EFFECT OF HIGHLY ACTIVE ANTIRETROVIRAL THERAPY (HAART) ON HIV-1 TAT PROTEIN-INDUCED NEUROTOXICITY

By

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Declaration

I, Simo Siyanda Zulu, student number: 207506590 hereby declare that the dissertation/thesis entitled:

EFFECT OF HIGHLY ACTIVE ANTIRETROVIRAL THERAPY (HAART) ON HIV-1 TAT PROTEIN-INDUCED NEUROTOXICITY

Is the result of my own investigation and research and that it has not been submitted in part or full for any other degree or to any other University or Tertiary Institution. The use of work by other was duly acknowledged in text.

The research conducted in this study was carried out under the supervision of Prof. W.M.U. Daniels and Dr. M.V. Mabandla.

S.S. Zulu
W.M.U. Daniels
M.V. Mabandla

Date 01/04/2015
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May the Almighty God richly bless all of you.
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<td>µg</td>
<td>microgram</td>
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<td>µl</td>
<td>Microliter</td>
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<td>4-HNE</td>
<td>4-Hydroxynonenal</td>
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<td>ADP</td>
<td>Adenosine Diphosphate</td>
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<td>AIDS</td>
<td>Acquired Immunodeficiency Syndrome</td>
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<td>AP</td>
<td>Anterior-Posterior</td>
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<td>ARV</td>
<td>Antiretroviral</td>
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<td>ART</td>
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<td>CD 4</td>
<td>Cluster of Differentiation 4</td>
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<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
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<td>DV</td>
<td>Dorsal-Ventral</td>
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<td>ELISA</td>
<td>Enzyme Linked Immunosorbent Assay</td>
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<td>Gram</td>
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<td>HAART</td>
<td>Highly Active Antiretroviral Therapy</td>
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<td>HAND</td>
<td>HIV-Associated Neurocognitive Disorder</td>
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<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
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<td>kg</td>
<td>Kilogram</td>
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<td>MAPK</td>
<td>Mitogen-Activated Protein Kinases</td>
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<td>mg</td>
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<td>mm</td>
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<td>NF-Κβ</td>
<td>Nuclear Factor kappa-beta</td>
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<tr>
<td>°C</td>
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<td>Abbreviation</td>
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<td>pERK</td>
<td>Phosphorylated Extracellular Signal-Regulated Kinase</td>
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<td>ROS</td>
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<td>Tat</td>
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<td>TNF-α</td>
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Abstract

Background: HIV-1-trans-activating (Tat) protein has been associated with development of HIV-associated neurocognitive disorder (HAND). Previous studies have demonstrated that Tat protein causes neurotoxicity through an increase in reactive oxygen species (ROS) leading to damage of proteins and other cellular components. Tat has also been shown to cause excessive production of pro-inflammatory cytokines. However, the role of antiretroviral agents in the neuropathology of HIV is not known. The objective of this study was to investigate whether a combination of antiretroviral drugs (Zidovudine, Lamivudine and Efavirenz, a highly active antiretroviral therapy, HAART) is effective in reducing the toxic effects of Tat protein in the rat hippocampus.

Materials and methods: Male Sprague-Dawley rats were divided into four groups (n=10 per group). Each rat received bilateral intrahippocampal injection of either Tat protein (5µg/10µL) or vehicle, followed 7 days later by a combination of antiretroviral drugs (Zidovudine 12mg/kg, Lamivudine 6mg/kg and Efavirenz 24mg/kg) or saline injected intraperitoneally, twice a day, for 7 days. After treatment, animals were sacrificed and hippocampal tissue was collected for analysis of cleaved caspase-3, 4-hydroxynonenal (NHE), tumor necrosis factor alpha (TNF-α), phosphorylated extracellular signal-regulated kinase (pERK) and Synaptophysin.

Results: Tat increased cleaved caspase-3 levels in the hippocampus. Antiretroviral treatment decreased the Tat-induced increase in cleaved caspase-3. Tat increased HNE, a marker of lipid peroxidation and reduced hippocampal synaptophysin. The latter Tat-induced effects were not reversed by antiretroviral treatment. The antiretroviral drug combination activated the pERK pathway and increased TNF-α levels in hippocampal tissue, independent of Tat infusion.

