7-day doxycycline-induction/washout schedule. As before, mice were randomly assigned to undergo a 15-min swim stress (or not) followed by an injection of saline (0.9%, i.p.) or oxycodone (3 mg/kg) 15 min prior to behavioral testing (Fig. 35A). Post behavioral testing, mice were immediately euthanized (~120 min from saline or oxycodone injection) and ELISA was performed to estimate circulating corticosterone (males and females), estradiol, and progesterone (in females) levels in plasma.

Experiment 5: Assessment of Acute Oxycodone Exposure following GR and/or CRF-R Blockade and HPG Blockade in females

To begin to identify the important receptor sites involved in HIV-1 Tat- or oxycodone-mediated disruption of the HPA axis, some mice were pretreated with the GR antagonist, RU-

486, and/or the CRF-R antagonist, antalarmin, and ovariectomized (in case of females) prior to testing. RU-486 was administered daily throughout the 7-day doxycycline-induction/washout schedule and 30 min prior to behavioral testing. Antalarmin was administered daily for 6-days during the doxycycline-induction/washout schedule (Fig. 37A, 40A) and 30 min prior to behavioral testing. Female mice were tested in proestrous phase of the estrous cycle. Some female mice were ovariectomized to remove the primary source of gonadal hormones and administered a daily vehicle injection (to account for potential injection stress). All mice received saline or oxycodone (3 mg/kg, i.p.) 15 min prior to behavioral testing (Fig. 37A, 40A). As in Experiments 3 and 4, mice were assessed for psychomotor and anxiety-like behavior. Post behavioral testing, mice were immediately euthanized (~120 min from saline or oxycodone injection) and ELISA was performed to estimate circulating corticosterone, estradiol and progesterone (in females) levels in plasma.

Experiment 6: Determination of hypothalamic allopregnanolone following exposure to Tat, Oxycodone, or OVX in Non-Stressed and Stressed mice

Hypothalamic allopregnanolone was determined from the hypothalamus region of the brain of behaviorally tested mice (stressed or non-stressed) exposed to Tat, oxycodone, and/or OVX. In brief, the hypothalamic region of the brain was dissected out and UPLC-MS was performed to estimate the levels of allopregnanolone (Fig.39A).

Experiment 7: Determination of HPA time course following Tat or Oxycodone Exposure

A subset of Tat(−) and Tat(+) mice were administered saline (0.9%, i.p.) or oxycodone (3 mg/kg) and had tail-blood collected 5, 30, and 120 min later (n = 5/group), and ELISA was

performed in order to assess the time-course for HPA activation (Fig.36).

Experiment 8: Assessment of Estradiol and Progesterone on HIV-1 Tat neurotoxicity in SH-SY5Y cells

Given that oxycodone administration and estrous cycle phase influence HIV-1 Tat's behavioral effects, the ability of E_2 or P_4 to protect against combination oxycodone/Tat neurotoxicity were investigated. Vehicle, Tat (100nM), a saturating dose of oxycodone (500nM), and low-to-high physiological E_2 (1 or 10 nM) or P_4 (10 or 100 nM) were exposed to differentiated SH-SY5Y human neuroblastoma cells for 20 hours and a number of dead cells and total cells were quantified using ImageJ Fiji software (Schindelin et al., 2012; Fig. 32) and the proportion of necrotic cells was calculated as [(dead cell # / total cell #) * 100].

Aim 1: Assess HPA activation by measurement of circulating corticosterone and corticotropin-releasing factor (CRF) at the levels of the adrenal (i.e. circulation) and hypothalamus, respectively.

1.3. Results

A. Female HIV-1 Tat transgenic mice

Acute oxycodone or Tat elevated circulating corticosterone, particularly among proestrus mice

HPA function was evaluated post-injection within 30 minutes [corresponding to peak corticosterone levels] by assessing the circulating corticosterone levels, in adult (2-6 months) female Tat transgenic mice via ELISA (Fig. 25A). Acute oxycodone exposure significantly increased circulating corticosterone [$F(1,64) = 10.83$, $p < 0.05$; $\eta^2 = 0.12$] (see †, Fig. 25A) and

interacted with estrous cycle phase and expression of HIV-1 Tat [$F(1,64) = 4.54$, $p < 0.05$; $\eta^2 = 0.05$] (see ‡, Fig. 25B). Exposure to Tat significantly increased circulating corticosterone in proestrous mice compared to their Tat(−) counterparts (see *, Fig. 25B). Among oxycodone-administered, proestrous, Tat(−) controls, corticosterone was significantly greater compared to all saline-administered groups with the exception of diestrous Tat(+) mice *(*$p = 0.005$ - 0.04; $d = 0.84$ - 1.65*;* see ‡, Fig. 25B*).* On diestrus, the combination of acute oxycodone and Tat significantly increased corticosterone compared to all saline-administered groups [with the exception of diestrous Tat(+) mice] and proestrous mice exposed to both oxycodone and Tat ($p = 0.0001$ - 0.03; $d = 0.95$ - 1.81; see ‡, Fig. 25A; Salahuddin et al., 2020a).

Repeated oxycodone exposure sensitized the circulating corticosterone levels

Among mice administered repeated saline (0.9%, i.p.) or oxycodone (3 mg/kg, i.p.), circulating corticosterone content was notably higher at baseline compared to those assessed in the acute-administration paradigm. No additional increase in corticosterone was observed with repeated oxycodone or Tat exposure (Fig. 25C; Salahuddin et al., 2020a).

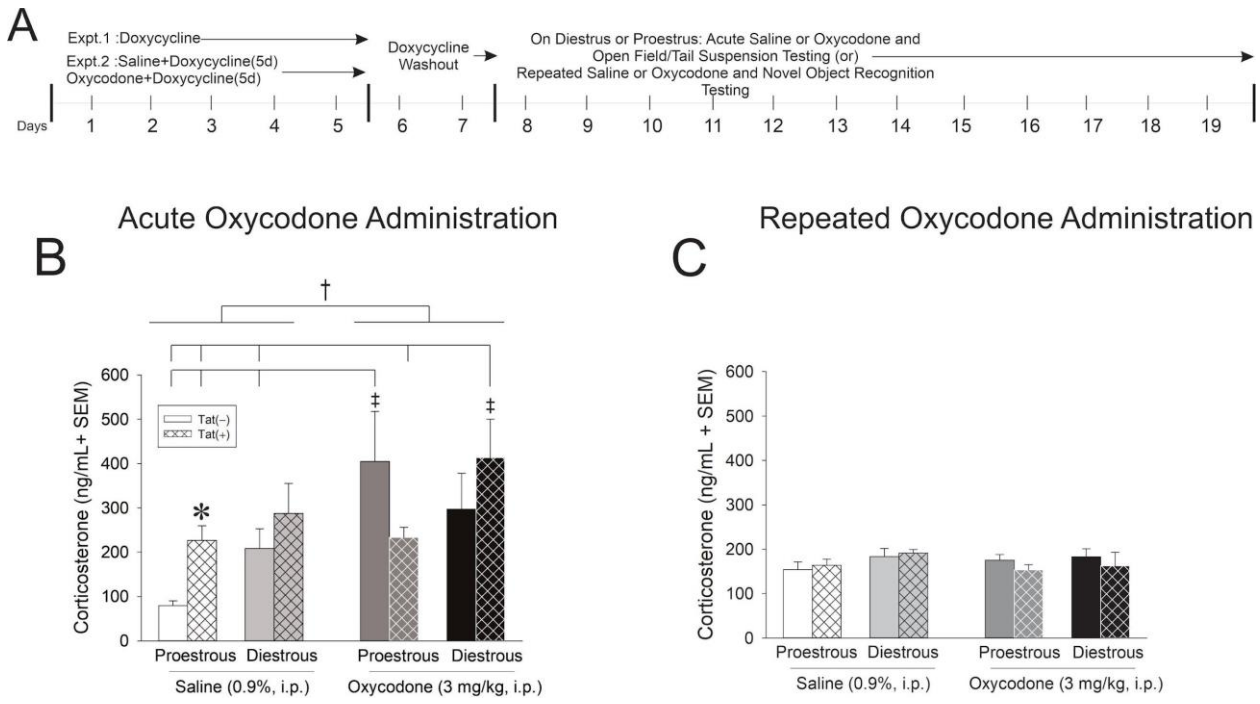

Figure 25: Effect of acute and repeated oxycodone exposure on circulating corticosterone in adult female HIV-1 Tat transgenic mice. Ref. Salahuddin et al., 2020a © 2019 Elsevier Inc. All rights reserved.

(**A**) In the first experiment HIV-1 Tat expression was induced in Tat(+) females, or not induced in Tat(−) controls, via administration of doxycycline (30 mg/kg, i.p., once daily for 5 d) with 2 days for washout. Estrous cycles were tracked for 12 d and mice were acutely-administered saline or oxycodone [(3 mg/kg, i.p., −15 min) and assessed in an open field and a tail suspension test on proestrus or diestrus (whichever came first). In a different experiment, Tat(+) and Tat(−) females were administered saline or oxycodone (3 mg/kg, i.p., once daily for 5 d) concurrent with the induction of HIV-1 Tat via doxycycline. Following 2 d of washout, estrous cycles were tracked and proestrous or diestrous mice were assessed in a novel object recognition test. (**B**) Circulating corticosterone ($n_{proestrous}$ = 6–12; $n_{diestrous}$ = 8–9) among Tat(−) and Tat(+) mice acutely-administered saline or oxycodone or (**C**) Circulating corticosterone ($n_{proestrous}$ = 10–11; $n_{diestrous}$ = 8–10) among Tat(−) and Tat(+) mice among repeatedly-administered saline or oxycodone (5d) as depicted in the (A) timeline. * indicates an interaction wherein saline-administered Tat(+) mice differ from respective Tat(−) controls; † indicates a main effect for oxycodone to differ from saline administration; ‡ indicates a 3-way interaction wherein the denoted group differs from those indicated, $p < 0.05$.

Acute oxycodone, Tat, and estrous cycle interacted to influence circulating estradiol levels.

Doxycycline (30 mg/kg, i.p., once daily for 5 days) was used to induce HIV-1 Tat expression in Tat(+) females or not in Tat(−) controls. The mice were given either saline or oxycodone (3 mg/kg, i.p., 15 min) and examined in an open field and a tail suspension test in the proestrus or diestrus phase (whichever came first; Fig. 26A). Similar to the effects observed on corticosterone, expression of HIV-1 Tat, estrous cycle phase, and exposure to acute oxycodone significantly interacted to influence the circulating E_2 levels [$F(1,53) = 7.41$, $p < 0.05$; $\eta^2 = 0.08$] (see ‡, Fig. 26B). Among diestrous mice, those exposed to Tat demonstrated significantly greater E_2 levels than all other groups, with the exception of their diestrous counterparts that were administered acute oxycodone ($p = 0.004 - 0.009$; $d = 1.20 – 1.42$; see ‡, Fig. 26B). Diestrous, Tat(−) controls administered acute oxycodone, demonstrated significantly greater E_2 levels than all other groups with the exception of their diestrous, Tat(+) counterparts ($p < 0.0001 - 0.02$; $d = 0.94 – 2.23$; see ‡, Fig. 26B; Salahuddin et al., 2020a).

Repeated oxycodone interacted with the estrous cycle to influence circulating estradiol, particularly among diestrous mice

Estradiol levels were notably higher among mice in the repeated-paradigm compared to levels previously observed in the acute-paradigm. Exposure to repeated oxycodone significantly increased circulating E_2 [$F(1,71) = 21.27$, $p < 0.05$; $\eta^2 = 0.15$] (see †, Fig. 26C) and interacted with the estrous cycle [$F(1,71) = 6.82$, $p < 0.05$; $\eta^2 = 0.05$] (see ^, Fig. 26C). Irrespective of exposure to Tat, diestrous mice demonstrated significantly greater E_2 than their proestrous counterparts and repeated oxycodone further increased this effect such that they significantly differed from all other groups ($p < 0.0001 - 0.008$; $d = 1.15 – 1.97$; see ^, Fig. 26C; Salahuddin et al., 2020a).

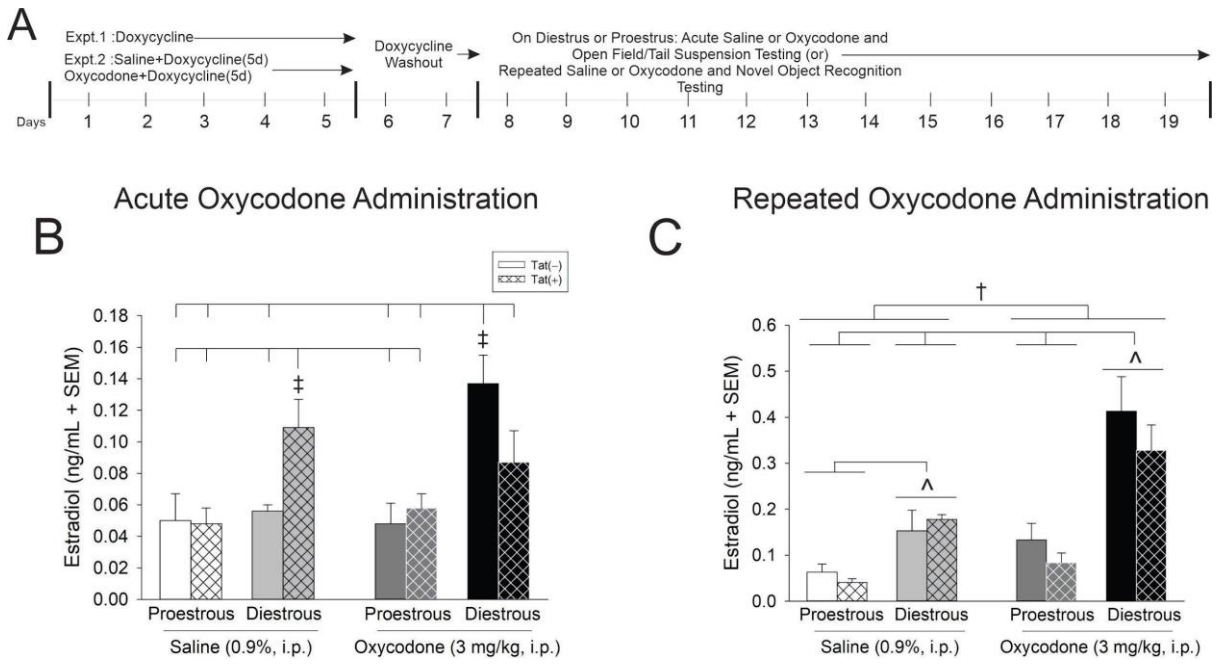

Figure 26: Effect of acute and repeated oxycodone exposure on circulating estradiol in adult female HIV-1 Tat transgenic mice. Ref. Salahuddin et al., 2020a © 2019 Elsevier Inc. All rights reserved.

(**A**) HIV-1 Tat expression was induced in Tat(+) females, or not induced in Tat(−) controls, via administration of doxycycline (30 mg/kg, i.p., once daily for 5 d) with 2 days for washout **(B)** Circulating estradiol ($n_{proestrous}$ =6-9; $n_{diestrous}$ = 8-9) among Tat(−) and Tat(+) mice acutely-administered saline (0.9%, i.p.) or oxycodone (3 mg/kg, i.p.) or **(C)** Circulating estradiol ($n_{proestrous}$ = 10–11; $n_{diestrous}$ = 7–10) among Tat(−) and Tat(+) mice repeatedly-administered saline (0.9%, i.p.) or oxycodone (3 mg/kg, i.p.) as depicted in the timeline. ‡ indicates a 3-way interaction wherein the denoted group differs from those indicated; ^ indicates a drug × estrous cycle interaction wherein the denoted group differs from those indicated. † indicates a main effect for oxycodone to differ from saline administration; # indicates a main effect for proestrous mice to differ from diestrous mice, $p < 0.05$.

Acute oxycodone interacted with estrous cycle to influence circulating progesterone

Doxycycline (30 mg/kg, i.p., once daily for 5 days) was used to induce HIV-1 Tat expression in Tat(+) females or not in Tat(−) controls. The mice were given either saline or oxycodone (3 mg/kg, i.p., 15 min) and examined in an open field and a tail suspension test in the proestrus or diestrus phase (whichever came first; Fig. 27A). Acute oxycodone and estrous cycle

phase influenced circulating P_4 content (Fig. 27B). There was a main effect for acute oxycodone to increase progesterone, irrespective of Tat exposure or cycle phase [$F(1,54) = 7.44$, $p < 0.05$; $\eta^2 = 0.10$] (see †, Fig. 27B). Concurrently, there was a main effect for diestrous mice to have significantly greater progesterone content than proestrous mice, irrespective of oxycodone administration or Tat exposure [$F(1,54) = 6.25$, $p < 0.05$; $\eta^2 = 0.08$] (see #, Fig. 27B; Salahuddin et al., 2020a).

Repeated oxycodone exposure influenced circulating progesterone during the rising HPA activation phase, particularly among proestrous phase.

Like other steroids examined, progesterone was elevated by oxycodone in the repeated-paradigm [$F(1,71) = 4.47$, $p < 0.05$; $\eta^2 = 0.05$] (see †, Fig. 27C) but the effect was most notable among proestrous controls [$F(1,71) = 4.65$, $p < 0.05$; $\eta^2 = 0.05$] (see ^, Fig. 27C). Irrespective of Tat exposure, proestrous mice repeatedly-administered saline demonstrated significantly greater circulating progesterone than all other groups ($p < 0.0009 - 0.003$; $d = 0.70 – 0.87$; see ^, Fig. 27C; Salahuddin et al., 2020a).

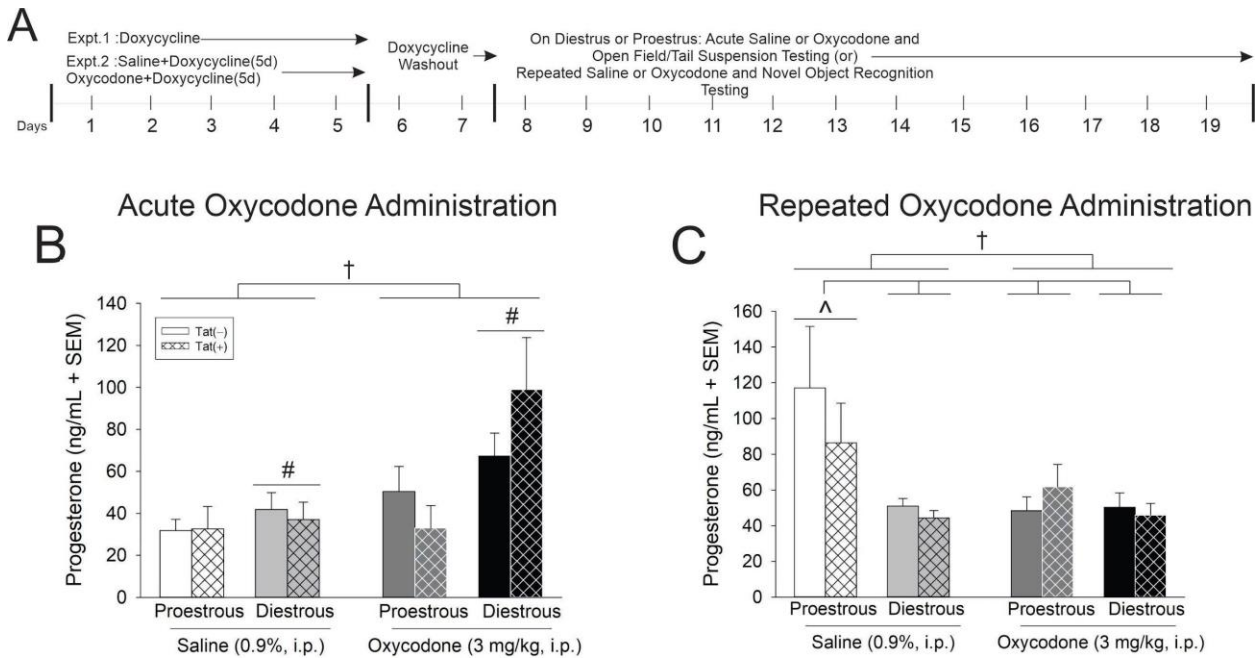

Figure 27: Effect of acute and repeated oxycodone exposure on circulating progesterone in adult female HIV-1 Tat transgenic mice. Ref. Salahuddin et al., 2020a © 2019 Elsevier Inc. All rights reserved.

(**A**) HIV-1 Tat expression was induced in Tat(+) females, or not induced in Tat(−) controls, via administration of doxycycline (30 mg/kg, i.p., once daily for 5 d) with 2 days for washout **(B)** Circulating progesterone *(*$n_{proestrous}$ =6-9; $n_{diestrous}$ = 8-9*)* among Tat(−) and Tat(+) mice acutely-administered saline (0.9%, i.p.) or oxycodone (3 mg/kg, i.p.) or **(C)** circulating progesterone ($n_{proestrous}$ = 9–11; $n_{diestrous}$ = 8–10) among Tat(−) and Tat(+) mice repeatedly-administered saline (0.9%, i.p.) or oxycodone (3 mg/kg, i.p.) as depicted in the timeline. ^ indicates a drug × estrous cycle interaction wherein the denoted group differs from those indicated; # indicates a main effect for proestrous mice to differ from diestrous mice; † indicates a main effect for oxycodone to differ from saline administration, $p < 0.05$.

Acute oxycodone, Tat, and estrous cycle interacted to influence E_2 to P_4 ratio

Doxycycline (30 mg/kg, i.p., once daily for 5 days) was used to induce HIV-1 Tat expression in Tat(+) females or not in Tat(–) controls. The mice were given either saline or oxycodone (3 mg/kg, i.p., 15 min) and examined in an open field and a tail suspension test in proestrus or

diestrus phase (whichever came first; Fig. 28A). Exposure to acute oxycodone, estrous cycle phase, and expression of HIV-1 Tat significantly interacted to influence the E_2 to P_4 ratio [$F(1,48) = 5.35$, $p < 0.05$; $\eta^2 = 0.09$] (see ‡, Fig. 28B). The E_2:P_4 ratio significantly favored E_2 among Tat(+) control mice in the diestrous phase of their estrous cycle compared to diestrous Tat(−) mice ($p = 0.03$; $d = 0.83$) or their proestrous, Tat(+) counterparts ($p = 0.04$; $d = 0.56$; see ‡, Fig. 28B). However, this difference was not observed when mice were administered acute oxycodone; diestrous, Tat(+) controls administered saline also significantly differed from diestrous Tat(+) and proestrous Tat(−) mice administered oxycodone ($p = 0.02 – 0.03$; $d = 0.53$ −0.85; see ‡, Fig. 28B; Salahuddin et al., 2020a).

Repeated oxycodone and estrous cycle interacted to influence E_2 to P_4 ratio

Repeated oxycodone and estrous cycle phase influenced the E_2 to P_4 ratio (Fig. 28C). There was a main effect for repeated oxycodone to increase the E_2:P_4 ratio, irrespective of Tat exposure or cycle phase [$F(1,68) = 13.50$, $p < 0.05$; $\eta^2 = 0.10$] (see †, Fig. 28C). Concurrently, there was a main effect for diestrous mice to have a significantly greater E_2:P_4 ratio than proestrus mice, irrespective of oxycodone administration or Tat exposure [$F(1,68) = 18.70$, $p < 0.05$; $\eta^2 = 0.15$] (see #, Fig. 28C; Salahuddin et al., 2020a).

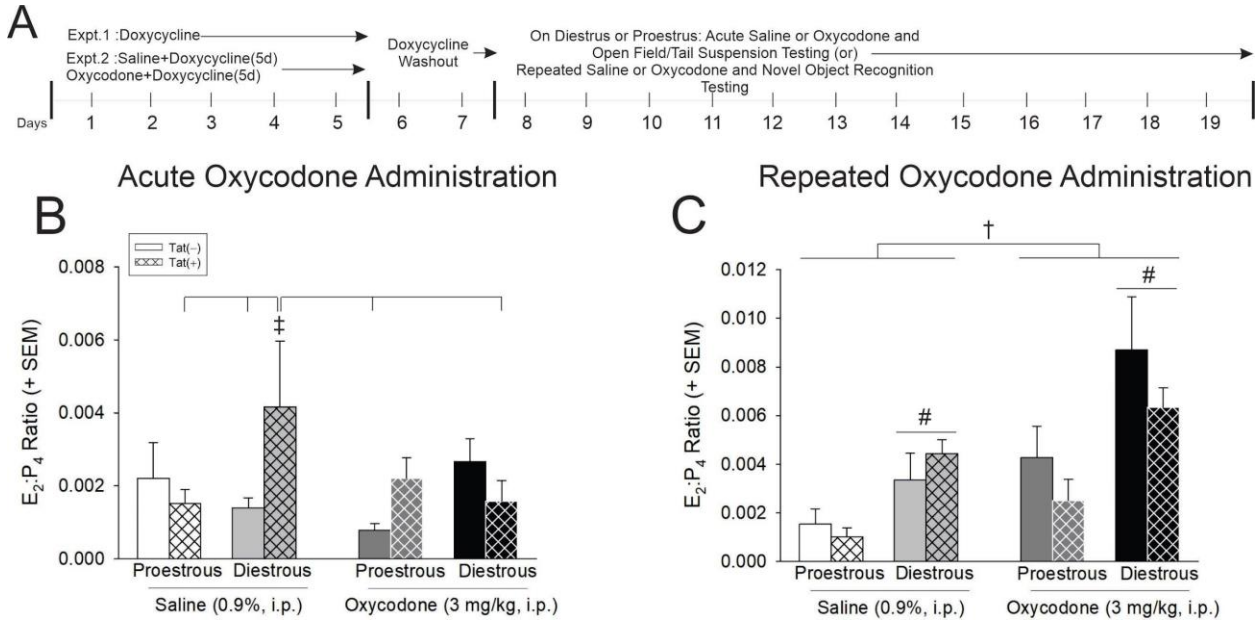

Figure 28: Effect of acute and repeated oxycodone exposure on circulating E2:P4 ratio in adult female HIV-1 Tat transgenic mice. Ref. Salahuddin et al., 2020a © 2019 Elsevier Inc. All rights reserved.

(**A**) HIV-1 Tat expression was induced in Tat(+) females, or not induced in Tat(−) controls, via administration of doxycycline (30 mg/kg, i.p., once daily for 5 d) with 2 days for washout **(B)** Circulating E_2:P_4 ratio *($n_{proestrous}$* = 5–8; $n_{diestrous}$ = 7–8*)* among Tat(−) and Tat(+) mice acutely-administered saline (0.9%, i.p.) or oxycodone (3 mg/kg, i.p.) or **(C)** Circulating E_2:P_4 ratio ($n_{proestrous}$ = 9–11; $n_{diestrous}$ = 7–10) among Tat(−) and Tat(+) mice repeatedly-administered saline (0.9%, i.p.) or oxycodone (3 mg/kg, i.p.) as depicted in the timeline. ‡ indicates a 3-way interaction wherein the denoted group differs from those indicated; # indicates a main effect for proestrous mice to differ from diestrous mice; † indicates a main effect for oxycodone to differ from saline administration; # indicates a main effect for proestrous mice to differ from diestrous mice, $p < 0.05$.

Acute oxycodone, and combined Tat exposure elevated, hypothalamic CRF

Western blot analysis of corticotropin-releasing factor (CRF)/GAPDH protein content in hypothalamus of Tat(−) and Tat(+) mice from acutely-administered saline (0.9%, i.p.) or oxycodone CRF was assessed in grossly-dissected hypothalamus via western blot (Fig. 29A). Acute oxycodone administration significantly interacted with estrous cycle phase, and Tat exposure to alter hypothalamic CRF protein expression [$F(1,39) = 4.68$, $p < 0.05$; $\eta^2 = 0.08$; Fig. 29B]. Compared to proestrous controls, CRF was significantly elevated on the diestrous phase or by exposure to oxycodone or HIV-1 Tat ($p = 0.001$ - 0.03; $d = 1.63$ - 3.03; see ‡, Fig. 29B; Salahuddin et al., 2020a).

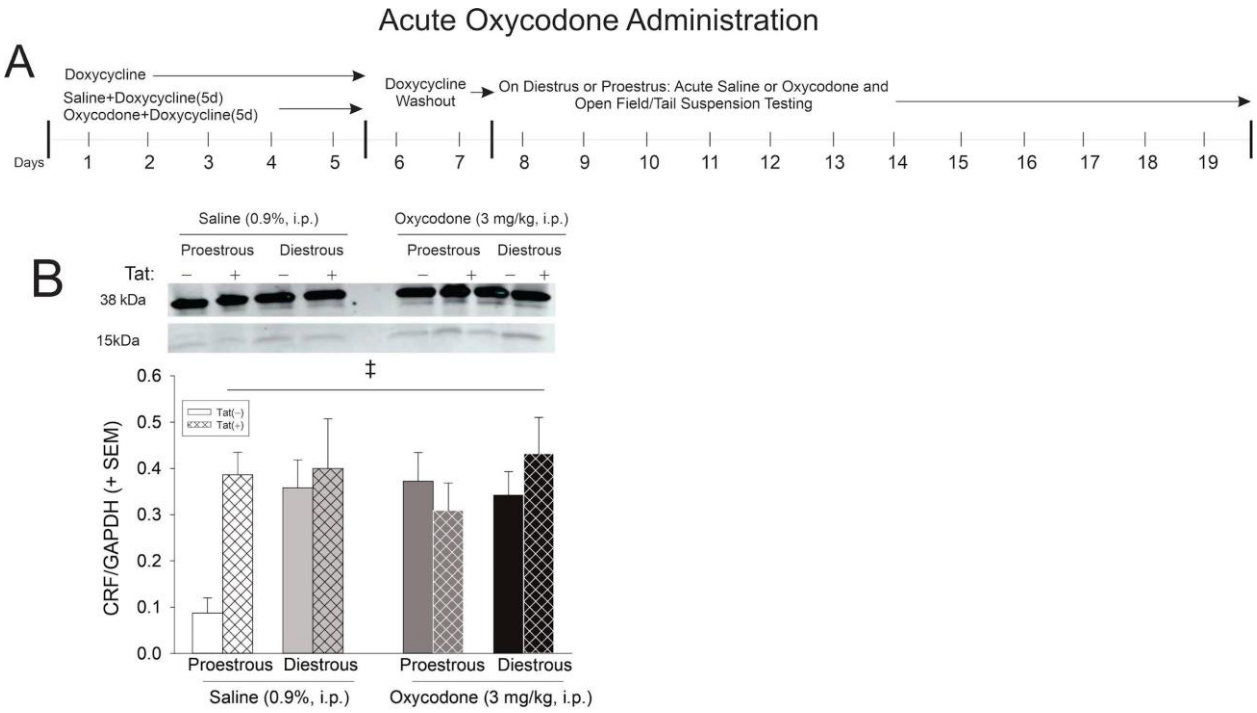

Figure 29: Western blot estimation of corticotropin-releasing factor (CRF)/GAPDH protein content in hypothalamus of adult female HIV-1 Tat transgenic mice. Ref. Salahuddin et al., 2020a © 2019 Elsevier Inc. All rights reserved.

Western blot analysis of corticotropin-releasing factor (CRF)/GAPDH protein content in hypothalamus of Tat(−) and Tat(+) mice acutely-administered saline (0.9%, i.p.) or oxycodone (3 mg/kg, i.p.; $n_{proestrous}$ = 5–6/group; $n_{diestrous}$ = 6/group) and behaviorally-assessed in an open field and tail suspension test in panels **A** and **B**. ‡ indicates a 3-way interaction wherein the denoted group differs from those indicated, $p < 0.05$.

Tat interacted with the estrous cycle to influence circulating corticosterone levels in non-stressed mice

Circulating steroid concentrations were assessed in serum ~2 h following saline or opioid injection when HPA activation is expected to be resolving. A 15 min forced swim was used to activate the HPA axis in the stressed paradigm (or not in the non-stressed paradigm). On the day

of testing, all mice received saline or oxycodone (3 mg/kg, i.p.) 15 min prior to behavioral assessment (Fig. 30A). In non-stressed female mice, expression of Tat interacted with estrous cycle phase to influence corticosterone concentrations [$F(1,69) = 4.60$, $p < 0.05$] (see ^, Fig. 30B). Irrespective of oxycodone administration, either Tat(+) or diestrous mice demonstrated significantly greater corticosterone than did proestrous Tat(−) controls ($p = 0.0035–0.0101$; Fig. 30B). Among swim stress-exposed female mice, circulating corticosterone was significantly greater in oxycodone-administered mice [$F(1,70) = 6.30$, $p < 0.05$] (see †, Fig. 30C) and was significantly reduced among diestrous mice [$F(1,70) = 37.05$, $p < 0.05$] (see #, Fig. 30C) compared to their respective saline-administered or proestrous counterparts. This data suggests unlike males; females did not demonstrate adrenal insufficiency when exposed to swim stressor implicating females are protected by gonadal hormones like estradiol progesterone to modulate the HPA axis (Salahuddin et al., 2021c).

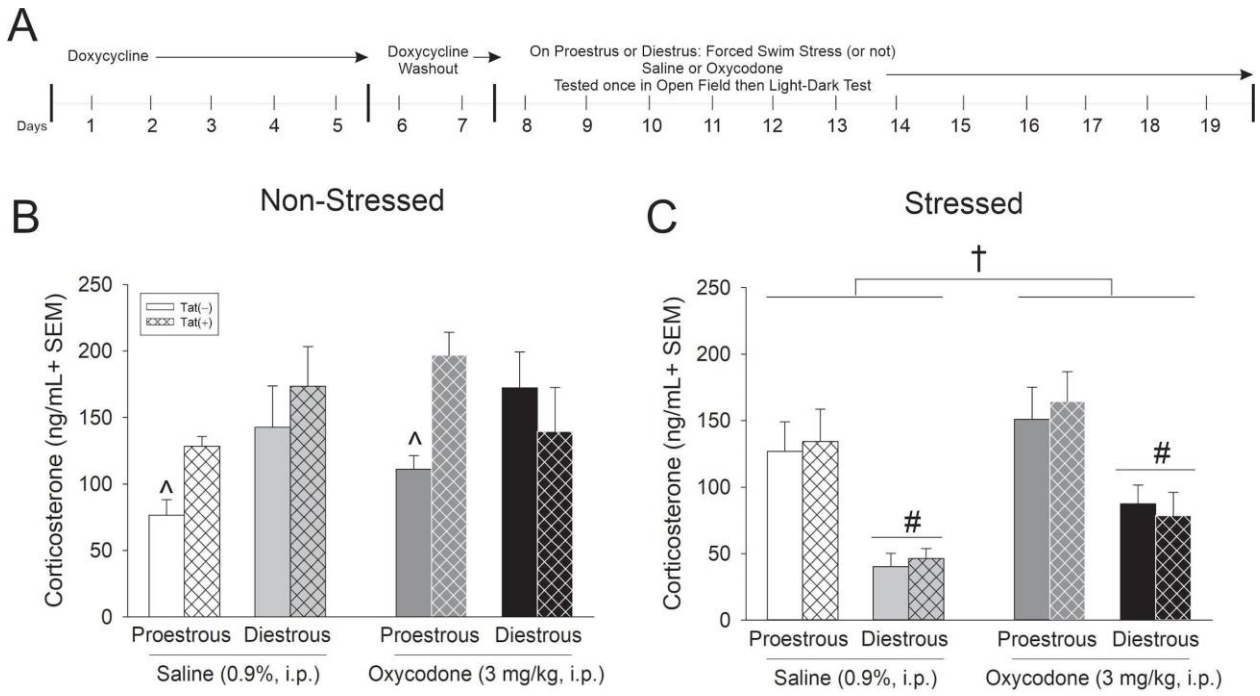

Figure 30: Effect of acute oxycodone exposure on circulating corticosterone in non-stressed and stressed adult female HIV-1 Tat transgenic mice. Ref. © 2021 Salahuddin et al., 2021c, Licensee MDPI, Basel, Switzerland distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

(**A**) Circulating corticosterone concentrations obtained from Tat-transgenic, female mice [hatched bars; Tat(+)] or control counterparts [open bars; Tat(−) controls] (n = 9–10/group). Corticosterone was assessed in (**B**) non-stressed, (**C**) stressed mice. ^ indicates an interaction wherein the denoted group differs from all other groups in panel B. † indicates a main effect for oxycodone to differ from saline administered mice in panel C. # indicates a main effect for proestrous mice to differ from diestrous mice in panel C, $p < 0.05$.

Diestrous mice revealed higher estradiol when stressed and higher basal progesterone levels

Circulating steroid concentrations were assessed in serum ~2 h following saline or opioid injection when HPA activation is expected to be resolving. A 15 min forced swim was used to activate the HPA axis in the stressed paradigm (or not in the non-stressed paradigm). On the day of testing, all mice received saline or oxycodone (3 mg/kg, i.p.) 15 min prior to behavioral assessment (Fig. 31A). No significant differences were observed in circulating estradiol (Fig. 31B).

Among swim stress-exposed mice, diestrous mice had significantly greater estradiol levels than did proestrous mice [$F(1,71) = 24.60$, $p < 0.05$] (see #, Fig. 31C). Circulating progesterone was significantly greater among diestrous, compared to proestrous, mice [$F(1,70) = 14.84$, $p < 0.05$] (see #, Fig. 31B'). No significant differences were observed in circulating progesterone among stressed mice (Fig. 31C'; Salahuddin et al., 2021c).

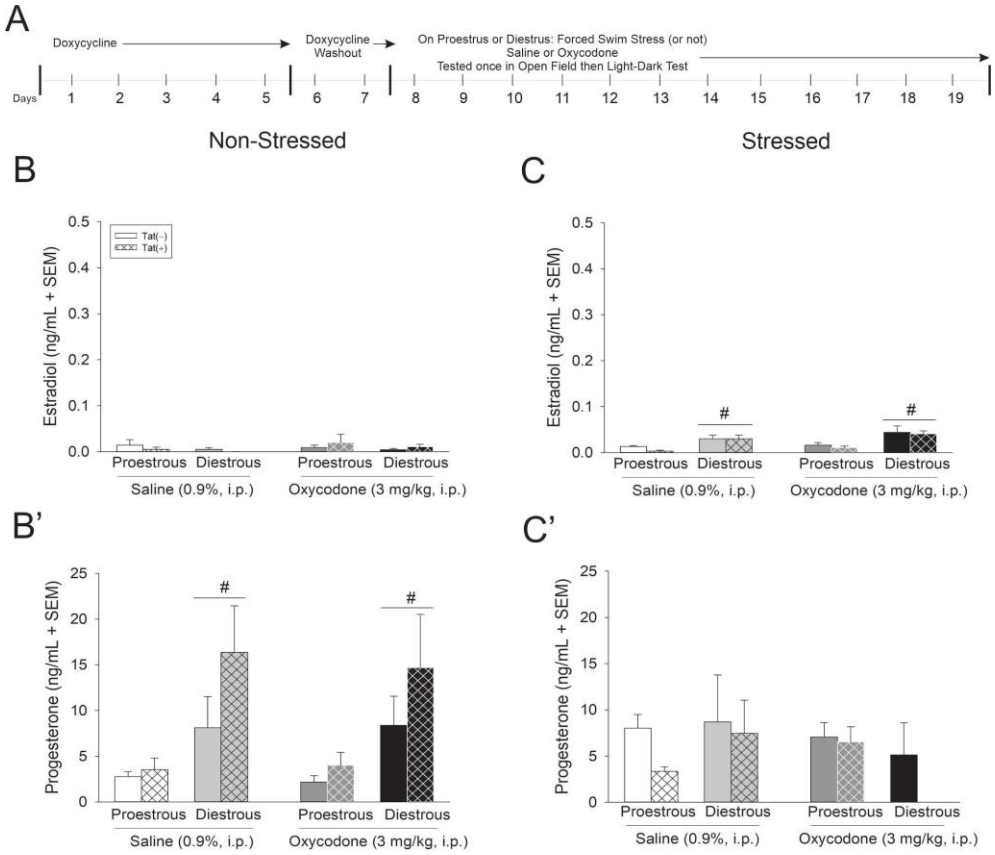

Figure 31: Effect of acute oxycodone exposure on circulating estradiol and progesterone in non-stressed and stressed adult female HIV-1 Tat transgenic mice.
(**A**) Circulating steroid concentrations obtained from Tat-transgenic, female mice [hatched bars; Tat(+)] or control counterparts [open bars; Tat(−) controls] described in timeline (n = 6–10/group). Estradiol and progesterone were assessed in (**B–B'**) non-stressed, (**C–C'**) stressed mice. # indicates a main effect for proestrous mice to differ from diestrous mice in panels **B'** and **C**, $p < 0.05$.

Either Estradiol or Progesterone can ameliorate the direct neurotoxic effects of HIV-1 Tat

Given that HIV-1 Tat's behavioral effects were influenced by oxycodone administration and estrous cycle phase, the capacity for E_2 or P_4 to protect against combined oxycodone/Tat neurotoxicity was assessed. Differentiated SH-SY5Y human neuroblastoma cells were exposed to vehicle, Tat (100nM), a saturating concentration of oxycodone (500nM), and low-to-high physiological E_2 (1 or 10 nM) or P_4 (10 or 100 nM) for 20 h (Fig. 32).

Steroid concentrations protected against HIV-1 Tat-mediated neurotoxicity in a concentration-dependent manner. Treatment with Tat and either E_2 [$F(2,155) = 3.36$, $p < 0.05$; $\eta^2 = 0.04$] or P_4 [$F(2,192) = 3.72$, $p < 0.05$; $\eta^2 = 0.04$] significantly interacted to influence cell death (Fig. 32). Tat significantly increased the proportion of cell death (see *, Fig. 32 A-D) and either 1 or 10 nM E_2 significantly attenuated this effect (see #, Fig. 32A,B). Whereas, treatment with 100 nM, but not 10 nM, P_4 significantly protected against Tat-mediated cell death (see #, Fig. 32C,D). Oxycodone did not significantly influence Tat-mediated death or steroid hormone protection (Salahuddin et al., 2020a).