Discussion: Our findings showed that antiretroviral drugs reversed Tat-induced cleaved caspase-3, reducing apoptosis but did not reverse Tat-induced increase in lipid peroxidation and the synaptic marker, synaptophysin. The evidence suggests that the combination of antiretroviral drugs may be toxic, elevating
hippocampal pERK and TNF-α levels. However, these effects could also be beneficial to the individual, since TNF-α has been shown to inhibit viral replication. The present results provide novel insight into the mechanism of antiretroviral action.

*Key words*: HIV-1-trans-activating protein, Tat, HAART, ERK, TNF-α, Synaptophysin, HAND
Chapter 1

1. Literature Review

1.1 Introduction

Neurological complications have become a major concern in people living with HIV and AIDS (Joska et al., 2011). These complications may range from deficits in motor function (loss of balance, motor activity slowness) to difficulties in learning, and memory loss (Nakku et al., 2013). These motor and cognitive impairments (mental slowing, attention/memory deficits) are collectively referred to as HIV-associated neurocognitive disorder (HAND) (Brew and Perdices, 1992). HAND has been classified into three forms i.e. HIV-associated asymptomatic neurocognitive impairments (ANI), HIV-associated mild neurocognitive disorder (MND) and HIV-associated dementia, also known as AIDS dementia complex (Brew and Perdices, 1992, Manji et al., 2013).

HIV enters the brain soon after infection and remains in the brain throughout the course of the disease regardless of immune response (An et al., 1999). HIV entry into the central nervous system (CNS) may occur in two ways, (i) direct uptake through the blood-brain-barrier (BBB) and (ii) through infected immune cells (macrophages and T-lymphocytes) which are considered to be the primary vehicles for HIV entry into the CNS. The presence of HIV in the CNS can subsequently lead to recruitment of additional immune cells which results in further disruption of the BBB, increased endothelial permeability and vascular leakage (Pu et al., 2003).
Infected monocytes and lymphocytes have the ability to release cellular and viral toxins into the extracellular space within the CNS (Dou et al., 2004). These toxins travel through anatomical pathways to subcortical areas such as the hippocampus and basal ganglia. Damage to these areas has been proposed to be responsible for the decline in cognitive function as seen in HAND (McArthur et al., 2010). Viral proteins such as glycoprotein 120 (gp120) and transactivator of transcription (Tat) protein, have been shown to promote the secretion of proinflammatory cytokines/chemokines that may cause neuropathogenesis (Lee et al., 2011). It has further been shown in mice that Tat itself may lead to neuronal damage which results in abnormalities in learning and memory (Carey et al., 2012).

**Figure1:** showing the mechanism of HIV entry into the central nervous system (Ivey et al., 2009).
1.2 HIV-TAT PROTEIN

1.2.1 HIV Tat protein (Tat) - structure and function

HIV Tat protein (Tat) is a transactivator protein that contributes to transactivation of viral and cellular genes (Ensoli et al., 1993, Song et al., 2007). Tat protein binds to the Trans-activation response element (TAR) of long terminal repeats and promotes expression of viral genes (Dingwall et al., 1990). It is a small protein of 86-104 amino acids encoded by two exons and its size varies between 14-16 kDa (Ruben et al., 1989).

Figure 2: Showing site which HIV Tat protein act to activate transcription (Zhou and Yik, 2006)

Tat is the first protein synthesized during HIV replication. When proviral DNA gets incorporated into the host cell genome, Tat synthesis is initiated (Romani et al., 2010). Once Tat is produced, it is often found within the nucleus and nucleolus (Ponti et al., 2008). It therefore has the potential to bind to different TAR in RNA (Dingwall et al., 1990). Tat drives viral replication through transactivation of the promoter region called long terminal repeat (LTR) of the virus (Li et al., 2009). The first exon of Tat is involved in transcription activation and the second exon mediates Tat binding to cellular surface intergrins (Shojania et al., 2010). Tat attracts host
positive transcription elongation factor b (p-TEFb) to the RNA hairpin formed at the 5’-end of the viral RNAs (TAR) (Feng and Holland, 1988). P-TEFb is a complex formed by two subunits called cdk9 and cyclin T1. The subunits control the RNA polymerase during transcription (Price, 2000). Tat recruits p-TEFb which then phosphorylate the C-terminal domain (CTD) repeats of RNA polymerase II (Jones and Peterlin, 1994).

It is known that HIV-1 Tat induces several inflammatory cytokines. However in vitro studies showed that recombinant HIV-1B Tat induces monocytes to secrete proinflammatory cytokines greater than HIV-1C Tat (Gandhi et al., 2009, Wong et al., 2010). A study performed on human primary neurons showed that HIV-1C Tat is relatively less neurotoxic compared to HIV-1B Tat. This was linked to variations in the dicysteine motif of both clades (Mishra et al., 2008).

Both in vivo and in vitro studies have demonstrated Tat to have neurotoxic effects (Agrawal et al., 2012, Capone et al., 2013). The actual half-life of Tat protein in the brain and peripheral circulation has not been well studied. Since factors such as proteases and those that regulate protein up-take control the half-life. However a study by Desfosses et al. (2005) has shown that the half-life of Tat was approximately 3 hours in HEK 293 cells. Notwithstanding uncertainty as to the half-life of tat, a study by Bansal et al. (2000) showed that 5µg/µl of Tat injected into the striatum caused a significant tissue loss and an increase in GFAP expression in astrocytes at the site of injection 7 days after injection. The deleterious effects of Tat were therefore evident 1 week after administration and hence suggested that tat may elicit long term damaging consequences such as the development of HAND (Carey et al., 2012).
1.2.2 Mechanisms of Tat-induced neuronal damage

HIV-infected cells release Tat into the extracellular fluid of the brain. From here Tat is able to enter non-infected target cells and induce its toxic effects intracellularly (Romani et al., 2010). After Tat is internalized it causes neurodegeneration through various ways that include oxidative stress and excitotoxic processes brought about by activation of glutamate receptors.

Tat has been shown to activate NADPH oxidase in microglial cells causing high glutamate release via the \( x_c \) cysteine-glutamate antiporter (Gupta et al., 2010). To protect itself from intracellular ROS accumulation, microglia cells take up cysteine for glutathione synthesis in exchange for glutamate (Piani and Fontana, 1994). Glutamate that is released into the extracellular space/synaptic cleft is then cleared by astrocytes through excitatory amino acid transporters (Bergles and Jahr, 1997). However excess glutamate in the synaptic cleft, can activate NMDA and non-NMDA receptors resulting in the opening of channels and subsequent influx of ions such as sodium and calcium into the cytoplasm (Bonavia et al., 2001). High

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**Figure 3:** Schematic illustration of astrocyte activation and excitotoxicity induce by HIV Tat protein (Ton and Xiong, 2013).
intracellular sodium levels often lead to necrotic death due to the hypertonic swelling of cells (Churchwell et al., 1996), while increased calcium influx may trigger the release of calcium ion from intracellular stores (e.g. endoplasm reticulum) to further flood the intracellular space with free calcium ions (Schinder et al., 1996). In response to protect the cells, mitochondria will take up large amounts of calcium through its electrochemical gradient, but this may lead to the disruption of the electrical potential across the mitochondria membrane (Schinder et al., 1996). Accumulation of calcium ions in the mitochondria may also cause osmotic swelling leading to the rapture of the outer membrane resulting in the release of the enzyme cytochrome c (Brustovetsky et al., 2002). Cytochrome c, proapoptosis 9 and apoptosis activating factor 9 (Apaf1) form a multi-protein complex apoptosome that activates caspase 9 (Garden et al., 2002). Activated caspase 9 cleaves procaspase 3 to produce cleaved caspase 3, one of the apoptosis executioner enzymes (Tenneti et al., 1998, Garden et al., 2002). Tat therefore may either cause necrotic or apoptotic cell death.