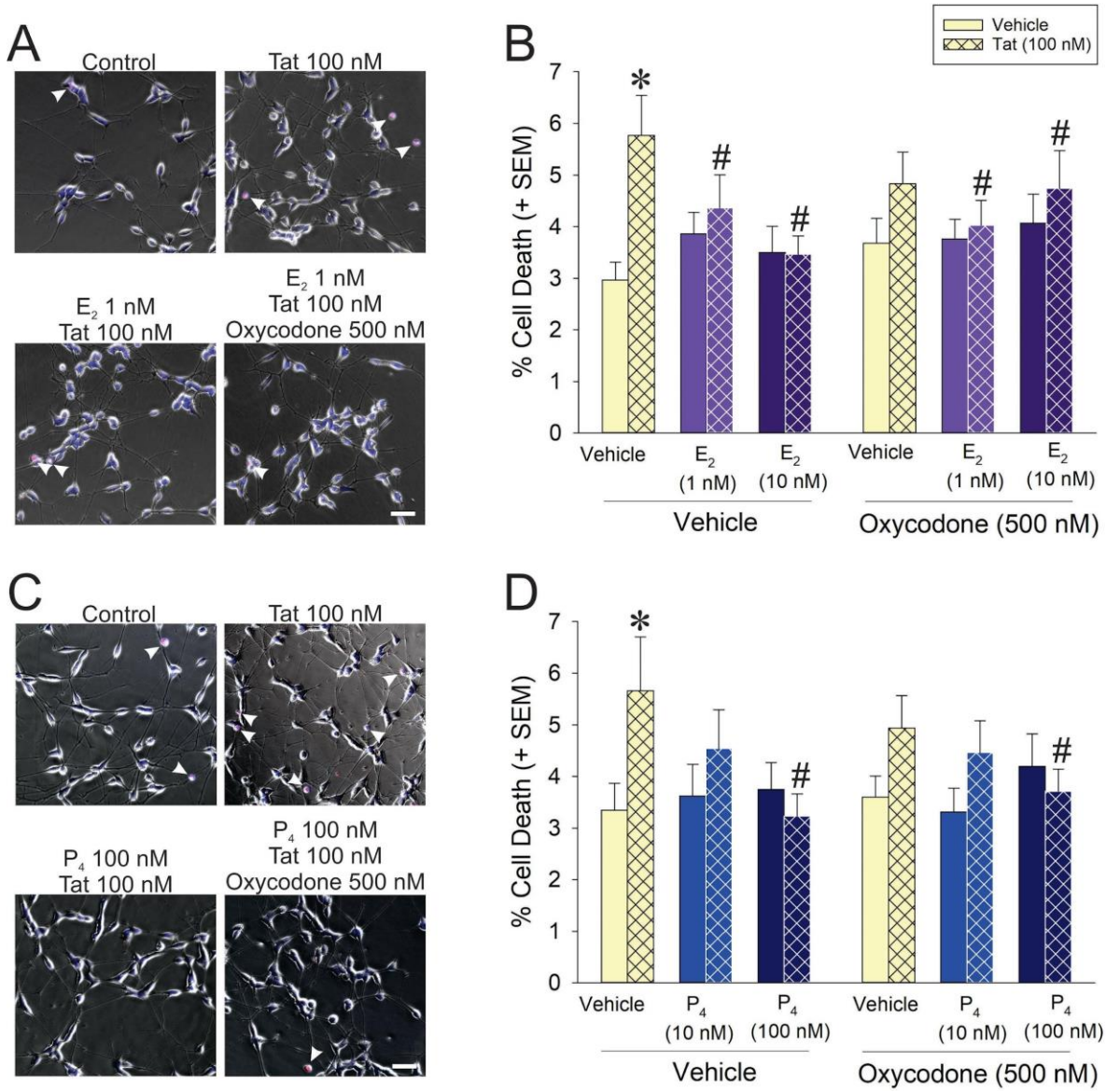

Figure 32: Proportion of cell death among differentiated SH-SY5Y human neuroblastoma cells. Ref. Salahuddin et al., 2020a

Proportion of cell death among differentiated SH-SY5Y human neuroblastoma cells that were exposed to vehicle, oxycodone, and/or Tat (100 nM) concurrent with (**A,B**) estradiol (E_2) 1 or 10 nM (n = 13–14) or (**C,D**) progesterone (P_4) 10 or 100 nM (n = 17). White arrows indicate dead cells. Scale bar = 50 μm. * indicates a significant increase in cell death compared to vehicle/vehicle control; # indicates a significant reduction in cell death compared to Tat/vehicle-exposed cells, $p < 0.05$.

Oxycodone downregulated expression of ERα, GPER1, and KOR in SH-SY5Y cells

To determine if oxycodone had an effect on the expression of steroid or opioid receptor targets that may be implicated in the observed neuroprotection, qRT-PCR was conducted on differentiated SH-SY5Y cells that had been treated to medium or oxycodone (500 nM) for 20 hours. Oxycodone significantly down-regulated gene expression of ERα normalized to β-actin [$F(1,10) = 9.23$, $p < 0.05$; $\eta^2 = 0.48$; Fig. 33A] or GAPDH [$F(1,10) = 7.97$, $p < 0.05$; $\eta^2 = 0.44$; Fig. 33A'], GPER1 normalized to β-actin [$F(1,10) = 23.20$, $p < 0.05$; $\eta^2 = 0.70$; Fig. 33B] or GAPDH [$F(1,10) = 13.19$, $p < 0.05$; $\eta^2 = 0.57$; Fig. 33B'], and KOR normalized to β-actin [$F(1,10) = 11.90$, $p < 0.05$; $\eta^2 = 0.54$; Fig. 33C] or GAPDH [$F(1,10) = 9.31$, $p < 0.05$; $\eta^2 = 0.48$; Fig 33C']. Oxycodone produced a small, but significant, up-regulation of mPRα when normalized to GAPDH [$F(1,10) = 5.36$, $p < 0.05$; $\eta^2 = 0.35$], but not when normalized to β-actin (Table 4). No significant differences in the expression of ERβ, PR, mPRβ, MOR, or DOR were observed (Salahuddin et al., 2020a).

Table 4: Fold changes in the mRNA expression of E2, P4 and opioid receptor targets in differentiated SH-SY5Y neuroblastoma cells. Ref. Salahuddin et al., 2020a

Differentiated SH-SY5Y neuroblastoma cell mRNA expression (mean ± SEM) of estrogen receptor (ER) α, ERβ, G-protein coupled estrogen receptor 1 (GPER1), progestin receptor (PR), membrane PR α (mPRα), mPRβ, μ opioid receptor (MOR), κ opioid receptor (KOR), and δ opioid receptor (DOR) normalized to β-actin or GAPDH (calculated $2^{-\Delta\Delta C_T}$ method).

	Normalized to β-actin		**Normalized to GAPDH**	
	Control	**Oxycodone (500nM)**	**Control**	**Oxycodone (500nM)**
β -actin	-	-	1.01±0.05	1.03±0.07
GAPDH	1.01±0.05	1.01±0.08	-	-
ERβ	1.00±0.03	1.05±0.13	1.00±0.03	1.02±0.06
PR	1.02±0.10	0.80±0.06	1.03±0.11	0.81±0.05
mPRα	1.01±0.07	1.2±0.06	1.01±0.07	1.21±0.05*
mPRβ	1.00±0.01	0.95±0.08	1.00±0.01	0.96±0.08
MOR	1.01±0.05	1.09±0.16	1.01±0.05	1.11 ±0.18
DOR	1.04±0.13	1. 13±0.12	1.05±0.15	1. 13±0.11

*indicates significant difference from respective GAPDH control, $p < 0.05$.

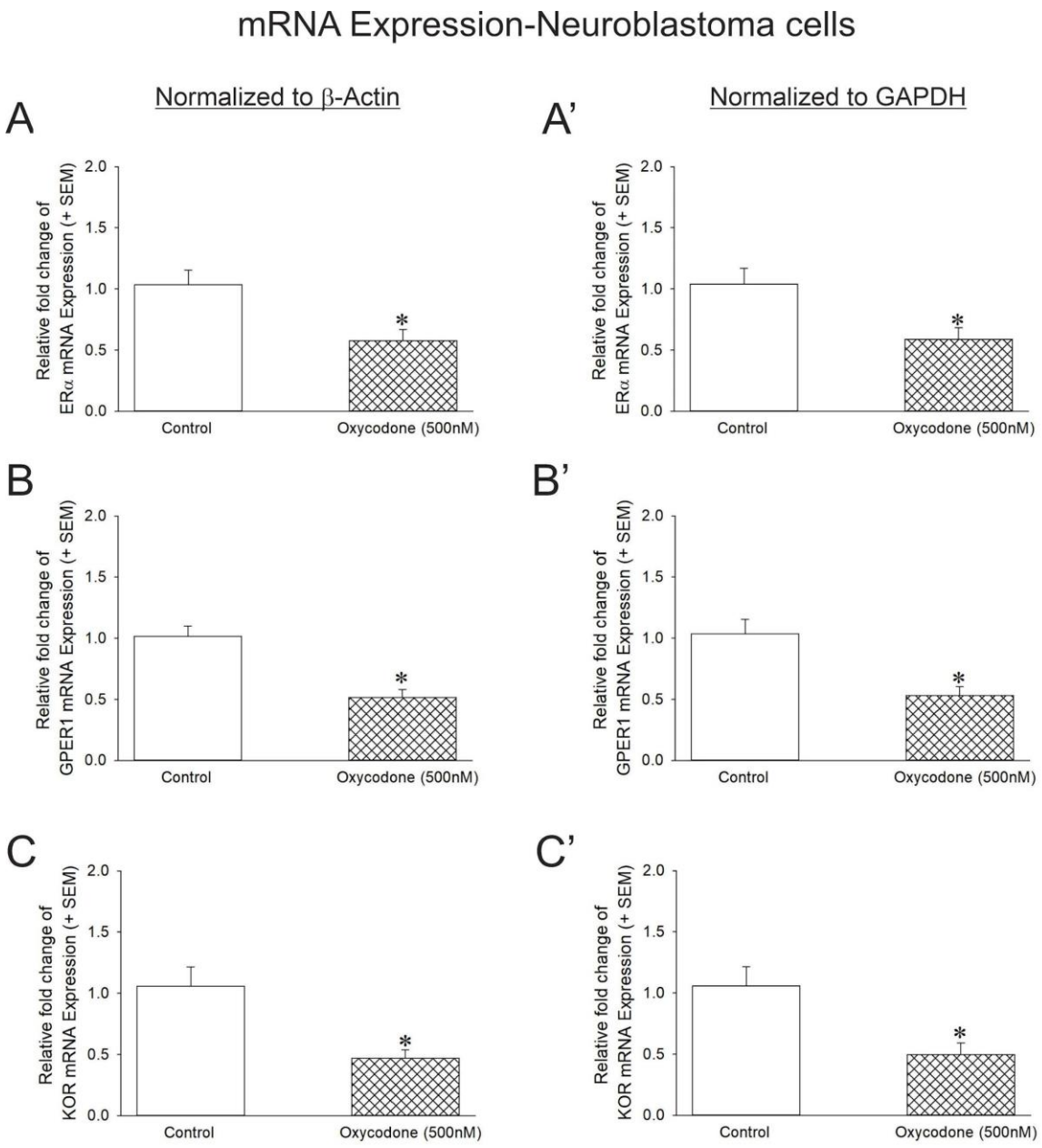

Figure 33: Fold changes of ERα, GPER1, KOR mRNA expression among differentiated SH-SY5Y human neuroblastoma cells that were exposed to vehicle and oxycodone.

Fold changes in the mRNA expression among differentiated SH-SY5Y human neuroblastoma cells that were exposed to vehicle and oxycodone (500nM) (**A, A'**) Estradiol receptor α (ER α) (n = 3) or (**B, B'**) G Protein-Coupled Estrogen Receptor 1 (GPER 1) (n = 3) or (**C, C'**) Kappa Opioid Receptor (KOR) (n = 3). * indicates a significant decrease in receptor expression compared to media control, $p < 0.05$.

B. Male HIV-1 Tat transgenic mice

Acute oxycodone interacts with HIV-1 Tat expression to elevate basal corticosterone and cause adrenal insufficiency on exposure to a natural stressor.

Tat-tg male mice had HIV-1 Tat expression induced (or not) via doxycycline administration for five days. After two days of doxycycline washout (to limit non-specific anti-inflammatory effects), mice were (or were not) exposed to 15-min swim stress. Following stress, mice were acutely administered an injection of saline (0.9%, i.p.) or oxycodone (3 mg/kg, i.p.), and psychomotor and anxiety-like behavior were assessed 15 min later. Following behavior testing, mice were sacrificed and trunk blood was collected and corticosterone was extracted via ether-steroid extraction protocol (Fig. 34A; Salahuddin et al., 2020b).

When circulating corticosterone was assessed in the non-stressed HIV-1 Tat males, a significant interaction between genotype and oxycodone administration was revealed [$F(1,31) = 5.15$, $p < 0.05$] (Fig. 34B). Tat(+) mice had significantly greater basal corticosterone compared to Tat(−) mice ($p = 0.02$; Fig. 34B; see *). Oxycodone increased corticosterone in Tat(−) mice such that this genotype difference was obviated following drug administration (Fig. 34B; Salahuddin et al., 2020b).

As expected, circulating corticosterone was greater among Tat(−) mice that underwent swim stress; however, their Tat(+) counterparts mounted a significantly reduced response in comparison [$F(1,29) = 13.88$, $p < 0.05$] (Fig. 34C; see *). Together, these data demonstrate that HIV-1 Tat expression in male mice increases basal corticosterone, but produces an adrenal insufficiency upon HPA activation, recapitulating the clinical phenotype reported among HIV^+

patients (Salahuddin et al., 2020b).

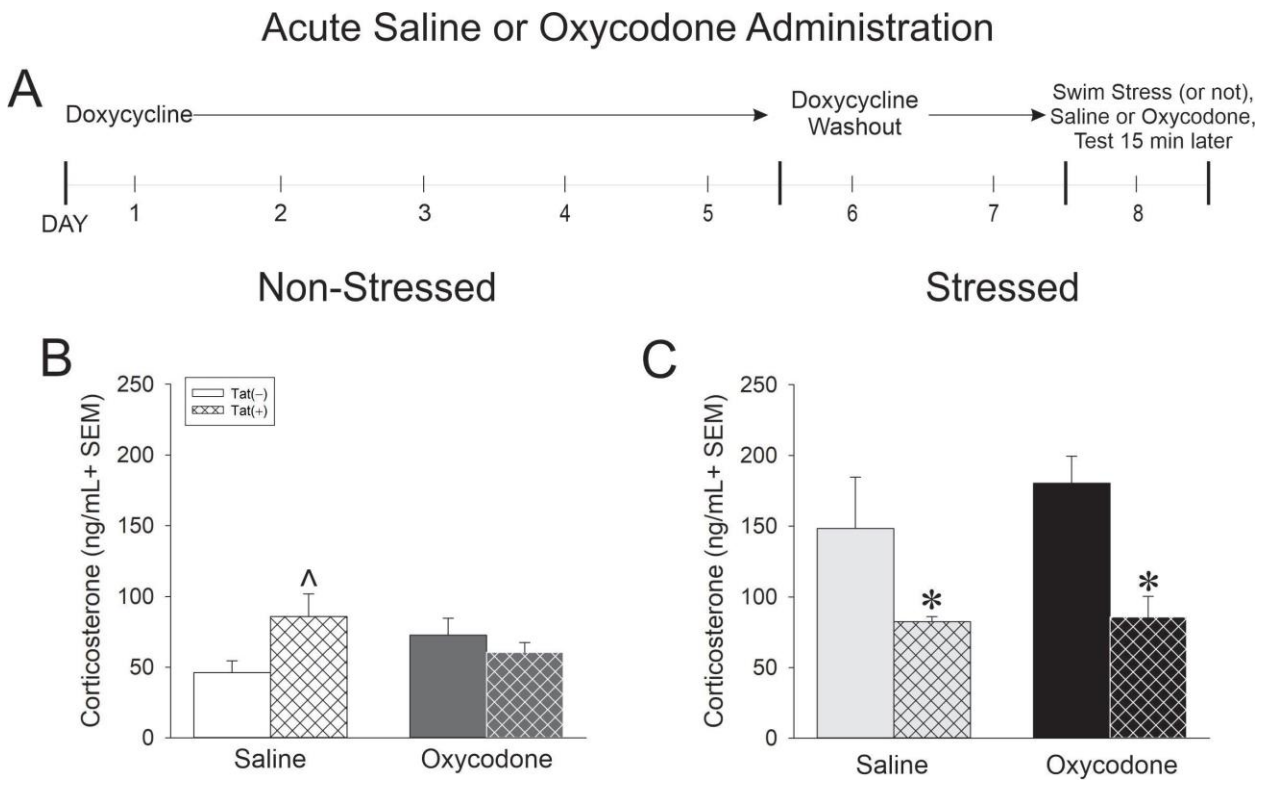

Figure 34: Effect of acute oxycodone exposure on circulating corticosterone in non-stressed and stressed adult male HIV-1 Tat transgenic mice. Ref. © 2020 Salahuddin et al., 2020b, Licensee MDPI, Basel, Switzerland distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

(**A**) Human immunodeficiency virus (HIV)-1 trans-activator of transcription (Tat) expression was induced in Tat(+) males (hatched bars), or not induced in Tat(−) controls (open bars), via administration of doxycycline (30 mg/kg, i.p., once daily for 5 days with 2 days of washout). Mice were either stressed via forced swim for 15 min (panel B) or not (panel C) and acutely-administered saline or oxycodone (3 mg/kg, i.p.) 15 min prior to assessment in an open field and light-dark transition test (n = 8–12/group). (**B**) circulating corticosterone among non-stressed mice. (**C**) circulating corticosterone among stressed mice. * indicates a main effect of genotype wherein Tat(+) mice differ from Tat(−) controls. ∧ indicates an interaction wherein saline-administered Tat(+) mice differ from respective Tat(−) controls, $p < 0.05$.

Repeated Exposure to Oxycodone increases the glucocorticoid stress response

While the initial opioid response is indicative of later abuse liability, many HIV^+ patients have been prescribed oxycodone and are exposed repeatedly. How repeated oxycodone exposure modifies the HPA stress response in Tat-exposed mice was of interest. Tat-tg male mice had HIV-1 Tat expression induced (or not) via doxycycline administration for five days (with two days of washout). During this time, mice received daily injections of saline (0.9%, i.p.) or oxycodone (3 mg/kg, i.p.). Mice were (or were not) exposed to 15-min swim stress prior to testing (Fig. 35A). Repeated oxycodone exposure significantly elevated circulating corticosterone levels among Tat(+) mice [$F(1,31) = 14.79$, $p < 0.05$; see *, Fig. 35B]. As observed in acutely administered saline/oxycodone males, swim stress increased circulating corticosterone; however, repeated oxycodone significantly interacted with genotype such that stress-exposed Tat(+) mice demonstrated a greater increase in corticosterone following repeated oxycodone injection [$F(1,29) = 9.60$, $p < 0.05$] (Fig. 35C; see §; Salahuddin et al., 2020b).

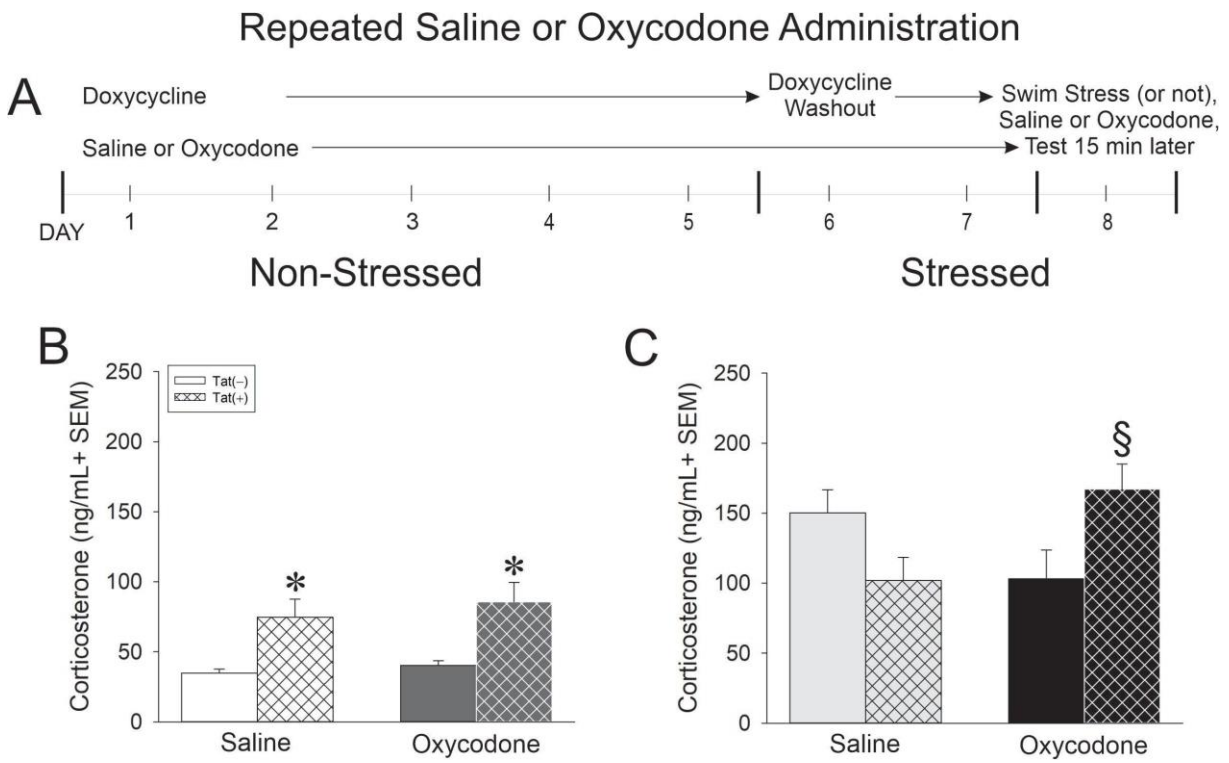

Figure 35: Effect of repeated oxycodone exposure on circulating corticosterone in non-stressed and stressed adult male HIV-1 Tat transgenic mice. Ref. © 2020 Salahuddin et al., 2020b, Licensee MDPI, Basel, Switzerland distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

(**A**) Tat(−) (see open bars) and Tat(+) (see hatched bars) mice were administered saline or oxycodone (3 mg/kg, i.p., once daily for 7 days) concurrent with the induction of HIV-1 Tat via doxycycline (30 mg/kg, i.p., once daily for 5 days with 2 days of doxycycline washout). Mice were stressed via forced swim for 15 min (panel **B**) or not (panel **C**), were administered the last treatment of repeated saline or oxycodone, and 15 min later were assessed in an open field and light dark transition test (n = 8–10/group). (**B**) circulating corticosterone in among non-stressed mice. (**C**) circulating corticosterone among stressed mice. * indicates a main effect of genotype wherein Tat(+) mice differ from Tat(−) controls. § indicates an interaction wherein oxycodone-administered Tat(+) mice differ from their respective Tat(−) controls & Tat(+) saline-administered mice, $p < 0.05$.

The Time-Course of HPA Axis Activation Was Influenced by Oxycodone Exposure

To account for differences in corticosterone levels across different time points, the concentration of circulating corticosterone was measured in plasma from blood samples collected at 5, 30, and 120 min post-injection of either saline or oxycodone. Oxycodone administration significantly interacted with the time from injection [$F(2,32) = 6.11$, $p < 0.05$]. Irrespective of genotype, at t_5 oxycodone-administered mice, exhibited significantly lower circulating corticosterone compared to saline-administered mice ($p = 0.03$; Fig. 36B; see †). At t_{30}, oxycodone produced peak plasma corticosterone, significantly differing from t_5 ($p = 0.002$; Fig. 36B; see #) and t_{120} ($p = 0.0006$; Fig. 36B; see #). At t_{120}, saline-administered mice demonstrated significantly lower circulating plasma corticosterone as compared to either t_5 ($p < 0.0001$; Fig. 36A; see #) or t_{30} ($p < 0.0001$; Fig. 36A; see #; Salahuddin et al., 2020b).

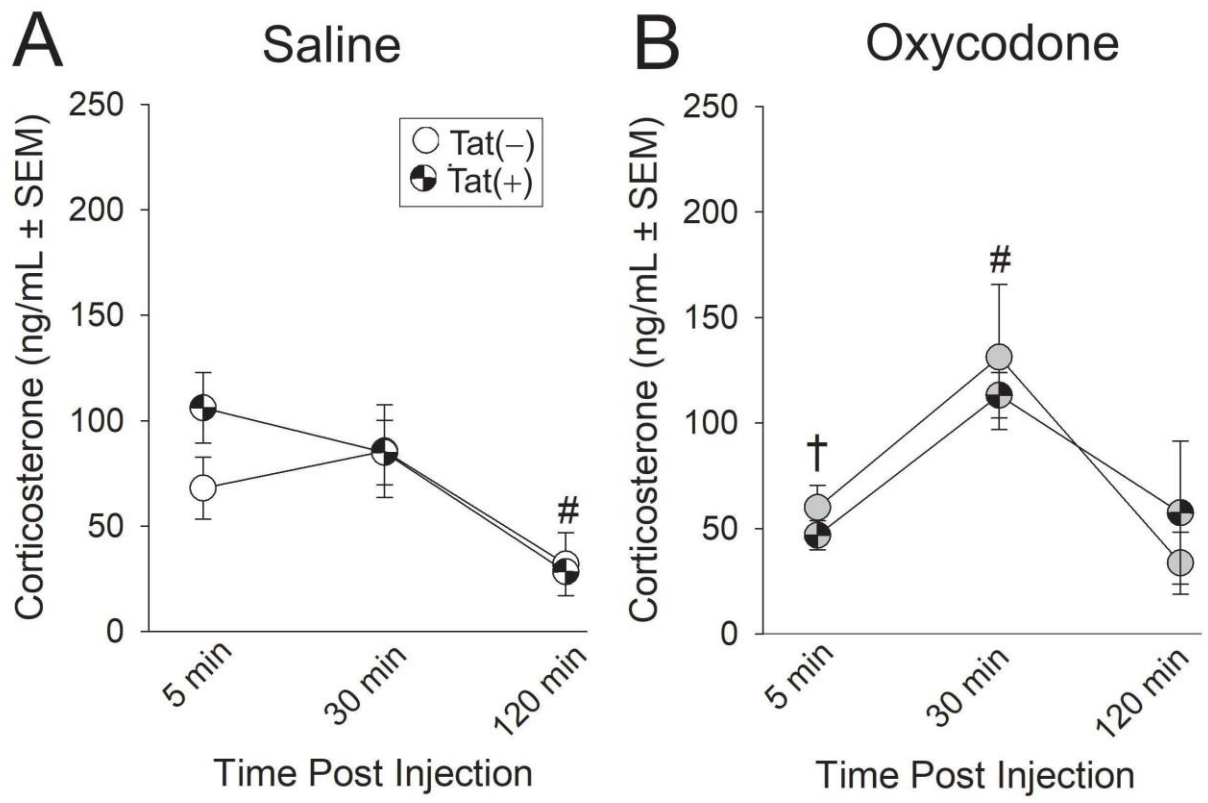

Figure 36: Time-course of HPA axis activation following acute saline or oxycodone exposure in adult male HIV-1 Tat transgenic mice. Ref. © 2020 Salahuddin et al., 2020b, Licensee MDPI, Basel, Switzerland distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

Tat(−) (see open circles) and Tat(+) (see hatched circles) mice (n = 5/group) were acutely-administered (**A**) saline (white circles) or (**B**) oxycodone 3 mg/kg, i.p., (gray circles) and circulating corticosterone was measured from serum collected by tail-bleed at 5, 30, and 120 min post-injection. † indicates an interaction wherein oxycodone-administered mice differ from saline-administered mice at t_5; # indicates an interaction wherein the indicated group differs from their respective t_5 and t_{30} time-points in panel **A** and t_5 and t_{120} time-points in panel **B**, $p < 0.05$.

Aim 2: Assess the pharmacodynamic targets (glucocorticoid receptor or CRF receptor) via systemic administration of pharmacological antagonists.

A. **Female HIV-1 Tat transgenic mice**

HPA/HPG blockade did not cause adrenal insufficiency in female HIV-1 Tat mice

Circulating corticosterone concentration was obtained from Tat-expressing (or not) mice behaviorally assessed in an open field and light-dark transition test following systemic HPA/HPG blockade (Fig. 37A). Pharmacologically antagonizing receptors that mediate HPA feedback or removing the primary source of gonadal steroids influenced circulating corticosterone. Either pretreating mice with Antalarmin (CRF-R blockade) or RU-486 (GR blockade), or conducting OVX (HPG blockade), significantly increased circulating corticosterone [$F(3,125) = 39.65$, $p < 0.05$] (see @, Fig. 37B). However, only OVX Tat(+) mice demonstrated a significant corticosterone increase compared to their respective Tat(−) controls [$F(1,125) = 6.1$, $p < 0.05$] (see *, Fig. 37B). Among pretreatments, GR inhibition via RU-486 increased circulating corticosterone to a greater degree than other manipulations [$F(3,125) = 39.65$, $p < 0.05$] (see §, Fig. 37B; Salahuddin et al., 2021c).

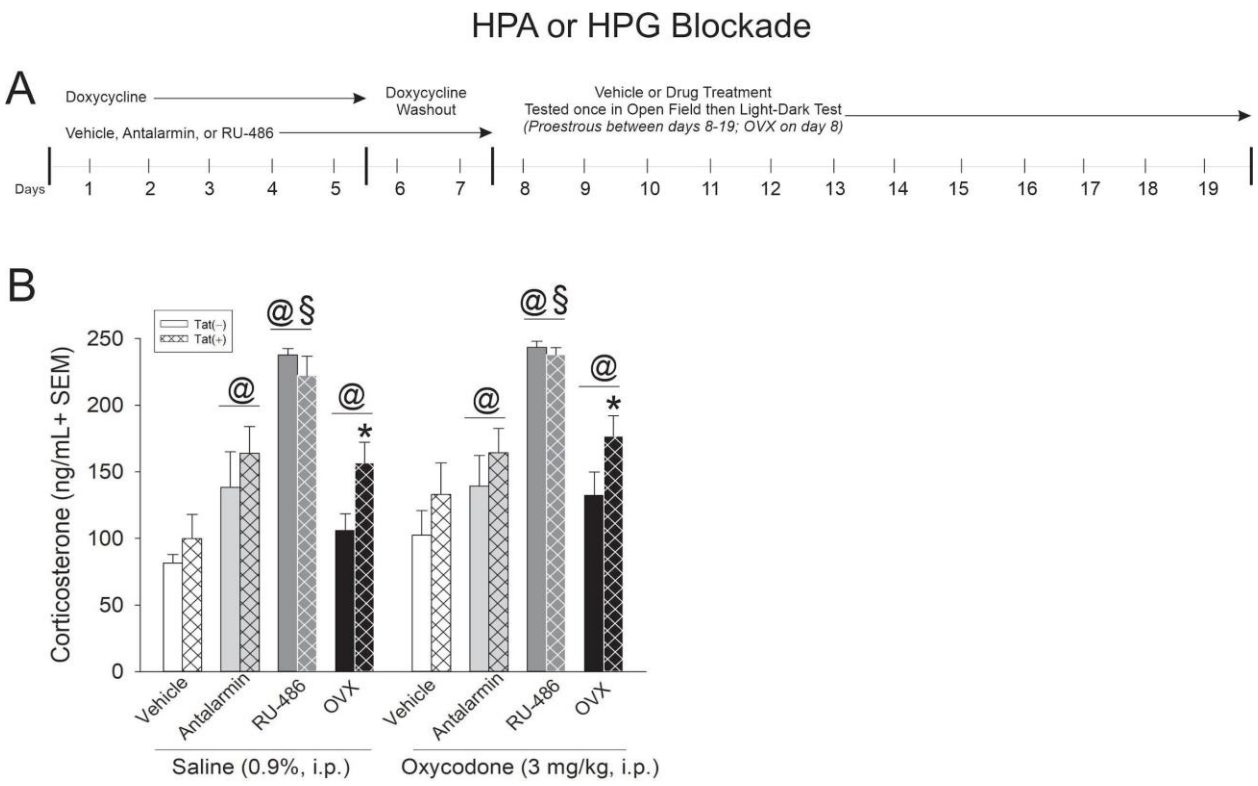

Figure 37: Effect of acute oxycodone exposure on circulating corticosterone via pharmacological HPA (CRF/GR) and HPG blockade (ovariectomy) in adult female HIV-1 Tat transgenic mice. Ref. © 2021 Salahuddin et al., 2021c, Licensee MDPI, Basel, Switzerland distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

(**A**) Circulating steroid concentrations obtained from Tat-transgenic, female mice [hatched bars; Tat(+)] or control counterparts [open bars; Tat(−) controls] as described in the timeline (n = 7–10/group). Corticosterone was assessed in (**B**) HPA or HPG manipulated mice. § indicates an interaction wherein mice pretreated with RU-486 differ from all other groups in panel B. @ indicates an interaction wherein the denoted group differs from their respective vehicle controls in panel B. * indicates a main effect of genotype wherein Tat(+) mice differ from Tat(−) controls in panel B, $p < 0.05$.

Tat and oxycodone exposure influenced circulating estradiol and progesterone levels in HPA manipulated female HIV-1 Tat mice

Circulating estradiol and progesterone concentrations was obtained from Tat-expressing (or not) mice behaviorally assessed in an open field and light-dark transition test following systemic HPA blockade (Fig. 38A). Pharmacologically antagonizing receptors that mediate HPA feedback influenced circulating estradiol and progesterone. When circulating estradiol was assessed, oxycodone-administered Tat(+) mice demonstrated greater circulating concentrations than did any other group [$F(2,97) = 3.23$, $p < 0.0001–0.0239$] (see ‡, Fig. 38B). Tat exposure and HPA receptor antagonism interacted to alter circulating progesterone [$F(2,97) = 3.43$, $p < 0.05$] (see ^, Fig. 38C). Blocking GRs via RU-486 increased circulating progesterone, irrespective of Tat exposure. However, blocking CRF-Rs via antalarmin only increased progesterone among Tat(−) control mice ($p = 0.0002–0.0020$; see ^, Fig. 38C). Additionally, HPA receptor antagonism and oxycodone administration interacted [$F(2,97) = 4.45$, $p < 0.05$] (see @, Fig. 38C). Blocking GRs via RU-486 increased progesterone, irrespective of oxycodone administration. Blocking CRF-Rs via antalarmin only increased progesterone among oxycodone-treated mice ($p < 0.0001–0.0493$; see @, Fig. 38C; Salahuddin et al., 2021c).

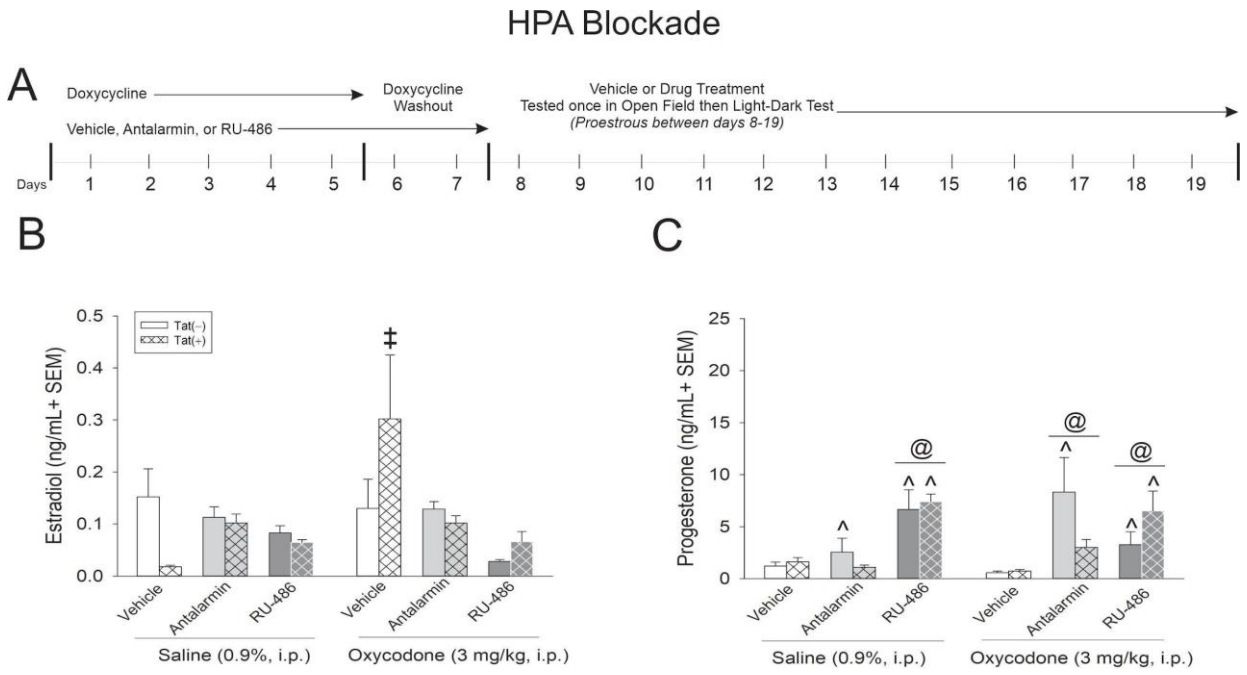

Figure 38: Effect of acute oxycodone exposure on circulating estradiol and progesterone via pharmacological HPA blockade (CRF/GR antagonism) in adult female HIV-1 Tat transgenic mice. Ref. © 2021 Salahuddin et al., 2021c, Licensee MDPI, Basel, Switzerland distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

(**A**) Circulating steroid concentrations obtained from Tat-transgenic, female mice [hatched bars; Tat(+)] or control counterparts [open bars; Tat(−) controls] as described in the timeline A, (n = 7–10/group). (**B**) Estradiol and (**C**) progesterone were assessed in HPA manipulated mice. ‡ indicates an interaction wherein the denoted group differs from all other groups in panel B, $p < 0.05$. @ indicates an interaction wherein the denoted group differs from their respective vehicle controls in panel C. ^ indicates an interaction wherein the denoted group differs from all other groups in panel C, $p < 0.05$.

Acute Oxycodone Interacted with Tat Exposure to Influence Hypothalamic Allopregnanolone

Hypothalamic allopregnanolone (ng/g) was measured from Tat(−) and Tat(+) mice exposed to a stressor (or not) when in proestrous, diestrous phase or ovariectomized (OVX) (Fig. 39A). Hypothalamic allopregnanolone content was greater among diestrous, compared to proestrous mice, in the non-stressed [$F(1,56) = 36.02$, $p < 0.05$] (see #, Fig. 39B) and stressed paradigms [$F(1,56) = 21.42$, $p < 0.05$] (see #, Fig. 39C). Moreover, Tat exposure interacted with oxycodone

and estrous cycle phase to influence hypothalamic allopregnanolone content among non-stressed [$F(1,56) = 4.02$, $p < 0.05$] (see §, Fig. 39B) and stressed mice [$F(1,56) = 4.09$, $p < 0.05$] (see § and ^, Fig. 39C). Among non-stressed mice, oxycodone-administered Tat(−) controls demonstrated greater allopregnanolone in the diestrous phase of their cycle than did their saline-administered counterparts or any proestrous group ($p < 0.0001$–0.0066, see §, Fig. 39B). Among stressed mice, Tat(+) saline-administered mice demonstrated greater allopregnanolone in the diestrous phase of their cycle than did any other proestrous group ($p < 0.0001$–0.0298, see §, Fig. 39C) or their oxycodone-administered diestrous counterparts ($p = 0.03$, see ^, Fig. 39C). No differences in hypothalamic allopregnanolone were observed among OVX mice (Fig. 39D), despite an apparent basal increase compared to naturally-cycling mice (Salahuddin et al., 2021c).

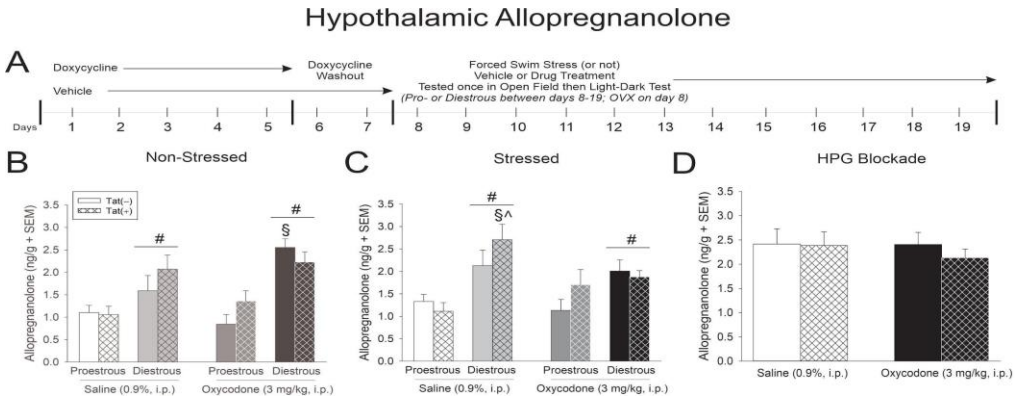

Figure 39: Effect of acute oxycodone exposure on hypothalamic allopregnanolone in non-stressed, stressed and ovariectomized adult female HIV-1 Tat transgenic mice. Ref. © 2021 Salahuddin et al., 2021c, Licensee MDPI, Basel, Switzerland distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

Proestrous, diestrous or ovariectomized (OVX) Tat(−) and Tat(+) mice had allopregnanolone content (ng/g) assessed in the hypothalamus in (**A**) non-stressed, (**B**) stressed, and (**C**) OVX mice (n = 8/group). # indicates a main effect for diestrous mice to differ from proestrous mice. § indicates an interaction wherein the denoted group differs from Tat(−) or Tat(+) proestrous mice. ^ indicates an interaction wherein the denoted group differs from oxycodone-administered, Tat(+) diestrous mice, $p < 0.05$.

B. **Male HIV-1 Tat transgenic mice**

HPA blockade cause adrenal insufficiency in male HIV-1 Tat mice

Transgenic mice expressing Tat (or not) were administered RU-486 and antalarmin and assessed in the behavioral battery of open field and light-dark transition test (Fig. 40A). GR and CRF-R inhibitors significantly interacted with genotype to influence circulating corticosterone concentrations [$F(3,121) = 11.54$, $p < 0.05$]. Pretreatment with RU-486 (alone or in conjunction with antalarmin) produced a significant and large increase in circulating corticosterone among Tat(−) mice ($p < 0.0001$; Fig. 40B; see #), presumably by blocking negative feedback within the HPA axis. However, among Tat(+) mice the RU-486-induced increase in corticosterone was present ($p < 0.0001$; Fig. 40B; see #), but significantly attenuated compared to that observed in Tat(−) controls ($p < 0.0001$–0.0006 Fig. 40B; see *), further supporting a Tat-induced adrenal insufficiency. Antalarmin did not influence corticosterone levels on its own (Salahuddin et al., 2020b).