Neuro-inflammatory cells like microglia/macrophages, when activated, release chemokines/cytokines and reactive oxygen species (Gupta et al., 2010) and accumulation of these agents may lead to neuronal dysfunction (Kim et al., 2008, Capone et al., 2013). Cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-1β released by infected macrophages/microglia can stimulate astrocytes to release of ROS (Buscemi et al., 2007, Sheng et al., 2013). Tat can also directly activate nitric oxide synthase (NOS) (Liu et al., 2002) to produce nitric oxide (NO), which is a nitrogen free radical (Stewart et al., 2000). In addition superoxide is released by HIV-infected macrophages and astrocytes (Sheng et al., 2013). The neurotoxicity of NO and superoxide is enhanced when they react with each other to form peroxynitrite, a potent oxidant that by damaging neurofilaments disrupts the structural stability
of the cell, leading to cellular dysfunction (Stewart et al., 2000). (Aksenov et al., 2001) demonstrated that a single injection of 5-50 µg/µl of recombinant Tat (1-72 amino acids) into the striatum of Sprague–Dawley male rats, caused tissue loss surrounding injection in a dose dependent manner. On the other hand it has been shown that HIV-1 clade B Tat induced increasing expression of CD11b, a phagocytosis receptor molecule in microglia that was associated with an increase in lipid peroxidation. Recently HIV-1 clade B Tat was reported to cause increase of spermine oxidase, an enzyme involved in the formation of hydrogen peroxidase, glutathione disulfide and a decline in glutathione (Capone et al., 2013).

In summary it appears that HIV-associated cellular damage may be caused by viral proteins such as Tat through mechanisms that may involve heightened glutamate release, influx of cations, the generation of ROS and the activation of necrotic and apoptotic pathways.

1.2.3 Neuro-inflammation and HAND

Soon after infection HIV-1 gain access to the central nervous system by passing through the blood-brain-barrier (BBB) via infected monocyte and lymphocytes (Gras and Kaul, 2010). Since infected monocyte/macrophages and lymphocyte actively support HIV-1 replication, these cells can release virions into the extracellular space (Fanales-Belasio et al., 2010). In the brain HIV-1 then infects neuroglia cells such as microglia and astrocytes. The entry of HIV-1 in microglia is mediated by CD4 receptors, whereas in astrocytes HIV-1 may enter via the chemokine co-receptor CCR5 (Liu et al., 2004). Microglial cells also support HIV-1 replication, further spreading the infection within the brain (Bagasra et al., 1996). Although astrocytes can get infected, they do not support HIV-1 replication (Gray et al., 2014). Astrocytes block HIV-1 replication by inhibiting the expression of HIV-1 regulatory proteins, Nef, Rev and Tat (Sabri et
Infected microglia and astrocytes are activated to release pro-inflammatory cytokines such as TNF-α and Interleukin-1β (Walsh et al., 2014), glutamate (Bezzi et al., 2001, Huang et al., 2011).

The pro-inflammatory cytokines such as TNF-α and interleukin-1β have been proposed to contribute to the death of neurons observed in HAND (Kaul et al., 2001). It has been demonstrated that TNF-α can disrupt the integrity of the BBB and increase its permeability (Fiala et al., 1997) by activating the expression of transmigration molecules such as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) on the cell surface of endothelial cells of the BBB (Brabers and Nottet, 2006), therefore TNF-α plays an important role in HIV-1 infection of the brain. In addition to BBB disruption and increased ICAM-1 and VCAM-1 expression, TNF-α also up-regulates the production and release of monocyte chemoattractant protein-1 (MCP-1) (Hurwitz et al., 1995). This chemokine serves to attract monocytes and macrophages towards the site of infection.

Studies have now showed that TNF-α causes excitotoxicity by influencing the glutamatergic system in the brain. TNF-α mediates neurotoxicity by over stimulating glutamate receptors such as NMDA receptors, leading to excessive calcium influx, dysregulation of mitochondrial function, generation of reactive oxygen species, and eventual neuron damage (Bezzi et al., 2001). A second mechanism by which TNF-α affects the glutamatergic system, is by inhibiting the uptake of this neurotransmitter by astrocytes, and thereby enhancing the synaptic concentration of glutamate resulting in excitotoxicity (Fine et al., 1996). Thirdly TNF-α, together with interleukin-1β co-operate to induce cytokine-inducible nitric oxide synthase (iNOS) (Jana et al., 2005). Activation of this enzyme produces nitric oxide leading to the development of oxidative stress and subsequent cell death (Chao et al., 1996).
1.3 ANTIRETROVIRAL THERAPY

1.3.1 Antiretroviral drugs mechanism

Since HIV mainly attacks the cells of the immune system, the primary function of antiretroviral (ARV) therapy has been to decrease the viral load. In general the approach is to inhibit the replication of the virus and in doing so, functioning of the immune system can be restored. In practice patients with HIV/AIDS are routinely treated with a combination of antiretroviral drugs that includes entry inhibitors (EI), nucleoside analogue reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), integrase inhibitors and protease inhibitor (PIs). The combinational approach is considered most effective for the treatment of HIV/AIDS since it is able to inhibit viral replication at various stages of the HIV life cycle (Thein et al., 2014). This approach is commonly referred to as highly active antiretroviral therapy (HAART).

Entry inhibitors are antiretroviral agents that prevent attachment of HIV to the target cell surface. Entry inhibition occurs in two ways, first through agents that binds to the gp41 envelope of the HIV membrane preventing fusion of the HIV membrane with the cellular membrane (Margolis et al., 2013). The second mechanism involves chemokine co-receptor 5 (CCR5) antagonists. CCR5 is a receptor found on most immune cells such as macrophages and T-cells. Binding of HIV to CCR5 has been shown to be important for HIV entry into the cell (Davies and Taylor, 2013).

Non-nucleoside and nucleoside/nucleotide reverse transcriptase inhibitors suppress HIV replication through blocking the production of double stranded DNA from virus RNA (Arts and Hazuda, 2012). NRTIs (Zidovudine and Lamivudine) terminate the addition of nucleotides to the DNA strand (Jochmans, 2008). NRTIs are deoxyribonucleosides that lack the 3’-OH group. They therefore block the addition of deoxyribose sugars into the newly formed DNA strand,
resulting in termination of viral DNA synthesis (Isel et al., 2001). Antiviral activity of NRTIs is dependent on cell metabolism. Host cell kinase enzymes phosphorylate NRTIs to NRTI-triphosphates and it is in this form that they bind to the newly synthesized DNA strand to prevent subsequent elongation (Balzarini et al., 2002).

NNRTIs (Efavirenz) are structurally different from NRTIs and do not require phosphorylation to perform their antiviral activity (Balzarini et al., 1998). Binding of NNRTIs to reverse transcriptase causes a conformational change in the substrate binding site, while simultaneously reducing the activity of the DNA polymerase enzyme (Arts and Hazuda, 2012). These two effects facilitate the inhibition of viral DNA production. HIV integrase is an essential enzyme for viral replication following reverse transcription of viral RNA into double-stranded DNA. HIV integrase processes the insertion of HIV proviral DNA into the cellular genome of the host (Summa et al., 2008). Integrase inhibitors therefore prevent the transfer of viral DNA strands into the nucleus and block the incorporation of the viral DNA strands into the host cellular genome (Mazumder et al., 1997, Davies and Taylor, 2013).

Protease inhibitors bind to the active site of the HIV protease enzyme preventing the action of proteases. Protease activity is important for the cleaving of viral proteins during virion maturation (Margolis et al., 2013).
1.3.2 Adverse effects of antiretroviral therapy (ART)

Since antiretroviral therapy to people living with HIV/AIDS is a life-long treatment, many studies have been conducted to evaluate effects of these drugs on the normal structure and function of various organs of the body. These investigations have been proven to be important as they indicated possible reasons for the poor treatment adherence of many patients to ART (Monforte et al., 2000). Initially systemic disorders such as diabetes and lipid dystrophy have been noted. (Rosenkranz et al., 2007). It shown that chronic treatment of murine adipocytes with some PIs and NRTIs decrease adipocyte lipid metabolism and increase secretion of proinflammatory cytokines (Lagathu et al., 2004, Vernochet et al., 2005). However in addition to these metabolic disorders, liver damage has also been reported as a common adverse effect (Rosenkranz et al., 2007). Subsequently it has been shown that the kidneys are also negatively
affected by ART with both tubulopathy (Labarga et al., 2009) and nephropathy (Kalyesubula and Perazella, 2011) been observed in patients with HIV/AIDS that are on ART.