The proportional change in corticosterone from baseline was also analyzed in order to account for differences in basal levels (Fig. 40C). Tat, oxycodone administration, and pharmacological inhibitors significantly interacted to influence the proportional increase in corticosterone. Among Tat(−) mice, oxycodone proportionally increased circulating corticosterone ($p = 0.002$–0.007; Fig. 40C; see †), and this was attenuated by RU-486 ($p = 0.002$–0.003; Fig. 40C; see #), but not antalarmin. Tat(+) mice did not generate the proportional increase observed in Tat(−) controls in response to oxycodone ($p = 0.0002$–0.001; Fig. 40C; see *) but did generate a modest, but significant increase following RU-486 ($p = 0.03$; Fig. 40C; see #; Salahuddin et al., 2020b).

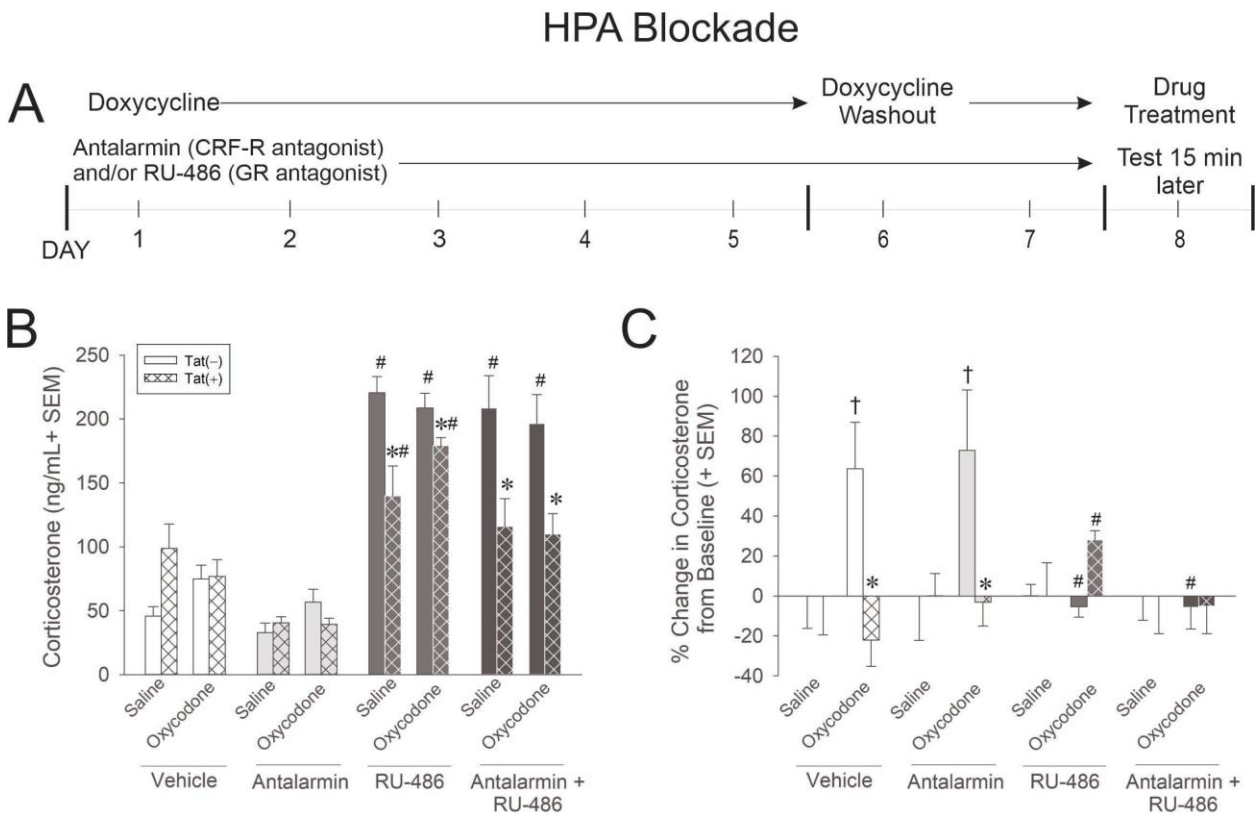

Figure 40: Effect of acute oxycodone exposure on circulating corticosterone and percentage change in corticosterone from baseline via pharmacological HPA blockade in adult male HIV-1 Tat transgenic mice. Ref. © 2020 Salahuddin et al., 2020b, Licensee MDPI, Basel, Switzerland distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

(**A**) Tat(−) (see open bars) and Tat(+) (see hatched bars) mice were administered antalarmin (corticotrophin-releasing factor receptor antagonist; 20 mg/kg, i.p. for 6 days) and/or RU-486 (glucocorticoid receptor antagonist; i.p., 20 mg/kg, i.p. for 7 days) concurrent with the induction of HIV-1 Tat via doxycycline (30 mg/kg, i.p., once daily for 5 days with 2 days of doxycycline washout). Mice were treated with the final dose of antalarmin and/or RU-486 and then challenged with saline or oxycodone (3 mg/kg, i.p.) and assessed in an open field and light-dark transition test (n = 8–10/group). (**B**) Circulating corticosterone and (**C**) the proportional change from baseline in circulating corticosterone. * indicates an interaction wherein Tat(+) mice differ from respective Tat(−) controls. # indicates an interaction wherein the denoted group differs from their respective vehicle controls, $p < 0.05$.

1.4. Discussion

The hypothesis that Tat expression in mice would dysregulate the HPA axis was upheld. This dissertation showed for the first time that Tat exposure was sufficient to dysregulate HPA confirmed by elevated basal corticosterone levels and seemingly paradoxical adrenal insufficiency in males on exposure to a swim stressor or pharmacological blockade of HPA feedback loop. Intriguingly females did not demonstrate adrenal insufficiency when exposed to a swim stressor or pharmacological blockade of HPA feedback loop, indicative of the protective role of gonadal hormones to offset the HPA insult. Oxycodone enhanced HPA activation markers acutely, but tolerance was developed with repeated administration. Cell culture studies reveal no direct cytotoxicity due to oxycodone, and E_2 or P_4 may mitigate Tat's neurotoxic effects without interfering with oxycodone. Particularly, oxycodone inhibited the expression of estrogen receptor targets (ER and GPER1) and KOR, which may be sites of direct or indirect interaction. These findings are consistent with the clinical presentation of increased basal cortisol coupled with secondary adrenal insufficiency, which occurs in up to 46% of HIV^+ patients (Marik et al., 2002). Moreover, Tat exposure dysregulated neurosteroidogenesis which occurred concurrent to HPA axis dysregulation. Thus, maintaining the neuroendocrine axis may be beneficial in combined HIV-1 and oxycodone-mediated neuropathology. Gonadal steroid-based therapies may be efficacious to offset these effects in HIV^+ patients who are prescribed clinical opioids or are opioid-naive.

The HPA axis and the immune system have complex interactions. As a result of innate and adaptive immune system activation, many inflammatory signals are generated, the majority of which are circulating cytokines (Zapanti et al., 2008). These cytokines have an important role in

the defense of the organism against external insults accounting for the majority of HPA axis activation during an infectious or inflammatory illness (Zapanti et al., 2008). Tumor necrosis factor-alpha (TNF-α), interleukins (IL-1, and IL-6) all stimulate the HPA axis separately, although synergistically (Imura et al., 1991; Rivest, 2010). Activation of the HPA axis, and therefore the production of glucocorticoids, plays an important role in adaptability during infection stress by regulating the immunological inflammatory response (Zapanti et al., 2008). Glucocorticoids decrease immunological activation of inflammatory cells, inhibit the generation of cytokines (TNF-α, IL-1, IL-6) and other inflammatory mediators (Chrousos, 1995; Chrousos and Gold, 1992), and suppress some lymphocyte subsets, namely Th1 lymphocytes.

Even when peripheral and CNS viral levels are well controlled, Tat has been detected in the CNS of people on cART (Henderson et al., 2019). Tat has been demonstrated to cause direct neurotoxicity (Sabatier et al., 1991), synaptic loss (Kim et al., 2008), and the activation of pro-inflammatory genes in the host (Buonaguro et al., 1992). Tat absorbed by bystander cells may enhance the production of proinflammatory chemokines and cytokines such as CCL2, TNF-α, IL-2, IL-6, IL-8, IL-1, and CXCL1 (Ambrosino et al., 1997; Conant et al., 1998; Kim et al., 2004; Kutsch et al., 2000; Mayne et al., 1998; Westendorp et al., 1994; Zou et al., 2010). In addition, Tat-expressing transgenic mice revealed increased levels of pro-and anti-inflammatory cytokines in the brain (Fitting et al., 2010a; Gandhi et al., 2009; Gonek et al., 2017). This evidence thus reveals the ability of Tat in HPA activation, and regulation of the immune system.

The hypercortisolemia observed in Tat-expressing mice may have various causes. Tat may enhance GR transcription by accumulating positive transcription elongation factor-b on GR-responsive promoters (Kino and Chrousos, 2004). Defensive activation of GR by Tat (alone or in

combination with other proinflammatory HIV proteins like Vpr) is also possible (Chrousos and Zapanti, 2014). Increased IL-2 and IL-4 levels reduce the GR's affinity for cortisol, leading to glucocorticoid resistance (Norbiato et al., 1992). In support, we and others have found Tat to elevate proinflammatory cytokines (Fitting et al., 2010b; Gonek et al., 2018) and promote glucocorticoid resistance in cultured splenocytes (Paris et al., 2020). Proinflammatory cytokines may also enhance the GRβ isoform that inhibits the active GRα isoform, decreasing GR signaling and increasing adrenal glucocorticoids (Bamberger et al., 1995; Charmandari et al., 2005; Leung et al., 1997). It is unknown whether high corticosterone levels are advantageous owing to the hormone's anti-inflammatory effects or harmful due to its immunosuppressive characteristics. However, it may be an adaptive, although allostatic, stress response.

Counterintuitively to popular belief, HIV^+ individuals have both hypercortisolemia and adrenal insufficiency (Chrousos and Zapanti, 2014). The causes for adrenal insufficiency are unknown, however, a depletion of the “adrenal reserve” has been proposed. HIV-1 Tat's ability to disrupt steroidogenesis may be part of the mechanism. Some of the plausible mechanisms are Tat-mediated dysregulation of the metabolism of bioavailable cholesterol (Bandaru et al., 2013). Inhibition of steroidogenic enzymes by ceramides produced by Tat protein (Haughey et al., 2004). Tat also disrupts mitochondrial function, the rate-limiting organelle in steroidogenesis. Tat promotes translocation of pro-apoptotic factors into mitochondria, disrupts oxidative phosphorylation, and thereby causes elevation of reactive oxygen species (Fields and Ellis, 2019). We have also shown Tat expression in mice reduces brain deoxycorticosterone concentrations (Paris et al., 2020). Thus, despite boosting basal glucocorticoids, Tat can promote adrenal insufficiency in males by impairing steroidogenesis.

The absence of adrenal insufficiency in female mice may be explained by decreased CRF-R internalization, enhancing sensitivity to CRF (Bangasser et al., 2010). Females have reduced GR receptor density and GR translocation in the hypothalamus, decreasing negative feedback (Solomon et al., 2015; Turner and Weaver, 1985). Females may also have greater corticosteroid-binding globulin (CBG), decreasing bioavailable corticosterone, and perhaps the reserve for negative HPA feedback (Tannenbaum et al., 1997). Finally, females have higher amounts of circulating and central pregnane steroids, which may protect them from greater HPA insults and associated neurological behavioral deficits (Frye et al., 2013).

Discussion for Cell Culture Studies

The neurotoxic effects of Tat/opioid interactions on the CNS may be prevented by utilizing steroid-based therapies. Tat may activate cation receptors such NMDA receptors and voltage-gated L-type Ca^{2+} channels, causing excitotoxicity and neuronal dendritic damage (Eugenin et al., 2007; Li et al., 2008; Mattson et al., 2005; Napier et al., 2014)., effects of which are exacerbated in presence of opioids like morphine (Fitting et al., 2014a). Herein we found Tat-mediated neurotoxicity increased SH-SY5Y cell death by 1.8 times (Salahuddin et al., 2020a). Given cell lines are insult-resilient, this rise in cell death is consistent with primary neuron findings (Kim et al., 2018). Pregnane steroids may reduce some of these direct neurotoxic effects by antagonizing NMDA receptors and L-type Ca^{2+} channels, while activating $GABA_A$ receptors to restore ion homeostasis, as earlier observed with allopregnanolone (Paris et al., 2016, 2020). Indirectly, combined Tat and/opioids increase neuroinflammatory signals from glial sources, including the production of NF-κB-regulated cytokines and chemokines (especially IL-6, TNF-α, CCL2, and CCL5), to cause cell damage/death (El-Hage et al., 2005;

Fitting et al., 2014b; Hauser et al., 2012). Moreover, Tat-mediated production of TNF-α and superoxide generation, phagocytic activation, and MAPK phosphorylation have all been shown to be reduced by estradiol (E_2) (Bruce-Keller et al., 2001), and either E_2 or P_4 has been observed to attenuate the Tat-mediated increase in the proinflammatory cytokines (Härkönen and Väänänen, 2006; Su et al., 2009). Herein we found, either E_2 or P_4 was able to significantly attenuate Tat-mediated cell death, and no detrimental interaction with oxycodone was observed (Salahuddin et al., 2020a). Nevertheless, the absence of glial inputs in these cultures does not rule out possible neuroinflammatory interactions. In primary cell cultures, future research should evaluate steroid-mediated protection against indirect Tat/opioid toxicity.

Discussion Opioid Receptors mRNA expression

The clinical opioid oxycodone reduced the expression of the novel estrogen receptor genes (ERα and GPER1) in human neuroblastoma cells (Salahuddin et al., 2020a). Others have shown that the ER is involved in the desensitization of MOR, demonstrating the importance of interconnections between these two systems (Conde et al., 2016; Lagrange et al., 1997; Micevych et al., 2009). While oxycodone had no impact on MOR or DOR gene expression, it did reduce KOR expression (Salahuddin et al., 2020a), a potential target for oxycodone's antinociceptive actions (Ross & Smith, 1997).

1.5. Conclusion

The present chapter revealed that both HIV-1 Tat-expressing male and female mice develop hypercortisolemia, and only males exhibited paradoxical adrenal insufficiency upon exposure to a natural stressor (Salahuddin et al., 2020b). These data recapitulate the clinical

phenotype of HPA dysfunction (evidenced by hypercortisolemia and adrenal insufficiency). Moreover, blocking both CRF and glucocorticoid receptors in males partially reinstated the HPA response, implicating these receptors in the pathogenesis of neuroHIV (Salahuddin et al., 2020b). Conversely, CRF or GR blockade in female mice did not produce adrenal insufficiency nor did attenuate the combined Tat and oxycodone potentiated psychomotor response (Salahuddin et al., 2021c). Notably, OVX mice showed a significant elevation in the corticosterone and hypothalamic allopregnanolone levels concurrent with attenuated Tat/oxycodone interactions (Salahuddin et al., 2021c). Overall this dissertation provides the first empirical evidence of the capacity of Tat to mediate dysregulation of the HPA axis and also emphasizes the critical role of the HPG axis in females (Salahuddin et al., 2020ab, 2021c). Disruption of HPA/HPG may increase susceptibility to neuroHIV symptomatology like mood and substance use disorders.

CHAPTER 2

Interaction of Human Immunodeficiency Virus (HIV) And Opioids to promote neuroHIV Behavior

This chapter was previously published by

1. [Hormones and Behavior] [Salahuddin MF, Qrareya AN, Mahdi F, et al. Combined HIV-1 Tat and oxycodone activate the hypothalamic-pituitary-adrenal and -gonadal axes and promote psychomotor, affective, and cognitive dysfunction in female mice. Horm Behav. 2020;119:104649. doi:10.1016/j.yhbeh.2019.104649].

2. [International Journal of Molecular Sciences] [Salahuddin MF, Mahdi F, Paris JJ. HIV-1 Tat Dysregulates the Hypothalamic-Pituitary-Adrenal Stress Axis and Potentiates Oxycodone-Mediated Psychomotor and Anxiety-Like Behavior of Male Mice. Int J Mol Sci. 2020;21(21):8212. Published 2020 Nov 3. doi:10.3390/ijms21218212]

3. [Viruses] [Salahuddin MF, Mahdi F, Sulochana SP, Paris JJ. HIV-1 Tat Protein Promotes Neuroendocrine Dysfunction Concurrent with the Potentiation of Oxycodone's Psychomotor Effects in Female Mice. Viruses. 2021;13(5):813. Published 2021 Apr 30. doi:10.3390/v13050813]

Abstract

Approximately 50% of the human immunodeficiency virus (HIV) infected individuals experience motor, affective and cognitive disorders collectively termed as "neuroHIV". We and others have shown that the neurotoxic HIV-1 regulatory protein, trans-activator of transcription (Tat) promoted neurotoxicity in cell cultures and neuropathology in Tat-expressing mice that can be exacerbated with opioids. These effects may vary based on the estrous cycle, however, the behavioral effects involving combined Tat/opioid interactions like oxycodone are not known. We hypothesized that Tat-mediated interactions with oxycodone are estrous cycle-dependent. We found conditional HIV-1 Tat expression in naturally-cycling transgenic female mice potentiated oxycodone-mediated psychomotor activity in a dose-dependent manner. In a tail-suspension test, Tat enhanced depression-like behavior in proestrous mice but lowered it in diestrous mice (who previously showed greater depression-like behavior) and oxycodone reversed these effects. On diestrus, a combination of Tat and oxycodone induced behavioral disinhibition of anxiety-like response, such that mice made more central entries, but spent less time in the center, and also had higher levels of circulating corticosterone. Anxiety-like behavior was enhanced by either Tat or oxycodone exposure. Glucocorticoid receptors (GRs) or corticotropin-releasing factor receptors (CRF-Rs) blockade did not attenuate combined Tat oxycodone potentiation of psychomotor behavior. However, OVX reduced the interaction between Tat and oxycodone, implicating the role of gonadal hormones to drive neuroHIV behavior in female mice. In male transgenic mice, HIV-1 Tat interacted with oxycodone to enhance psychomotor and anxiety-like behavior, whereas repeated exposure sensitized stress-related psychomotor activity and the HPA stress response. In Tat-expressing male mice,

pharmacological inhibition of GRs partially reinstated the stress response and reduced oxycodone-mediated psychomotor activity, suggesting the role of GR in these effects. Blocking CRF-Rs decreased anxiety-like behavior in oxycodone-administered mice. These findings add to the growing body of evidence that HIV proteins like Tat and opioids can drive neuroHIV behavior in both female and male mice and that the HPA axis in males and the HPG axis are important in females. Disruption of HPA/HPG axes may increase the vulnerability to mood and substance use disorders.

2.1. Introduction

The availability of combination antiretroviral therapy (cART) has considerably prolonged the lives of people living in developed nations who are infected with the human immunodeficiency virus type 1 (HIV-1). However, the neurological consequences of long-term viral infection are increasingly becoming apparent. Approximately half of the HIV^+ population have neuroHIV, a constellation of disorders that include affective, cognitive, antinociceptive, and motor dysfunction (reviewed in Sanchez and Kaul, 2017; Saylor et al., 2016). Although cART has suppressed the peripheral and CNS viral load to undetectable levels, its poor retention inside the CNS compartment and inability to target latent reservoirs like microglia and to a lesser extent astrocytes have little effect on these neurological consequences. Furthermore, a history of substance abuse, particularly opioid usage, may exacerbate neuroHIV symptomology (alone or in combination with other drugs of abuse; Anthony et al., 2008; Byrd et al., 2011; Hauser et al., 2005; Nath et al., 2002; Soontornniyomkij et al., 2016). Opioids are increasingly being prescribed for intractable pain to HIV^+ patients, with the majority receiving hydrocodone-acetaminophen or oxycodone, expanding these issues beyond illegal misuse to licit opioid users

as well (Edelman et al., 2013; Jeevanjee et al., 2014; Koeppe et al., 2013; Merlin et al., 2016; Silverberg et al., 2012). As a result, it is crucial to identify adjunct treatments that may improve neuroHIV symptoms in individuals who are either opiate-dependent or naive.

The mechanisms underlying neuroHIV symptomatology are hypothesized to involve neurotoxic viral proteins. The trans-activator of transcription (Tat) is the best characterized of them. Tat is a multifunctional viral regulatory protein that promotes HIV transcription (Das et al., 2011). It is present in post-mortem brain tissues and remains in the CSF of aviremic HIV-1 patients despite cART (Henderson et al., 2019). Tat causes neuronal injury and excitotoxicity by directly or indirectly activating cation channels and releasing proinflammatory cytokines (Dreyer et al., 1990; Eugenin et al., 2007; Henderson et al., 2019; Hu, 2016; Li et al., 2008; Mattson et al., 2005; Napier et al., 2014; Wayman et al., 2012). Given that opioids influence drug reward via activation of mesolimbic dopaminergic system (dorsal and ventral striatum) (Massaly et al., 2016; Nestler and Carlezon, 2006; Sinha, 2008), and Tat's ability to alter the dopaminergic homeostasis as a DAT transporter (Gaskill et al., 2017), suggesting a biological mechanism by which Tat might alter drug reward, though behavioral mechanisms are still unclear, which is why this study is important.

In order to assess the role that HIV-1 Tat may play in the HPG/HPA response to a predominantly prescribed, licit opioid namely oxycodone and the resulting neurological sequelae, transgenic mice that conditionally expressed the Tat protein were tested in a behavioral battery of psychomotor, affective, and cognitive responses to oxycodone. We expected that HIV-1 Tat expression would enhance oxycodone-mediated psychomotor responses, affective dysfunction, and cognitive impairment in conjunction with alterations in circulating E_2, P_4, and

corticosterone levels. We also anticipated that HPA blockade via pharmacological inhibition of GR and/or CRF receptors and HPG blockade via ovariectomy would restore HPA function and alleviate behavioral impairments.

In order to achieve the general objective, I have subdivided Chapter 2 into two aims.

Aim 1: Assessment of depression- and anxiety-like behavior (e.g., tail suspension and open field/light-dark transition), cognitive and psychomotor behaviors in response to Tat or clinical opioid (e.g. oxycodone) exposure.

Aim 2: Assessment of the behavior endpoints following HPA blockade via systemic antagonism of pharmacodynamic targets (glucocorticoid receptor or CRF receptor) or HPG blockade.

2.2. Materials and Methods

Behavioral Assessment

Mice were behaviorally tested on the 8th day of the protocol (in case of males & OVX females) and in the proestrous or diestrous phase of their estrous cycle (whichever came first) within 14 days of completing doxycycline treatment or were excluded from the study without testing if they were irregularly cycling (fewer than one proestrus per 5 days; n=19, in case of females). We have not previously observed doxycycline or Tat induction to influence cycle length (Paris et al., 2014bd), nor did we observe this presently. Affective behavioral changes induced by Tat are observed to be stable for at least 14 days *(*Paris et al., 2014c). Notably, the length of time from the end of doxycycline treatment to testing was not observed to influence any dependent measure in the present study. All mice were acclimated to the behavioral testing

room for 30 minutes prior to testing and were assessed approximately 2–3 h into the dark phase of their light cycle. Behavior was tracked and encoded by an ANY-maze behavioral tracking system (Stoelting Co., Wood Dale, IL).

Open Field

The open field test was used to assess motor and anxiety-like behavior as previously described (Hall and Ballachey, 1932; Paris et al., 2014c). In brief, mice were placed in the corner of a square Plexiglas box (40 × 40 × 35 cm; Stoelting Co.) with a brightly-lit center (inner 20 cm) and allowed to behave for 5 min. Their mean velocity (meters/sec) and total distance traveled (meters) were used as indices for motor behavior. Entry into the center of the open field was used as an index of anxiety-like behavior (longer latencies to enter, fewer entries, and less time spent in the center indicated greater anxiety-like behavior; Hall and Ballachey, 1932; Paris et al., 2014c).

Tail Suspension Test (TST)

Immediately following the open field test, mice were assessed in the tail suspension test as previously described (McLaughlin et al., 2017; Steru et al., 1985). Briefly, mice were suspended vertically and their tails were fastened to a horizontal surface 18 inches above the floor with laboratory tape. Prior to taping, a small clean plastic cup was placed over the tails to avoid tail climbing. Six minutes of behavior were recorded (with the initial 2 min discarded for acclimation). The duration spent motionless (i.e. adopting a completely fixed posture with the exception of whole-body swaying due to the momentum of the previous movement) was quantified by two blinded investigators. Increased time spent immobile was considered to be an

indicator of depression-like behavior (McLaughlin et al., 2017; Steru et al., 1985).

Light-dark transition test

The light-dark transition test was used to assess anxiety-like behavior as previously described (Bourin and Hascoët, 2003). In brief, mice were placed in the brightly-lit corner of a square Plexiglas box (40 × 40 × 35 cm; Stoelting Co., Wood Dale, IL, USA) that was evenly divided into two compartments (one brightly-lit side and one enclosed dark side) and allowed to explore for 5 min. The latency to enter the dark compartment and the time spent in the light chamber was considered an index of anxiety-like behavior. The number of transitions between compartments was used as an index of motor activity (Salahuddin et al., 2020b, 2021c).

Porsolt Forced Swim Stress Stimulus

The Porsolt forced swim test was used to activate the HPA stress axis (Porsolt et al., 1977). In brief, mice were placed in room temperature water (~22 °C) and allowed to swim for 15 min. Following swimming, mice were dried with paper towels and returned to their home cages (Salahuddin et al., 2020b, 2021c).

Experiment 1: Oxycodone Dose-Response

To determine the optimal concentration of oxycodone for use in behavioral experiments, mice were administered a cumulative dose-response regimen of oxycodone hydrochloride (Sigma-Aldrich, St. Louis, MO) dissolved in sterile 0.9% saline (0.0, 0.1, 0.3, 1.0, 3.0, and 10.0 mg/kg, i.p.) 15 minutes prior to behavioral assay (plasma half-life is 3-5h; Ordóñez Gallego et al., 2007). Briefly, mice were first administered saline and then permitted to explore the open

field for 15 minutes. The mice were then removed from open field and administered oxycodone 0.1mg/kg and allowed to explore the open field for another 15 minutes. Similarly, a cumulative dosing regimen of 0.3, 1.0, 3.0, and 10.0 mg/kg was completed with 15 minutes of open field exploration following each dose. The distance traveled by the mice was determined, as well as the ED50.

Experiment 2: Assessment of Acute Oxycodone Exposure on Psychomotor, Anxiety- and Depression-like behavior

To assess the interaction between HIV-1 Tat expression and acute oxycodone exposure, mice were randomly assigned to receive vehicle (sterile saline, 0.9%, i.p.) or oxycodone hydrochloride (3 mg/kg, i.p.; Sigma-Aldrich, St. Louis, MO; Rubin et al., 2020), either in their proestrous or diestrous phase of the estrus cycle, once 15 mins prior to open field and tail suspension behavioral testing.

Experiment 3: Assessment of Repeated Oxycodone Exposure on Cognitive Behavior

To assess the interaction between HIV-1 Tat expression and repeated oxycodone exposure to influence cognitive-behavioral outcomes, mice were randomly assigned to receive vehicle (sterile saline, 0.9%, i.p.) or oxycodone hydrochloride (3 mg/kg, i.p.; Sigma-Aldrich, St. Louis, MO) once daily for 5 days, and assessed for novel object recognition behavioral testing either in their proestrous or diestrous phase of the estrous cycle.

Experiment 4: Assessment of Acute Oxycodone Exposure in Non-Stressed and Stressed mice

To begin to determine the HPA-axis interactions involved in exposure to HIV-1 Tat and acute oxycodone, mice were randomly assigned to undergo 15-min swim stress (or not) followed

by administration of vehicle (saline, 0.9%, i.p.) or oxycodone (3 mg/kg, i.p.) only once prior to behavioral testing. Fifteen minutes after drug administration, mice were assessed in an open field to determine their psychomotor response followed immediately by assessment in a light-dark transition test to determine anxiety-like behavior.

Experiment 5: Assessment of Repeated Oxycodone Exposure in Non-Stressed and Stressed Mice

Given that most patients are exposed to opioids on a repeated dosing schedule, some mice were administered sterile saline (0.9%) or oxycodone (3 mg/kg) daily throughout the 7-day doxycycline-induction/washout schedule. As before, mice were randomly assigned to undergo a 15-min swim stress (or not) followed by an injection of saline (0.9%, i.p.) or oxycodone (3 mg/kg) 15 min prior to behavioral testing and assessed for psychomotor and anxiety-like behavior in an open field and light-dark transition tasks.

Experiment 6: Assessment of Acute Oxycodone Exposure Following GR and/or CRF-R Blockade and HPG Blockade in females

To begin to identify the important receptor sites involved in HIV-1 Tat- or oxycodone-mediated disruption of the HPA axis, some mice were pretreated with the GR antagonist, RU-486, and/or the CRF-R antagonist, antalarmin, and ovariectomized (in case of females) prior to testing. RU-486 was administered daily throughout the 7-day doxycycline-induction/washout schedule and 30 min prior to behavioral testing. Antalarmin was administered daily for 6-days during the doxycycline-induction/washout schedule and 30 min prior to behavioral testing. Female mice were tested in proestrous phase of estrous cycle. Some female mice were ovariectomized to remove the

primary source of gonadal hormones and administered a daily vehicle injection (to account for potential injection stress). All mice received saline or oxycodone (3 mg/kg, i.p.) 15 min prior to behavioral testing and were assessed for psychomotor and anxiety-like behavior in an open field and light-dark transition tasks.

2.3. Results

Aim 1: Assessment of depression- and anxiety-like behavior (e.g., tail suspension and open field/light-dark transition), psychomotor and cognitive behavior in response to Tat or clinical opioid (e.g. oxycodone) exposure.

A. Female HIV-1 Tat transgenic mice

Oxycodone Dose-Response Curve

In order to establish optimal oxycodone dosing in the present transgenic model, diestrous and proestrous Tat-tg mice had HIV-1 Tat expression induced (or not) via doxycycline administration for five days. After two days of doxycycline washout (to limit non-specific effects), the estrous cycle was assessed daily and diestrous or proestrous mice were acutely administered a cumulative dose-response regimen of oxycodone (0.0, 0.1, 0.3, 1.0, 3.0, and 10.0 mg/kg, i.p.) prior to the assessment of psychomotor response in an open field.

Expression of HIV-1 Tat significantly interacted with oxycodone dosing to increase the distance travelled [$F(5,140) = 24.29$, $p < 0.05$; $\eta^2 = 0.13$] (Fig. 41). Compared to Tat(−) controls, Tat(+) mice had a significantly greater psychomotor response to oxycodone at 3 and 10 mg/kg ($p < 0.0001 - 0.003$; $d = 1.16 - 2.05$), irrespective of estrous cycle phase (Fig. 41). These data

demonstrated that Tat expression could shift the oxycodone psychomotor response to the left and identified the 3 mg/kg dose as optimal [ED_{50} : $Tat(-)_{proestrous}$ = 1.45, $Tat(-)_{diestrous}$ = 1.32, $Tat(+)_{proestrous}$ = 0.91, $Tat(+)_{diestrous}$ = 0.80; Salahuddin et al., 2020a; Fig. 41].

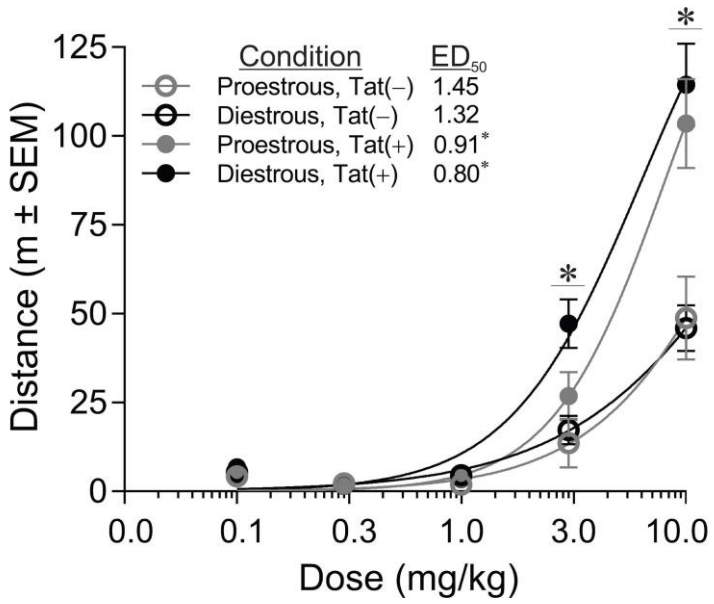

Figure 41: Determination of oxycodone dose-response curve in an open field test in naturally-cycling female HIV-1 Tat transgenic mice. Ref. Salahuddin et al., 2020a

Proestrous and diestrous, Tat(−) and Tat(+) mice (n=8/group) were administered a cumulative dose-response regimen of oxycodone (0 – 10 mg/kg, i.p.) and were assessed for the distance travelled in an open field. * indicated Tat(+) group significantly differs from Tat(−) control, $p < 0.05$.

It is thus critical to understanding the notion of dose extrapolation from mice to humans when conducting new animal or human tests. Allometric scaling technique is used when extrapolating medicinal agent doses between species to account for changes in body surface area, which is proportional to animal weight (Nair & Jacob, 2016). According to allometric conversion, the current dose of 3.0 mg/kg of oxycodone used in behavioral tests is commensurate to the 17.0 mg/kg human equivalent dose (HED) prescribed to opioid-naive human subjects (Ordóñez

Gallego et al., 2007; Roth et al., 2000; U.S. Food and Drug Administration, 2016).

Calculation of Human Equivalent Dose via Allometric scaling technique (Nair & Jacob, 2016)

HED (mg/kg) = Multiply dose used in mice by 0.081

HED (mg/kg) = 3 x 0.081 = 0.243mg/kg = 0.243mg x 70kg = ~17 mg in 70 kg adult.

Psychomotor and Affective Response to Acute Oxycodone

HIV-1 Tat expression potentiated oxycodone psychomotor behavior

With dosing established, a separate set of mice were assessed for psychomotor, anxiety-, and depression-like behavior following the induction (or not) of HIV-1 Tat and administration of saline (0.9%, i.p.) or oxycodone (3 mg/kg, i.p.; Fig. 42A).

As expected, oxycodone significantly increased the distance [$F(1,84) = 106.39$, $p < 0.05$; $\eta^2 = 0.32$] (see †, Fig. 42B) and velocity [$F(1,84) = 106.51$, $p < 0.05$; $\eta^2 = 0.31$] (see †, Fig. 42C) travelled by mice in an open field and these effects were potentiated by expression of Tat ([$F_{Distance}(1,84) = 59.74$, $p < 0.05$], [$F_{Velocity}(1,84) = 59.85$, $p < 0.05$]. Irrespective of estrous cycle phase, oxycodone-administered Tat(+) mice travelled further (see §, Fig. 42B) and with greater speed (see §, Fig. 42C) than did any other group including oxycodone-administered Tat(−) mice ($p < 0.0001$; $d_{Distance} = 2.53 – 3.43$; $d_{Velocity} = 2.55 – 3.45$; Salahuddin et al., 2020a).

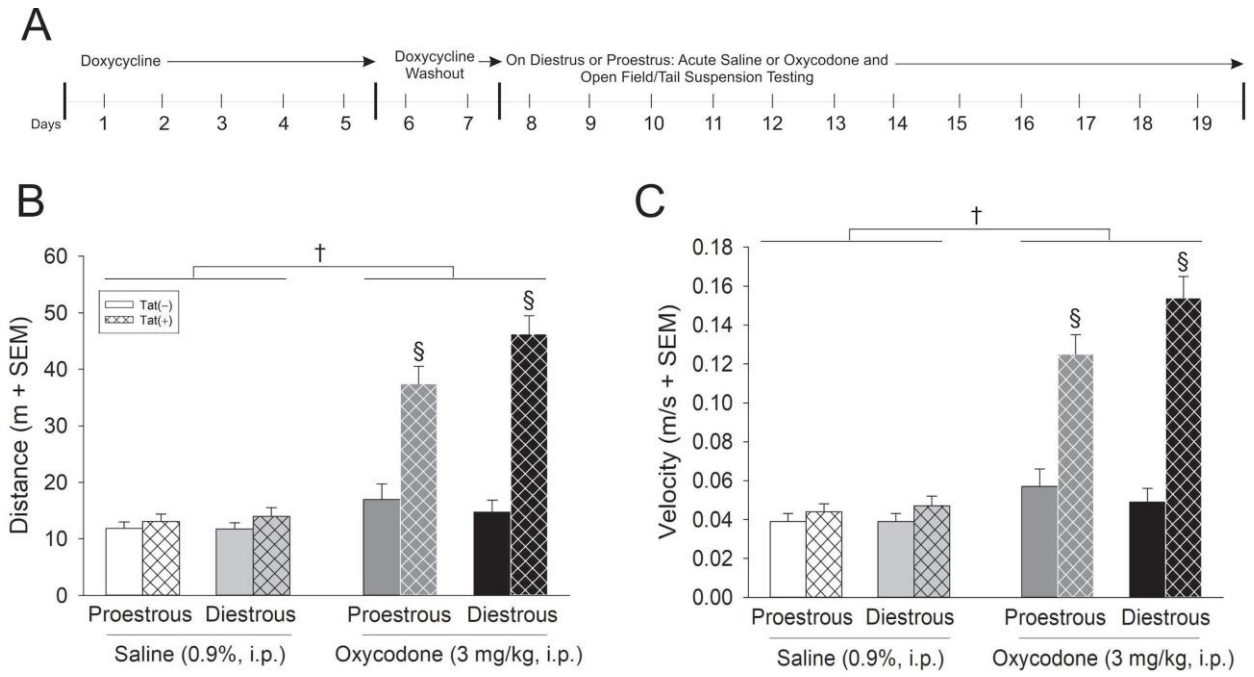

Figure 42: Effect of acute oxycodone exposure on psychomotor response and velocity of travel in an open field test in adult naturally-cycling female HIV-1 Tat transgenic mice. Ref. Salahuddin et al., 2020a

(**A**) HIV-1 Tat expression was induced in Tat(+) females, or not induced in Tat(−) controls, via administration of doxycycline (30 mg/kg, i.p., once daily for 5 d) with 2 days for washout. Estrous cycles were tracked for 12 d and mice were acutely-administered saline or oxycodone (3 mg/kg, i.p., −15 min) and assessed in an open field and a tail suspension test on proestrus or diestrus (whichever came first; $n_{\text{proestrous}}$ = 12–13; $n_{\text{diestrous}}$ = 10–11). (**B**) Distance (m) traveled in an open field. (**C**) Velocity (m/s) traveled in an open field. † indicates a main effect for oxycodone to differ from saline administration; § indicates an interaction wherein oxycodone-administered, Tat(+) mice differ from all other groups; $p \leq 0.05$.

Anxiety-like behavior is increased by acute oxycodone and Tat, particularly on diestrus

To assess anxiety-like behavior, entries into the brightly-lit center of the open field were calculated following the HIV-1 Tat induction (or not) and administration of saline (0.9%, i.p.) or oxycodone (3 mg/kg, i.p.; Fig. 43A). Oxycodone significantly increased the number of central

entries made [$F(1,84) = 5.81$, $p < 0.05$; $\eta^2 = 0.04$] (see †, Fig. 43B) and expression of Tat significantly interacted with both acute oxycodone administration [$F(1,84) = 11.35$, $p < 0.05$; $\eta^2 = 0.09$] and estrous cycle phase [$F(1,84) = 4.91$, $p < 0.05$; $\eta^2 = 0.04$] (see §, Fig. 43B). Either Tat(+) mice administered acute oxycodone, or Tat(+) mice in the diestrous phase of the estrous cycle, made significantly more entries into the center of the open field than did any other group ($p < 0.0001 - 0.005$; $d = 0.77–1.08$; Fig. 43B). The amount of time spent in the center of the open field was significantly reduced by acute oxycodone administration compared to saline, irrespective of Tat exposure or estrous cycle phase [$F(1,84) = 5.23$, $p < 0.05$; $\eta^2 = 0.05$] (Fig. 43C), indicating greater velocity when entering the center of the open field. No significant differences were observed in the latency to enter the center of the open field (Salahuddin et al., 2020a; Table 5).

Depression-like behavior is influenced by HIV-1 Tat exposure and estrous cycle phase

Following testing in the open field, mice were immediately assessed for depression-like behavior in a tail suspension test. Estrous cycle phase, expression of HIV-1 Tat, and exposure to acute oxycodone significantly interacted to influence the time spent immobile [$F(1,84) = 13.22$, $p < 0.05$; $\eta^2 = 0.06$] (Fig. 43D). Among Tat(−) controls, those in the diestrous phase demonstrated greater immobility time than did their proestrous counterparts ($p = 0.007$; $d = 1.17$; see #, Fig. 43D) consistent with prior observations on this task (Kastenberger and Schwarzer, 2014). However, these effects were reversed among Tat(+) mice who demonstrated greater immobility on proestrus ($p = 0.02$; $d = 1.09$), and lesser immobility on diestrous, compared to their Tat(−) counterparts ($p = 0.04$; $d = 0.92$; see*, Fig. 43D). Given that acute oxycodone was psychostimulatory, immobility behavior was reduced compared to all groups administered saline ($p < 0.0001 - 0.001$; $d = 1.64 – 3.55$; see †, Fig. 43D). Among those administered acute oxycodone

Tat(−) controls had lower immobility on diestrous compared to proestrous (p = 0.04; d = 1.22; see #, Fig. 43D; Salahuddin et al., 2020a).

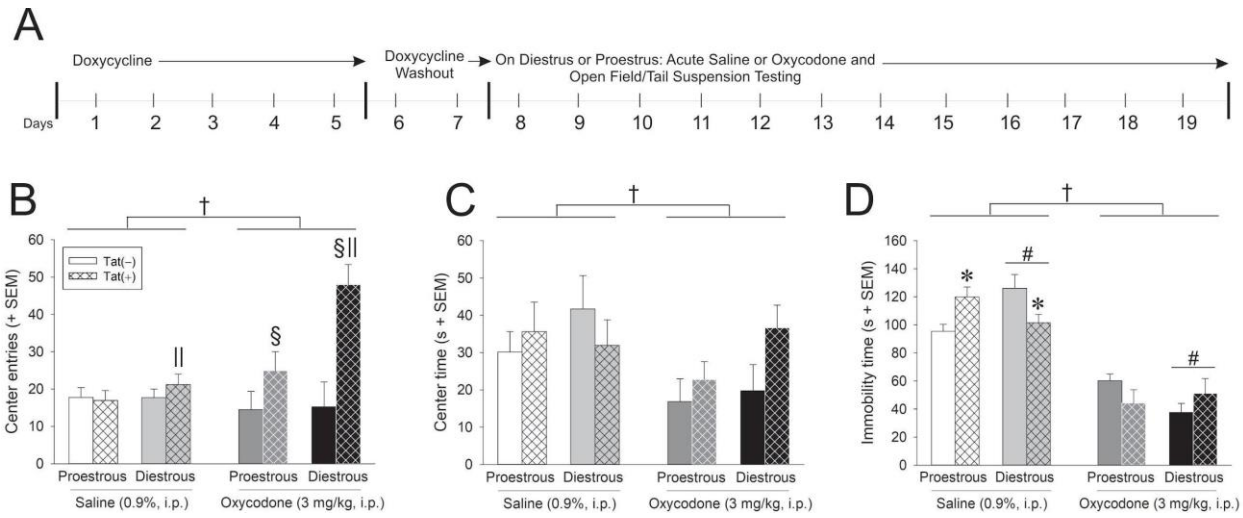

Figure 43: Effect of acute oxycodone exposure on anxiety- and depression like behavior in adult naturally-cycling female HIV-1 Tat transgenic mice. Ref. Salahuddin et al., 2020a

(**A**) In experiment 1 (Expt 1), HIV-1 Tat expression was induced in Tat(+) females, or not induced in Tat(−) controls, via administration of doxycycline (30 mg/kg, i.p., once daily for 5 d) with 2 days for washout. Estrous cycles were tracked for 12 d and mice were acutely-administered saline or oxycodone (3 mg/kg, i.p., −15 min) and assessed in an open field and a tail suspension test on proestrus or diestrus (whichever came first; $n_{\text{proestrous}}$ = *12–13;* $n_{\text{diestrous}}$ = *10–11).* (**B**) The frequency of entries into the brightly-lit center of an open field. (**C**) The time (s) spent in the center of an open field. (**D**) Mean time spent immobile (s) in a tail suspension test. † indicates a main effect for oxycodone to differ from saline administration; § indicates an interaction wherein oxycodone-administered, Tat(+) mice differ from all other groups; || indicates an interaction wherein diestrous, Tat(+) differ from all other groups; * indicates an interaction wherein saline-administered Tat(+) mice differ from respective Tat(−) controls; # indicates a main effect for proestrous mice to differ from diestrous mice; $p \leq 0.05$.

Table 5: The latency to enter the center of a brightly-lit open field in cycling female adult transgenic mice. Ref. Salahuddin et al., 2020a © 2019 Elsevier Inc. All rights reserved.

The latency to enter the center of a brightly-lit open field among Tat(−) or Tat(+) mice (n = 10 - 13) administered saline or oxycodone and assessed in the pro- or diestrous phase of their estrous cycle.

	Saline (0.9%, i.p.)				Oxycodone (3 mg/kg, i.p.)			
	Proestrous		Diestrous		Proestrous		Diestrous	
	Tat(−)	Tat(+)	Tat(−)	Tat(+)	Tat(−)	Tat(+)	Tat(−)	Tat(+)
Latency to Center of the Open Field (s)	20 ± 6	13 ± 3	11 ± 4	23 ± 11	51 ± 26	27 ± 10	31 ± 13	7 ± 2

Cognitive behavioral response to Repeated Oxycodone exposure

Recognition memory is influenced by HIV-1 Tat exposure and estrous cycle phase

To assess the effect of estrous cycle, oxycodone and HIV-1 Tat exposure, Tat(−) and Tat(+) mice were administered doxycycline for 5 days with concurrent saline (0.9 %, i.p.) or oxycodone (3 mg/kg, i.p.) administration in order to assess the effects of repeated oxycodone exposure (Fig. 44A). Mice were allowed two days to washout doxycycline and had their estrous cycles tracked for the next 14 days. Mice were assessed for short-term memory performance in a novel object recognition test on the next instance of proestrus or diestrus (whichever came first; Fig. 44A).

The proportion of time spent with the novel object was significantly increased in the retention trial indicating that mice were able to discern the novel object [$F(1,93) = 38.16$, $p < 0.05$; $\eta^2 = 0.27$] (see ¶, Fig. 44B). Estrous cycle phase significantly interacted with Tat exposure

to influence short-term memory performance [$F(1,93) = 11.58$, $p < 0.05$]; $\eta^2 = 0.10$; see ||, Fig. 44B). Irrespective of repeated oxycodone administration, proestrous mice exposed to Tat spent significantly less time investigating the novel object than did proestrous, Tat(−) controls (see ||, Fig. 44B). Repeated oxycodone appeared to reduce short-term memory performance among diestrous, compared to proestrous controls; however, this did not reach statistical significance ($p = 0.07$; $d = 0.51$; Salahuddin et al., 2020a).

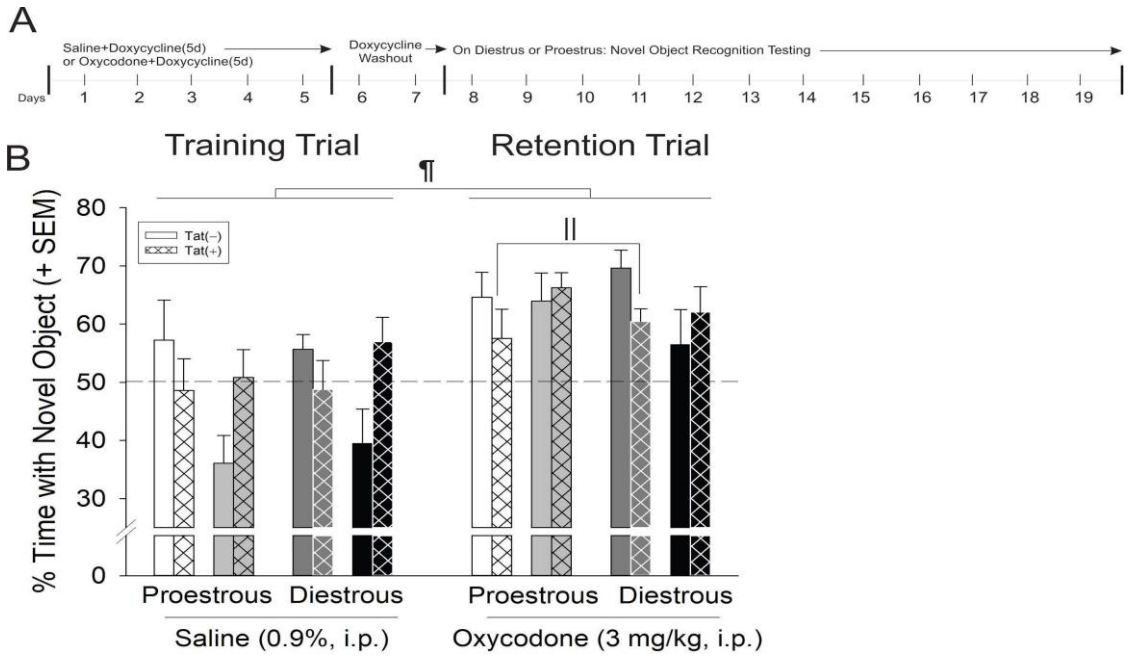

Figure 44: Effect of acute oxycodone exposure on short-term memory behavior in adult naturally-cycling female HIV-1 Tat transgenic mice. Ref. Salahuddin et al., 2020a

(**A**) Tat(+) and Tat(−) female mice were administered saline or oxycodone (3 mg/kg, i.p., once daily for 5 d) concurrent with the induction of HIV-1 Tat via doxycycline. Following 2 d of washout, estrous cycles were tracked and mice in their proestrous or diestrous cycle were assessed in a novel object recognition test ($n_{proestrous} = 10–17$; $n_{diestrous} = 7–15$). (**B**) The proportion of time spent investigating a novel object (dashed line indicates equal time spent with the familiar and novel objects); $p \leq 0.05$.

HIV-1 Tat and Oxycodone-Mediated Psychostimulation is Moderated by Stress and Estrous Cycle

In order to assess the influence of stress on combined HIV-1 Tat interactions with oxycodone, a 15 min forced swim test was used to activate the HPA axis in the stressed paradigm (or not in the non-stressed paradigm). As reported earlier, Tat was induced via systemic administration of doxycycline for 5 days (with two days of washout; Fig. 45A). The estrous cycle was assessed daily over the next 12 days and mice were behaviorally tested when in the proestrous or diestrous phase of their estrous cycle (whichever came first). On the day of testing, all mice received saline or oxycodone (3 mg/kg, i.p.) 15 min prior to behavioral assessment (Fig. 45A).

In the non-stressed paradigm, oxycodone significantly increased the distance [$F(1,70) = 69.07$, $p < 0.05$] (see †, Fig. 45B) and velocity [$F(1,70) = 69.42$, $p < 0.05$] (see †, Table 6) travelled by the mice in an open field test compared to saline administered controls. There was an interaction wherein oxycodone-administered Tat(+) mice travelled a significantly greater distance ($p < 0.0001$; see §, Fig. 45B) and speed ($p < 0.0001$–0.0002; see §, Table 6) than any other group, irrespective of estrous cycle phase. Oxycodone-administered Tat(−) controls also travelled a greater distance than their saline-administered counterparts ($p = 0.0001$–0.0002). When anxiety-like behavior was assessed in a light-dark transition test, estrous cycle, oxycodone, and Tat exposure interacted to influence the time spent in the light zone [$F(1,67) = 5.34$, $p < 0.05$]. Diestrous Tat(+) mice administered saline demonstrated the least anxiety-like behavior, spending significantly more time in the light zone than any other group with the exception of proestrous Tat(+) mice administered saline ($p = 0.0003$–0.0478; Table 6). Diestrous Tat(+) mice administered oxycodone demonstrated the most anxiety-like behavior on this test, significantly differing from

proestrous, Tat(+) controls (p = 0.0329; Table 6). As expected, oxycodone also influenced motor behavior in the light-dark test, significantly increasing the number of chamber transitions [F(1,69) = 16.10, p < 0.05] (see †, Table 6); whereas Tat(+) mice made significantly fewer transitions than Tat(−) controls [F(1,69) = 4.10, p < 0.05] (see *, Table 6). Estrous cycle also influenced motor/exploratory behavior in this test, with diestrous mice rearing more than proestrous mice [F(1,69) = 6.60, p < 0.05] (see #, Table 6; Salahuddin et al., 2021c).

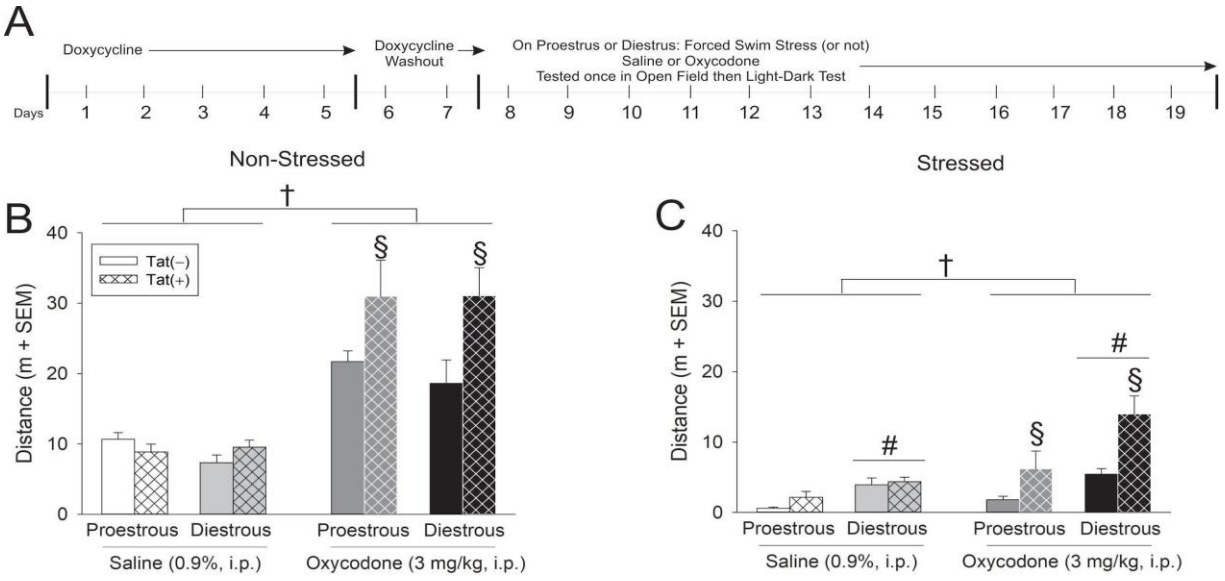

Figure 45: Effect of acute oxycodone exposure on psychomotor response in non-stressed and stressed adult naturally-cycling female HIV-1 Tat transgenic mice. Ref. © 2021 Salahuddin et al., 2021c, Licensee MDPI, Basel, Switzerland distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

(**A**) HIV-1 Tat-transgenic, female mice [hatched bars; Tat(+)] or control counterparts [open bars; Tat(−) controls] had transgene expression induced via doxycycline (30 mg/kg, i.p., once daily for 5 days with 2 days for washout; n = 8–10/group). On the day of testing, proestrous or diestrous mice were exposed to a forced swim stress (or not) and administered saline or oxycodone 15 min prior to assessment in an open field and light-dark transition test. (**B**) Distance (m) travelled in an open field among (**B**) non-stressed mice, (**C**) stressed mice. † indicates a main effect for oxycodone-administered mice to differ from saline-administered controls. § indicates an interaction wherein oxycodone-administered Tat(+) mice differ from respective Tat(−) controls and saline-administered controls. # indicates a main effect of estrous cycle wherein diestrous mice differ from proestrous mice, p < 0.05.

Table 6: Motor and anxiety-like measures for non-stressed Tat(−) and Tat(+) female mice assessed in open field and light-dark transition tests following acute oxycodone exposure. Ref.

Motor and anxiety-like measures for Tat(−) and Tat(+) female mice assessed in open field and light-dark transition tests. Proestrus or diestrous mice were administered saline or oxycodone prior to behavioral assessment. * indicates a main effect of genotype wherein Tat(+) mice differ from Tat(−) controls. † indicates a main effect of drug condition wherein oxycodone-administered mice differs from saline-administered controls. # indicates a main effect of estrous cycle wherein diestrous mice differ from proestrous mice. § indicates an interaction wherein oxycodone-administered Tat(+) mice differ from Tat(−) controls and saline-administered controls. ^ indicates an interaction wherein saline-administered, diestrous Tat(+) mice differ from all other groups except for their respective proestrous counterparts, $p < 0.05$.

Behavioral Measure	Non-Stressed							
	Saline (0.9% *w/v*)				Oxycodone (3 mg/kg)			
	Proestrous		Diestrous		Proestrous		Diestrous	
	Tat(−)	Tat(+)	Tat(−)	Tat(+)	Tat(−)	Tat(+)	Tat(−)	Tat(+)
Light Zone Time (s)	78 ± 14	88 ± 34	24 ± 5	141 ± 42^	61 ± 19	49 ± 13	71 ± 13	20 ± 4
Mean Velocity (m/sec)	0.036±0.003	0.030±0.004	0.024±0.004	0.032±0.003	0.072±0.005†	0.103±0.017§†	0.062±0.011†	0.104±0.013§†
Number of transitions	15 ± 2	8 ± 1*	6 ± 1	5 ± 1*	19 ± 4†	15 ± 3†*	19 ± 5†	14 ± 3†*
Rearing Time (s)	19 ± 4	20 ± 3	27 ± 6#	26 ± 7#	5 ± 1	15 ± 4	60 ± 23#	33 ± 20#

In the stressed paradigm, motor activity was notably reduced compared to the non-stressed paradigm; however, the influence of estrous cycle phase became apparent after forced swim stress. As anticipated, oxycodone-administered mice traveled a significantly greater distance [$F(1,71) = 16.51$, $p < 0.05$] (see †, Fig. 45C) and velocity [$F(1,71) = 16.46$, $p < 0.05$] (Table 7) than saline-administered controls. Following the forced swim, diestrous mice traveled a significantly greater distance than their proestrous counterparts [$F(1,71) = 17.83$, $p < 0.05$] (see #, Fig. 45C). There was an interaction wherein Tat exposure potentiate oxycodone's psychomotor effects, irrespective of estrous cycle phase [$F(1,71) = 7.40$, $p < 0.05$]. Oxycodone-administered Tat(+) mice traveled a significantly greater distance than did any other group ($p < 0.0001$–0.0001; see §, Fig. 45C). Expression of Tat also influenced rearing time such that Tat(+) mice significantly spent more time rearing than their respective Tat(−) controls [$F(1,69) = 7.50$, $p < 0.05$] (see *, Fig. 45C). When assessed in the light-dark transition test, significant differences in anxiety-like behavior were not observed (Table 7). However, oxycodone administration interacted with Tat expression to influence motor/exploratory behavior [$F(1,70) = 5.15$, $p < 0.05$] such that oxycodone-administered Tat(+) mice made more transitions than Tat(−) oxycodone- and Tat(+) saline-administered mice ($p = 0.0200$–0.0313; see §, Table 7). Estrous cycle also interacted with Tat expression [$F(1,70) = 8.52$, $p < 0.05$] such that Tat(−) mice in the proestrous phase made significantly fewer transitions than did Tat(+) mice in the proestrous phase or Tat(−) mice in the diestrous phase ($p < 0.0079$–0.0407; Table 7; Salahuddin et al., 2021c).

Table 7: Motor and anxiety-like measures for forced-swim stressed Tat(−) and Tat(+) female mice assessed in open field and light-dark transition tests following acute oxycodone exposure.

Motor and anxiety-like measures for Tat(−) and Tat(+) female mice assessed in open field and light-dark transition tests. Proestrus or diestrous mice were exposed to a forced swim stress prior to administration of saline or oxycodone. * indicates a main effect of genotype wherein Tat(+) mice differ from Tat(−) controls. # indicates a main effect of estrous cycle wherein diestrous mice differ from proestrous mice. § indicates an interaction wherein oxycodone-administered Tat(+) mice differ from respective Tat(−) controls and saline-administered controls. ‡ indicates an interaction wherein proestrous Tat(−) mice differ from respective Tat(+) and diestrous Tat(−) controls, $p < 0.05$.

Behavioral Measure	Stressed							
	Saline (0.9% *w/v*)				Oxycodone (3 mg/kg)			
	Proestrous		Diestrous		Proestrous		Diestrous	
	Tat(−)	Tat(+)	Tat(−)	Tat(+)	Tat(−)	Tat(+)	Tat(−)	Tat(+)
Light Zone Time (s)	60 ± 16	43 ± 4	112 ± 30	81 ± 28	79 ± 36	38 ± 9	45 ± 6	44 ± 10
Mean Velocity (m/sec)	0.002±0.001	0.007±0.003	0.013±0.003$^{\#}$	0.015±0.002$^{\#}$	0.006±0.002	0.021±0.008§	0.018±0.003$^{\#}$	0.047±0.009$^{\#\S}$
Number of transitions	10 ± 2	12 ± 2‡	14 ± 3‡	7 ± 1	5 ± 1	19 ± 5‡§	14 ± 2‡	14 ± 3§
Rearing Time (s)	0.35±0.15	1.97±0.82*	1.56±0.44	2.10±0.66*	0.01±0.01	1.65±1.11*	0.21±0.13	1.02±0.50*

In summary, Tat expression potentiated oxycodone-mediated psychomotor behavior. Stress enhanced this effect among diestrous, compared to proestrous, mice implicating HPG factors to influence behavioral outcomes (Salahuddin et al., 2021c).

B. Male HIV-1 Tat transgenic mice

HIV-1 Tat expression promotes anxiety-like and potentiates oxycodone psychomotor behavior.

In this experiment, Tat-tg male mice had HIV-1 Tat expression induced (or not) via doxycycline administration for five days. After two days of doxycycline washout (to limit non-specific anti-inflammatory effects), mice were (or were not) exposed to 15-min swim stress. Following stress, mice were acutely administered an injection of saline (0.9%, i.p.) or oxycodone (3 mg/kg, i.p.) and psychomotor and anxiety-like behavior were assessed 15 min later (Fig. 46A).

The induction of HIV-1 Tat significantly potentiated the psychomotor response to acute oxycodone, increased anxiety-like behavior in a light-dark task, and dysregulated the HPA stress axis. Compared to Tat(−) controls, Tat(+) mice traveled a significantly greater distance [$F(1,34) = 5.00$, $p < 0.05$] (Fig. 46B; see *) and velocity [$F(1,34) = 5.00$, $p < 0.05$] (Table 8) in an open field and spent less time engaged in rearing behavior [$F(1,34) = 5.04$, $p < 0.05$] (Table 8). Irrespective of genotype, oxycodone significantly increased the distance [$F(1,34) = 27.16$, $p < 0.05$] (Fig. 46B; see †) and speed [$F(1,34) = 27.34$, $p < 0.05$] of travel and decreased the frequency [$F(1,34) = 5.60$, $p < 0.05$] and time spent rearing [$F(1,34) = 68.97$, $p < 0.05$], compared to saline administration (Table 8). In a light-dark transition task, Tat(+) mice spent significantly less time in the brightly-lit compartment [$F(1,32) = 8.69$, $p < 0.05$] and made fewer transitions between compartments [$F(1,32) = 4.99$, $p < 0.05$] compared to Tat(−) controls (Table 8). No significant difference was observed in the latency to transition to the dark compartment (Table 8; Salahuddin et al., 2020b).

Activation of the HPA axis via 15-min swim stress altered the psychomotor and anxiety-like behavior of mice. Following swim stress, motor behavior in the open field was notably reduced among all groups compared to that previously observed in non-stressed mice. As seen before, oxycodone significantly increased the distance [$F(1,30) = 13.83$, $p < 0.05$] and speed [$F(1,30) = 13.50$, $p < 0.05$] of travel; however, no differences were observed between Tat(−) and Tat(+) mice (Fig. 46C), nor were any differences observed in the frequency or time spent rearing (Table 8). Similarly, swim stress attenuated any prior anxiety-like differences observed on the light-dark transition test (Table 8; Salahuddin et al., 2020b)

Table 8: Motor and anxiety-like measures for forced-swim stressed (or not) Tat(−) and Tat(+) male mice assessed in open field and light-dark transition tests following acute oxycodone exposure. Ref. © 2020 Salahuddin et al., 2020b, Licensee MDPI, Basel, Switzerland distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

Motor measures acquired in an open field and anxiety-like/motor measures acquired in a light-dark transition task from Tat(–) and Tat(+) males that were exposed (or not) to a 15 min forced swim stress prior to administration of saline or oxycodone. * indicates a main effect of genotype wherein Tat(+) mice differ from Tat(–) controls. † indicates a main effect for oxycodone to differ from saline administered mice, $p \leq 0.05$.

Behavioral Measure	Non-Stressed				Stressed			
	Saline (0.9% *w/v*)		Oxycodone (3 mg/kg)		Saline (0.9% *w/v*)		Oxycodone (3 mg/kg)	
	Tat(−) (*n* = 8-9)	Tat(+) (*n* = 7-8)	Tat(−) (*n* = 12)	Tat(+) (*n* = 8-9)	Tat(−) (*n* = 8)	Tat(+) (*n* = 9)	Tat(−) (*n* = 8)	Tat(+) (*n* = 9)
Mean Velocity (m/s)	0.025 ± 0.004	0.027 ± 0.002*	0.048 ± 0.008†	0.076 ± 0.009†*	0.005 ± 0.001	0.002 ± 0.001	0.012 ± 0.003†	0.015 ± 0.004†
Rearing number	39.4 ± 6.2	23.8 ± 3.2	18.1 ± 11.4†	6.8 ± 2.2†	4.5 ± 2.4	0.9 ± 0.7	0.8 ± 0.3	0.7 ± 0.2
Rearing Time (s)	31.13 ± 4.74	20.31 ± 2.58*	3.66 ± 1.56†	2.14 ± 0.92†*	2.61 ± 1.53	0.63 ± 0.60	0.29 ± 0.15	0.19 ± 0.09
Latency to first enter dark (s)	61 ± 32	28 ± 19	14 ± 4	22 ± 9	77 ± 45	89 ± 36	63 ± 38	37 ± 33
Light zone time (s)	106 ± 32	17 ± 5*	75 ± 17	43 ± 9*	102 ± 40	121 ± 27	112 ± 32	30 ± 5
Number of transitions	9 ± 3	5 ± 1*	13 ± 2	8 ± 1*	9 ± 3	7 ± 1	13 ± 3	11 ± 3

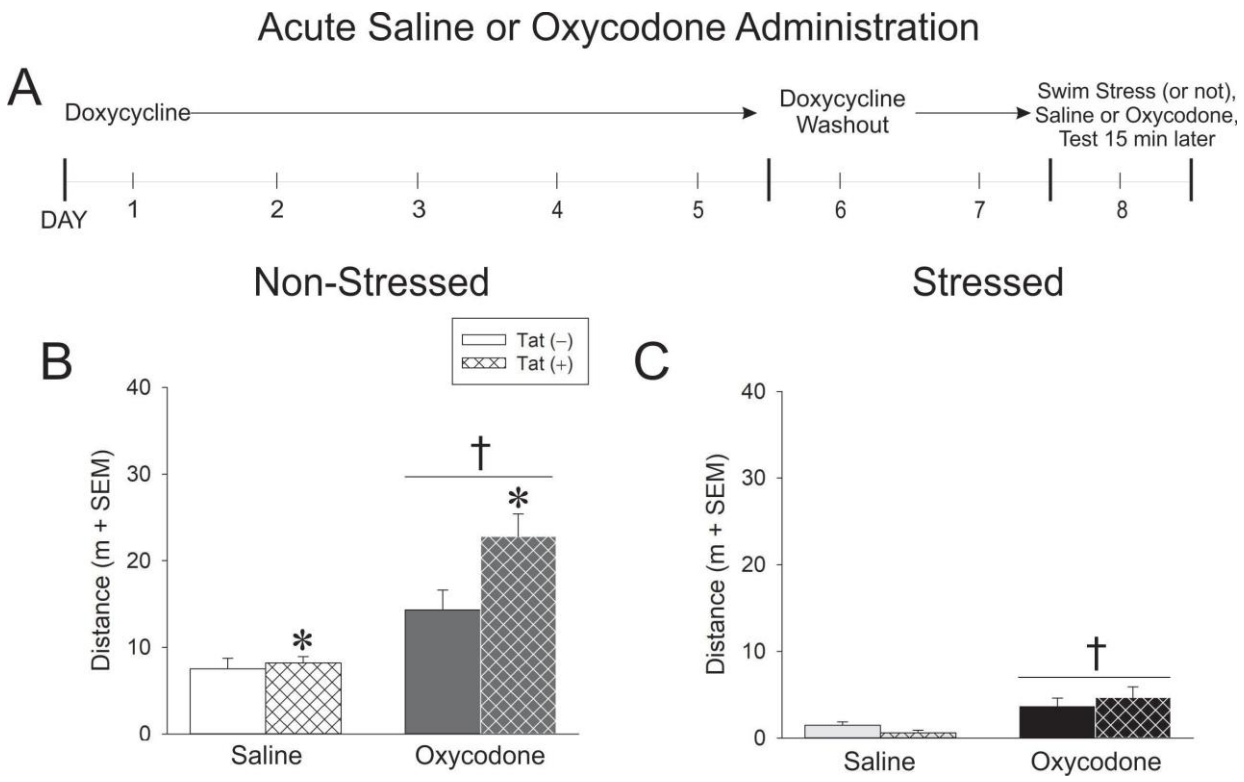

Figure 46: Effect of acute oxycodone exposure on psychomotor response in non-stressed and stressed adult male HIV-1 Tat transgenic mice. Ref. © 2020 Salahuddin et al., 2020b, Licensee MDPI, Basel, Switzerland distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

(**A**) Human immunodeficiency virus (HIV)-1 trans-activator of transcription (Tat) expression was induced in Tat(+) males (hatched bars), or not induced in Tat(−) controls (open bars), via administration of doxycycline (30 mg/kg, i.p., once daily for 5 days with 2 days of washout). Mice were either stressed via forced swim for 15 min (panel C) or not (panel B) and acutely-administered saline or oxycodone (3 mg/kg, i.p.) 15 min prior to assessment in an open field and light-dark transition test (n = 8–12/group). (**B**) Distance (m) traveled in an open field among non-stressed mice. (**C**) Distance (m) traveled in an open field among stressed mice. * indicates a main effect of genotype wherein Tat(+) mice differ from Tat(−) controls. † indicates a main effect for oxycodone to differ from saline-administered mice, $p < 0.05$.

Repeated Exposure to Oxycodone Increases the Tat-mediated Psychomotor behavior

While the initial opioid response is indicative of later abuse liability, many HIV+ patients have been prescribed oxycodone and are exposed repeatedly. It was of particular interest to investigate how repeated oxycodone administration alters the psychomotor behavioral response in Tat-exposed animals. In the present experiment, Tat-tg male mice had HIV-1 Tat expression induced (or not) via doxycycline administration for five days (with two days of washout). During this time, mice received daily injections of saline (0.9%, i.p.) or oxycodone (3 mg/kg, i.p.). Mice were (or were not) exposed to 15-min swim stress prior to testing (Fig. 47A).

Repeated oxycodone significantly increased the distance [$F(1,32) = 46.98$, $p < 0.05$] (Fig. 47B; see †) and velocity [$F(1,32) = 46.68$, $p < 0.05$] (Table 9) of travel among mice while reducing the frequency [$F(1,32) = 6.08$, $p < 0.05$] (Table 9) and time [$F(1,32) = 11.18$, $p < 0.05$] (Table 9) spent rearing, irrespective of their genotype. An interaction was observed for anxiety-like behavior in the light-dark transition test wherein Tat(+) mice demonstrated a significant decrease in the latency to transition to the dark compartment when administered repeated oxycodone [$F(1,32) = 4.05$, $p = 0.05$]; no such effect was observed on Tat(−) controls (Table 9). Likewise, Tat(+) mice spent significantly less time in the brightly-lit compartment, compared to Tat(−) mice [$F(1,32) = 3.99$, $p = 0.05$] (Table 9). Irrespective of genotype, repeated oxycodone induced more light-dark compartmental transitions than did repeated saline [$F(1,32) = 4.93$, $p = 0.05$] (Table 9). When considered in light of the data collected in the acutely-administration paradigm, repeated oxycodone attenuated HPA axis activation among control mice and commensurately potentiated their psychomotor response to the drug, similar to that of Tat(+) mice. These data demonstrate the effects of repeated opioid exposure on the HPA axis and related behavior (Salahuddin et al.,

2020b).

Repeated oxycodone injection also altered the psychomotor response to a stressor, particularly among Tat(+) mice. As seen in the acute administration paradigm, overall motor behavior was reduced following swim stress compared to that observed in non-stressed mice. Repeated oxycodone and genotype interacted such that oxycodone–administered Tat(+) mice traveled a significantly greater distance [$F(1,29) = 7.01$, $p < 0.05$] (Fig. 47C; see ‡) and velocity [$F(1,29) = 7.10$, $p < 0.05$] (Table 9) than did any other group. No differences in rearing frequency or time were observed. Notably, repeated oxycodone decreased the time spent in the brightly-lit compartment of the light-dark transition test [$F(1,29) = 3.86$, $p = 0.05$] (Table 9). No differences were observed in the latency to the dark compartment or the number of transitions between compartments (Table 9; Salahuddin et al., 2020b).

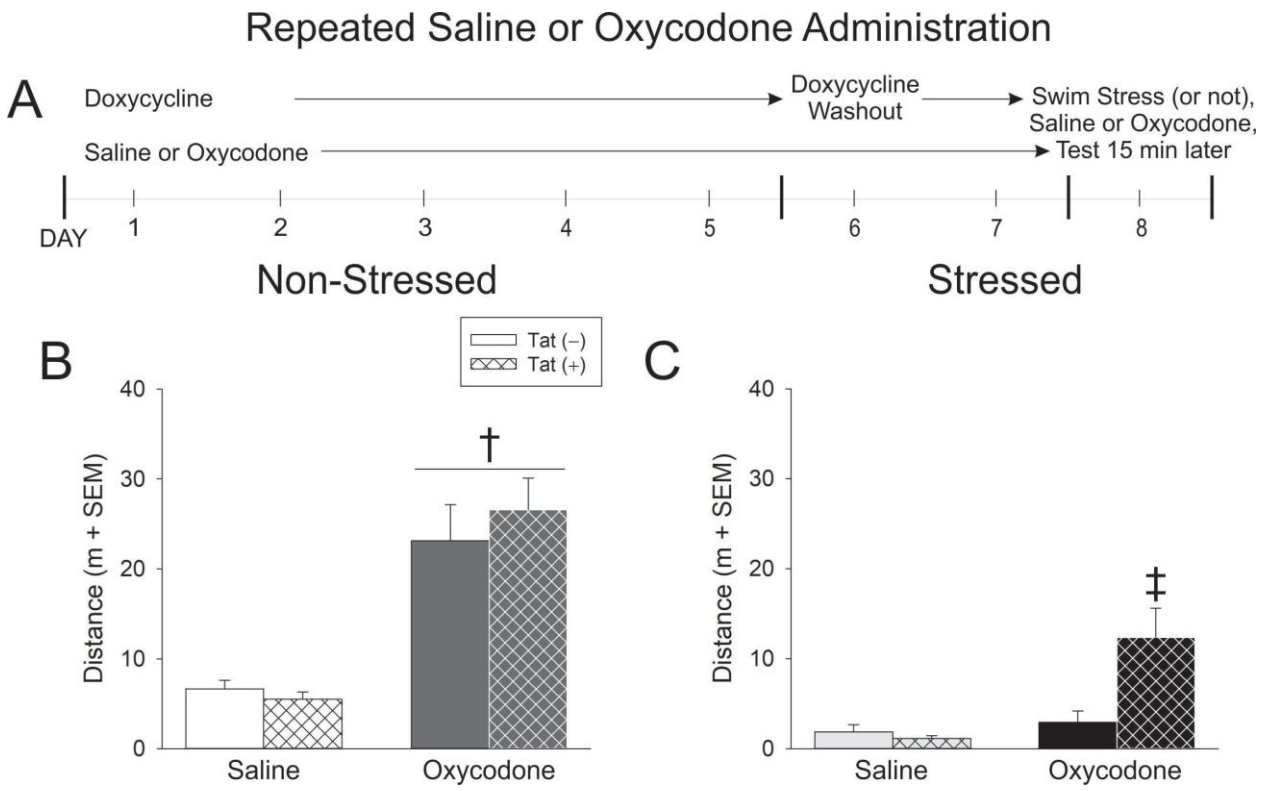

Figure 47: Effect of repeated oxycodone exposure on psychomotor response in non-stressed and stressed adult male HIV-1 Tat transgenic mice. Ref. © 2020 Salahuddin et al., 2020b, Licensee MDPI, Basel, Switzerland distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

(**A**) In the above experiment, Tat(−) (see open bars) and Tat(+) (see hatched bars) mice were administered saline or oxycodone (3 mg/kg, i.p., once daily for 7 days) concurrent with the induction of HIV-1 Tat via doxycycline (30 mg/kg, i.p., once daily for 5 days with 2 days of doxycycline washout). Mice were stressed via forced swim for 15 min (panel C) or not (panel B), were administered the last treatment of repeated saline or oxycodone, and 15 min later were assessed in an open field and light dark transition test (n = 8–10/group). (**B**) Distance (m) traveled in an open field among non-stressed mice. (**C**) Distance (m) traveled in an open field among stressed mice. † indicates a main effect for oxycodone to differ from saline-administered mice. ‡ indicates an interaction wherein oxycodone-administered Tat(+) mice differ from all other mice, $p < 0.05$.

Table 9: Motor and anxiety-like measures for forced-swim stressed (or not) Tat(−) and Tat(+) male mice assessed in open field and light-dark transition tests following repeated oxycodone exposure. Ref. © 2020 Salahuddin et al., 2020b, Licensee MDPI, Basel, Switzerland distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

Motor measures acquired in an open field and anxiety-like/motor measures acquired in a light-dark transition task from Tat(−) and Tat(+) males that were exposed (or not) to a 15-min forced swim stress with repeated administration of saline or oxycodone. * indicates a main effect of genotype wherein Tat(+) mice differ from Tat(−) controls. † indicates a main effect for oxycodone to differ from saline-administered mice. ‡ indicates an interaction wherein the denoted group significantly differs from Tat(+), saline-administered controls. § indicates an interaction wherein the denoted group significantly differs from all other groups, $p \leq 0.05$

Behavioral Measure	Non-Stressed				Stressed			
	Saline (0.9% *w/v*)		Oxycodone (3 mg/kg)		Saline (0.9% *w/v*)		Oxycodone (3 mg/kg)	
	Tat(−) (*n* = 8)	Tat(+) (*n* = 10)	Tat(−) (*n* = 8)	Tat(+) (*n* = 10)	Tat(−) (*n* = 8)	Tat(+) (*n* = 8)	Tat(−) (*n* = 8)	Tat(+) (*n* = 9)
Mean Velocity (m/s)	0.022 ± 0.003	0.018 ± 0.003	0.077 ± 0.013 †	0.089 ± 0.012 †	0.007 ± 0.003	0.004 ± 0.001	0.01 ± 0.004	0.041 ± 0.011 §
Rearing number	34.8 ± 6.6	25.3 ± 6.2	13.8 ± 4.0 †	19.7 ± 4.1 †	1.1 ± 0.6	1.6 ± 0.5	1.4 ± 0.9	2.0 ± 1.2
Rearing Time (s)	23.3 ± 5.6	19.5 ± 5.5	5.2 ± 1.8 †	9.2 ± 2.3 †	0.4 ± 0.3	0.6 ± 0.2	0.6 ± 0.5	1.0 ± 0.6
Latency to first enter dark (s)	30 ± 12	81 ± 32	38 ± 17	8 ± 2 ‡	44 ± 37	38 ± 19	17 ± 9	14 ± 5
Light zone time (s)	116 ± 32	91 ± 25 *	119 ± 25	46 ± 12 *	102± 38	68 ± 19	53 ± 12 †	33± 7 †
Number of transitions	10 ± 2	9 ± 2	17 ± 3 †	11 ± 1 †	10 ± 2	11± 2	15 ± 3	11 ± 2

Aim 2: Assess the behavior endpoints above following systemic antagonism of pharmacodynamic targets (glucocorticoid receptor or CRF receptor).

A. Female HIV-1 Tat transgenic mice

Gonadal Steroids Are Necessary for Tat to Potentiate Oxycodone-Mediated Psychostimulation

In order to determine the importance of the HPA and HPG axes in Tat-potentiated psychomotor and/or anxiety-like behavior, HPA axis receptor sites were pharmacologically blocked, and circulating gonadal hormones were surgically attenuated. To achieve this, some mice were administered a vehicle, antalarmin (CRF-R blocker), or RU-486 (GR blocker) concurrent with Tat induction for seven days. Some mice were OVX to remove the primary source of circulating gonadal hormones. Gonadally-intact mice were tested when in proestrus. All mice received an acute injection of saline or oxycodone (3 mg/kg) 15 min prior to behavior testing (Fig. 48A).

Oxycodone significantly increased the distance traveled for all mice compared to saline administration [$F(3,128) = 2.73$, $p > 0.05$] (see †, Fig. 48B). HIV-1 Tat exposure interacted with oxycodone administration to influence psychomotor behavior as assessed by the distance [$F(1,128) = 14.42$, $p < 0.05$] and velocity [$F(1,128) = 14.56$, $p < 0.05$] travelled in an open field (Fig. 48B; Table 10). Irrespective of treatment with vehicle, antalarmin or RU-486, oxycodone-administered Tat(+) mice travelled a significantly greater distance ($p < 0.0001$; see §, Fig. 48B) and speed ($p < 0.0001$; Table 10) than did Tat(−) controls or their saline administered counterparts. As well, there was an interaction for OVX to attenuate the Tat-potentiated increase in oxycodone-mediated distance [$F(3,128) = 2.66$, $p < 0.05$] and velocity [$F(3,128) = 2.66$, $p < 0.05$] traveled (see ‡, Fig.

48B). OVX/Tat(+) mice administered oxycodone demonstrated a significant attenuation in the distance ($p < 0.0023–0.0033$; see ‡, Fig. 48B) and speed ($p < 0.0023–0.0033$; Table 10) of travel compared to vehicle- or inhibitor-treated Tat(+) mice administered oxycodone, indicating the significant role ovarian hormones play modulating these effects (Salahuddin et al., 2021c).

In the light-dark transition test, either oxycodone [$F(1,124) = 4.29$, $p < 0.05$] (see †, Table 10) or Tat expression [$F(1,124) = 16.66$, $p < 0.05$] (see *, Table 10) significantly increased anxiety-like behavior by reducing the amount of time spent in the light zone. Pretreatment with RU-486 also increased anxiety-like behavior, reducing the amount of time spent in the light zone compared to antalarmin administration or OVX [$F(3,124) = 2.70$, $p < 0.05$] (see #, Table 10). Any pharmacological pretreatment or OVX significantly reduced the time spent rearing compared to vehicle pretreatment [$F(3,123) = 6.30$, $p < 0.05$] (see #, Table 10). When transitions were assessed in light dark test, oxycodone administration, estrous cycle phase and Tat expression interacted [$F(3,126) = 2.75$, $p < 0.05$] (see #, Table 10) such that antalarmin-treated Tat(+) mice made significantly fewer transitions than their respective Tat(−) control group ($p = 0.046$; Table 10). Conversely, antalarmin-treated Tat(−) mice made significantly more transitions than their respective Tat(−) control group ($p = 0.0151$; Table 10; Salahuddin et al., 2021c).

In summary, OVX attenuated Tat's capacity to potentiate oxycodone-mediated psychostimulation. Neither CRF nor GR blockade influenced these effects, further supporting the influence of HPG factors in female mice (Salahuddin et al., 2021c).