These adverse effects have mainly been seen in patients on NNRTIs, NRTIs and PIs (Monforte et al., 2000, Rosenkranz et al., 2007) and have been suggested to result from mitochondrial impairment (Dalakas, 2001, Gerschenson and Brinkman, 2004). Evidence associating NRTIs and NNRTIs with mitochondrial malfunction has been convincing with a possible mechanism of action been proposed. ARVs selectively inhibit mitochondrial DNA polymerase gamma leading to a depletion of mitochondrial DNA (Lim and Copeland, 2001). This depletion of mitochondrial DNA results in reduction of mitochondrial enzymes necessary for oxidative phosphorylation (Gegg et al., 2009). Disruption of oxidative phosphorylation may lead to the leakage of electrons (Beyer, 1992), promoting the generation of ROS. The subsequent formation of ROS then cause oxidative damage resulting in a decrease in ATP production. (Gegg et al., 2009). Studies conducted in human primate and rodents have shown that conditions such as lip dystrophy and insulin resistance may results after prolong exposure to NRTIs and NNRTIs (Waters and Nelson, 2007). Although the toxicity of ARVs has been studied in several organs, the toxic effect of ARVs on the brain and their role in HAND remains poorly understood.

The standard therapeutics used for HIV management consists of two NRTIs and NNRI or PI (Hammer et al., 2008). Since these drugs are taken over long periods of time, interest has been raised to its effects on the central nervous system (CNS) (Husstedt et al., 2002), as such HIV-infected patients on ART showed a complete suppression of HIV replication, but simultaneously exhibited characteristics of cognitive malfunction (Giunta et al., 2011). Drug-related CNS toxicity has subsequently become a cause of concern for many researchers especially in conditions such as HIV/AIDS where viral-induced brain abnormalities are also expected (Carr
and Cooper, 2000). This concern has been highlighted by recent reports showing ARTs affecting brain function (Liner et al., 2010) and observations of patients who discontinue ART to display an improvement in cognitive function (Robertson et al., 2010). However reports such as these are not only limited at present, they also do not reveal a mechanism of action as to how ART mediates its effects on the CNS. Interestingly some clinicians prefer the use of drugs with a high BBB penetration rate in order to prevent the brain from becoming a HIV reservoir (Marra et al., 2009, Tozzi et al., 2009). It is therefore obvious that the impact of ART on the brain is controversial and more studies are required to improve our current knowledge in this area of HIV/AIDS research.

1.3.3 Antiretroviral drugs and blood-brain-barrier permeability

The blood-brain-barrier (BBB) is a selective permeable barrier that separates the circulating blood from the extracellular fluid in the central nervous systems. BBB is found along all central nervous system capillaries and consists of endothelial cells which are connected by tight junctions (Abbott et al., 2010). The access of the brain by various molecules is dependent on their ability to penetrate the BBB. The penetration of drugs including antiretroviral drugs across BBB is linked to several factors. These include physical and chemical characteristics of both drugs and the BBB such as the molecular weight of the drug, whether the drug is bound to plasma proteins, the degree of ionization and lipid solubility of molecules and the presence of molecular pumps within the BBB (Mangas-Sanjuan et al., 2010).

The link between antiretroviral drugs, BBB penetration and HAND has been reported by many authors (Caniglia et al., 2014) has recently reported that initiation of a combined antiretroviral therapy regimen with a high CNS Penetration Effectiveness score increases the risk of HIV
dementia. A study conducted in South Africa showed that there was no significant difference in cognitive outcomes between higher and lower CPE regimen groups (Cross et al., 2013). In addition to drug regimens comparing, researchers have investigated the CPE of individual drugs from all classes of antiretroviral drugs.

In the NRTIs class, Zidovudine has been shown to possess a high penetration rate followed by Stavudine, Didanosine and Lamivudine (Strazielle and Ghersi-Egea, 2005). The high CPE is associated with its lipid solubility and less plasma protein binding (Burger et al., 1993). Nevirapine has a good CPE among NNRTIs followed by Efavirenz (Antinori et al., 2005). Poorer CPE observed with Efavirenz is associated with it molecular weight and also high plasma protein binding affinity (Ene et al., 2011). Most PIs are substrates of P-glycoprotein-1, a cell membrane protein that pumps many foreign substances out of cells (Kim et al., 1998, Choo et al., 2000). Plasma protein binding has been shown to be high among PIs. These are linked to PIs having a poor CPE and their low cerebrospinal fluid concentration (Kageyama et al., 1994).