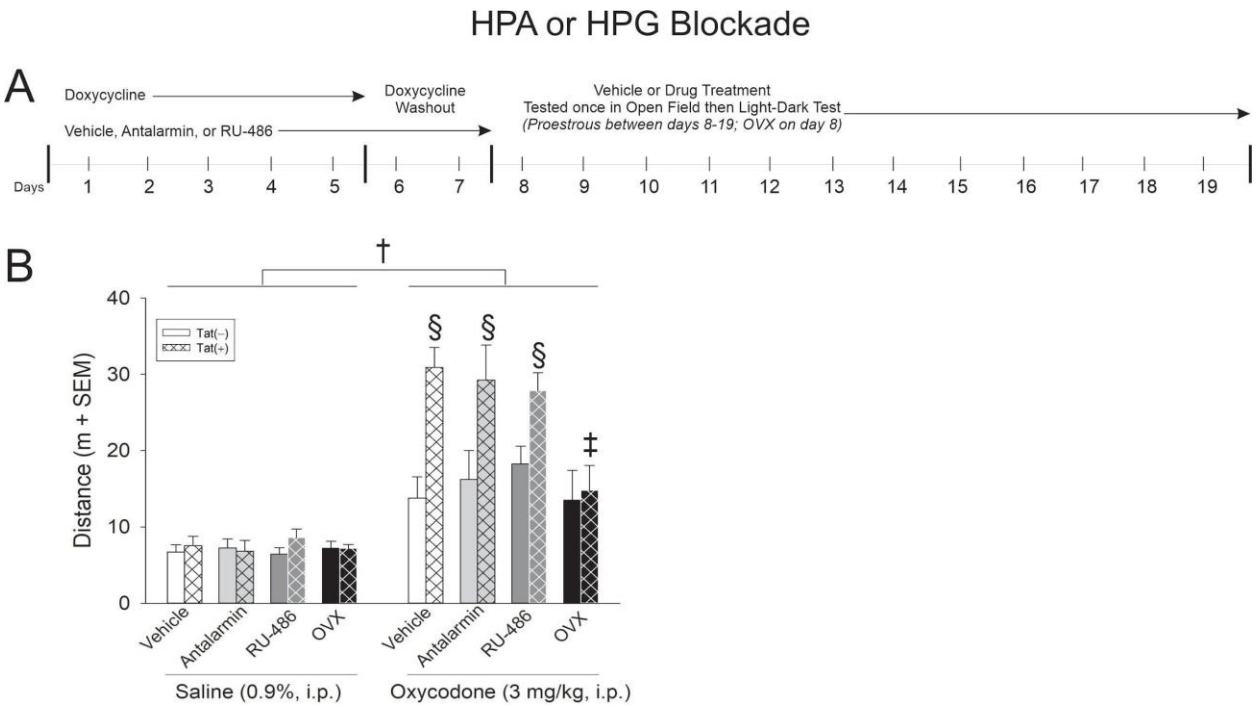

Figure 48: Effect of acute oxycodone exposure on psychomotor response in HPA or HPG blockade adult naturally-cycling female HIV-1 Tat transgenic mice. Ref. © 2021 Salahuddin et al., 2021c, Licensee MDPI, Basel, Switzerland distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

(**A**) HIV-1 Tat-transgenic, female mice [hatched bars; Tat(+)] or control counterparts [open bars; Tat(−) controls] had transgene expression induced via doxycycline (30 mg/kg, i.p., once daily for 5 days with 2 days for washout; n = 8–10/group) concurrently mice were also pretreated with either vehicle, antalarmin (20 mg/kg, i.p.), RU-486 (20 mg/kg, i.p.) or were ovariectomized (OVX). On the day of testing mice were administered saline or oxycodone 15 min prior to assessment in an open field and light-dark transition test. (**B**) Distance (m) travelled by mice in an open field. † indicates a main effect for oxycodone-administered mice to differ from saline-administered controls. § indicates an interaction wherein oxycodone-administered Tat(+) mice differ from respective Tat(−) controls and saline-administered controls. ‡ indicates an interaction wherein oxycodone-administered Tat(+) OVX mice differ from all other oxycodone- administered Tat(+) groups, $p < 0.05$.

Table 10: Motor and anxiety-like measures for HPA/HPG blockade Tat(−) and Tat(+) female mice assessed in open field and light-dark transition tests following acute oxycodone exposure. Ref. © 2021 Salahuddin et al., 2021c, Licensee MDPI, Basel, Switzerland distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

Motor and anxiety-like measures for Tat(–) and Tat(+) female mice assessed in open field and light-dark transition tests. Proestrus mice were pretreated with vehicle, antalarmin, RU-486 or were ovariectomized (OVX) prior to administration of saline or oxycodone. * indicates a main effect of genotype wherein Tat(+) mice differ from Tat(–) controls. † indicates a main effect of drug condition wherein oxycodone-administered mice differ from saline-administered controls. # indicates a main effect for any HPA or HPG manipulation to differ from vehicle-pretreated mice. ^ indicates a main effect for RU-486 to differ from antalarmin or OVX. § indicates an interaction wherein wherein oxycodone-administered Tat(+) mice differ from respective Tat(–) and saline-administered controls. @ indicates an interaction wherein antalarmin treated Tat(–) mice differ from respective Tat(+) mice and vehicle-treated Tat(–) controls. ‡ indicates an interaction wherein oxycodone-administered OVX mice differ from oxycodone-administered mice pretreated with vehicle, RU-486 and Antalarmin, $p < 0.05$.

Behavioral Measure	Vehicle				Antalarmin			
	Saline (0.9% *w/v*)		Oxycodone (3 mg/kg)		Saline (0.9% *w/v*)		Oxycodone (3 mg/kg)	
	Tat(−)	Tat(+)	Tat(−)	Tat(+)	Tat(−)	Tat(+)	Tat(−)	Tat(+)
Light zone time (s)	112 ± 38	95 ± 43*	119 ± 35†	23 ± 7†	165 ± 34	100 ± 35*	85 ± 31†	31 ± 12†
Mean Velocity (m/s)	0.022± 0.003	0.025± 0.004	0.046± 0.009†	0.103± 0.009†§	0.024± 0.004	0.023± 0.005	0.054± 0.013†	0.098± 0.015†§
Number of transitions	3.4 ± 0.3	7.5 ± 1.5	14.3 ± 2.9	7.7 ± 2.3	11.8 ± 2.3@	5.1 ± 1.1	11.2 ± 3.3	12.5 ± 4.7
Rearing Time (s)	62 ± 33	15 ± 4	61 ± 37	34 ± 17	9 ± 3#	12 ± 2#	4 ± 2#	7 ± 3#

Behavioral Measure	RU-486				OVX			
	Saline (0.9% *w/v*)		Oxycodone (3 mg/kg)		Saline (0.9% *w/v*)		Oxycodone (3 mg/kg)	
	Tat(−)	Tat(+)	Tat(−)	Tat(+)	Tat(−)	Tat(+)	Tat(−)	Tat(+)
Light zone time (s)	50 ± 12$^{\#\wedge}$	29 ± 8*$^{\wedge}$	83 ± 28$^{\dagger\wedge}$	29 ± 7$^{\dagger\wedge}$	129 ± 39	88 ± 30*	137 ± 39†	27 ± 6†
Mean Velocity (m/s)	0.022± 0.003	0.029± 0.004	0.061± 0.008†	0.093± 0.008†§	0.024± 0.003	0.024± 0.002	0.045± 0.013†‡	0.049± 0.011†§‡
Number of transitions	4.9 ± 0.6	4.3 ± 0.6	9.3 ± 2.6	10.5 ± 1.5	9.4 ± 2.5	6.8 ± 1.0	9.1 ± 2.5	10.8 ± 2.3
Rearing Time (s)	9 ± 2$^{\#}$	17 ± 4$^{\#}$	3 ± 1$^{\#}$	11 ± 2$^{\#}$	11 ± 2$^{\#}$	15 ± 2$^{\#}$	16 ± 11$^{\#}$	2 ± 1$^{\#}$

B. Male HIV-1 Tat transgenic mice

Glucocorticoid and CRF Receptors Are Involved in the Psychomotor, Anxiety-Like, and HPA Axis Response to Oxycodone

In order to begin identifying the important aspects of the HPA axis that are involved in the psychomotor, anxiety-like, and glucocorticoid response to oxycodone and Tat, mice were administered glucocorticoid receptor (GR) and/or corticotropin-releasing factor-receptor (CRF-R) inhibitors concurrent with doxycycline administration (Fig. 49A). To block GR and CRF-R, mice were administered RU-486 (a.k.a. mifepristone) and/or antalarmin, respectively. Mice received an injection of saline (0.9%, i.p.) or oxycodone (3 mg/kg, i.p.) 15 min prior to testing (Fig. 49A).

HIV-1 Tat exposure, oxycodone administration, and pharmacological inhibitors

significantly interacted to influence psychomotor behavior as assessed by the distance [$F(3,122) = 2.86$, $p < 0.05$] and speed [$F(3,122) = 3.49$, $p < 0.05$] traveled. As before, oxycodone significantly increased the distance ($p < 0.0001$–0.03; Fig. 49B; see †) and speed ($p < 0.0001$–0.03; Table 11) traveled among all mice compared to saline administration. Compared to Tat(−) controls, Tat(+) mice exhibited a significant potentiation in oxycodone-induced distances ($p < 0.0001$–0.007; Fig. 49B; see *) and speed ($p < 0.0001$–0.006; Table 11) traveled irrespective of pre-treatment with vehicle, antalarmin, or RU-486. However, when Tat(−) mice were treated with both antalarmin and RU-486 (blocking GRs and CRF-Rs), they demonstrated a potentiation of oxycodone-induced psychostimulation that was commensurate to Tat(+) mice ($p = 0.003$; see #). All Tat(+) mice administered a GR and/or CRF-R inhibitor demonstrated a modest, but significant, reduction in the distance ($p < 0.0001$–0.04; Fig. 49B; see #) and speed ($p < 0.0001$–0.002; Table 11) of travel. RU-486 notably reduced the distance ($p = 0.04$; Fig. 49B; see #) and speed ($p = 0.04$; Table 11) of travel among Tat(−) controls. These data support the notion that GR- and CRF-mediated feedback plays an important role in the acute behavioral response to opioids and the capacity for Tat to potentiate these effects in male mice (Salahuddin et al., 2020b).

Table 11: Motor and anxiety-like measures for HPA blockade Tat(−) and Tat(+) male mice assessed in open field and light-dark transition tests following acute oxycodone exposure. Ref. © 2020 Salahuddin et al., 2020b, Licensee MDPI, Basel, Switzerland distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

Motor measures acquired in an open field and anxiety-like/motor measures acquired in a light-dark transition task from Tat(−) and Tat(+) males that were pretreated with the corticotrophin-releasing factor receptor antagonist, antalarmin, and/or the glucocorticoid receptor antagonist, RU-486, prior to administration of saline or oxycodone. * indicates an interaction wherein Tat(+) mice differ from respective Tat(−) controls. † indicates an interaction wherein oxycodone-administered mice differ from respective saline-administered controls. # indicates an interaction wherein the denoted group differs from their respective vehicle controls, $p < 0.05$.

Behavioral Measure	Vehicle				Antalarmin			
	Saline (0.9% *w/v*)		Oxycodone (3 mg/kg)		Saline (0.9% *w/v*)		Oxycodone (3 mg/kg)	
	Tat(−) (*n* = 8)	Tat(+) (*n* = 7–8)	Tat(−) (*n* = 8–9)	Tat(+) (*n* = 9)	Tat(−) (*n* = 8)	Tat(+) (*n* = 8–9)	Tat(−) (*n* = 8–9)	Tat(+) (*n* = 10)
Mean Velocity (m/s)	0.031 ± 0.006	0.031 ± 0.005	0.053 ± 0.008 †	0.11 ± 0.01 †*	0.018 ± 0.003	0.016 ± 0.002	0.051 ± 0.007 †	0.077 ± 0.005 †*#
Rearing number	34 ± 5	32 ± 8	8 ± 4	38 ± 12	16 ± 3	21 ± 6	10 ± 4	12 ± 5
Rearing Time (s)	27.3 ± 4.5	27.7 ± 7.5	1.9 ± 0.7 †	8.6 ± 2.0 †	11 ± 2.4 #	13.6 ± 4.5 #	6.6 ± 3.3 †	2.7 ± 0.9 †
Latency to first entry to dark zone (s)	40.7 ± 14.2	5.3 ± 1.8	10.7 ± 4.9	7.3 ± 1.7	86.9 ± 43.3	125.5 ± 36.4	7.0 ± 1.3 †	6.8 ± 1.9 †
Light zone time (s)	177 ± 31	38 ± 11 *	32. ± 7 †	32 ± 5	104 ± 40	148 ± 34 #	98 ± 31	74 ± 26 †

Number of transitions	14.9 ± 3.6	11.4 ± 2.9	8.4 ± 1.2	9.1 ± 1.3	6.0 ± 2.5	6.1 ± 1.3	14.2 ± 3.5	11.8 ± 2.3

Behavioral Measure	**RU-486**				**Antalarmin + RU-486**			
	Saline (0.9% *w/v*)		**Oxycodone (3 mg/kg)**		**Saline (0.9% *w/v*)**		**Oxycodone (3 mg/kg)**	
	Tat(−) (*n* = 8–9)	**Tat(+) (*n* = 7–8)**	**Tat(−) (*n* = 8–9)**	**Tat(+) (*n* = 7–8)**	**Tat(−) (*n* = 9)**	**Tat(+) (*n* = 8)**	**Tat(−) (*n* = 9–10)**	**Tat(+) (*n* = 9–10)**
Mean Velocity (m/s)	0.01 ± 0.001 #	0.021 ± 0.003	0.035 ± 0.006 †	0.065 ± 0.011 †*#	0.020 ± 0.003	0.018 ± 0.004	0.082 ± 0.012 †#	0.075 ± 0.007 †#
Rearing number	9 ± 2	17 ± 4	25 ± 20	15 ± 7	12 ± 3	16 ± 5	57 ± 29	11 ± 4
Rearing Time (s)	7.8 ± 2.1 #	13.8 ± 3.5 #	2.9 ± 1.9 †	4.0 ± 1.2 †	8.6 ± 1.9 #	11.0 ± 4.0 #	9.1 ± 2.9 †	1.8 ± 0.4 †
Latency to first entry to dark zone (s)	78.0 ± 35.5	97.3 ± 52.4	16.1 ± 4.5	3.2 ± 0.7	110.0 ± 47.9	23.9 ± 6.4	14.8 ± 4.0 †	3.8 ± 0.9 †
Light zone time (s)	120 ± 43	107 ± 43	116 ± 378 #	8 ± 1 †*	169 ± 40	34 ± 5 *	200 ± 17 #	61 ± 13 *
Number of transitions	3.4 ± 0.7 #	3.1 ± 0.9 #	9.0 ± 2.1	5.0 ± 0.9	7.4 ± 2.4	4.0 ± 0.5	27.4 ± 6.0 †#	33.2 ± 15.5 †#

To account for differences in baseline psychostimulation that were caused by inhibitors, the proportional change in distance from each group's baseline was also analyzed (Fig. 49C). Oxycodone did not initially increase the distance traveled among Tat(−) controls; however, blocking either GRs and/or CRF-Rs significantly increased the proportional distance traveled ($p <$ 0.0001–0.002; Fig. 49C; see †), supportive of an inhibitory role for these receptors in this process. Tat(+) mice demonstrated a proportional oxycodone-mediated increase in psychostimulation

irrespective of GR and/or CRF-R inhibition ($p < 0.0001$; Fig. 49C; see †; Salahuddin et al., 2020b).

Compared to vehicle-administration, antalarmin and/or RU-486 significantly increased oxycodone-mediated psychostimulation in Tat(−) mice ($p < 0.0001$–0.02; Fig. 49C; see #), suggesting that CRF-R and GR signaling are intact and typically inhibitory of this behavior. Among Tat(+) mice, only antalarmin significantly increased oxycodone-mediated psychostimulation ($p = 0.01$; Fig. 49C; see #), suggesting that CRF-Rs remain functional; however, the sensitivity or function of GRs may be perturbed (Salahuddin et al., 2020b).

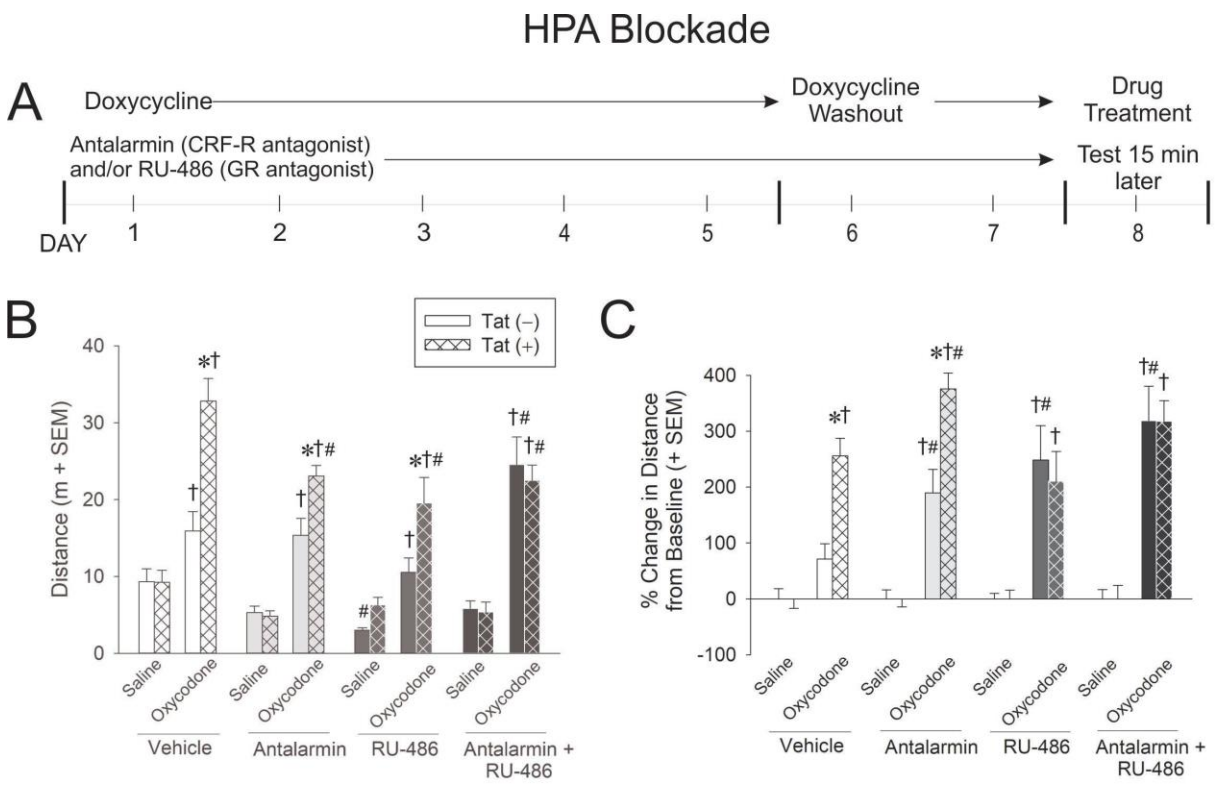

Figure 49: Effect of acute oxycodone exposure on psychomotor response in HPA blockade adult male HIV-1 Tat transgenic mice. Ref. © 2020 Salahuddin et al., 2020b, Licensee MDPI, Basel, Switzerland distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

In the above experiment, (**A**) Tat(−) (see open bars) and Tat(+) (see hatched bars) mice were administered antalarmin (corticotrophin-releasing factor receptor antagonist; 20 mg/kg, i.p. for 6 days) and/or RU-486 (glucocorticoid receptor antagonist; i.p., 20 mg/kg, i.p. for 7 days) concurrent with the induction of HIV-1 Tat via doxycycline (30 mg/kg, i.p., once daily for 5 days with 2 days of doxycycline washout). Mice were treated with the final dose of antalarmin and/or RU-486 and then challenged with saline or oxycodone (3 mg/kg, i.p.) and assessed in an open field and light-dark transition test (n = 8–10/group). (**B**) Distance (m) traveled in an open field and (**C**) the proportional change from baseline in distance traveled in open field. * indicates an interaction wherein Tat(+) mice differ from respective Tat(−) controls. † indicates an interaction wherein oxycodone-administered mice differ from respective saline-administered controls. # indicates an interaction wherein the denoted group differs from their respective vehicle controls, $p < 0.05$.

2.4. Discussion

The hypothesis that HIV-1 Tat exposure would promote anxiety- and depression-like behavior, cognitive impairment and potentiate oxycodone-induced psychomotor responses, together with alterations in circulating steroidal hormones (i.e. corticosterone, estradiol, and progesterone), were largely upheld (Salahuddin et al., 2020a, 2020b; 2021c). Oxycodone initially enhanced indicators of HPA activation, although there was an apparent tolerance after repeated exposure (Salahuddin et al., 2020a, 2020b). Repeated oxycodone exposure sensitized psychomotor activity in Tat-exposed mice and, when coupled with a natural stressor, increased sensitivity even more (Salahuddin et al., 2020b). Tat had a cycle-dependent effect on oxycodone's HPA activation and simultaneously potentiate oxycodone's psychostimulatory effects (Salahuddin et al., 2021c). Tat exposure impaired both affective and cognitive function, and cognition was significantly reduced when corticosterone levels were substantially elevated (Salahuddin et al., 2020a). The behavioral findings occurred along with sensitization of the HPA response, indicating that these changes were not mutually exclusive (Salahuddin et al., 2020b). The pharmacological inhibition of GR and/or CRF-R enhanced the psychomotor effects of oxycodone in Tat(−) control mice, indicating their role in opioid-mediated psychostimulation (Salahuddin et al., 2020b). Blocking GR enhanced circulating corticosterone while decreasing oxycodone-mediated psychomotor activity in Tat(+) male mice (Salahuddin et al., 2020b). Blocking CRF-R reduced combined Tat and oxycodone mediated anxiety-like behavioral effects (Salahuddin et al., 2020b). Significant sex differences were observed, unlike males, CRF-R and GR were not involved in mediating in opioid-mediated psychostimulation in female mice (Salahuddin et al., 2021c). However, in OVX mice, Tat's ability to enhance oxycodone's motor

effects was completely diminished, concomitant with an increase in adrenal glucocorticoids and pregnane steroids like allopregnanolone, implicating the HPG axis in these effects (Salahuddin et al., 2021c). These results extend on previous findings, suggesting that Tat exposure dysregulates the HPA axis, and neurosteroidogenesis, thereby promoting neuroHIV like phenotype and increasing susceptibility to oxycodone's psychostimulant effects (Salahuddin et al., 2020a, 2020b, 2021c). Thus, maintaining the neuroendocrine axis may benefit HIV-1 and oxycodone-mediated neuropathological outcomes.

Psychomotor locomotion has been shown to be a reliable test for assessing the effectiveness of opioids in rats (Zhang and Kong, 2017). To corroborate this, we demonstrated that exposure to Tat substantially exacerbated the psychomotor effects of opioids like oxycodone (Salahuddin et al., 2020a, 2020b; 2021c). In Tat(−) proestrous and diestrous mice, oxycodone enhanced locomotor activity by 1.4 and 1.3 times, respectively, when compared to saline-administered controls (Salahuddin et al., 2020a). When mice were exposed to Tat, there was a 2.9-fold rise in proestrous and a 3.3-fold increase in the diestrous phase, respectively (Salahuddin et al., 2020a). These findings corroborate earlier observations of increased psychostimulant locomotion in male mice treated to a similar Tat-induction paradigm (Paris et al., 2014a). These effects are clinically significant since the initial potency of a substance of abuse is crucial in the addiction cycle (Koob and Schulkin, 2018). The present opioid problem is fueled in part by very powerful synthetic opioids (i.e. fentanyl; Volkow et al., 2019). The clinical significance of this study underlies the capacity of HIV-1 Tat to increase the potency of oxycodone, and contribute to HAND. Based on prior findings that opioid analgesia is reduced in HIV^+ patients (Smith, 2011) and preclinical models such as mice exposed to HIV-1 Tat protein (Gonek et al., 2018) or coat

protein gp120 (Minami et al., 2003), increases in opioids' psychomotor or rewarding effects (Gonek et al., 2018) may be indicative of later abuse risk.

The emotional/affective dysregulation observed in HIV^{+} patients (Bing et al., 2001; Evans et al., 2005) is replicated in male mice following exposure to HIV-1 Tat (Makhathini et al., 2018a, 2018b; Paris et al., 2014a, 2014c; Schier et al., 2017). Female models are underrepresented in the preclinical research; however, the development of affective disorder may vary by sex. Women may be less prone to HIV-related anxiety and depression than males (Goggin et al., 1998; Lopes et al., 2012), as seen in Tat-exposed mice (Hahn et al., 2015). Also, some studies showed male gender may predict HIV affective disorder (Orlando et al., 2002). We believe that females' protection to affective dysregulation may be due to circulating steroid variation, however further research is needed. Animal models may aid in controlling for the hormonal differences and assess for behavioral outcomes. As such, we found Tat enhanced depression-like behavior in proestrous mice and decreased it in diestrus animals (Salahuddin et al., 2020a; who already exhibited greater depression-like behavior). Prior studies demonstrated male mice exposed to Tat had similar heightened depression-like responses (Fu et al., 2011; Kesby et al., 2016a; Lawson et al., 2011; McLaughlin et al., 2017). The opioid's motor effects reduced overall immobility, but the pattern of depression-like behavior was reversed and was higher in Tat-treated diestrous mice than in Tat(−) counterparts (Salahuddin et al., 2020a). Among diestrous, Tat-exposed mice, higher corticosterone correlated with depression-like behavior (Salahuddin et al., 2020a). Likewise, clinical studies revealed, HIV infected patients using opioids show unsuppressed viral loads (Denis et al., 2021) and predicted depression symptoms (Pilowsky et al., 2011; German and Latkin, 2012). HIV-1 Tat was found to reduce

the inhibitory synapses and pre and postsynaptic proteins, namely synaptotagmin 2 and gephyrin in the anterior cingulate cortex (Nass et al., 2020), which may explain a pattern suggestive of behavioral disinhibition observed when depression-like and HPA-related effects occurred concurrently (Salahuddin et al., 2020a).

The HPA axis activation was reduced with repeated oxycodone administration, but Tat exposure impacted novel object recognition in mice (Salahuddin et al., 2020a). Pre- and post-cART opioid usage has been associated with cognitive impairment in clinical populations (Bell et al., 1998; Byrd et al., 2011; Martin-Thormeyer and Paul, 2009). Lifetime abuse is generally associated with polysubstance use and several episodes of abstinence and withdrawal periods. A 5-day oxycodone exposure may not have these substantial impacts (Salahuddin et al., 2020a). Other studies show Tat exposure impairs short-term memory performance in male rodent models (Carey et al., 2012; Marks et al., 2016), although not necessarily when the animals are drug-naive (Kesby et al., 2016a, 2016b; Kesby et al., 2018). We found that Tat impairs novel object recognition (Salahuddin et al., 2020a) and others have shown Tat suppress LTP (Behnish et al., 2004; Li et al., 2004); however, this pattern was only seen in female mice in their proestrous cycle (when circulating levels favored P_4 over E_2; Salahuddin et al., 2020a). However, when assessed for object recognition in the diestrus phase cognitive function was maintained, indicating Tat-mediated short-term memory impairments may be reversible in certain circumstances (Salahuddin et al., 2020a). These findings are further supported by clinical evidence which revealed a two- to three-fold increase in the risk of learning impairment in HIV^+ women on learning tasks during their transition from premenopause to peri and postmenopause, when estradiol levels are at their lowest (Maki et al., 2021). These findings corroborated with *in*

vitro studies that demonstrate E_2 or P_4 can decrease Tat-mediated neurotoxicity (Salahuddin et al., 2020a). As such future studies should assess chronic oxycodone use/abuse and its interactions with HIV proteins on memory function.

HPA dysregulation may increase vulnerability to addiction and mood disorders (Koob and Volkow, 2016). The sensitizing (Salahuddin et al., 2020ab; Kesby et al., 2017) and rewarding effects of illegal substances (Gonek et al., 2018; Salahuddin et al., 2022a Unpublished*) were observed to be augmented. Other studies have found that HIV-tg rats self-administer psychostimulants more than non-HIV-tg rats (de Guglielmo et al., 2020; McIntosh et al., 2015), however, these observations are not made consistently (Huynh et al., 2020; Kesby et al., 2019; Wayman et al., 2016). Maintaining the HPA stress axis may help prevent vulnerability to substance use disorders. Chronic stress increases the conditioned place preference for illicit drugs (Bali et al., 2015). Stress frequently triggers drug relapse and desire in human addicts (Mantsch et al., 2016). Tat's propensity to dysregulate the HPA axis may increase sensitivity to sensitizing and rewarding effects of illicit drugs and also promote affective disorders. Similarly, clinical reports suggest stressful life events, subjective stress, anxiety, and depressive symptoms were associated with elevated cortisol-to-DHEA ratios in HIV^+ population (Mukerji et al., 2021; Qiao et al., 2017). Likewise, Tat expression causes anxiety and depression in mice (reviewed in Gaskill et al., 2017). Stressed mice in their diestrous phase (greater estradiol: progesterone ratio) were more susceptible to Tat/oxycodone behavioral interactions (Salahuddin et al., 2021c), which corroborates similar findings of higher estradiol levels to boost psychostimulant reactions to drugs of abuse in rat models (Hu and Becker, 2003; Calipari et al., 2017; Vandegrift et al., 2017; Ramôa et al., 2013). Hence, reversing Tat-mediated HPA dysfunction may improve

outcomes across a number of physiological and behavioral markers.

2.5. Conclusion

The present dissertation chapter revealed HIV-1 Tat-expressing mice demonstrated neuroHIV-like symptomatology including anxiety/depression-like behavior, disinhibition, cognitive impairment, and a potentiated psychomotor response to oxycodone (Salahuddin et al., 2020a, 2020b; 2021c). Systemic injection of antalarmin (CRF receptor blocker) and RU-486 (glucocorticoid receptor blocker) attenuated psychomotor and anxiety-like behavior in male transgenic mice (Salahuddin et al., 2020a), but OVX reduced the oxycodone and Tat-mediated interactions in female mice (Salahuddin et al., 2021c). Taken together, these findings support the notion that Tat exposure might disrupt the HPA axis, increasing sensitivity to stress-related substance use and affective disorders.

CHAPTER 3

Neuroendocrine Modulators in the restoration of HIV-1 Tat-mediated hypothalamus-pituitary-adrenal stress axis dysregulation and neurological behavioral deficits

This chapter is under preparation

1. Salahuddin MF,, Mahdi F, Paris JJ. Neuroendocrine Modulators in restoration of HIV-1 Tat mediated Hypothalamus-pituitary-adrenal stress axis dysregulation. 2022b.

Abstract

Human immunodeficiency virus (HIV) is associated with comorbid affective, stress-sensitive neuropsychiatric and neuroendocrine complications that afflict ~50% of infected individuals, but the mechanisms are not known. One factor that may contribute to hypothalamic-pituitary-adrenal (HPA) stress axis dysfunction is the neurotoxic HIV-1 regulatory protein, trans-activator of transcription (Tat). We previously demonstrated that HIV-1 Tat promotes anxiety-like behavior in mice concurrent with an elevation of basal corticosterone (seen in males and females) and adrenal insufficiency (seen only in males). The HPA axis is tightly regulated by GABAergic signaling, therefore impairments in GABAergic signaling may contribute to neuroendocrine dysfunction, conferring vulnerability to stress-sensitive disorders. Given that neurosteroids are potent allosteric modulators of $GABA_A$ receptor, enhancing neurosteroidogenesis may ameliorate Tat-mediated HPA dysfunction. Adult male transgenic mice that expressed Tat_{1-86} protein [Tat(+)] or not [Tat(-)] were administered i.c.v. vehicle, the neurosteroid allopregnanolone (AlloP; 100nM), or the neurosteroid-enhancing compound FGIN-1-27 (5μg/μL). Mice were exposed to swim stress (or not) and behaviorally-tested in an open field. qRT-PCR was performed to assess expression of steroidogenic enzymes in the hypothalamus. AlloP reduced the latency to enter the brightly-illuminated center of an open field concurrent with normalizing Tat-mediated downregulation of the 3α-HSD-synthesizing enzyme. FGIN-1-27 reduced corticosterone in all mice. These data provide proof-of-principle that enhancing neurosteroidogenesis in the central nervous system can influence the HPA axis and related affective dysfunction. Reinstatement of central neurosteroid content may restore HPA function and reduce vulnerability to psychiatric disorders.

3.1. Introduction

Human immunodeficiency virus type 1 (HIV-1) remains a major public health problem, with over 1 million infected people in the United States. (CDC, 2020). Access to combined antiretroviral therapeutics (cART) transformed HIV/AIDS from a fatal disease to a manageable chronic illness with a normal life expectancy (Fauci and Marston, 2005; Tan and McArthur, 2012). HIV's primary target is the patient's immune system, but it also affects the central nervous system (CNS) to produce a range of neurocognitive dysfunctions (Saylor et al., 2016). Conceivably, patients continue to experience a constellation of neurological symptoms (i.e., neuroHIV), most likely as a result of poor retention capacity of cART in the CNS and failure to target neurotoxic HIV-1 proteins and latent viral reservoirs predominantly microglia and astrocytes (Alvarez-Carbonell et al., 2019; Letendre et al., 2004; Maban et al., 2016; Rao et al., 2009; Wallet et al., 2019; Zhang et al., 2015). Hence, HIV-1 brain reservoirs are safe viral sanctuaries where cART is ineffective and new adjunct therapeutics are required for functional cure.

Disruption of the hypothalamic-pituitary-adrenal (HPA) stress axis is a frequent but under-investigated neurological consequence of HIV (Nicolaides et al., 2020). Despite cART therapy, 14–46% of HIV^+ patients have a dysregulated HPA axis, as indicated by increased baseline glucocorticoids and seemingly counterintuitive adrenal insufficiency in response to a stressor (Chrousos and Zapanti, 2014; Marik et al., 2002; reviewed in Mayo et al., 2001). While it is widely documented that acute and/or chronic stress impairs HPA axis activity (Chrousos and Gold, 1992), recent evidence supports the notion that HIV-1 infection directly impairs the HPA axis (Chrousos and Zapanti, 2014). The majority of this evidence is based on molecular similarities found between several HIV genomic sequences and transcripts associated with the HPA axis (Kumar et al., 2003).

Thus, HIV-mediated HPA axis dysfunction may increase susceptibility to affective and substance use disorders (Koob and Volkow, 2016).

One of the viral proteins namely transactivator of transcription (Tat), crucial for HIV transcription (Das et al., 2011) may contribute to neuroHIV like symptomatology (Paris et al., 2020; Salahuddin et al., 2020ab, 2021c). Tat promotes excitotoxicity by direct activation of calcium channels resulting in the injury of the dendrites thereby interrupting neurotransmission (Fields et al., 2015; Hahn et al., 2015; Hu, 2016), or indirectly by release of proinflammatory cytokines (Ajasin and Eugenin, 2020; Ben Haij et al., 2015; Langford et al., 2015). Our prior studies show Tat to promotes HPA dysfunction (confirmed by elevated cortisol and adrenal insufficiency; Salahuddin et al., 2020ab, 2021c). Other studies show increased vulnerability of GABAergic neurons to Tat and HIV-1 infection (Barbour et al., 2020; Buzhdygan et al., 2016; Gelman et al., 2012). Indeed, GABA plays an important role in the control of the HPA axis at the PVN level, and GABA modulators, such as neurosteroids, may modulate the HPA axis (Maguire, 2019). In support, prior studies demonstrated HIV^+ infected patients with impaired circulating neurosteroid levels to be associated with depressive symptomatology (Mukerji et al., 2021). Consistently, other evidence shows HIV-infected [HIV(+)] human brains revealed a reduction in CYP450scc, 5α-reductase, and 3α-hydroxysteroid dehydrogenase compared to seronegative controls (Maingat et al., 2013). Given the stress sensitivity circuits are dysregulated in HIV^+ patients, manipulation of the HPA axis with GABAergic modulators like neurosteroids may have significant therapeutic implications.

We hypothesized that HIV-1 Tat would promote HPA dysfunction concurrent with promoting anxiety-like behavior in a conditionally-inducible Tat transgenic mouse model. We

further expected that neuroendocrine modulators like allopregnanolone or FGIN-1-27 would restore HPA function and ameliorate the behavioral deficits produced by HIV-1 Tat.

Aim: Assess the protective effects of neuroendocrine modulators in an HIV-1 transgenic mice model.

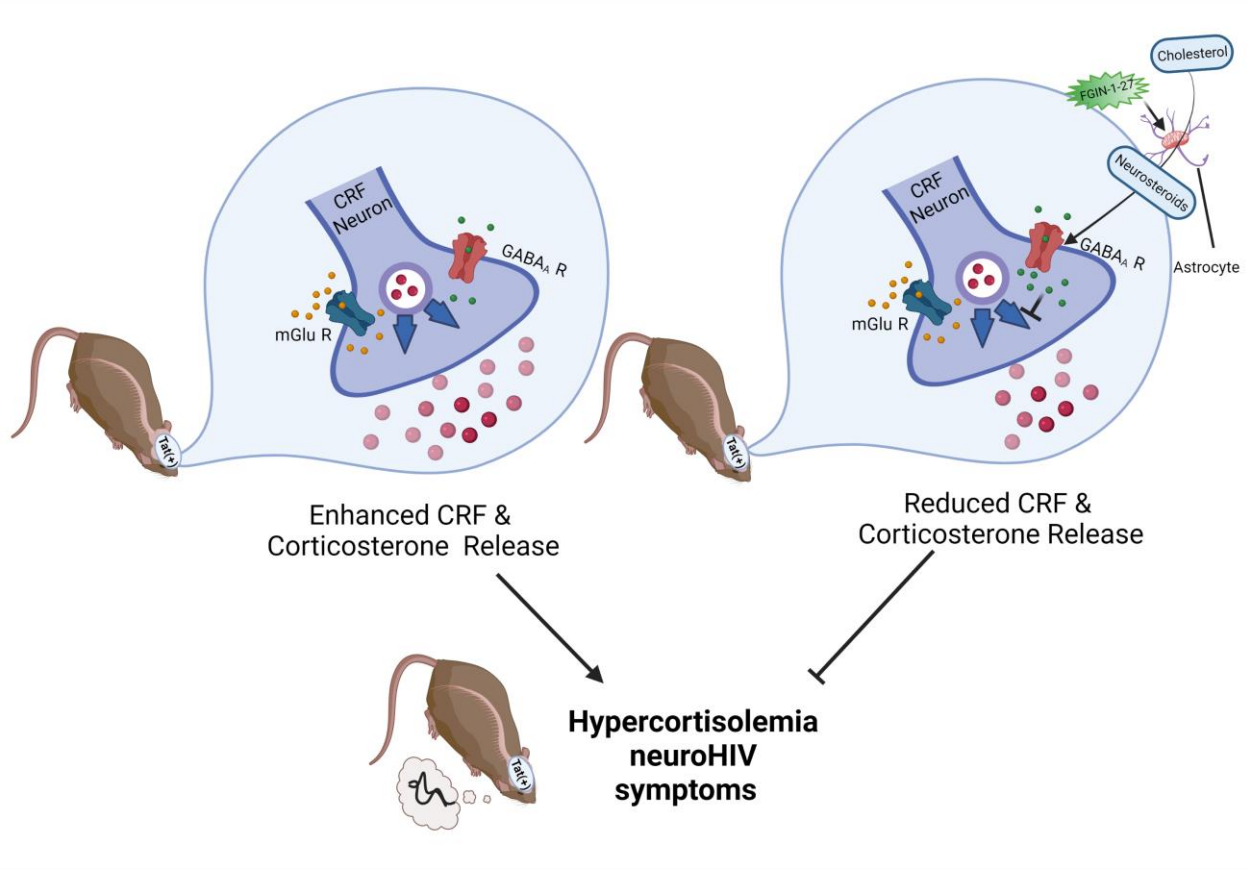

Figure 50: Schematic diagram of neurosteroids to reinstate HIV-1 Tat mediated HPA dysfunction and ameliorate neuroHIV phenotype in HIV-1 Tat transgenic mice.

HIV-1 Tat-expressing mice exhibit hypercortisolemia (elevated circulating corticosterone) due to imbalance in the glutamatergic and GABAergic transmission at CRF neuron concurrent with neuroHIV like phenotype. Neurosteroids increase GABAergic transmission and directly reduce CRF levels thereby reinstating HPA homeostasis and neuroHIV-like symptomatology.

3.2. Materials and Methods

Animal procedures were pre-approved by the University of Mississippi Institutional Animal Care and Use Committee (#18-004 & 21-005; approved October 2017 & September 2020) and were in accordance with National Institutes of Health's ethical standards (NIH Publication No. 85-23).

Subjects & Housing

Male adult doxycycline (DOX)-inducible, GFAP-driven, HIV-1 Tat-transgenic mice (n = 139) were bred in the vivarium at the University of Mississippi (University, MS, USA). Mice were housed in groups of two to five in temperature- and humidity-controlled environment with a 12:12 h light:dark cycle (lights switched off at 09:00 h) with *ad libitum* access to food and drinking water. Mice expressing the Tat and rtTA (reverse tetracycline transactivator) transgenes [(Tat+)] or mice lacking the tat transgene, but expressing the rtTA transgene only (Tat−), were administered doxycycline to turn the conditional expression of HIV-1 $Tat_{1–86}$ (Bruce-Keller et al., 2008).

Brain-targeted Tat induction with Doxycycline treatment

$Tat_{1–86}$ protein was conditionally expressed in HIV-1 Tat transgenic mice [Tat(+) or Tat(−)] by administration of doxycycline intraperitoneal (i.p.) for five consecutive days (30 mg/kg in 0.9% saline, 0.3 ml/30 g body weight). The doxycycline dose utilized for this study (30 mg/kg/d, i.p.) was based on prior studies confirming the effectiveness of Tat expression (Salahuddin et al., 2020ab; Salahuddin et al., 2021c). Given doxycycline may reduce neuroinflammation and therefore mask Tat's potential effects, a 1 to 2-day doxycycline washout interval was carried out (doxycycline $t_{1/2}$ = 5–6 h in mice; Lucchetti et al., 2019).

Intracerebroventricular administration technique

Transgenic mice that expressed the Tat_{1-86} protein [Tat(+)] or not [Tat(−)] were administered either vehicle (DMSO:Saline(0.9%) in a ratio of 1:10,000) or FGIN-1-27 (5 µg/µL) or Allopregnanolone (AlloP;100ng) directly into the lateral ventricle via an ALZET osmotic minipump (7 days). All mice underwent intracerebroventricular (i.c.v.) infusion modified from prior studies (Liebrand et al., 2017). Briefly, osmotic pump implantation was performed under anesthesia using inhaled isoflurane (4 percent), while the mouse was mounted to a stereotaxic frame (Stereotaxic Alignment System, Kopf Instruments). The osmotic pump was targeted to the lateral ventricle using the following coordinates from the mouse brain atlas (Bregma: AP: -0.5 mm, Lat: ±1.5 mm, DV: 2 mm). A hole was drilled in the skull to accommodate for drug infusion from the osmotic pump. For subcutaneous placement of the pump, a tiny incision was made between the scapulae. A tiny pocket was created by spreading the subcutaneous connective tissues apart with a hemostat. The pump is placed in the pocket, with the flow moderator pointed away from the incision (Alzet Osmotic pump, Cupertino, CA, USA). The brain infusion kit (attached to the pump on one side and open on the other) was inserted into the drilled hole using 'super glue' and secured with acrylic bone cement around the pump to create a 'cap'. A surgical nylon suture was used to seal the skin incision. Following surgery, mice were given acetaminophen (2mg/mL concentration) and underwent post-op monitoring for 96h to ensure weight gain, muscular tone, and appropriate neurological response and general health (Crawley and Paylor, 1997).

Behavioral Assessment

Mice were assessed in a behavioral battery of an open field test and then subjected to a light-dark transition test. All mice were placed 30 minutes prior to testing in a behavioral testing

room equipped with white noise (70 dB) and tested 2–3 hours into the dark phase of the light-dark cycle. An ANY-maze behavioral tracking system was used to monitor and encode activity (ver. 5, Stoelting Co., Wood Dale, IL, USA).

Behavioral Assays

Open Field: As previously mentioned, the open field test was utilized to evaluate both novelty-induced locomotor activity (Hall & Ballachey, 1932; Salahuddin et al., 2020a). Mice were placed in the corner of a square Plexiglas box (40 × 40 × 35 cm; Stoelting Co.) with a highly illuminated center (inner 20 cm) and permitted to explore the box for 5 minutes. Their mean velocity (meters/sec) and total distance traveled (meters) were utilized as indices for their motor activity. Latency to first enter into the open field's center, center entries, and center time were considered as indices for anxiety-like behavior (longer latencies to enter the center, fewer entries, and less time spent in the center indicated greater anxiety-like behavior; Hall and Ballachey, 1932; Paris et al., 2014c). Testing occurred under incandescent lighting.

Light-dark transition test: Light-dark transition test was utilized to measure anxiety-like behavior based on approach-avoidance conflict principle. Mice were placed in a highly illuminated corner of a square Plexiglas box (40×40×35 cm; Stoelting Co., Wood Dale, IL, USA) that was evenly divided into two compartments [one brightly lit (unprotected) and one completely dark (protected)] and allowed to explore for 5 minutes. The time required to enter the dark compartment and time spent in the light compartment were considered as indices of anxiety-like behavior (longer latencies to enter the dark compartment, less time spent in the light compartment were indicative of greater anxiety-like behavior; Hall and Ballachey, 1932; Salahuddin et al., 2020b). Testing occurred under incandescent lighting.

Forced Swim Stressor: Given, mice consider swimming in water stressful, Porsolt forced swim test was performed to activate the HPA axis, as previously reported (Salahuddin et al., 2020b). Briefly, mice were placed in room temperature (22 °C) water for 15 minutes and permitted to swim. Following swimming, mice were cleaned and returned to their home cages using paper towels.

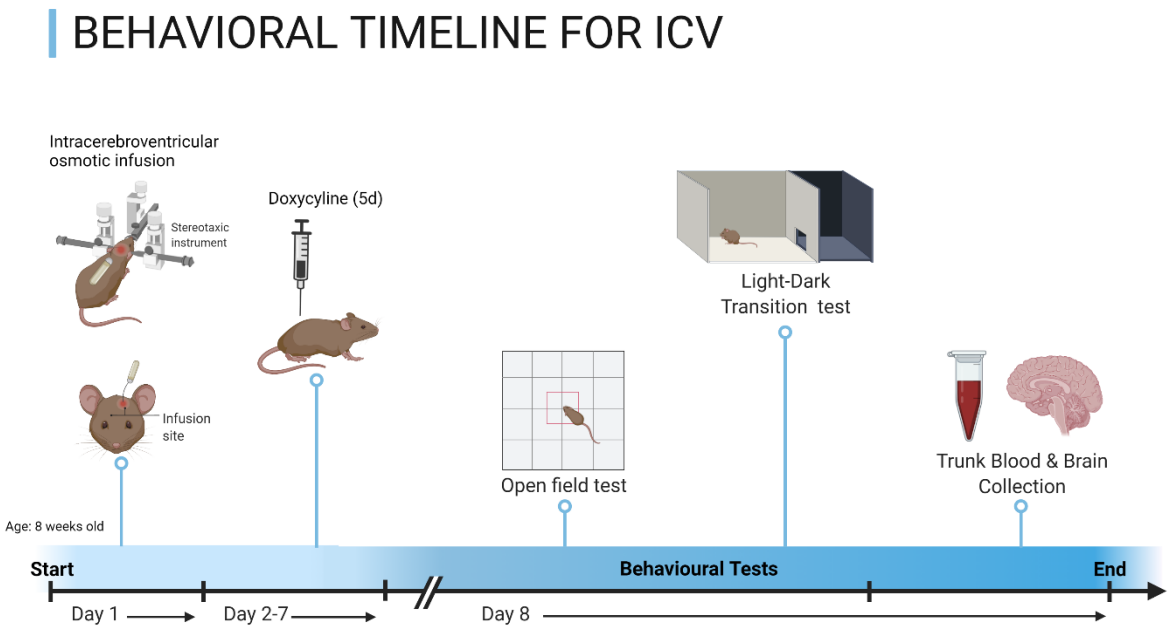

Figure 51: Experimental timeline for intracerebroventricular osmotic infusion of neurosteroids.

Timeline of the experimental design- Intracerebroventricular osmotic infusion of Vehicle/AlloP/FGIN-1-27, Doxycycline (30mg/kg; i.p. 5 days; Tat induction), 1-2 days of doxycycline washout, Behavioral testing (open field, light-dark transition test), and tissue collection for corticosterone estimation in blood and steroidogenic enzyme expression in brain.

Assessment of neurosteroid infusion in Non-stressed and Stressed mice.

To begin elucidating the HPA-axis interactions involved in HIV-1 Tat exposure and vehicle, allopregnanolone or FGIN-1-27 treatment, surgically infused mice were randomly assigned to endure 15-minute swim stress (or not) followed by injection of saline once before the behavioral testing. Mice were tested in an open field 15 minutes after saline administration followed by an assessment in a light-dark transition test to determine anxiety-like behavior (Fig. 51). In the non-stressed paradigm, the mice were euthanized 60 minutes after receiving saline, and in the stressed paradigm, they were euthanized 120 minutes after exposure to a stressor.

Steroidal Assay

Tissue Collection

Mice were euthanized immediately after behavioral testing by cervical dislocation followed by rapid decapitation. Blood was drawn from the trunk and centrifuged at 13,500 *g* for 20 minutes at 4°C. Serum was kept at -80°C until further use. Following 24 hours of fixation in 4% paraformaldehyde, the fixed brains were suspended in 15% sucrose for 24 hours, followed by 30% sucrose for 24 hours, after which the brains were removed and embedded in Tissue-Tek O.C.T. compound (Sakura Finetek, Torrance, CA). Some other brains were flash-frozen in dry ice for future mRNA estimation.

Tissue Dissection

Briefly, the frozen brains were thawed for roughly 5 minutes on an ice-cold inverted glass beaker to mimic a dissection plate. Once thawed, the hypothalamus region of the brain was

dissected. The tissue was then flash-frozen using TRizol in a 1.5 ml microtube. The tissues in the TRizol solution were stored at -80°C until utilized for mRNA estimation.

Steroid Extraction:

As previously described (Salahuddin et al., 2020a), circulating steroids were extracted from serum using an ether extraction technique. Serum samples were incubated with 1 mL of ice-cold anhydrous ethyl ether before being snap-frozen in an acetone/dry ice combination (Salahuddin et al., 2020a). The supernatant was collected and evaporated to dryness overnight in a fume hood, followed by reconstitution in the extraction buffer to 50 times of the original volume. The enzyme-linked immunosorbent test was conducted as directed by the manufacturer (Neogen Life Sciences, Lexington, KY, USA).

Enzyme-Linked Immunosorbent Assay (ELISA)

Corticosterone levels in the blood were determined using an ELISA according to the manufacturer's instructions (Neogen Life Sciences; #402810) and as previously described (Salahuddin et al., 2020a). The absorbance of all tests was measured at 650 nm using a CLARIOstar microplate reader (BMG Labtech Inc., Cary, NC, USA). The antibody cross-reactivity with corticosterone is reported to be 100%. Additionally, the corticosterone antibody exhibits cross-reactivity with deoxycorticosterone (38%), 6-hydroxycorticosterone (19%), and progesterone (5.1%) with only a little cross-reactivity to other steroids (2.7%). The intra- and inter-assay variations were 12.9 and 23.1 percent, respectively.

RNA isolation and quantitative real-time polymerase chain reaction:

The hypothalamus region of mouse brain for each subject was isolated and homogenized

in TRizol reagent according to manufacture protocol (Invitrogen) followed by Qiagen Clean up kit (Qiagen, Germantown, MD). Total RNA concentration was determined by Nanodrop spectrophotometer (NanoDrop 2000c, Thermo Fisher Scientific, Waltham, MA). 1μg of RNA was used using RevertAid First Strand cDNA Synthesis (#K1651, Fisher Scientific) to make cDNA. All primers were purchased by IDT (Coralville, IA; See Methods for primer sequences). qRT-PCR reactions were performed using a Bio-Rad CFX Connect Real-Time System (Bio-Rad, Hercules, CA) in 96 well plates (Applied bioSystems). 1 mg of cDNA in a final volume of 25 mL containing 400nM primers using SYBR Green master mix (Thermo Fisher Scientific, Waltham, MA) was used for each reaction. The three-steps PCR thermal cycling reaction for each set of primers are as follow;

5α reductase 1 and 5α reductase 2 primers:

Step 1: 95°C for 2 mins (denaturation); Step 2: 95°C for 30s (denaturation), 64°C for 30s (annealing) followed by 72°C for 60s (extension) for a total of 40 cycles; Step 3: 72 °C for 7 mins.

Cyp 11a1 and 3α-HSD primers:

Step 1: 95°C for 2 mins (denaturation); Step 2: 95°C for 30s (denaturation), 56°C for 30s (annealing) followed by 72°C for 60s (extension) for a total of 40 cycles; Step 3: 72 °C for 7 mins.

GABA- γ2 and GABA- δ receptors:

Step 1: 95°C for 2 mins (denaturation); Step 2: 95°C for 30s (denaturation), 62°C for 30s (annealing) followed by 72°C for 60s (extension) for a total of 40 cycles; Step 3: 72°C for 7 mins.

The qRT-PCR for the housekeeping gene, GAPDH, was performed in parallel for all the

reactions. The results are presented as the average of three independently conducted trials. Additionally, melt curve analysis [95°C, 1min, 70 cycles(1s)] was carried out immediately following amplification to identify any nonspecific product. As a negative control, we performed parallel reactions without template. All the resulting curves were sigmoidal and amplification efficiency was calculated to be 100%.

Chemicals

Allopregnanolone and FGIN-1-27 Preparation

Doxycycline hyclate was prepared fresh daily and dissolved in sterile saline (0.9%) to the desired concentration, as described previously (30 mg/kg, i.p.; Cayman Chemical, Ann Arbor, MI, USA). Allopregnanolone (Aliquots of stock concentration of 1mM in DMSO were primarily prepared and stored at -20°C; #P8887, Sigma-Aldrich). This stock solution (1mM) was diluted 1:10,000 in saline to make the physiological concentration of 100ng. Allopregnanolone dosing was chosen based on prior demonstrations to produce physiological concentrations in the brain (Frye et al., 2020). FGIN-1-27 (5µg/µl; #18461; Cayman Chemical, Ann Arbor, MI, USA) was dissolved in 10% DMSO and diluted 1:10 in saline). FGIN-1-27 dosing was chosen based on prior studies showing levels comparable to those seen in naturally sexually responsive rats (Frye et al., 2009). Vehicle (DMSO diluted 1:10,000 in saline) was prepared as a control for AlloP and FGIN 1-27.

Osmotic pump preparation

Following preparation of the drug solution, 200µl of drug solution was loaded into the ALZET® osmotic pump (Model 2001, 1 µl/h delivery; Alzet Osmotic pump, Cupertino, CA, USA). The pump was connected to the brain infusion kit (ALZET Brain Infusion Kit 3 #0008851;

Alzet Osmotic pump, Cupertino, CA, USA) through polyethylene or vinyl catheter tubing (1.5cm long; provided with the kit). The penetration depth from the skull surface was adjusted with 2 spacers provided with the kit. The drug preparations were made 24h prior to the surgeries in batches for reducing any dosing variability.

Statistical analyses

All the behavioral and steroid analyses were performed separately using two-way analyses of variance (ANOVA) with drug condition (vehicle or AlloP or FGIN-1-27) and genotype [Tat(–) or Tat(+)] as between-subject factors. Simple main effects were followed by Fisher's protected least significant difference post hocs to delineate the group differences. Interactions were followed by simple main effects and main effect contrasts while controlling for family-wise error. Data from qRT-PCR were computed using the $2^{-\Delta\Delta C}{}_{T}$ method (Livak and Schmittgen, 2001) and analyzed using the two-way ANOVA with drug condition (Vehicle or Allopregnanolone), genotype [Tat(–) or Tat(+)] and paradigm (stressed or non-stressed) as between-subject factors.

3.3. Results

Anxiety-like behavior is reduced by Allopregnanolone

Intracerebroventricular osmotic infusion of Vehicle/AlloP/FGIN-1-27 is followed by induction of HIV-1 transactivator of transcription (Tat) expression in Tat(+) males (hatched bars) or Tat(−) controls (open bars) via doxycycline (30 mg/kg, i.p., once daily for 5 days with 1-2 days washout). Mice were either stressed for 15 minutes (or not) and acutely administered saline 15 minutes prior to being assessed for anxiety-like behavior in an open field test (Fig. 52A). To assess the anxiety-like behavior, latency to first enter center of open field, entries into the brightly-lit

center, and time spent in the brightly-lit center was calculated. As anticipated, stressed mice significantly took more time to enter the center of the open field [$F(1,118) = 87.44$, $p < 0.05$; see §, Fig. 52B]. Hormone treatment significantly influenced the latency to first enter center of open field [$F(2,118) = 4.71$, $p < 0.05$; see †, Fig. 52B]. Expression of Tat significantly increased the latency to first enter the center of the open field [$F(1,118) = 7.43$, $p < 0.05$]. Exposure to stress interacted with Tat expression to significantly influence the latency to first enter the center of the open field [$F(1,118) = 4.32$, $p < 0.05$] such that, stressed Tat(+) and Tat(−) mice significantly took more time to enter the center of open field than any other mice (p <0.0001-0.0090).

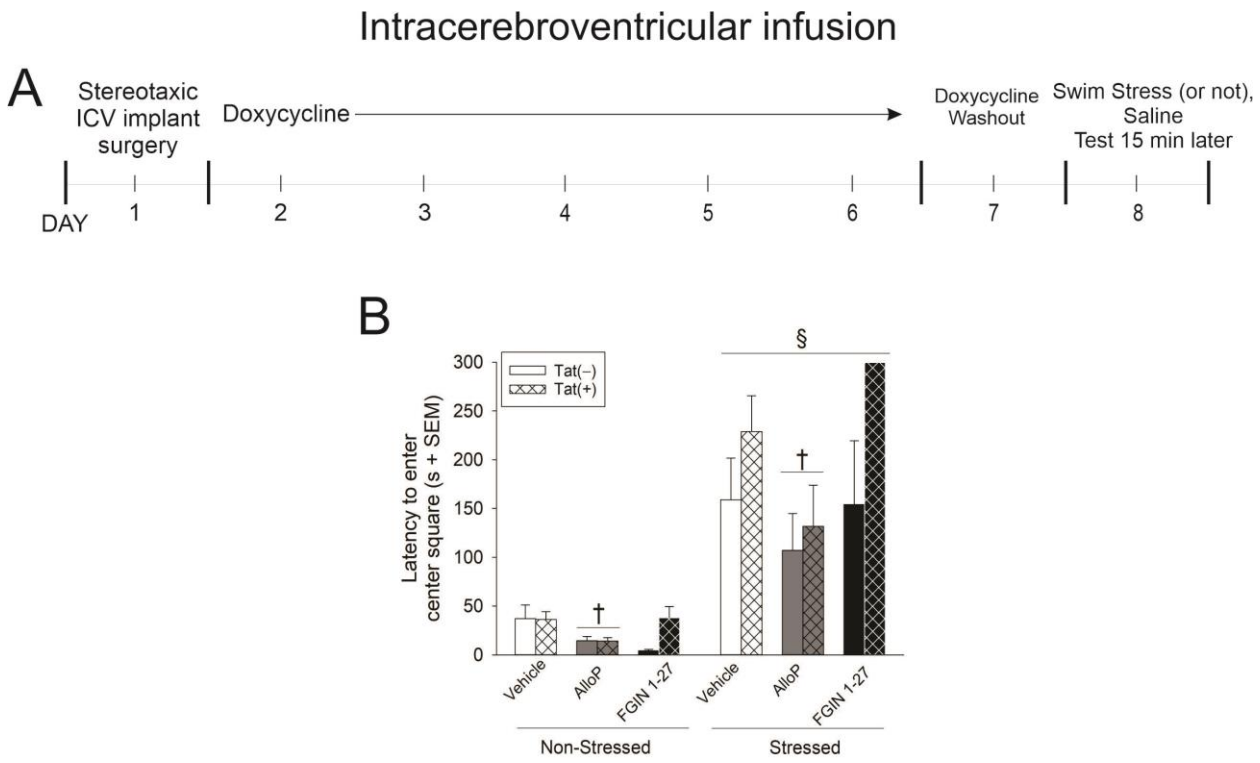

Figure 52: Effect of intracerebroventricular exposure of AlloP or FGIN 1-27 on anxiety-like behavior in adult male HIV-1 Tat transgenic mice.

(**A**) Intracerebroventricular osmotic infusion of Vehicle/AlloP/FGIN-1-27 is performed followed by HIV-1 trans-activator of transcription (Tat) expression induction in Tat(+) males (hatched bars), or not in Tat(−) controls (open bars), via administration of doxycycline (30 mg/kg, i.p., once daily for 5 days with 1-2 days of washout). Mice were either stressed for 15 min (or not) and acutely-administered saline 15 min prior to assessment in an open field test for anxiety-like behavior (**B**) Latency to enter center square. † indicates main effect of AlloP to be different from other groups in panel B; § indicates main effect of stressed group to differ from non-stressed counterparts in panel B, $p \leq 0.05$.

Neurosteroids lack soporific effect

The distance traveled by the mice in an open-field test was used to determine any soporific effects of neurosteroids. As expected, stressed mice significantly traveled less distance than their non-stressed counterparts [$F(1,121) = 155.20$, $p < 0.05$]. The distance traveled between treatment

groups was not significantly different, indicating that the neurosteroids administered at this dose have no soporific effect.

FGIN-1-27 attenuated elevated circulating corticosterone

Intracerebroventricular osmotic infusion of Vehicle/AlloP/FGIN-1-27 is performed, and followed by induction of HIV-1 transactivator of transcription (Tat) expression in Tat(+) males or Tat(−) controls via doxycycline (30 mg/kg, i.p., once daily for 5 days with 1-2 days washout). Mice were either stressed for 15 minutes (or not) and acutely administered saline 15 minutes prior to being assessed for anxiety-like behavior in an open field test and light-dark transition test (Fig. 53A). When circulating corticosterone was assessed in behaviorally-tested mice via ELISA, hormone treatment influenced the circulating corticosterone [$F(2,119) = 4.02$, $p < 0.05$; see †, Fig. 53B] such that FGIN 1-27 treatment significantly reduced the circulating corticosterone compared to vehicle ($p = 0.0061$) and allopregnanolone ($p = 0.0122$) treatment groups.

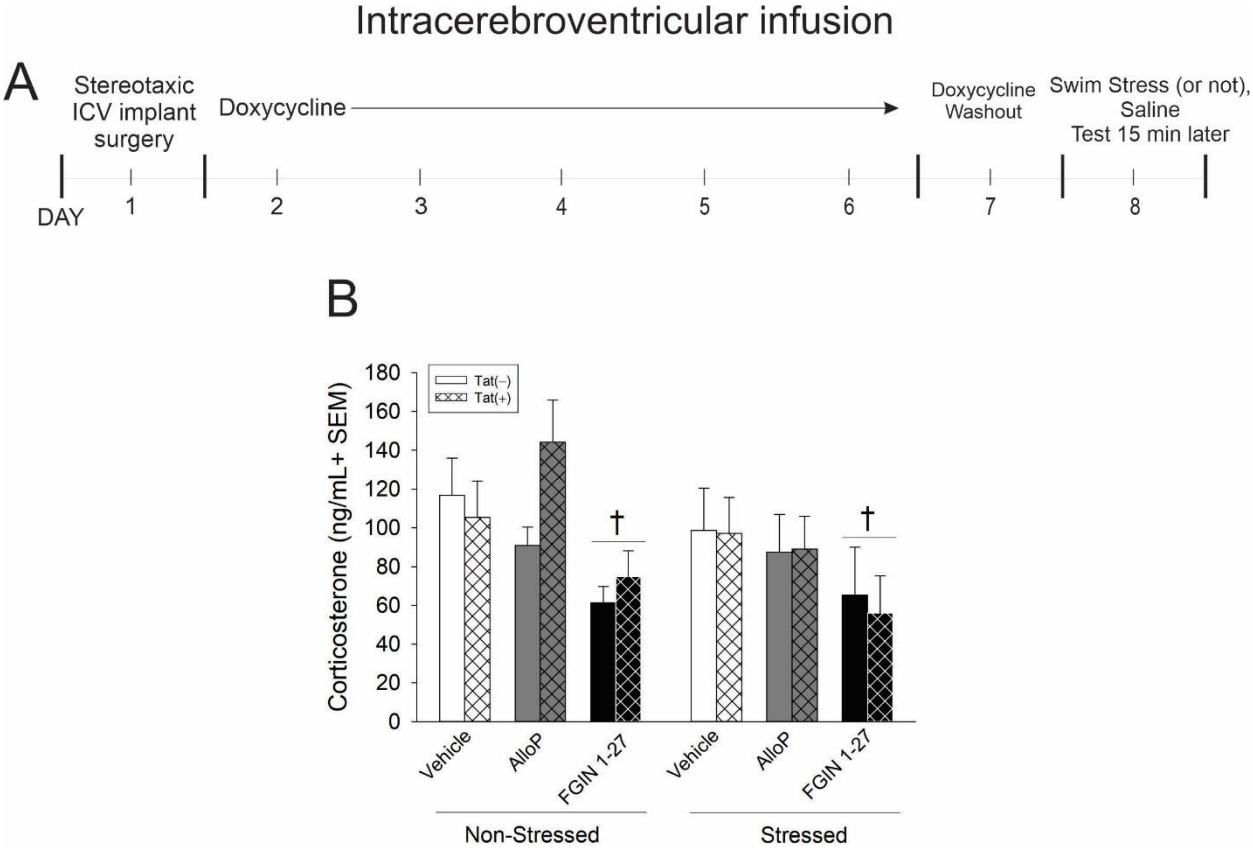

Figure 53: Effect of intracerebroventricular exposure of AlloP or FGIN 1-27 on circulating corticosterone in adult male HIV-1 Tat transgenic mice.

(**A**) Intracerebroventricular osmotic infusion of Vehicle/AlloP/FGIN-1-27 is performed followed by HIV-1 trans-activator of transcription (Tat) expression induction in Tat(+) males (hatched bars), or not in Tat(−) controls (open bars), via administration of doxycycline (30 mg/kg, i.p., once daily for 5 days with 1-2 days of washout). Mice were either stressed via forced swim for 15 min and acutely-administered saline 15 min prior to assessment in an open field and light-dark transition test (n = 5–17/group). (**B**) Circulating corticosterone was measured following behavioral testing. † indicates main effect of FGIN-1-27 to be different from other groups (AlloP and Vehicle), $p < 0.05$.

Tat (+) mice exhibited enhanced neurosteroidogenesis, counterintuitively showed reduced formation of AlloP

To determine if vehicle or AlloP pretreatment influenced expression of different enzymes of steroidogenesis in brain or neurosteroidogenic receptor targets, implicated to promote neurosteroidogenesis, qRT-PCR was performed on hypothalamus region of behaviorally tested mice (Fig. 54A). Similar to models of neural injury, Tat exposure significantly elevated the gene expression of *cyp11a1* (encode for cyp450scc enzyme) compared to Tat (–) counterparts [$F(1,37) = 91.91$, $p < 0.05$; see *, Fig. 54B,C].

When gene expression levels of *akr1c4* (encode for 3α-HSD enzyme) expression was assessed, hormone pretreatment interacted with Tat exposure [$F(1,37) = 8.13$, $p < 0.05$; see ‡, Fig. 54B] such that Tat-expressing mice demonstrated significant lower expression of *akr1c4* mRNA expression compared to Tat(–) controls ($p = 0.0025$); AlloP treated Tat (+) mice exhibited a significant increase in the *akr1c4* mRNA expression compared to Tat(+) vehicle controls ($p = 0.0383$); Tat(–) vehicle controls revealed a higher expression of *akr1c4* mRNA compared to Tat(–) AlloP controls ($p = 0.0365$).

When gene expression levels of *srd5a2* (encode for 5α-reductase 2 enzyme) expression was assessed, the main effect of paradigm was observed [$F(1,37) = 4.70$, $p < 0.05$; see §, Fig. 54B,C], such that stressed mice demonstrated significantly higher *srd5a2* mRNA expression compared to their non-stressed counterparts.

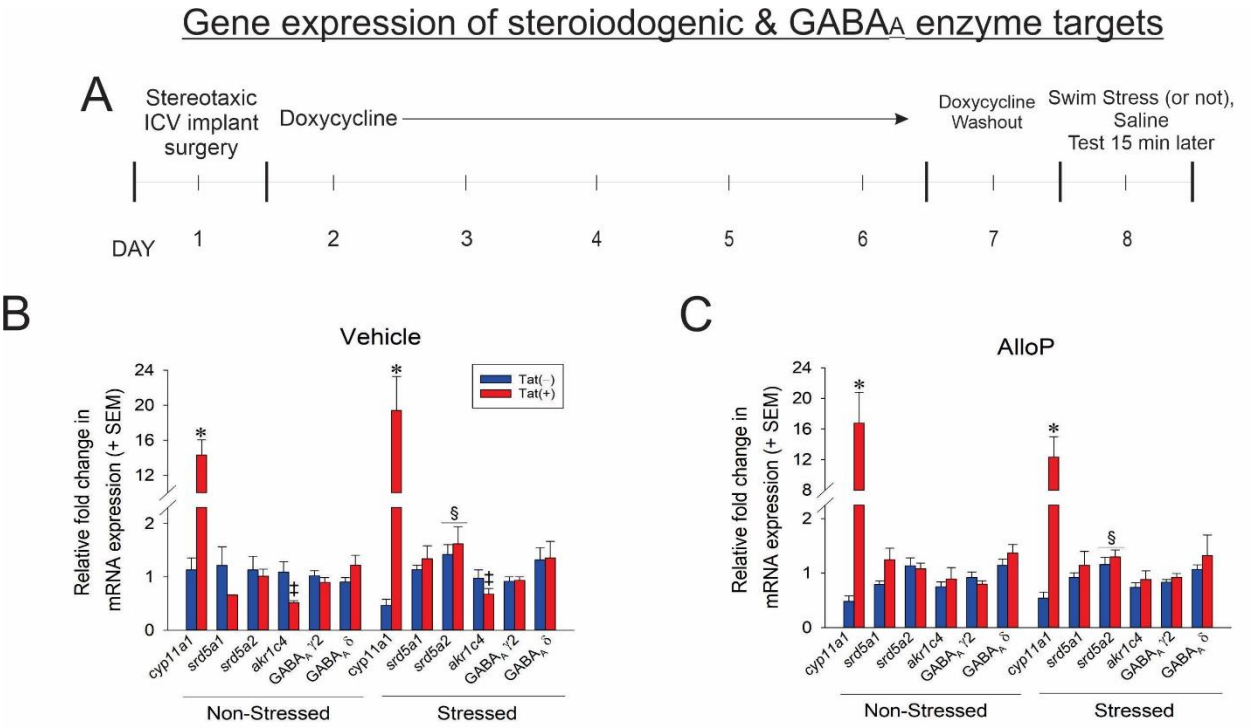

Figure 54: Effect of intracerebroventricular exposure of AlloP or FGIN 1-27 on hypothalamic steroidogenic enzyme targets in adult male HIV-1 Tat transgenic mice.

(**A**) Intracerebroventricular osmotic infusion of Vehicle or AlloP is performed followed by HIV-1 trans-activator of transcription (Tat) expression induction in Tat(+) males (red color bars), or not in Tat(−) controls (blue color bars), via administration of doxycycline (30 mg/kg, i.p., once daily for 5 days with 1-2 days of washout). (**B**) Steroidogenic enzymes target mRNA expression (mean + SEM) of cytochrome P450 enzyme (*cyp11a1*), 3α-hydroxysteroid dehydrogenase (*akr1c4*), 5α-reductase 1 (*srd5a1*), 5α-reductase 2 (*srd5a2*), and $GABA_A$ receptor isoform targets like $GABA_A\gamma2$ and $GABA_A\delta$ receptor normalized to GAPDH (calculated by $2^{-\Delta\Delta CT}$ method). * indicates significant effect of Tat (+) to exhibit higher levels of *cyp11a1* hypothalamic expression than Tat(–) controls in panel **B & C**. § indicates main effect of paradigm , wherein stressed *srd5a2* demonstrated higher levels when compared to non-stressed counterparts in panel **B & C**. ‡ indicates an interaction between hormone exposure and genotype such that, Tat (+) mice exhibited significantly lower expression of *akr1c4* when compared to Tat (–) controls, regardless of stress induction in panel **B**, $p < 0.05$

3.4. Discussion

The hypothesis that exogenous allopregnanolone would restore Tat-mediated HPA dysregulation and anxiety-like behavior in Tat-expressing transgenic male mice were upheld. These findings extend prior reports demonstrating that Tat promotes HPA dysregulation concurrent with inducing anxiety-like behavior in male mice (Salahuddin et al., 2020b; Paris et al., 2014c). Among the possible underlying mechanisms are, change in neurosteroid levels and GABAergic signaling, which may contribute to neuroendocrine (HPA) dysfunction and increase sensitivity to affective behaviors. In support, clinical evidence show HIV^+ patients which exhibited reduced neurosteroid and higher cortisol levels, predicted greater depressive symptoms (Mukerji et al., 2021). Consistently, the present study showed for the first time intracerebral (site to study the central effects) infusion of allopregnanolone reinstated HPA homeostasis and alleviated the anxiety-like behaviors in HIV-1 Tat transgenic male mice. These data extend prior findings to reveal that exogenous progesterone, and not Finasteride (5α-reductase inhibitor) administration would attenuate Tat-mediated anxiety-like behavior (Paris et al., 2016). In the present experiments, we have observed FGIN-1-27 attenuated elevated corticosterone levels in male mice. The mechanisms that may underlie these effects are not known, but parallel *ex vivo* experiments assessed molecular signatures of neurosteroidogenesis and revealed neurosteroids like allopregnanolone administration attenuated Tat-mediated anxiety-like behavior (reduced the latency to enter the brightly illuminated center of the open field) concurrent with normalizing Tat-mediated downregulation of 3α-HSD enzyme expression, implicating the role of 5α-reduced pregnane steroids in these protective effects. These data provide proof of principle that enhancing neurosteroidogenesis in the central nervous system can influence the HPA axis and related

affective dysfunction.

HPA dysregulation (evidenced by elevated baseline cortisol levels) is commonly observed in ~50% of HIV^+ infected patients (reviewed in Chrousos and Zapanti et al., 2014; George and Bhangoo, 2013) and has been implicated to promote vulnerability to the pathogenesis of affective and neurocognitive decline (Jacobs et al., 2018; Mukerji et al., 2021; Qiao et al., 2017; Semniuk et al., 2001). The mechanisms underlying HIV-mediated hypercortisolemia may involve a) stress-induced shift from adrenal androgens to cortisol (Zapanti et al., 2008). In support, we found neurotoxic proteins like HIV-1 Tat to promote elevated basal corticosterone and paradoxical adrenal insufficiency in response to a natural stressor (Salahuddin et al., 2020ab; Salahuddin et al., 2021c) b) elevated cytokines such as IL-1 and IL-6 may confer glucocorticoid resistance, thereby reducing the sensitivity of GR to regulate the HPA negative feedback (Goleva et al., 2002; Pariante et al., 1999; Raddatz et al., 2001). Consistent with these, we and others have shown the capacity of Tat to promote an increase in IL-2 and IL-4 in the brain (Fitting et al., 2010b; Gonek et al., 2018;) and thereby confer corticosterone insensitivity in murine cultured splenocytes (Paris et al., 2020); c) Given GRβ is a negative inhibitor of bioactive GRα, a surge in Tat-mediated proinflammatory cytokines may produce an increase in GRβ:GRα ratio to mediate a glucocorticoid resistance phenotype and block the glucocorticoid signaling to reinstate HPA homeostasis (Taniguchi et al., 2010; Webster et al., 2001). One other clinical phenotype observed in HIV patients is adrenal insufficiency (Membreno et al., 1987). Although severe adrenal insufficiency is uncommon in AIDS patients, modest adrenal deficits might occur (George and Bhangoo, 2013). In the pre-cART era, adrenal atrophy was quite common owing mostly to opportunistic infections (Glasgow et al., 1985). However, in the post-cART era, depletion of the adrenal reserve may

underlie insufficiency. The mechanism is unknown, but evidence show a)Tat to dysregulate gene expression associated with lipid and cholesterol trafficking (Cotto et al., 2018; Mohseni Ahooyi et al., 2018) and b) Tat mediated increase in production of ceramides that inhibit steroidogenic enzymes (Haughey et al., 2004), c) Tat-mediated disruption of mitochondrial steroidogenesis (Field and Eliis, 2019). Other possible mechanisms that may underlie HPA dysfunction include perceived stress (Hand et al., 2006; Reif et al., 2011), downstream effects of cytokines (Ancuta et al., 2008; Edén et al., 2007), and antiretroviral medication side effects (Costa et al., 2000; George and Bhangoo, 2013). Consistently, we have shown earlier Tat's capacity to promote adrenal insufficiency in Tat-expressing male mice in response to stressor challenge or pharmacological HPA blockade (Salahuddin et al., 2020b). Henceforth, Tat may enhance glucocorticoid resistance through a variety of pathways, raising basal corticosterone levels in a manner similar to that seen in the HIV^+ population, and promote adrenal insufficiency perhaps via the impairment of steroidogenesis.

Chronic dysregulation of the HPA axis may predispose individuals to develop stress-related psychiatric disorders (Jacobs et al., 2018; Porter and Gallagher, 2006; Varghese and Brown, 2001). A preponderance of data shows the ability of Tat to promote neuroHIV like phenotype which encompasses affective dysregulation (anxiety and depression-like behaviors), deficits in pre-attentive filter processing, and cognitive decline as observed in HIV^+ patients (Carey et al., 2012; Fitting et al., 2006, 2013; Langford et al., 2018; Marks et al., 2016; Paris et al., 2015; Qrareya et al., 2021). To this end, our prior studies have demonstrated Tat to promote anxiety-and depression-like behaviors concurrent with HPA stress axis dysregulation (Salahuddin et al., 2020ab; 2021ac; Qrareya et al., 2021). Preservation of the HPA axis may confer protection against affective and

cognitive decline (Herman et al., 2016). In support, increased cortisol-to-DHEA ratios in HIV^+ individuals predict greater levels of stressful life events, subjective stress, anxiety, and depressive symptomatology scores (Qiao et al., 2017; Mukerji et al., 2021). Thus, HPA function is crucial to reinstate central and peripheral homeostasis, therefore reversing Tat-mediated HPA dysregulation may improve neurological outcomes.

Given the well-established role of GABAergic transmission in HPA axis control, it is not surprising that neurosteroids have been shown to influence HPA axis function (Crowley and Girdler, 2014; Wirth et al., 2011). Some of the underlying mechanisms for altered GABAergic signaling in chronic illness like HIV include increases in inflammatory cytokines, notably interferon (IFN)-alpha (Ries et al., 2012) resulting in a shift in the excitatory/inhibitory neurotransmission balance in the brain (Boero et al., 2019), which can lead to neuropsychiatric disorders and depression (Felger and Lotrich, 2013). Moreover, further evidence show CRF neurons express L-type calcium channels (Xie et al., 1999), which is one of the targets via which Tat mediates its excitotoxic effects (Hu et al., 2016), thus, it is plausible to assume that Tat may act on calcium channels to disrupt HPA homeostasis, impairing the neuronal functioning and promote HPA dysregulation. These changes in the HPA axis signaling, likely contribute to the pathogenic effects of stress, including neuronal damage, and death of neurons or non-neuronal cells, as well as related tissue loss in HIV-infected brain areas, leading to depression and other neuropsychiatric (e.g., cognitive or psychomotor) symptoms (Payne et al., 2012; Treisman et al., 1998, Wojna et al., 2006). Additionally, the capacity of Tat to produce alterations in excitatory/inhibitory neurotransmission and production of proinflammatory cytokines (Fitting et al., 2014a, 2014b) may underlie the loss of GABAergic control thereby leading to behavior

disinhibition (Nass et al., 2020; Salahuddin et al., 2020a). According to one clinical study in HIV^+ drug users, HIVE in the hypothalamus is associated with altered levels of D2 and D3, which may confer to altered hypothalamic signaling, and contribute to worsening cognitive deficits, poor medication adherence, and faster disease progression (Langford et al., 2011). Furthermore, another mechanism that may underlie the loss of GABAergic inhibition, may involve downregulation of the K+/Cl co-transporter (KCC2) in the hypothalamus of male rats (Hewitt et al., 2009; Sarkar et al., 2011), which may promote a switch in the GABAergic transmission on CRF neurons from inhibitory to excitatory, resulting in ineffective HPA axis inhibition (Boero et al., 2019). In support HIV proteins like Tat exposure caused KCC2 loss specifically in the D2 MSNs which may result in disruption of $GABA_AR$-mediated hyperpolarization and inhibition (Barbour et al., 2020, 2021). Furthermore, whole-cell recordings of GABAergic neurotransmission in mouse striatal neurons indicated that Tat promotes a concentration-dependent decrease in the frequency and amplitude of spontaneous and miniature inhibitory postsynaptic currents (Xu and Fitting, 2016) Henceforth, under chronic perceived stressful situations, impaired GABAergic signaling may underlie the HPA axis dysregulation observed in HIV^+ patients.

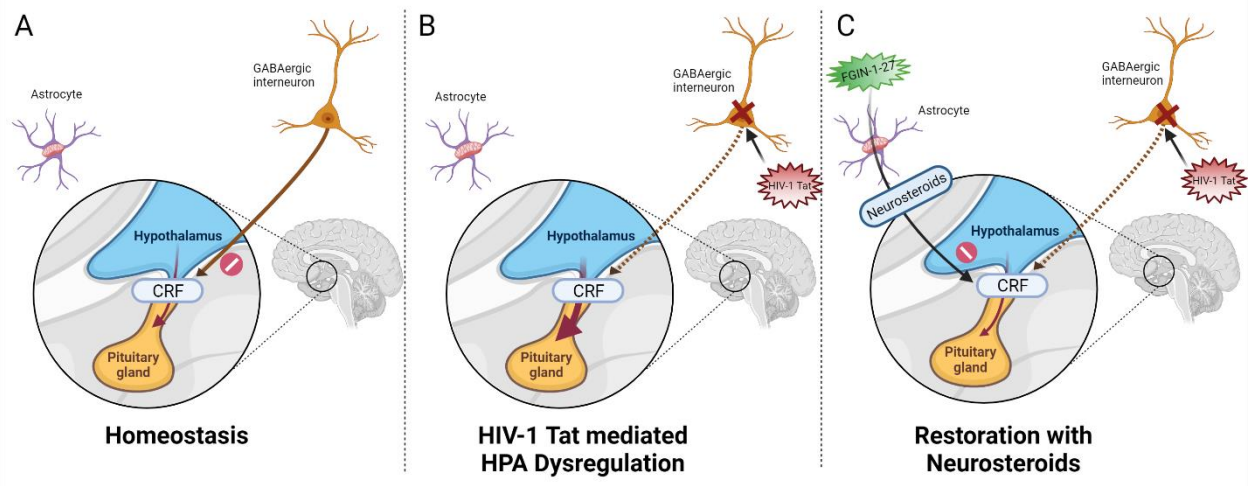

Figure 55: Schematic diagram of restoration of HPA dysregulation in HIV-1 Tat transgenic mice by GABAergic steroids.

A) GABAergic transmission and thus CRF signaling are normal during homeostasis. **B**) On exposure to Tat, there is reductions in the GABAergic interneuron subpopulations, as a result CRF signaling is increased due to an imbalance in the GABAergic tone at the CRF neuron. **C**) FGIN-1-27 increase the GABAergic transmission and directly reduce CRF levels, thereby restoring HPA homeostasis.

Animal studies show neuroactive steroids may be an effective therapeutic strategy for resolving GABAergic inhibition abnormalities with stress-related illness observed in HIV^{+} patients. In support, in both animals (Bitran et al., 1991; Crawley et al., 1986; Khisti et al., 2000) and humans (Kanes et al., 2017; Meltzer-Brody et al., 2018), allopregnanolone has exhibited anxiolytic and antidepressant-like effects, which may be beneficial for the prevention and recovery from stress-related disorders. Acute exposure to a stressor, activates the HPA axis, to release corticosterone and allopregnanolone, as a compensatory mechanism that restores GABAergic regulation of the hypothalamic PVN, thereby shutting down HPA axis activity (Biggio et al., 2007; Gunn et al., 2015). Consistently, our earlier reports show, Tat(+) mice exhibit a substantial rise in

pregnenolone and AlloP levels throughout the brain (Paris et al., 2020), as well as elevated AlloP levels in the hypothalamus (Salahuddin et al., 2021c). In the present study, we found Tat-expressing mice demonstrated downregulation of *akr1c4* gene (encode for 3α-HSD enzyme) expression indicating dysregulation in the neurosteroidogenesis pathway that would load on DHP with a bias against the formation of AlloP (Fig. 54A, 56A). Thus, exogenous administration of neuroactive steroids may be a viable approach to enhance neurosteroidogenesis and reinstate HPA homeostasis (Fig. 55). Consistently, others have shown allopregnanolone or 3α,5α-THDOC treatment before stress reduces the stress-induced increase in ACTH and corticosterone (Owens et al., 1992; Patchev et al., 1996). Additional studies in both prepubertal and adult rats showed intracerebroventricular allopregnanolone antiserum increased the corticosterone response to stress without changing basal levels (Guo et al., 1995). Similarly, systemic administration of allopregnanolone to non-stressed adult male rats increased hypothalamic CRF content, serum ACTH, and corticosterone levels (Naert et al., 2007). Consistent with these reports, the present dissertation showed, under non-stressed and stressful conditions, AlloP reinstated Tat-mediated downregulation of *akr1c4* gene expression (Fig. 56B), concurrent to amelioration of anxiety-like behavior in open field task, indicating AlloP to act as a regulator of HPA function. Intriguingly, TSPO activation (rate-limiting step of neurosteroidogenesis) by FGIN 1-27 (Porcu et al., 2016; Zorumski et al., 2019), completely attenuated increase in corticosterone which imply the combined role of various neuroactive steroids to regulate the HPA axis.