1.4 BRAIN AREAS

1.4.1 Hippocampus and the HIV

Clinical studies have shown that the limbic system is important for normal cognitive function (Shaw and Alvord, 1997) Brain regions that make up the limbic system include thalamus, hypothalamus, frontal lobe, olfactory bulb, amygdala and hippocampus.
Of these the role of the hippocampus in learning and memory has extensively been studied (Alvarez et al., 1995, Assunção et al., 2007).

Implanting Tat-producing cells into the striatum or dentate gyrus of rats, Bruce-Keller and colleagues (2003) could demonstrate how this protein is transported through anatomical pathways to various areas in the brain where it caused neurotoxic damage. It is therefore conceivable that HIV may enter the brain, where it produces and releases tat that is able to locate to brain regions involved in cognition. Support for this notion comes from studies where difficulties in learning, memory and poor attention had been observed in patients with HIV (Nakku et al., 2013). In addition a study conducted by Fitting et al. (2008) showed rats pups that received intracerebral injection of Tat into the hippocampus performed poor in learning and memory tests.
1.5 Aims of the study

In view of the uncertainties in the current literature, the following aims were formulated for the present study:

Aim 1: - to confirm the neurotoxic properties of Tat,

Aim 2: - to investigate whether ART may be toxic, and

Aim 3: - to investigate whether ART may block the neurotoxic effects of Tat

1.6 Hypothesis:

We hypothesized that antiretroviral therapy (ART) will not display any toxic effects and that the administration of ART will protect the brain against Tat-induced neurotoxicity.

1.7 Objectives:

The objectives of our study were:

1. To inject Tat into the hippocampus of rats and assess the effects thereof on specific molecular markers of oxidative stress and apoptosis,

2. To determine in which way ART may affect these molecular markers, and

3. To assess whether ART may reverse or exacerbate the effects of Tat on these molecular markers.
EFFECT OF HIGHLY ACTIVE ANTIRETROVIRAL THERAPY (HAART) ON HIV-1 TAT PROTEIN-INDUCED NEUROTOXICITY

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Abstract

Background: HIV-1-trans-activating (Tat) protein has been associated with development of HIV-associated neurocognitive disorder (HAND). Previous studies have demonstrated that Tat protein causes neurotoxicity through an increase in reactive oxygen species (ROS) leading to damage of proteins and other cellular components. Tat has also been shown to cause excessive production of pro-inflammatory cytokines. However the role of antiretroviral agents in the neuropathology of HIV is not known. The objective of this study was to investigate whether a combination of antiretroviral drugs (Zidovudine, Lamivudine and Efavirenz, a highly active antiretroviral therapy, HAART) is effective in reducing the toxic effects of Tat protein in the rat hippocampus.

Materials and methods: Male Sprague-Dawley rats were divided into four groups (n=10 per group). Each rat received bilateral intrahippocampal injection of either Tat protein (5µg/10µL) or vehicle, followed 7 days later by a combination of antiretroviral drugs (Zidovudine 12mg/kg,
Lamivudine 6mg/kg and Efavirenz 24mg/kg) or saline injected intraperitoneally, twice a day, for 7 days. After treatment, animals were sacrificed and hippocampal tissue was collected for analysis of cleaved caspase-3, 4-hydroxynonenal (NHE), tumor necrosis factor alpha (TNF-α), phosphorylated extracellular signal-regulated kinase (pERK) and Synaptophysin.

**Results:** Tat increased cleaved caspase-3 levels in the hippocampus. Antiretroviral treatment decreased the Tat-induced increase in cleaved caspase-3. Tat increased HNE, a marker of lipid peroxidation and reduced hippocampal synaptophysin. The latter Tat-induced effects were not reversed by antiretroviral treatment. The antiretroviral drug combination activated the pERK pathway and increased TNF-α levels in hippocampal tissue, independent of Tat infusion.