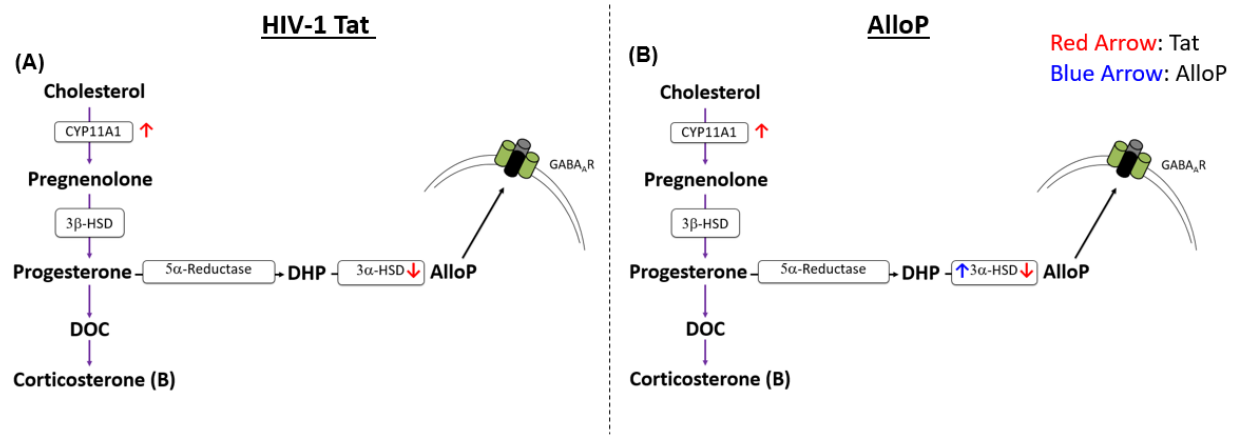

Figure 56: Schematic diagram of restoration of neurosteroidogenesis in HIV-1 Tat transgenic mice by Allopregnanolone.

(**A**) HIV-1 Tat expressing mice exhibited downregulation of gene expression levels of 3α-HSD enzyme and promote dysregulation in neurosteroidogenesis, and contribute to HPA dysfunction. (**B**) AlloP enhanced gene expression levels of 3α-HSD enzyme in Tat-expressing mice thereby increasing neurosteroidogenesis and correcting the GABAergic transmission and may help restore HPA homeostasis.

3.5. Conclusion

Modulating neurosteroidogenesis in order to restore the normal endogenous neuroactive steroid tone may be a viable therapeutic approach to curtail stress-induced HPA activation in Tat-expressing mice. The present study demonstrated that attempts to enhance neurosteroidogenesis by targeting the neuroactive steroid biosynthetic pathway at various stages may result in beneficial HPA effects. Briefly, intracerebroventricular administration of AlloP or FGIN-1-27 augmented neurosteroidogenesis to attenuate HPA activation concurrent with a decrease in anxiety-like behavior. Increased neurosteroidogenesis to counteract HPA dysregulation through these pathways may benefit not only HIV but can extend its application to other neuropsychiatric disorders such as major depressive disorder, post-traumatic stress disorder, and substance use disorders.

SUMMARY AND CONCLUSIONS

HIV infection and hypothalamus pituitary adrenal stress axis

With the advent of combinative antiretroviral therapeutics, HIV/AIDS is evolving from a condition marked by early mortality owing to opportunistic infections and malignancies to a chronic sickness defined by insulin resistance, metabolic abnormalities, and cardiovascular disease (Maartens et al., 2014). HIV infection causes multi-system dysfunction including the endocrine system (Zaid and Greenman, 2019). Among the endocrine abnormalities, pituitary, adrenals, gonads, thyroid, bone, and metabolic disorders have been reported (Brown, 2011; Zaid and Greenman, 2019). HIV-encoded proteins directly affect the HPA axis, while HIV-associated opportunistic infections and drug side effects, including those utilized in highly active antiretroviral therapy (HAART), indirectly affect the HPA axis (Nicolaides et al., 2020). Among these, alterations in the hypothalamic-pituitary-adrenal (HPA) axis is the most common (Zaid and Greenman, 2019). In support, we found HIV proteins promote HPA dysfunction in Tat-expressing mice (Salahuddin et al., 2020ab, 2021c). The HIV mediated HPA axis dysfunction may manifest as adrenal insufficiency and hypercortisolemia (Membreno et al., 1987; Mayo et al., 2002,). Adrenal insufficiency is caused by HIV, opportunistic infections, or cancers that affect the adrenal glands or the hypothalamus/pituitary gland (Glasgow et al., 1985). Additionally, an increase in serum cortisol (hypercortisolemia) and adrenocorticotropic hormone (ACTH) is also reported (Membreno et al., 1987). Some of the underlying mechanisms for hypercortisolemia is stimulation of the HPA axis by HIV infection or pro-inflammatory cytokines (IL-1, IL-2, IL-6, TNF-α),

activation of 11-hydroxysteroid dehydrogenase (11-HSD) type 1 enzyme in the periphery most common in adipose tissue, leading to increase in cortisone conversion to cortisol, or decrease in cortisol metabolism (Bons et al., 2013). Adrenal insufficiency manifests when greater than 80% of the adrenal gland is damaged (Bhatia, 2018). The frequency of adrenal insufficiency varies depending on the method of assessing adrenal function and the diagnostic criteria used. The illness affects 10-20% of patients during the advanced stage of disease and also those with multiple comorbidities (Gonzalez-Gonzalez et al., 2001; Wolff et al., 2001). Combinative ART has reduced the occurrence of adrenal insufficiency (Bons et al., 2013). In addition to the infection by HIV, adrenal glands could also be infected by other opportunistic infections including *cytomegalovirus* (CMV), *Mycobacterium avium-intracellulare* and *M. tuberculosis*, and fungi (*Histoplasma* and *Cryptococcus*), *Pneumocystis carinii*, and *Toxoplasma gondii* (Glasgow et al., 1985). The most common cause is CMV infection (Bons et al., 2013). In the pre-cART era, about 80% of HIV-infected CMV patients had adrenal gland involvement (Pulakhandam and Dincsoy, 1990). Certain AIDS-related comorbidity drugs can directly impair adrenal steroidogenesis (Bhatia, 2018), such as ketoconazole, which inhibits multiple steps in adrenal steroid production (Pont et al., 1982). Other drugs, such as rifampicin, enhance hepatic steroid metabolism or inhibit corticotropin secretion via its glucocorticoid action (megestrol acetate) (Hofbauer and Heufelder, 1996; Leinung et al., 1995). For diagnostic purposes, many clinical symptoms of adrenal insufficiency (namely, anorexia) overlap with those of HIV-infected patients who do not have this illness, making the clinical diagnosis challenging (Bhatia, 2018). Additionally, a decrease in endogenous and exogenous glucocorticoids metabolism caused by several ART medicines, notably protease inhibitors such as ritonavir, might result in an iatrogenic Cushing's syndrome with secondary

adrenal insufficiency (Hyle et al., 2013; Norbiato et al., 1992; Samaras et al., 2005). Some of the HIV patients develop peripheral glucocorticoid resistance, with increased cortisol and ACTH, but no clinical signs of adrenal insufficiency (Norbiato et al., 1992). These patients have low-affinity glucocorticoid receptors due to HIV or exposure to cytokines. Likewise, we found exposure to neurotoxic proteins like HIV-1 Tat produces glucocorticoid resistance in cultured primary splenocytes (Paris et al., 2020). Unlike the majority of HIV^+ patients with elevated cortisol levels, those with adrenal insufficiency require continued glucocorticoid and mineralocorticoid supplementation in addition to antiretroviral therapy as a primary stay until clinical trials on steroid replacement in HIV^+ patients are conducted (Marik et al., 2002).

The HIV-1 Tat, HPA, and immune-inflammatory response

The HPA axis and the immune system have well-defined and dynamic interactions that likely contribute to the current findings. HIV-1 Tat has been found in the CNS of individuals on cART, even when peripheral and CNS viral levels are effectively controlled (Henderson et al., 2019; Johnson et al., 2013). Tat protein is involved in neuropathogenesis by attracting peripheral mononuclear phagocytes (MPs) to the CNS (Albini et al., 1998; Weiss et al., 1999), resulting in an increased HIV load in the CNS. Tat has been shown to produce direct neurotoxicity (Sabatier et al., 1991), synaptic loss via low-density lipoprotein receptor-related protein (LRP) mediated mechanism (Kim et al., 2008), and the upregulation of pro-inflammatory genes in the host (Buonaguro et al., 1992). Tat protein is produced non-canonically from infected cells (Rayne et al., 2010) and may be picked up by uninfected bystander cells (Frankel and Pabo, 1988). In addition to its regulatory role, Tat promotes transcellular signaling and acts as a powerful transactivator of gene expression (Fittipaldi and Giaca, 2005; Hellband et al., 1991; Thomas et al., 1994) in HAND-

relevant cells such as microglia, macrophages, and neurons (D'Aversa et al., 2004; Kolson et al., 1994; Nath et al., 1999; Vendeville et al., 2004), thereby spreading inflammation beyond the CNS's relatively limited population of HIV-infected cells (Lambotte et al., 2003). Similar to the direct Tat's neurotoxic effects on infected cells, uninfected bystander cells internalized by Tat, may increase the expression of proinflammatory chemokines and cytokines such as CCL2, TNF-α, IL-2, IL-6, IL-8, IL-1, and CXCL1 (Ambrosino et al., 1997; Conant et al., 1998; Kim et al., 2004; Kutsch et al., 2000; Mayne et al., 1998; Westendorp et al., 1994; Yang et al., 2010). Moreover, Tat expressing transgenic mice also showed elevation of pro-and anti-inflammatory cytokines in the brain (Fitting et al., 2010b; Gonek et al., 2018). *In vitro* studies showed primary monocytes treated with HIV-1 Tat demonstrated elevation of proinflammatory cytokines like IL-6 (Yim et al., 2009) and TNF-alpha (Gandhi et al., 2009). Western blot analysis revealed elevation of proinflammatory cytokines like TNF-α and IL-1β concurrent to decrease in the mRNA expression levels of GR and MR, indicating the role of cytokines to differentially modulate glucocorticoid signaling at the GR receptor in Tat treated rats (bilateral injections of Tat in the dorsal hippocampus) (Makhathini et al., 2018a). Other evidence reveals GR signaling inhibition by cytokine-producing second messengers and transcription factors as STAT5, p38 MAPK, and NF- κB (Pace and Miller, 2009). The GR nuclear translocation is blocked by STAT5 phosphorylation, pro-inflammatory cytokines like IL-2 and IL-1, and/or anti-inflammatory cytokines like IL-4 (Goleva et al., 2002; Pariante et al., 1999; Raddatz et al., 2001). These cytokines' activity may cause GR insensitivity and thus promote glucocorticoid resistance. Conversely, synthetic glucocorticoid receptor agonist, Dexamethasone (anti-inflammatory mediator) impair the capacity of rat dendritic (microglial) cells to generate IL-1 and TNF-α (Avenant et al., 2010), resulting in suppression of HIV transcription. Thus, dysfunctional GR signaling may contribute to proinflammatory cytokines surge in CNS,

promoting HIV replication and progression of the HAND symptomatology (Alvarez-Carbonell et al., 2019). Consequently, activating the HPA axis can modulate immunological signals. Phagocytic activation of monocyte-derived macrophages is enhanced by glucocorticoids which downregulate the proinflammatory cytokine surge (Bellavance and Rivest, 2014). Glucocorticoids foster a Th2 profile, reducing Th1 and Th17 polarization (Franchimont, 2004; Leung et al., 1995), and may also increase regulatory T cell differentiation (Baschant and Tuckerman, 2010). As such, HAND-relevant brain monocyte-derived cells, present as a novel glucocorticoid target and a source of putative GR-mediating cytokines. Given this dynamic link, it's plausible that HIV-1 proteins may contribute to HPA axis dysfunction; however, the mechanisms are unknown.

HIV-1 Tat and hypercortisolemia endophenotype

Hypercortisolemia is one of the main clinical endophenotypes reported in the majority of HIV^+ patients (Membreno et al., 1987). The present dissertation showed for the first time, Tat protein is sufficient to promote high baseline corticosterone levels (hypercortisolemia) concurrent with increased hypothalamic CRF expression in mice (Salahuddin et al., 2020a). Several underlying mechanisms may underlie this endophenotype. Firstly, an enzymatic switch toward a shift of cholesterol metabolism from DHEA, aldosterone, to increased cortisol production as an adaptive response to stress (Brown et al., 1991; Grinspoon and Bilezikian,1992; Hofbauer and Heufelder, 1996). In support, cortisol to DHEA levels was shown to be elevated in HIV infected patients (Mukherji et al., 2021; Qiao et al., 2017), which was proposed as a surrogate measure for poor HIV outcomes (Christeff et al., 1997). Secondly, HIV^+ infected individuals show higher cortisol binding globulin binding sites than respective controls, prompting a compensatory increase in the circulating cortisol levels (Schürmeyer et al., 1997). Thirdly, higher cortisol production

without increased corticotropin-releasing factor (CRF) indicates that non pituitary factors released from infected immune cells such as IL-1 and IL-6 may contribute (Biglino et al.,1995; Tauveron et al., 1994; Villette et al., 1990) and directly activate the adrenal cortex. Moreover, a significant positive correlation was observed between IL-1 and IL-6 levels and serum cortisol implicating the role of cytokines to modulate the HPA axis (Biglino et al., 1995). Besides, those patients who show hypercortisolemia with elevated CRF levels, these abnormalities may be caused due to cytokines (eg, interferon, IL-1, IL-2, and IL-6) (Raber et al., 1998) or the HIV envelope protein gp120 (Costa et al., 2000) stimulating hypothalamic corticotropin-releasing hormone release. Consistently, Tat was shown to elevate IL-2 and IL-4 in the brain in rodents (Fitting et al., 2010b; Gonek et al., 2018), which induced p38 MAPK to phosphorylate GR and thereby reduce the ligand-binding affinity, promoting a GR resistance state (Irusen et al., 2002; Leung et al., 1995). Likewise, some of the AIDS patients show glucocorticoid resistance phenotype, due to acquired glucocorticoid receptor (GR) abnormalities, defined by increased GR density and decreased GR affinity for substrate (Norbiato et al., 1992). In support, we have found the highest concentration of Tat to produce glucocorticoid resistance in cultured splenocytes of mice (Paris et al., 2020). Fourthly, negative GR regulation may also raise basal glucocorticoids. Generally, the GRβ isoform, a strong negative inhibitor of the bioactive GRα, is considerably less abundant in the nucleus than GRα (Nicolaides et al., 2010). Proinflammatory cytokines may increase the GRβ:GRα ratio, inactivating GR (Taniguchi et al., 2010; Webster et al., 2001). Negative GR regulators like Fkbp5 may also reduce GR expression (as shown in HIV^+ women; Bekhbat et al., 2018). Finally, other proteins like the HIV Vpr gene product acts as a GR coactivator in human lymphoid and muscle-derived cell lines (Kino et al., 1999), perhaps increasing tissue glucocorticoids sensitivity. Thus, Tat may confer a glucocorticoid resistance state, raising baseline corticosterone levels proportionate to

those seen in the HIV^+ population.

HIV-1 Tat and adrenal insufficiency

Intriguingly, HIV-infected individuals show elevated cortisol and low CRF with paradoxical Addisonian characteristics, although a clinical range extending from asymptomatic changes to overt adrenal insufficiency as disease transition to AIDS was observed. Likewise, when subjected to a natural or pharmacological HPA stimulus, Tat induced adrenal insufficiency concurrent to elevated basal corticosterone in Tat-expressing male mice (Salahuddin et al., 2020b). Consistently, similar findings of hypercortisolemia and adrenal insufficiency have been described in HIV^+ individuals (Chrousos and Zapanti, 2014; Zapanti et al., 2008). A loss of "adrenal reserve" has been proposed as one possible cause. These effects may be mediated in part by HIV-1 Tat's ability to disrupt steroidogenesis. Bioavailable cholesterol is required for all of the steroidogenesis, and metabolism of cholesterol is dysregulated in HIV^+ patients (Bandaru et al., 2013). Tat-protein expression is sufficient for the disruption of genes involved in the transport of fats and cholesterol as well as for maintaining homeostasis (Cotto et al., 2018; Mohseni Ahooyi et al., 2018), and it also stimulates the synthesis of ceramides, which block the activity of steroidogenic enzymes (Haughey et al., 2004). Moreover, Tat also confers mitotoxic effects on mitochondria (which is the organelle responsible for the majority of steroidogenesis; Fields and Ellis, 2019). Tat promotes disruption of oxidative phosphorylation, resulting in the formation of reactive oxygen species, depolarization of mitochondrial membranes, leading to mitochondrial swelling as well as the transfer of pro-apoptotic factors like Bax2 and Cyt C into the cytosol (Fields and Ellis, 2019). We found earlier, Tat expression in mice to lower brain concentrations of the glucocorticoid precursor, deoxycorticosterone (Paris et al., 2020). As a result, despite the fact that it increases basal

glucocorticoids, Tat contributes to adrenal insufficiency, perhaps via the inhibition of steroidogenesis.

Sex differences and adrenal insufficiency on HIV Tat exposure

Adrenal insufficiency is another feature of HPA dysregulation seen in HIV patients (up to 46 percent (González-González et al., 2001; Prasanthai et al., 2007; Marik et al., 2002; Afreen et al., 2017; Sharma et al., 2018; Chrousos and Zapanti, 2014). Consistent to elevated basal corticosterone levels observed in male mice (Salahuddin et al., 2020b), we observed Tat expressing female mice also revealed higher basal corticosterone compared to its counterparts (Salahuddin et al., 2021c). However, unlike Tat-tg males, notably, Tat expressing female mice on exposure to a swim stressor or pharmacological inhibition of the HPA feedback loop did not demonstrate adrenal insufficiency (Salahuddin et al., 2021c). However, sex as a biological determinant has not been thoroughly characterized and reported in the HIV clinical population due to its multifactorial characteristics (Chrousos and Zapanti, 2014; Eledrisi and Verghese, 2001; Freda and Bilezikian, 1999; González-González et al., 2001; Mayo et al., 2002). Potential mechanisms for the lack of adrenal insufficiency in Tat- expressing female mice may be explained by decreased CRF-R internalization, enhancing sensitivity to CRF (Bangasser et al., 2010). Females have reduced GR receptor density and GR translocation in the hypothalamus, decreasing negative feedback (Turner and Weaver, 1985; Solomon et al., 2015). Conversely, male mice have a higher level of hypothalamic GR-mediated negative feedback than female mice (Solomon et al., 2015), which may make them more susceptible to HPA insults. Female mice had greater CBG, decreasing bioavailable corticosterone, and perhaps the reserve to mediate negative HPA feedback (Tannenbaum et al., 1997). These sex differences may also be explained by the role of gonadal

steroid hormones. Females have higher amounts of pregnane steroids than men, which may confer resilience to HPA insults. Likewise, neurosteroids like allopregnanolone have been shown to counteract stress-induced HPA activation (Crowley et al., 2014; Girdler et al., 2007; Gunn et al., 2015). In support, we found either OVX or combined Tat and oxycodone exposure increased endogenous hypothalamic allopregnanolone levels (Salahuddin et al., 2021c). This may be a fundamental adaptive stress response, as shown earlier in males exposed to Tat and morphine (Paris et al., 2020). These findings indicate that Tat may disrupt glucocorticoid neuroendocrine function, resulting in increased endogenous neurosteroids such as allopregnanolone (Salahuddin et al., 2021c). Finally, females have higher amounts of circulating and central pregnane steroids, which may confer protection against HPA insults (Frye et al., 2013). These results have clinical implications, wherein dysregulated HPA/HPG axis may promote vulnerability to neuropsychiatric complications and opioid addiction liability (Sinha, 2008).

Sex differences and HPA-mediated behavioral interactions in HIV

Sex or gender variations in HIV are understudied, leading to some inconsistencies in the literature. While some studies show that HIV^+ women have better immune responses to cART and slower viral progression than HIV^+ males (Finkel et al., 2003; Grinsztejn et al., 2011), others show that women are more susceptible to HIV-associated neurocognitive problems (Maki et al., 2018; Rubin et al., 2019; Qiao et al., 2019). However, neuroHIV susceptibility varies with dimensionality assessment. Compared to HIV^+ men, HIV^+ women had a lower incidence of severe depression and any anxiety condition (Bing et al., 2001; Lopes et al., 2012). Unfortunately, many of these studies do not account for gender, HIV acquisition method, or endocrine variables, which adds to the results' variability. Some other studies show no gender differences (Sewell et al., 2000; Tsao et al.,

2004). Stress vulnerability may be one of the factors underlying cognitive and affective behavioral gender differences (Wang et al., 2007). In HIV^+ women, when HPA axis mediated behavioral outcomes are considered, anxiety and depression favor women (Goggin et al., 1998; Lopes et al., 2012; Orlando et al., 2002). Given the HPA axis interacts with HPG factors, it is plausible to assume gonadal hormones play a pivotal role in stress-related neuropsychiatric illnesses (Andreano et al., 2018; Goldstein et al., 2010). In rodent models, HPA/HPG axes are tightly regulated, hence females show a stronger neuroendocrine response to stress than males (Babb et al., 2013; Handa et al., 1994; Iwasaki-Sekino et al., 2009; MacLusky te al., 1996; Viau et al., 2005), with higher hypothalamic CRF release and circulating glucocorticoids (Duncko et al., 2001; Iwasaki-Sekino et al., 2009; Viau et al., 2005). In support, we found that stressed Tat transgenic female mice exhibited increased vulnerability to Tat/oxycodone behavioral interactions in the diestrous phase of the estrous cycle when the circulating estrogens and progestogens are low (Salahuddin et al., 2021c). Notably, in HIV^+ women, higher E_2 and P_4 levels predicted lower cortisol reactivity and emotional responses (Maki et al., 2015). Together, these findings show the potential of HIV-1 Tat and estrous cycle interactions to influence the HPA axis and behavioral outcomes.

Addictive and affective behavioral dysregulation by HIV Tat exposure

HPA dysregulation may promote susceptibility to addiction- and affective-related behaviors (Koob and Volkow, 2016). We and others found Tat enhances the sensitizing (Kesby et al., 2017; Salahuddin et al., 2020a) and rewarding effects of illicit drugs (Gonek et al., 2018; Salahuddin et al., 2022a *Unpublished**) in mice models, and other reports suggest that HIV-tg rats self-administer psychostimulants to a higher extent (de Guglielmo et al., 2020; McIntosh et al., 2015) implicating HIV^+ individuals are more prone to increased drug consumption; however,

these findings are not uniform (Huynh et al., 2020; Kesby et al., 2019; Wayman et al., 2016). Preserving the HPA stress axis may help decrease the vulnerability to substance use disorders (Wand, 2008). Numerous preclinical studies demonstrate that acute stressors reduce conditioned place preference for illicit drugs, while chronic stressors amplify these effects (Bali et al., 2015). Consistently, stress may often trigger drug relapse and craving in human addicts (Mantsch et al., 2016). Tat's potential to dysregulate the HPA axis may promote vulnerability to the sensitizing and rewarding effects of illicit substances, which may co-occur with affective disorders (Salahuddin et al., 2020a, 2020b, 2021c). Increased cortisol-to-DHEA ratios are associated with higher scores on stressful life events, subjective stress, anxiety, and depression symptomatology in HIV^+ individuals (Mukerji et al., 2021; Qiao et al., 2017). Similarly, Tat expression in mice produces elevated circulating corticosterone levels concurrent with promoting anxiety- and depression-like behavior (reviewed in Gaskill et al., 2017). Moreover, sex differences were also reported, wherein stressed mice showed increased susceptibility to Tat/oxycodone behavioral interactions while in their diestrous phase (higher estradiol: progesterone ratio; Salahuddin et al., 2021c). This is in line with other studies in rat models, wherein increased estradiol levels promote psychostimulant responses to drugs of abuse (Calipari et al., 2017; Hu and Becker, 2003; Ramôa et al., 2013; Vandegrift et al., 2017). Given the HPA axis's widespread involvement in central and peripheral homeostasis, reversing Tat-mediated HPA dysregulation may improve outcomes across a range of physiological and behavioral measures.

Cognitive decline by HIV Tat exposure

When oxycodone was administered repeatedly, its stimulation of the HPA axis was diminished concurrently with a lesser impact on short-term memory function; nevertheless, Tat

affected novel object recognition in mice (Salahuddin et al., 2020a). Opioid use has been linked to cognitive impairment in clinical populations in both the pre-and post-cART periods (Bell et al., 1998; Byrd et al., 2011; Martin-Thormeyer and Paul, 2009). Lifetime abuse, on the other hand, is usually linked with polysubstance use as well as several periods of abstinence and withdrawal. It is possible that oxycodone exposure over a short period of time (5 days) will not have these significant influences on short-term memory outcomes in Tat-expressing mice. In accordance with this, herein we found oxycodone did not enhance Tat-mediated cell death in cell culture experiments (Salahuddin et al., 2020a). Cognitive function has been shown to be impaired by Tat exposure in male rodent models (Carey et al., 2012; Marks et al., 2016); however, this is not always the case when the animals are drug-naive (Kesby et al., 2016ab, 2018). Herein, we found that Tat-expressing mice have working memory deficits, revealed by a significant reduction in proportionate time spent with the novel object (Salahuddin et al., 2020a). Given, the parahippocampal regions of the brain are involved in the visual object recognition memory (Hammond et al., 2004), prior reports suggest the capacity of Tat to cause selective loss of network of connected hippocampal CA1 interneurons (Marks et al., 2016), and Tat-mediated disruption of dopaminergic transmission may suppress LTP, which is crucial for learning and memory (Behnish et al., 2004; Li et al., 2004); however, this pattern was only seen in female mice in their proestrous cycle (when circulating levels favored P_4 over E_2). Increased estradiol levels, similar to those seen on diestrus, were found to be linked with maintenance of short-term memory function, indicating that Tat-mediated cognitive deficits may be reversible in certain cases (Salahuddin et al., 2020a). Notably, mice exposed to repeated oxycodone had a reduced HPA response, while the HPG axis was increased in favor of E_2 (Salahuddin et al., 2020a). Consistently, clinical reports of HIV^+ women performing a learning task had a two- to three-

fold higher odds of learning impairment, which persisted throughout the menopausal transition and into the postmenopausal period, when estradiol levels are nadir (Maki et al., 2021). In support we found, irrespective of oxycodone exposure, E_2 or P_4 reduced neurotoxicity caused by the Tat protein in SH-SY5Y neuroblastoma cell culture studies (Salahuddin et al., 2020a). It needs to be emphasized that use of clinical opioids for a longer period of time is linked with HPA attenuation and consequent hypogonadism (Katz and Mazer, 2009). Continued research on the long-term cognitive effects of chronic oxycodone use/abuse, as well as their interactions with HIV-1 proteins, should be conducted.

Tat-mediated neurosteroidogenesis and HPA axis

Non-traditional acting steroid hormones generated *de novo* in the brain regulate the HPA axis (i.e., neurosteroids Crowley and Girdler, 2014). Stress-induced neurosteroids such as allopregnanolone restore HPA-axis balance by potently modulating inhibitory $GABA_A$ receptors (Majewska et al., 1986; Morrow et al., 1987; Lambert et al., 2009; Reddy and Rogawski, 2002). The PVN of the hypothalamus contains innervations of GABAergic interneurons (Miklós and Kovács, 2002) and $GABA_A$ receptors (Cullinan et al., 2000). Prior evidence shows Tat promotes loss of GABAergic interneurons in the hippocampus, especially those that are nNOS+, SST+, or PV+ (Marks et al., 2016). Loss of GABAergic interneurons may cause inhibitory deficiencies in the hypothalamus. The loss of GABAergic interneurons may compensate by an increase in AlloP concentration in the hypothalamus. As such we found, combined Tat and oxycodone exposure and OVX promoted an increase in hypothalamic AlloP levels and lowered oxycodone psychomotor effects, indicating its function in maintaining HPA homeostasis (Salahuddin et al., 2021c). Further evidence shows AlloP downregulates CRF mRNA expression in adrenalectomized mice, and

suppresses the firing of PVN CRF neurons to stimulate CRF release following stress (Cullinan et al., 2000; Gunn et al., 2013; Patchev et al., 1994, 1996). Additionally, neurosteroids inhibit downstream ACTH release and subsequent corticosterone production in rats (Bitran et al., 1999; Carboni et al., 1996; Crowley and Girdler, 2014; Owens et al., 1992; Patchev et al., 1994, 1996). While neurosteroids suppress the HPA response, a dysfunctional HPA axis may influence neurosteroidogenesis (Matsumoto et al., 2005; Romeo et al., 1998; Serra et al., 2000; Uzunova et al., 1998). In mice, like other models of traumatic brain injury, and ischemic stroke, HIV-1 Tat expression promotes neurosteroidogenesis, thereby increasing pregnenolone and 5**α**-reduced metabolites, including allopregnanolone (Paris et al., 2020), as a central adaptive response to stress. In addition to AlloP's positive allosteric modulating effects at $GABA_AR$, it inhibits L-type calcium channels (Earl and Tietz, 2011; Hu et al., 2007), and sulfated metabolite of AlloP (AlloP-S) is a negative allosteric modulator at NR2B subunit of NMDA receptor (Johansson and Le Grevès, 2005), which may alleviate Tat-mediated excitotoxicity mediated by cationic channels like LRP, L-type calcium channels, and NMDA receptor (Paris et al., 2016, 2020). In support, we found neurosteroids administration augmented neurosteroidogenesis to attenuate Tat-mediated HPA activation concurrent with a decrease in anxiety-like behavior (Salahuddin et al., 2022b *Unpublished**). These effects occurred concurrent to AlloP capacity to restore Tat-mediated downregulation of 3α-HSD enzyme expression, implicating the role of 5α-reduced pregnane steroids in these protective effects (Salahuddin et al., 2022b *Unpublished**).

The present dissertation revealed neurosteroids like FGIN-1-27 and AlloP reduced elevated corticosterone levels and anxiety-like behavior. AlloP also reinstated Tat-mediated downregulation of 3α-HSD neurosteroidogenic enzymes, thereby promoting neurosteroidogenesis

and ameliorating Tat-mediated HPA dysfunction. Taken together, these findings support the notion that FGIN-1-27 and AlloP are viable adjuncts to cART for HIV treatment for neurological and neuroendocrine complications. The schematic diagram describing the results and proposed future directions is depicted in Figure 57.

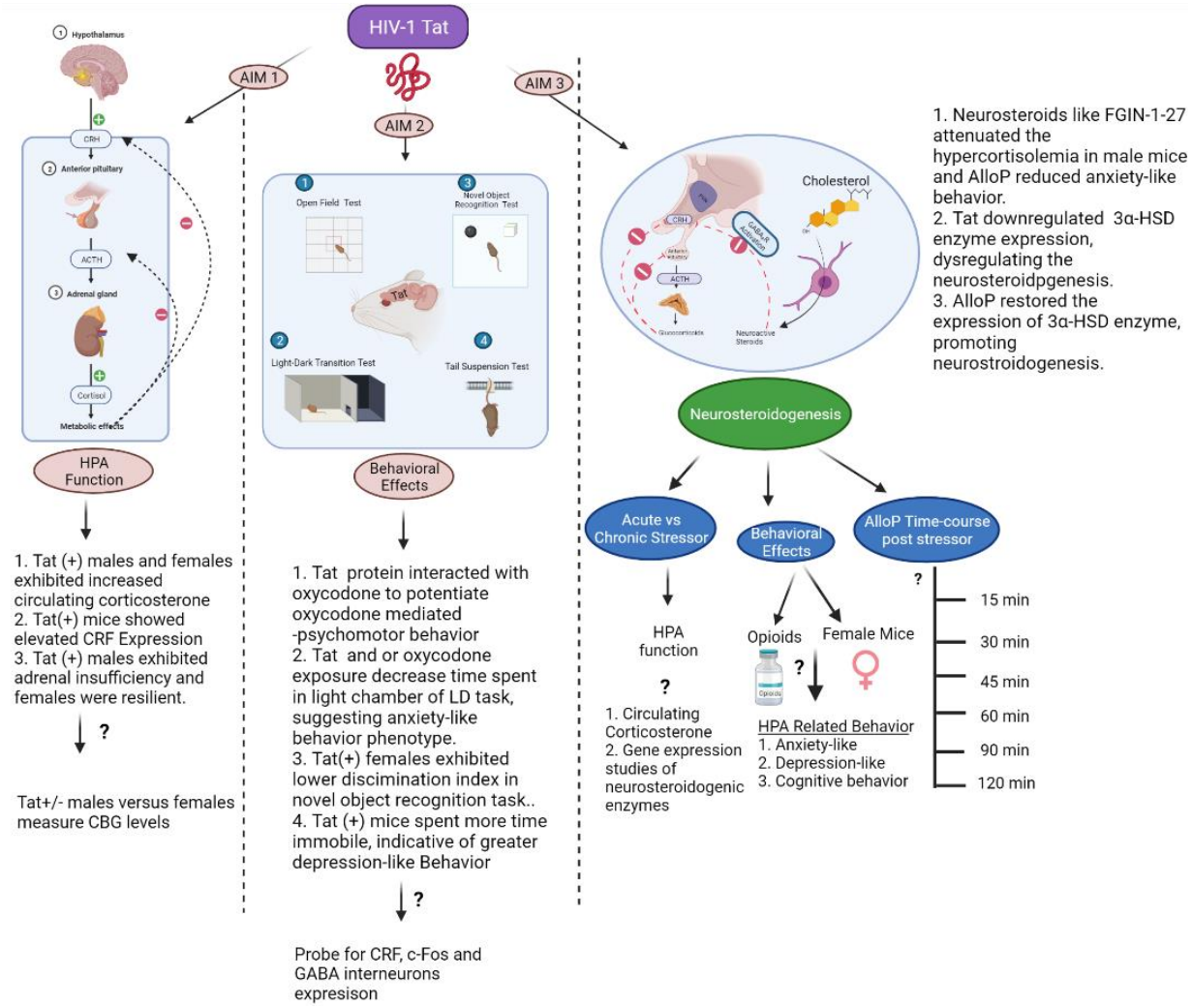

Figure 57: Schematic representation of conclusions and future directions.

HPA related effects: Irrespective of sex, Tat (+) mice exhibited elevated basal corticosterone levels and higher CRF protein expression, mimicking the clinical phenotype of HPA dysregulation. Unlike males, females did not demonstrate adrenal insufficiency. Behavioral effects: HIV-1 Tat expression interacted with oxycodone to potentiate oxycodone-mediated psychomotor behavior. Tat and/or oxycodone promoted anxiety-like behavior. Tat-expressing mice exhibited lower discrimination index in novel object recognition task. Tat(+) mice spent more time immobile, indicative of depression-like behavior. Neurosteroidogenesis: Neurosteroids like AlloP or 18kDa translocator protein, namely FGIN 1-27 attenuated hypercortisolemia and rescued anxiety-like behavior. Moreover, AlloP reinstated Tat-mediated downregulation of steroidogenic enzymes which partly explains reduced neurosteroidogenesis, responsible for HPA mediated-neuroHIV like behavior.

Limitations of Dissertation

Our conditional expression model of Tat (HIV- Tat_{1-86}) was generated via targeting GFAP-expressing cells (Bruce-Keller et al., 2008). The Tat expression is regulated via astrocyte-specific GFAP promoter and doxycycline promoter (Fitting et al., 2010a). The GFAP relegated Tat expression would target astrocytes throughout the CNS, including the hippocampus, hypothalamus, cerebellum, cortex, and spinal cord, as well as other parts of the brain (Uhlén et al., 2015). While astrocytes are the most abundant GFAP-expressing cells, however, GFAP-expressing cells are not exclusive to the CNS, since peripheral Schwann cells such as those found in the sertroli, atria, and liver also express GFAP, (Mancardi et al., 1991; Messing and Brenner, 2020), and can act as potential targets of Tat. While this model has been frequently utilized in the research and has provided valuable insight into the neurobehavioral and neuropathogenesis of HAND (Fitting et al., 2010a; Kim et al., 2003), it does have limitations. To begin, the Tat_{1-86} model is not infectious, and while one could argue that it replicates Tat persistence in the brain (as observed in infected individuals receiving cART), the major limitation is that it is Tat-centric, neglecting the role of other residual proteins including gp120, Vpr, Nef, and Rev in the central nervous system. Secondly, to what extent does this model relate to cART-treated HAND neuropathology? While HIV-1 infects up to 19% of perivascular astrocytes in HAND patients (Churchill et al., 2009), these cells are not the principal generators of viral Tat protein in HIV-infected brains in the clinical population. Hence better models which emulate neuropathology and behavioral characteristics of HIV need to be explored.

Other limitations may include the capacity of other virotoxic proteins produced by HIV in HPA dysfunction. To this end, studies showed HIV proteins like viral protein R to activate

the GR and enhance glucocorticoid effects, thereby causing glucocorticoid resistance (Kino et al., 1999, 2004). When expressed in a transgenic mouse model, the HIV envelope protein, gp120, increases plasma ACTH and corticosterone levels (Raber et al., 1996). In *ex vivo* mouse or rat hypothalamus explants, gp120 stimulates CRF mRNA expression and release (Costa et al., 2000; Pozzolli et al., 2001). Hence, viral proteins like Tat, gp120, and/or viral protein R may work alone or in conjunction to produce glucocorticoid resistance (Chrousos and Zapanti, 2014). The characteristic phenotype of HPA axis dysfunction observed in HIV^+ patients may thus contribute to the neuroHIV-like phenotype.

While the focus of the present dissertation was exclusively on assessing the molecular underpinnings of neuroHIV, other stresses such as socioeconomic and environmental factors may also have a role in HPA response and HIV outcomes. In support, evidence shows HIV^+ patients experience stigma-related psychosocial stressors which may impede their medication adherence, complicate diagnosis and treatment, augment HIV viral load and contribute to HIV-related psychiatric symptomatology (Katz et al. 2013; Nyblade et al., 2019; Siefried et al., 2017; Tomori et al., 2014).

Given oxycodone is a psychostimulatory compound, it may particularly confound the anxiety-like behavior in open field. Light-dark transition test was thus used as a an additional behavioral task for the assessment of anxiety-like behavior, albeit it doesn't preclude the motor component associated with oxycodone. Thus, anxiety-like behavior should be interpreted prudently.

The behavioral endpoints like locomotion and corticosterone response vary throughout the day (Velasco et al., 1993). In almost all the behavioral experiments, the number of animals

to be tested each day and the restricted number of testing devices required testing animals at various times throughout their active dark cycle phase. Although all drugs administered are represented at all time points, the circadian influence on physiological and behavioral measures may not be entirely eliminated.

Future Directions

The present dissertation established the effectiveness of the forced swim stressor as a technique of stress inoculation to promote HPA dysfunction in these mice. Future studies should examine the HPA axis function using other stressors such as restraint and immobilization stress, electric foot shock-induced stress, social defeat stress, chronic unpredictable stress.

Given that the circadian rhythm of the AlloP response to psychological stressor may be different in rodents when compared to humans (Crowley and Girdler, 2014; Girdler et al., 2001), future studies should explore the time course of AlloP response and corticosterone levels to various psychological stressors.

While the present dissertation was able to assess the influence of exogenous AlloP and FGIN-1-27 on the HPA axis and behavioral response, the fluctuating levels of gonadal hormones in females is a major factor to contribute to sex differences (Oyola and Handa, 2017), henceforth future research should examine the effect of exogenous AlloP on stress axis responsiveness and behavioral performance in female mice. Moreover, the capacity of exogenous neurosteroids to restore the HPA axis in presence of clinical opioids like morphine and oxycodone needs to be explored.

While the present dissertation focus on the role of AlloP and FGIN-1-27 to influence the

HPA axis, it has to be appreciated that other neurosteroids like TH-DOC, DHEA, DHEA-S, may also regulate the HPA axis function (Crowley and Girdler, 2014; Girdler et al., 2001). Future studies should assess the role of progesterone-derived neurosteroids and androstenedione-derived neurosteroids on the stress axis and behavioral function.

Given, the HPA axis and behavioral response vary from the time of stressor, future research may be able to assess the time-course of drug administration, time of year, acute stressor versus chronic stressor, and its correlation to stress axis response and behavioral endpoints.

The varying amplitude, sensitization, and habituation of allostatic stress response, as well as the intricate interplay between HPG and HPA axes, may further need to be considered for each behavioral outcome (Patchev and Patchev, 2006). Besides adherence to maintaining similar experimental conditions like mouse strain, sex, age, cycle phase, ambient humidity conditions, animal handling skills, etc preemptive considerations of study design and stressor model may enhance the reproducibility and replicability of the data.

Moreover, the present dissertation used the pharmacological blockade of HPA feedback via systemic injection of antalarmin and RU-486. Future studies should test the region-specific delivery of antalarmin and RU-486 and assess the recapitulation of HPA related functional effects.

Given that the HPA axis and the immune system have dynamic interactions and the capacity of HIV-1 Tat to dysregulate the HPA axis concurrent to an increase in the cytokines (Fitting et al., 2010b; Salahuddin et al., 2020a, 2020b, 2021c). Further studies should assess the capacity of Tat to moderate or mediate the relationship between HPA markers like cortisol,

cytokines both in the periphery and cerebrospinal fluid.

Furthermore, future research should be conducted to determine the effectiveness of HPG/HPA endocrine modulators' role in primary and infectious HIV cell cultures.

Clinical Significance of This Dissertation

When considering the neuroendocrine dysregulation seen in HIV^+ patients, as well as the prospective advantages of novel steroid-based therapies, the clinical significance of this dissertation is most evident. Estimates may vary, but the prevalence of HPG and HPA dysfunction varies between 20 and 88 percent across different cohorts (Clark et al., 2001; Rietschel et al., 2000; Rochira et al., 2011; Tripathy et al., 2015). Novel steroid-based therapies are being increasingly investigated for their potential role to decrease Tat-mediated neuropathology. Didehydro-cortistatin A (dCA), a glucocorticoid found in marine sponges, has been shown to limit Tat's ability to transactivate the HIV-1 LTR, to alleviate Tat-mediated inflammation, and to reduce Tat's ability to enhance cocaine-induced conditioned place preference (Mediouni et al., 2019, 2015; Mousseau et al., 2012). In a similar context, the isoflavone equol, which acts on the estrogen receptor (ERβ), has been shown to inhibit Tat-mediated dendritic synaptic loss, with or without potentiation by cocaine, in an ER-β dependent manner (Bertrand et al., 2015). It is possible that steroidal backbone therapeutics like neurosteroids may help combat combined Tat and opioid-mediated synergy. We have found neurosteroids like FGIN 1-27 and allopregnanolone reinstated HPA dysfunction and ameliorated anxiety-like behavior. Hence, new therapies with a steroid backbone may thus be significant adjuvant components for future antiretroviral therapy regimens that seek to prevent neuroHIV alone or in conjunction with clinical opioids.

Conclusion

The present dissertation proposed and tested the hypothesis that HIV- Tat and oxycodone interact to modulate the HPA axis and contribute to affective and cognitive decline, and novel adjunct therapeutics, such as the neurosteroid, allopregnanolone, restore the stress axis function and ameliorate underlying neuroHIV symptoms. These data showed for the first time, the capacity of neurotoxic proteins like HIV-1 Tat to dysregulate the stress axis (as evidenced by elevated basal corticosterone and adrenal insufficiency in males) concurrent with potentiation of psychomotor and anxiety-like behavior in male and female transgenic mice. Additionally, results indicate blockade of the stress-responsive corticotropin receptor and glucocorticoid receptors in males partially reinstated normative HPA responding, implicating these receptors in the pathogenesis of neuroHIV. Ovariectomy, but not CRF or GR blockade attenuates the psychomotor response, implicating the role of gonadal hormones to promote these behaviors in female mice. We also find the intracerebral infusion (site-targeted delivery) of allopregnanolone to restore HPA stress axis dysfunction and to alleviate anxiety-like behaviors in male mice. Overall this dissertation demonstrated the efficacy of these novel therapeutics in a model of neuroHIV and may provide the basis for potential HIV treatment in HIV^{+} patients.

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Zorrilla, E. P., Logrip, M. L., & Koob, G. F. (2014). Corticotropin releasing factor: a key role in the neurobiology of addiction. *Frontiers in neuroendocrinology*, *35*(2), 234–244. https://doi.org/10.1016/j.yfrne.2014.01.001

Zorumski, C. F., Paul, S. M., Covey, D. F., & Mennerick, S. (2019). Neurosteroids as novel antidepressants and anxiolytics: GABA-A receptors and beyond. *Neurobiology of stress*, *11*, 100196. https://doi.org/10.1016/j.ynstr.2019.100196

Zou, W., Wang, Z., Liu, Y., Fan, Y., Zhou, B. Y., Yang, X. F., & He, J. J. (2010). Involvement of p300 in constitutive and HIV-1 Tat-activated expression of glial fibrillary acidic protein in astrocytes. *Glia*, *58*(13), 1640–1648. https://doi.org/10.1002/glia.21038

VITA

EDUCATION

PhD, Pharmacology, University of Mississippi, United States May, 2022
Major Advisor: Dr Jason Paris
cGPA: 3.98/4.00
Dissertation: HIV Tat and Opioids- Implications in Stress related psychopathology

M.Sc., Pharmacology, Aston University, Birmingham, United Kingdom 2010
Major Advisor: Dr Gavin Woodhall
cGPA: 3.23/4.00
Thesis: Effect of presynaptic mGluRs in gamma oscillations in rat medial entorhinal cortex

Bachelor of Pharmacy, Osmania University, India 2008
cGPA: 3.83

PROFESSIONAL POSITIONS

Graduate Research Assistant, University of Mississippi 2018- Present
Department of BioMolecular Sciences, Oxford, MS

- Research focused on exploring the role of pregnane and estrane steroids in presence of opioids in ameliorating HIV neurotoxicity.
- Performed a variety of **behavioral assays** to assess learning and memory, locomotion, muscular co-ordination and anxiety and depression-like behavior in rodents.
- Performed murine surgical procedures like **stereotaxic**, **subcutaneous implant**, and **ovariectomy**.
- Performed a variety of cell-based assays using different techniques in **biochemistry** and **molecular biology**, and **microscopy**.
- Participated in preparing and writing grant applications, scientific publications and conference abstracts.
- Trained and supervised undergraduate students in mouse dissection and surgical procedures.

Instructional Professor of Pharmacology, Mizan Tepi University, Ethiopia 2012-2018

- Teach undergraduate and graduate pharmacology courses to health science and medicine students each semester.
- Prepare and implement daily lesson plans to foster student learning.
- Attend regular conferences, training, and professional development seminars.
- Represent department and college at symposium and career fairs.
- Pursue and acquire research grants in order to enhance scientific advancement.
- Meet with students outside of class to reinforce and mentor on tough concepts.

COMPLETED RESEARCH SUPPORT

(**PI: Salahuddin Mohammed**) **University of Mississippi, Total Costs: $1000**
Sex differences in steroidogenic response to Hypothalamic Pituitary Adrenal Stress Axis Dysregulation in HIV-1 Tat Female Mice **04/27/21 - 03/08/22**

(**PI: Salahuddin Mohammed**) **University of Mississippi, Total Costs: $1000**
Assessment of underlying neurocircuitry in dysregulation of the Hypothalamic Pituitary Adrenal Stress Axis in HIV-1 Tat Male Mice **05/30/20 - 11/01/20**
https://egrove.olemiss.edu/gsc_researchgrants/13

(**Co-I: Salahuddin Mohammed**) **Mizan Tepi University, Total Costs: $1000**
Epidemiology & Predictors of drug related problems among ambulatory Type 2 Diabetes mellitus patients at Mizan Tepi Teaching Hospital and Gebresadik Shawo General hospital

TECHNICAL APPROACHES

In Vivo/Behavioral	• Screening anxiety-like, depression-like, cognitive, reward, social, nociceptive, motor, (attentional processes and impulse control behavior via 5-CSRTT) • Aseptic surgery (Stereotaxic, Adrenalectomy, Ovariectomy and Subcutaneous implant) • Rodent husbandry (transgenic mice), Brain Dissections
Biochemical	• Enzyme Immunoassays (ELISA; EIA), Immunocyto-/ Immunohistochemistry, Western Blot • Polymerase Chain Reaction (PCR) • Microscopy (Fluorescence, Visible Light)
In Vitro/ Culture	• Primary human/murine cell culture and human/murine cell lines
Statistical/ Analytic	• Behavioral encoding via ANY-Maze • *In vitro* encoding via ImageJ • Analyses via GraphPad Prism, R, SigmaPlot, SPSS, StatView

COMPETITIVE AWARDS, GRANTS, AND HONORS

2022 3rd Place Podium Presentation Award, 12th Annual GSC Symposium, UM $400
2021 Dissertation Fellowship Award, UM $6500
2021 BioMolecular Sciences Student Advocates Student Excellence Award $500
2021 Edith Pritchard Graduate Student Award, University of Mississippi (UM) $500
2021 Graduate Achievement Award, University of Mississippi (UM)
2021 2nd Place Poster Award, UM Neuroscience showcase $100
2020 Trainee Professional Development Award, Society of Neuroscience (SfN) $100
2020 Honorable Poster Award, UM Neuroscience showcase $95
2020 Young Investigator's Educational Enhancement (YIEE) Travel Award, American Society of Neurochemistry $1000
2019 Marvin Davis Graduate Student Award, University of Mississippi (UM) $500
2008 Merit bursary, Aston University, United Kingdom £1000

PROFESSIONAL MEMBERSHIPS AND COMMUNITY OUTREACH

2020-2021	Journal Club Chair, University of Mississippi, USA	Chair
2020-2021	Sigma Xi, The Scientific Research Honor Society	Member
2019-2023	Phi Kappa Phi, University of Mississippi, USA	Member
2019-	Rho Chi, School of Pharmacy, University of Mississippi, USA	Life Member
2019-present	American Society of Neurochemistry	Member
2019-present:	World Sleep Society	Member
2019-present	Society for Neuroscience	Member

CERTIFICATIONS

2021	NIH Grant Writing Workshop (7.5hours), UM Certificate	
2021	Responsible conduct of research (15 hours), UM	Certificate
2020	COVID-19 Special Update; Harvard Medical School	Certificate
2019-Present	American Chemical Society (ACS) Reviewer Certification	Reviewer
2019-Present	Elsevier Reviewer Certification	Reviewer
2018	Biomedical Responsible conduct of Research; CITI program	Certificate
2018	Responsible conduct of research (RCR); CITI program	Certificate
2014	SPSS- Improving Quality of Teaching in Higher Education Institution	Certificate

DEDICATED SECTIONS

Diversity, Equity and Inclusion (DEI)

Personal Statement: I am a strong advocate for DEI in and outside my teaching, research and service activities, which is well-reflected in my classroom composition, guest lectures attendance, and workshops. I also support UM mission of research and creative achievement to advance society, engage and transform communities, offer enriching opportunities outside the classroom; supports lifelong learning; and develops a sense of global responsibility.

DEI activities:

2021	Certified Search Committee Training	Certificate
2020	Certified Implicit Bias and Microaggressions Training	Certificate
2020	Certified Exploring Gender and Sexuality	Certificate
2020	Panelist, Graduate Student Survival Strategies	

Mental Health

Personal Statement: My experience as a mentor in both official and informal settings demonstrates my willingness to devote time in mental health problems and the stigma that surrounds them. I've grown to be aware of and knowledgable about impostor syndrome, patience, and empathy through time, and I advocate for the critical role of mental health wellness for a healthy, inclusive community.

LEADERSHIP ROLE & EXTRACURICCULAR ACTIVITIES

2021-	Planning, designing and execution of pre-clinical research studies, India	Oral Talk
2021-	Manuscript Writing Tips, UM	Oral Talk
2021-	Judge- Rho Chi Student Research Day, UM	

2021- Professional Development - Keep Educating Yourself, UM Oral Talk
2021- Member- Dean Search Committee, School of Pharmacy, UM
2020- Judge- Rho Chi Student Research Day, UM
2020- Graduate Student Survival Panel, School of Pharmacy, UM
2020- Graduate Student Life: Need of the Hour Oral Talk
2020- COVID-19 and Substance Use Disorders Oral Talk
2020- Hosted Dr Roshni Rao, Professional Development Speaker
(Director of Phutures from John Hopkins University;
Weblink: https://pharmacy.olemiss.edu/bms/bsa-calendar-of-events/ for Talk on:
"Establishing an Online Presence to Design Your Career" on July 22, 2020
2020- Hosted Dr. Rehana Leak, Associate Professor of Pharmaceutical Sciences from Duquesne University; Weblink: https://pharmacy.olemiss.edu/bms/fall-2020/ for BioMolecular Sciences department (BMS) external seminar speaker for talk on "Sex differences in Lewy bodies in Parkinson's animal model" on August 25, 2020
2020- Hosted Dr. James Prestegard, Emeritus Professor of Analytical Chemistry from University of Georgia;
Weblink: https://pharmacy.olemiss.edu/bms/bsa-calendar-of-events/ for BioMolecular Sciences department (BMS) external seminar speaker for talk on "Glycoprotein Structure and function from NMR Methodology" on November 12, 2020

PEER-REVIEWED PUBLICATIONS (*h*-index =14)

Current Most Relevant Contributions:

https://www.ncbi.nlm.nih.gov/myncbi/1dQSgFRdi-tQs8/bibliography/public/

https://scholar.google.com/citations?user=8wO-6FEAAAAJ&hl=en

1. **Salahuddin MF**, Qrareya AN, Mahdi F, Li J, Le H, Paris JJ. Allopregnanolone and NeuroHIV: Potential Benefits of Neuroendocrine Modulation in the Era of Antiretroviral Therapy. *J Neuroendocrinol*. https://doi.org/10.1111/jne.13047
2. **Salahuddin MF**, Mahdi F, Paris JJ. HIV-1 Tat Protein Promotes Neuroendocrine Dysfunction Concurrent with the Potentiation of Oxycodone's Psychomotor Effects in Female Mice. *Viruses* 2021; 13(5): 813. PMID: 33946474
3. **Salahuddin MF**, Mahdi F, Paris JJ. HIV-1 Tat Dysregulates the Hypothalamic-Pituitary-Adrenal Stress Axis and Potentiates Oxycodone-Mediated Psychomotor and Anxiety-Like Behavior of Male Mice. *Int J Mol Sci.* 2020;21(21):8212. PMID: 33153023
4. **Salahuddin MF**, Qrareya AN, Mahdi F, Jackson D, Foster M, Vujanovic T, Box JG, Paris JJ. Combined HIV-1 Tat and oxycodone activate the hypothalamic-pituitary-adrenal and -gonadal axes and promote psychomotor, affective, and cognitive dysfunction in female mice. *Hormones and Behavior*, 2020; 119:104649. PMID: 31821792
5. Paris JJ, Liere P, Kim S , Mahdi F , Buchanan ME , Nass SR, Qrareya AN , **Salahuddin MF** , Pianos A , Fernandez N , Shariat-Madar Z , Knapp PE , Schumacher M, Hauser KF. Pregnane steroidogenesis is altered by HIV-1 Tat and morphine: Physiological allopregnanolone is

protective against neurotoxic and psychomotor effects. *Neurobiology of Stress.* 2020; 12: 100211. PMID: 32258256

6. **Salahuddin MF**, Manzar MD, Hassen HY, Unissa A, Hameed UA. Spence DW, Pandi-Perumal SR. Prevalence and Predictors of Neurocognitive Impairment in Ethiopian Population Living with HIV. *HIV/AIDS - Research and Palliative Care.* 2020,12: 559–572. PMCID: PMC7568595

Publications under preparation

7. **Salahuddin MF,** Mahdi F, Paris JJ. Neuroendocrine Modulators in restoration of HIV-1 Tat mediated Hypothalamus-pituitary-adrenal stress axis dysregulation. *Intended Submission to Hormones and Behavior**

8. **Salahuddin MF**, Mahdi F, Paris JJ. HIV-1 Tat potentiates the psychostimulatory and rewarding effects of clinically-used opioids via actions preferentially involving MOR and DOR. *Intended Submission to Drug Alcohol Dependence**

9. Mahdi F, **Salahuddin MF**, Liere P, McLane VD, Pianos A, Fernandez A, Qrareya AN, Foster M, Cook, D, Schumacher M, Kanpp PE, Hauser KF, Paris JJ, Combined HIV-1 Tat and Morphine Exposure Alter Androgen Steroid Formation, Immune Response, and Withdrawal Behaviors of Male Mice. *Intended Submission to Brain Behavior Immunity**

Clinical Collaborative Contributions:

10. **Salahuddin MF**, Manzar MD, Unissa A, Pandi-perumal SR, Bahammam AS. The global shortage of essential drugs during the COVID-19 pandemic: evidence based on aggregated media and social media reports. *Journal of Nature of Science and Medicine*, 2022; 5(1): 23-28. DOI: 10.4103/jnsm.jnsm_61_21

11. **Salahuddin MF**, Manzar MD, Pandi-perumal SR, Bahammam AS. Emerging challenges in COVID-19 with Substance use disorders. Addictive Disorders & Their Treatment. April 06, 2021 - DOI: 10.1097/ADT.0000000000000266

12. Khan M, Manzar MD, Alghadir AH, **Salahuddin MF**, Hassen HY, Mansour AL, Nureye D, Tekalign E, Shah SA, Pandi-perumal SR, Bahammam AS. Poor sleep in community-dwelling polysubstance users: Association with khat dependence, metacognition, socio-demographic factors. *Accepted**

13. Manzar MD, **Salahuddin MF,** Alghadir AH, Anwer S, Peter S, Bahammam AS, Pandi-perumal SR. Psychometric properties of the Generalized Anxiety Disorder-7 Scale in Ethiopian university students. December 2021. *Bull Menninger Clin*. DOI: 10.1521/bumc.2021.85.4.405

14. Manzar MD, Alghadir AH, Khan M, **Salahuddin MF,** Manaiago JD, Vasquez B, Pandi-perumal SR, Bahammam AS. Anxiety symptoms are associated with higher psychological stress, poor sleep, and inadequate sleep hygiene in collegiate young adults-a cross-sectional study. *Frontiers in Psychiatry.*2021; 12:677136. PMCID: PMC8280471

15. Manzar MD, Alghadir AH, Alqahtani M, **Salahuddin MF**, Addo HA, Jifar WW, Alasmee NA. Psychometric Properties of the General Anxiety Disorders-7 Scale Using Categorical Data Methods: A Study in a Sample of University Attending Ethiopian Young Adults. Neuropsychiatric Disease and Treatment 2021,17:893-903.PMID: 33790558

16. Manzar MD, **Salahuddin MF**, Pandi-Perumal SR, Bahammam AS. Insomnia May Mediate the Relationship Between Stress and Anxiety: A Cross-Sectional Study in University Students. Nature and Science of Sleep. 2021:13 31–38; PMID: 33447116
17. Legesse M., Ali JH, Manzar MD., **Salahuddin MF** Hassen HY. Level of physical activity during the third trimester of pregnancy and its association with birthweight at term in South Ethiopia: A prospective cohort study. PLoS One. 2020;15(7):e0236136. PMID: 32687541
18. Manzar MD, Albougami A, Alamri M, **Salahuddin MF**, Pandi-Perumal SR. Psychometric analysis of sleep hygiene index and correlation With stress and anxiety among Saudi university students. *Nature and Science of Sleep*, 2019;325-332. PMID: 31807105
19. Manzar MD, Hameed UA., **Salahuddin MF**, Khan MYA, Nureye D, Wakene W, Alamri M, Albougami A, Pandi-Perumal SR, Bahammam AS, Migraine Screen Questionnaire: Further psychometric evidence from categorical data methods. Health and Quality of life Outcomes. 2020; 28:18(1):113 PMID: 32345313
20. Manzar MD, Noohu MM, **Salahuddin MF**, Nureye D, Albougami A, Spence DW, Pandi-Perumal SR, Bahammam AS. Insomnia Symptoms and Their Association with Anxiety and Poor Sleep Hygiene Practices Among Ethiopian University Students. Nature and Science of Sleep 2020:12 575–582. PMID: 32884384
21. Hameed UA., Al-Jarrah MD, Manzar MD, Albougami A, Alrasheadi BA, Noohu MM, **Salahuddin MF.** Leeds sleep evaluation questionnaire in Jordanian university students: A psychometric investigation using comparative confirmatory factor analysis. Saudi Med J 2020, 41 (7):179-185.PMID: 32601644
22. Anwer S, Manzar MD, Alghadir A, **Salahuddin MF,** Hameed UA. Psychometric analysis of the perceived stress scale among healthy university students. Neuropsychiatric Disease and Treatment 2020. 16: 2389-2396. PMID: 33116538
23. Alghadir A, Manzar MD, Anwer S, Albougami A, **Salahuddin MF.** Psychometric Properties of the Generalized Anxiety Disorder Scale Among Saudi University Male Students. Neuropsychiatric Disease and Treatment 2020:16 1427–1432 PMID: 32606696
24. Manzar MD, Hameed UA, Alqahtani M, **Salahuddin MF**. Morgan P. Bahammam AS., Pandi-Perumal SR. Obstructive sleep apnea screening in young people: Psychometric validation of a shortened version of the STOP-BANG questionnaire using categorical data methods. Ann Thorac Med. PMID: 33381236
25. Engiso H, Nureye D, Worku T, **Salahuddin MF**, W/selassie W, Hambisa S., Sharif NM. Antibacterial Activity of Ritchiea albersii Gilg (Capparidaceae) and Cynoglossum ampldolium (Boraginaceae,) leaves against selected bacteria. Saudi Journal of Medicine & Medical Sciences, 2020;8:201-7 PMID: 32952512
26. Manzar MD, **Salahuddin MF,** Shah SA, Khan TA, Otaibi JA, Almansour AM, Alrasheadi BM, Pandi-Perumal SR, Bahammam AS. Mizan Khat Use Disorder Index (MiizKUDI) tool development and validation in habitual khat users. Pakistan Journal of Medical Sciences. 2020; 14(1):478-482.
27. Manzar MD, **Salahuddin MF**, Khan TA, Sharief NM, Shah SA, Nureye D, Wakene W, Addo HA, Albougami A. Psychometric properties of a brief metamemory and metaconcentration scale in substance use problem. 2020 International Journal of mental health and addiction. DOI: 10.1007/s11469-020-00256-6

28. Manzar MD, **Salahuddin MF**, Kedir MS, Chattu VK, Warren Spence D, Pandi-Perumal SR. How We Do Harm: Do Copyrighted Scales Benefit Research in the Developing World? Front Public Health. 2019;18:7:377. PMID: 31921742
29. Manzar MD, Noohu M, Begashaw B, **Salahuddin MF,** Bahammam AS, Albougami A, Spence DW, Pandi-Perumal SR. Prevalence of poor sleep quality in the Ethiopian population: A systematic review and meta-analysis. Sleep & Breathing. 2019;1-8. PMID:31183743; DOI:10.1007/s11325-019-01871-x
30. Manzar MD, **Salahuddin MF**, Alamri M, Albougami A, Khan MYA, Nureye D, Spence DW, Pandi-Perumal SR. Psychometric properties of the Epworth sleepiness scale in Ethiopian university students. Health and Quality of Life Outcomes 2019; 17(1):30. PMID: 30732633
31. Manzar MD, **Salahuddin MF**, Peter S, Alghadir A, Anwer S, Bahammam AS. Psychometric properties of the perceived stress scale in Ethiopian university students. BMC Public Health. 2019; 19:1. PMID: 30626438
32. Manzar MD, Albougami A, **Salahuddin MF**, Peter S, Spence DW, Pandi-Perumal SR The Mizan meta-memory and meta-concentration scale for students (MMSS): a test of its psychometric validity in a sample of university students. BMC Psychology 2018; 6:59. PMID: 30563573
33. Manzar MD, **Salahuddin MF**, Tesfaye T, Ahmad Alghadir, Shahnawaz Anwer, Bahammam AS, Pandi-Perumal SR. Validation of the adapted Leeds sleep evaluation questionnaire in Ethiopian university students. Health and Quality of Life Outcomes, 2018; 16:49. PMID: 29534726
34. Manzar MD , **Salahuddin MF**, Tesfaye T, Pandi-Perumal SR, Bahammam AS. Poor sleep in concurrent users of alcohol, khat, and tobacco smoking in community-dwelling Ethiopian adults. Annals of Thoracic Medicine, 2018; 13:4:220. PMID: 30416593
35. Manzar MD, Majid AS, **Salahuddin MF**, Khan MYA, Chattu VK, Pandi-Perumal SR, Bahammam AS. Psychometric properties of the severity of the dependence scale for Khat (SDS-Khat) in polysubstance users. BMC Psychiatry. 2018; 18:343. PMID: 30340476
36. Manzar MD, **Salahuddin MF**, Khan TA, Shah SA, Alamri M, Bahammam AS, Pandi-perumal SR. Psychometric properties of the Insomnia severity index in Ethiopian adults with substance use problems. Journal of Ethnicity in Substance Abuse. 2018; 5:1-15. PMID:30183562
37. Manzar MD, **Salahuddin MF**, Tesfaye T, Pandi-Perumal SR, Bahammam AS, Sleep correlates of substance use in community-dwelling Ethiopian adults. Sleep Breath, 2017; 1-7. PMID:28884262; DOI: 10.1007/s11325-017-1567-5
38. Manzar MD, **Salahuddin MF**, Sony P, Tesfaye T, Moscovitch A, Pandi-perumal SR, Bahammam AS. Sleep disturbances and memory impairment among pregnant women consuming Khat: An under-recognized problem. Annals of Thoracic Medicine, 2017; 12(4):247-251. PMID: 29118856
39. Manzar MD, Sony P, **Salahuddin MF**, Kumalo A, Geneto M, Pandi-perumal SR, Moscovitch A, Bahammam AS. Electrolyte imbalance and sleep problems during anti-retroviral therapy: an under-recognized problem. Sleep Science. 2017; 10(2:64-67). PubMed PMID: 28966741
40. **Salahuddin MF**, Tesfaye T , Kumalo A, Pandi-Perumal SR, Bahammam AS, Manzar MD. Validation of the Pittsburgh Sleep quality Index in community dwelling Ethiopian adults. Health and Quality of Life Outcomes. 2017; 15:58. PMID: 28347341
41. Tadesse A, Hymete A, Bekhit AA, **Salahuddin MF**. Quantification of Total Polyphenols, Catechin, Caffeine, L-theanine, Determination of Antioxidant Activity and Effect on

Antileishmanial Drugs of Ethiopian Tea Leaves Extracts, Pharmacognosy Research, 2015; 7(5):7-14. PMID: 26109792

42. Berheto TM, Haile DB, **Salahuddin MF**. Predictors of loss to follow-up in patients living with HIV/AIDS after initiation of antiretroviral therapy. North Am J Med Sci, 2014;6:453-459. PMID: 25317390

Global Burden/Local Burden of Disease (GBD/LBD) Collaborative Contributions:

43. GBD 2019 HIV Collaborators. Global, regional, and national sex-specific burden and control of the HIV epidemic, 1990-2019, for 204 countries and territories: the Global Burden of Diseases Study 2019 Lancet HIV. 2021; 8 (10): e633-e651. PMCID: PMC8491452

44. LBD Double Burden of Malnutrition Collaborators, **Salahuddin MF.** Mapping local patterns of childhood overweight and wasting in low- and middle-income countries between 2000 and 2017. Nat Med. 2020;26(5):750-759. PMID: 32313249

45. GBD 2017 US Neurological Disorders Collaborators, **Salahuddin MF.** Burden of Neurological Disorders Across the US From 1990-2017: A Global Burden of Disease Study. JAMA Neurol 2020; e204152. PMCID: PMC7607495

46. LBD HIV Collaborators, **Salahuddin MF.** Subnational mapping of HIV incidence and mortality among individuals aged 15-49 years in sub-Saharan Africa, 2000-18: a modelling study. Lancet HIV 2021; e363-e375. PMCID: PMC8187986

47. LBD 2017 Africa Onchocerciasis Project. **Salahuddin MF.** Predicting the environmental suitability for onchocerciasis in Africa as an aid to elimination planning. PLOS Neglected Tropical Diseases. 2021;15(7):e0008824. PMID: 34319976

48. GBD 2019 Dementia Collaborators. **Salahuddin MF**. Use of multidimensional item response theory methods for dementia prevalence prediction: an example using the Health and Retirement Survey and the Aging, Demographics, and Memory Study. BMC Med Inform Decis Mak 2021; 21(1):241. PMCID: PMC8356410

49. LBD 2018 Anemia Collaborators, **Salahuddin MF.** Anemia Prevalence in women of reproductive age in low- and middle-income countries between 2000 and 2018 DOI: 10.1038/s41591-021-01498-0

50. GBD 2019 Dementia Collaborators. **Salahuddin MF**. The Burden of Dementia due to Down Syndrome, Parkinson's Disease, Stroke, and Traumatic Brain Injury: A Systematic Analysis for the Global Burden of Disease Study 2019. Neuroepidemiology. 2021;55(4):286-296. DOI: https://doi.org/10.1159/000515393

51. GBD 2019 Ethiopia Subnational-Level Disease Burden Initiative Collaborators. Progress in health among regions of Ethiopia, 1990-2019: a subnational country analysis for the Global Burden of Disease Study 2019 [published online ahead of print, 2022 Mar 11]. Lancet. 2022;S0140-6736(21)02868-3. DOI: https://10.1016/S0140-6736(21)02868-3

Additional Peer-Reviewed Contributions:

52. Hussain Z, Sana A, **Salahuddin MF**, Mohammed AR: "Patterns of drug therapy among diabetic hypertensive patients with other complications", International Journal of Pharmacy and Pharmaceutical Sciences, 2014, 6(6): 270-277

53. **Salahuddin MF**, Motbaynor B, Haile DB: A review on the impact of the various environmental adversities on the developmental disorders of brain in children, International

Journal of Pharmacy and Pharmaceutical Sciences, 2014, 6(6): 161-164

54. **Salahuddin MF**, Kedir MS, Maru TT. Lipohypertrophy- The neglected area about the much talked about Diabetes. International Journal of Diabetes Research, 2015,4(2):38-42.

Publications under review

55. Akins NS, **Salahuddin MF**, Pandey P, Kim SJ, Mahdi F, Khan MIH, Moss E, Worth C, Keanne MM, Chittiboyina AG, Doerksen RJ, Paris JJ, Le HV. Alleviation of Cocaine Withdrawal and Pertinent Interactions between Salvinorin-based Antagonists and Kappa Opioid Receptor. *ChemRxiv**.

CONFERENCE/ABSTRACT PRESENTATIONS (ORAL/POSTER)

Oral Talks

1. **Salahuddin MF***, Mahdi F, Paris JJ. HIV- Neurosteroids Biosynthesis Upregulation: A Novel Promising Strategy For HIV-1 Tat Mediated Anxiety Disorders. Annual Graduate Student Council Research Symposium, University of Mississippi on March 8, 2022.
2. **Salahuddin MF***, Mahdi F, Paris JJ. HIV-1 Tat alters the Hypothalamic-Pituitary-Adrenal Stress Axis and Potentiates Oxycodone-mediated Behavior of Male Mice partly. Annual Graduate Student Council Research Symposium, University of Mississippi on March 15, 2021.
3. **Salahuddin MF***, Mahdi F, Paris JJ. Sex differences in the hypothalamic–pituitary–adrenal axis' response to stress in adult HIV Tat transgenic mice. University of Mississippi Neuroscience showcase on March 26, 2021. https://egrove.olemiss.edu/neuro_showcase/16/
4. **Salahuddin MF***. HIV-1 Tat dysregulate HPA axis and contribute to neurological behavior deficits. 3-minute Thesis competition, University of Mississippi on January 9, 2021
5. **Salahuddin MF***, Paris JJ. HIV-1 Tat Dysregulates the Hypothalamic-Pituitary-Adrenal Stress Axis and Potentiates Oxycodone-mediated Behavior of Male Mice partly via Actions at the Glucocorticoid Receptor. Oral Presentation at University of Mississippi on March 24, 2020.
6. **Salahuddin MF*.** Planning, designing and execution of pre-clinical research studies, India Oral Talk. Pulla Reddy College of Pharmacy, India on December 22, 2021.
7. **Salahuddin MF*.** HIV-1 Tat and Opioids. Implication for Stress, Shadan College of Pharmacy, India on January 14, 2020.

Posters

International Level

1. **Salahuddin MF***, Mahdi F, Paris JJ. Neurosteroids influence HIV-1 Tat-mediated HPA dysregulation and anxiety-like behavior. Society of Neuroscience conference, Chicago, IL, USA. Poster Presentation on November 8-11, 2021.
2. Qrareya A*, **Salahuddin MF**, Vujanovic T, Serrano P, Mahdi F, Paris JJ HIV-1 Tat influences morphine reward and voluntary oral morphine consumption among ovariectomized female mice. Society of Neuroscience conference, Chicago, IL, USA. Poster Presentation on November 8-11, 2021.
3. Khan MIH*, **Salahuddin MF**, Akins N, Mahdi F, Kim SJ, Li J, Paris JJ, Le H. Discovery of 5α-pregnan-2β,3α-diol-20-one as a neuroHIV protective agent. ACS National Meeting

September 2021.
4. Akins N, **Salahuddin MF**, Mahdi F, Kim SJ, Li J, Paris JJ, Le H. Synthesis, computational studies and behavioral evaluation of novel salvinorin-based opioid receptor agonists and antagonists. ACS National Meeting. September 2021.
5. **Salahuddin MF***, Mahdi F, Paris JJ. HIV-1 Tat Protein Promotes Neuroendocrine Dysfunction Concurrent with the Potentiation of Oxycodone's Psychomotor Effects in Female Mice. Poster presentation at American Society of Neurochemistry, United States of America on June 28-July1, 2021.
6. **Salahuddin MF***, Mahdi F, Paris JJ. HIV-1 Tat protein alters the Hypothalamic pituitary adrenal stress axis (HPA), hypothalamus Allopregnanolone and potentiates the effects of oxycodone. Accepted for Poster presentation at Steroid and Nervous System Virtual Conference at Torino, Italy on February 11-14, 2021*
7. Mahdi F*, **Salahuddin MF***, Paris JJ. HIV-1 proteins promote mitochondrial dysfunction in human and murine primary cells that can be rescued by exogenous allopregnanolone or 3a-androstanediol. Steroid and Nervous System Virtual Conference at Torino, Italy on February 11-14, 2021.
8. Jackson D*, **Salahuddin MF**, Qrareya A, Mahdi F, Foster M, Vujanovic T, Box G, Paris JJ. Neuroprotective effects of estrane and pregnane steroids in response to combined exposure of HIV-1 Tat and oxycodone. AS Microbiology 2019.
9. **Salahuddin MF***, Qrareya AN, Mahdi F, Paris JJ. HIV-1 Tat protein and oxycodone dysregulate adrenal and gonadal endocrine axes and promote affective and cognitive dysfunction in mice. Society of Neuroscience conference, Chicago, IL, USA. Poster Presentation on October 23, 2019
10. Paris JJ*, Mahdi FM, **Salahuddin MF**, Qrareya AN, Shariat-Madar, Z. HIV-1 Tat and morphine promote neuroendocrine dysfunction that may involve disruption of mitochondrial complexes I and II. Society of Neuroscience conference, Chicago, IL, USA. Oral Presentation on October 23, 2019.
11. Qrareya AN*, **Salahuddin MF,** Mahdi FM, Paris JJ. HIV-1 Tat interacts with selective estrogen receptor modulators to influence conditioned place-preference. Society of Neuroscience conference, Chicago, IL, USA. Poster Presentation on October 23, 2019.
12. **Salahuddin MF**. Assessment, diagnosis and treatment of HIV associated neurocognitive disorders (HAND). 4th Annual Research conference, Mizan Tepi University, Ethiopia on May 14, 2018.
13. **Salahuddin MF**. Pharmacological simulations: An Adjuvant to Practical Pharmacology Teaching. 3rd Annual Research conference, Mizan Tepi University, Ethiopia on May 13, 2017

National Level
14. Paris JJ*, Qrareya AN, Mahdi F, **Salahuddin MF.** HIV-1 Tat Promotes Age-Related Cognitive, Anxiety-like, and Antinociceptive Impairments in Mice that are Moderated by Aging Endocrine Status. Poster presentation at 16th International Symposium on NeuroVirology on November 12, 2019.

Regional Level

15. **Salahuddin MF***, Mahdi F, Paris JJ. Enhancing Neurosteroidogenesis Attenuates HIV-1 Tat Mediated Stress-Related Outcomes. Poster Presentation at University of Mississippi Neuroscience

showcase on March 24, 2022.

16. **Salahuddin MF***,Mahdi F,Paris JJ. HIV-1 Tat Protein Promotes Neuroendocrine Dysfunction Concurrent with Potentiation of Oxycodone's Psychomotor Behavioral Effects in Female Transgenic Mice 2021. https://egrove.olemiss.edu/pharm_annual_posters_2021/6/

17. Khan MIH*, **Salahuddin MF**, Akins N, Mahdi F, Kim SJ, Li J, Paris JJ, Le H. Discovery of 5α-pregnan-2β,3α-diol-20-one as a neuroHIV Protective Agent. 2021 SOP Annual Poster Symposium https://egrove.olemiss.edu/pharm_annual_posters_2021/10/

18. **Salahuddin MF***, Mahdi F, Paris JJ. HIV-1 Tat Dysregulates the Hypothalamic-Pituitary-Adrenal Stress Axis and Potentiates Oxycodone-mediated Psychomotor and Anxiety-like Behavior of Male Mice. 2020 SOP Annual Poster Symposium https://egrove.olemiss.edu/pharm_annual_posters/1/

19. **Sneed L***, Mahdi F, **Salahuddin MF,** Paris JJ, Shariat-Madar Z. HIV Tat protein activates plasma kallikrein-kinin system in the doxycycline-inducible astrocyte specific HIV-1 Tat transgenic mice.2020. https://egrove.olemiss.edu/pharm_annual_posters/3/

20. **Salahuddin MF***, Mahdi F, Qrareya, Alaa N. Paris JJ. HIV-1 Tat Dysregulates the Hypothalamic-Pituitary-Adrenal Stress Axis and Potentiates Oxycodone-mediated Behavior of Male Mice (2020). Poster Presentation at University of Mississippi Neuroscience showcase on April 13, 2020. https://egrove.olemiss.edu/neuro_showcase/7

21. Cook D*, **Salahuddin MF**, Mahdi F, Qrareya AN, Foster M, Paris JJ. HIV-1 The Neurotoxic HIV-1 Tat Protein Potentiates Morphine's Psychomotor Effects which can be Ameliorated by the Neuroprotective Steroid, 3a-diol. Poster presentation at Mississippi Academy of Sciences, 84th Annual Meeting, Hattiesburg, University of Southern Mississippi on February 21, 2020.

22. Cook D***, Salahuddin MF,** Mahdi F, Qrareya AN, Vujanovic T, Foster M. HIV-1 Tat Potentiates Morphine's Effects and the Steroid, 3a-Diol, is Protective. Oral presentation for STEMS REU program, MS, USA on August 23, 2019.

23. **Salahuddin MF*,** Mahdi F, Liere P, Kim S, Buchanan ME , Qrareya AN, Pianos A , Fernandez N, Shariat-Madar Z , Knapp PE , Schumacher M, Hauser KF, Paris JJ,. The neurosteroid, allopregnanolone, may ameliorate HIV-1 Tat associated mitotoxicity. Poster presentation at Jackson Avenue Center, Mississippi on April 26, 2019

https://egrove.olemiss.edu/researchday/2019/posters/33/

24. **Salahuddin MF*,** Mahdi F, Liere P, Kim S, Buchanan ME, Qrareya AN, Pianos A , Fernandez N, Shariat-Madar Z , Knapp PE , Schumacher M, Hauser KF, Paris JJ,. The neurosteroid, allopregnanolone, may ameliorate HIV-1 Tat associated mitotoxicity. Poster presentation at Mississippi Academy of Sciences, 83rd Annual Meeting, Hattiesburg, University of Southern Mississippi on February 21, 2019

ONLINE RELEASES

- Pharmacology graduate student striving for excellence. https://news.olemiss.edu/pharmacology-graduate-student-striving-for-excellence/
- Hyderabadi Student in US identifies underlying mechanism of HIV's neurological symptoms. *Siasat** https://www.siasat.com/hyderabadi-student-in-us-identifies-underlying-mechanism-of-hivs-neurological-symptoms-2176627/

- Neurosteroidogenesis as therapeutic strategy to combat Hormonal Dysregulation in HIV-1 patients (2021). https://brainstorm267.wordpress.com/2021/08/19/neurosteroidogenesis-as-therapeutic-strategy-to-combat-hormonal-dysregulation-in-hiv-1-patients/

- HIV-1 Tat Protein and Oxycodone Dysregulate Hypothalamic Pituitary Adrenal axis and Promote Affective and Cognitive Dysfunction in Mice (2020) https://brainstorm267.wordpress.com/2020/01/06/hiv-1-tat-protein-and-oxycodone-dysregulate-hypothalamic-pituitary-adrenal-axis-and-promote-affective-and-cognitive-dysfunction-in-mice/

- "Cathinone Associated Memory Deficit and Neurodegeneration" (2019): https://brainstorm267.wordpress.com/2019/04/12/cathinone-associated-memory-deficit-and-neurodegeneration/

- "Opioids abuse and Efavirenz in the prognosis of neuroAIDS: The overlooked facet" (2018): https://brainstorm267.wordpress.com/2018/09/20/opioids-abuse-and-efavirenz-in-the-prognosis-of-neuroaids-the-overlooked-facet/

SERVICE TO PROFESSIONAL PUBLICATIONS

Associate Editor: BMC Psychiatry, Journal of Concurrent Disorders, All Life Pharmacology & Pharmaceutics Journal.

Ad hoc journal reviewer: Nature and Science of Sleep, Sleep and Vigilance, Frontiers in Psychiatry, Frontiers in Psychology, Behavioral Brain Research, SAGE Open, Therapeutic Innovation and Regulatory Science, Current HIV Research, Journal of Multidisciplinary Healthcare, Clinical Epidemiology, Substance Abuse Research and Treatment. HIV/AIDS- Research & Palliative Care.

TEACHING EXPERIENCE

UNDERGRADUATE COURSES -

Graduate Teaching Assistant, University of Mississippi, United States

Semester	Course Number	Course Name	Students Enrolled
Spring 2019	Phcl 344	Physiological foundation of Therapeutics	120
Spring 2020	Phcy 412	Human Physiology/ Pathophysiology II	118

Instructional Professor of Pharmacology, Mizan Tepi University, Dept. Pharmacy, Ethiopia

Semester	Course Number	Course Name	Students Enrolled
Fall 2017	Phar 3102	Pharmacology II	120

Fall 2016	Phar 422	Applied Toxicology	66
Fall 2015	Phar 2054	Pharmacology for Public Health	75
Fall 2015	Phar 2071	Pharmacology for Midwives	149
Fall 2014	Phar 2071	Pharmacology for Nurses	69
Fall 2013	Phar 3102	Pharmacology I	38
Spring 2013	Phar 421	Complementary & Alternative Medicine	38
Fall 2012	Phar 2101	Pharmacology II	40

RESEARCH MENTORSHIP

UNDERGRADUATE: Mizan Tepi University, Clinical Pharmacology

Mentor to 9 undergraduate honors theses

2017 Gatluak Dong, Retrospective study on adherence to anti-Retroviral Therapy among HIV positive pregnant women at Gambella General Hospital, Gambella Region, West Ethiopia.

2017 Mihiretu Ashuro, Assessment of HIV-Associated Dementia/Cognitive Impairment in HIV-Infected Adults in Mizan Tepi University Teaching Hospital.

2016 Biruk Tafesse, A study of Ruta chalepensis for its phytochemical and antibacterial activity.

2016 Hamid Sinba, Self-management practices among adult type 2 diabetic patients.

2016 Argeta Dufera, Assessment of knowledge, attitude and practice toward social drug use among productive people in berber-gallessa town, Metekle zone, benishangul gumuz region, western Ethiopia

2015 Mohammed Yimam, Assessment of Diabetic Mellitus treatment outcomes in Mizan-Tepi University Teaching Hospital

2015 Dawit Hailegorgis, Assessment of Drug Prescription Pattern Among pregnant women attending antenatal outpatient department of MTUTH, South West Ethiopia.

2015 Haileyesus Kifle, Assessment of the perception of the community towards the use of modern medicine in Mizan Teferi, Ethiopia.

2015 Nibu Mogos, Assessment of potential drug-drug interactions in inpatients treated in medical ward of MAGH of SNNP in South-West Ethiopia