**Discussion:** Our findings showed that antiretroviral drugs reversed Tat-induced formation of cleaved caspase-3, reducing apoptosis but did not reverse Tat-induced increase in lipid peroxidation and the synaptic marker, synaptophysin. The evidence suggests that the combination of antiretroviral drugs may be toxic, elevating hippocampal pERK and TNF-α levels. However, these effects could also be beneficial to the individual, since TNF-α has been shown to inhibit viral replication. The present results provide novel insight into the mechanism of antiretroviral action.

**Key words:** HIV-1-trans-activating protein, Tat, HAART, ERK, TNF-α, Synaptophysin, HAND
Introduction

Despite significant advances, the current number of people living with HIV has been estimated to be a staggering 35.3 million (UNAIDS report, 2013). Development of neurocognitive disorders in many HIV-infected individuals (Nakku et al., 2013) has added another dimension to the complexity of HIV/AIDS, and notwithstanding extensive research, many questions regarding its pathophysiology and treatment remain unanswered.

HIV-associated neurocognitive disorder (HAND) is a neurodegenerative disorder that is characterized by cognitive deficit and behavioral dysfunction. Brain autopsies performed on people who were HIV positive, showed that 90% of these individuals had severe neuron damage (Joska et al., 2011b). The HIV-1-trans-activating protein (Tat) has been strongly linked to the neuropathology observed in HAND patients (Bagashev and Sawaya, 2013). Tat is synthesized early after HIV infection and is responsible for viral replication (Sierra et al., 2005). HIV rarely infects neuronal cells, but other brain cells such as macrophages and microglia, are infected by the virus (Wiley et al., 1986). These infected macrophages and microglia produce and shed Tat into the intercellular space from where it can exert its toxic effects (Williams et al., 2012).

Tat has been shown to be transported along anatomical pathways to reach subcortical areas such as the hippocampus, basal ganglia and striatum - brain regions that are important in cognitive function (McArthur et al., 2010). Tat employs a number of strategies to induce neuron damage. These include the activation of glutamate receptors (Dou et al., 2004), calcium overload (Agrawal et al., 2012), oxidative stress (Potter et al., 2013), apoptosis (Capone et al., 2013; Ramataur et al., 2012) and the release of pro-inflammatory cytokines (Williams et al., 2012).
The introduction of highly active antiretroviral therapy (HAART) which is aimed at targeting HIV at various stages of its life cycle, has effectively suppressed viral replication and partially restored immune function in humans (Ellis, 2010). HAART has also reduced the prevalence of HAND from 50% to 20% (Manji et al., 2013). However, people living with HIV/AIDS are exposed to antiretroviral agents (ARVs) for long periods of time. The effects of long-term exposure to HAART have therefore been a major concern for clinicians and scientists worldwide. There is some evidence to suggest the drugs used in HAART cause metabolic disorder, lipodystrophy and diabetes (Leow et al., 2003, Waters and Nelson, 2007). Similarly nucleoside reverse transcriptase inhibitors and non-nucleoside reverse transcriptase inhibitors have been suggested to induce mitochondrial dysfunction through the inhibition of DNA polymerase-gamma, resulting in increased production of ROS, causing oxidative damage and eventual organ malfunction (Lim and Copeland, 2001, Opio et al., 2007). With respect to brain function, there is controversy as to whether HAART helps to restore cognitive function in people living with HIV/AIDS (Cohen et al., 2001, Obiabo et al., 2012) or if antiretroviral agents in fact worsen cognitive decline (Giunta et al., 2011, Tovar-y-Romo et al., 2012). In support of the latter, a study by Robertson et al. (2010) showed that patients suffering from HAND who discontinue HAART, tend to regain their neuropsychological ability. Since the role of HAART in HIV-related neuropathogenesis appears to be unclear, our study subsequently investigated the impact of a currently used regime of ARVs on various molecular markers of cellular stress and/or damage in saline and Tat-treated animals.
**Materials and methods**

**Animals**

Male Sprague-Dawley rats (250 g - 300 g) were obtained from the Biomedical Research Center at the University of KwaZulu-Natal. Animals were housed under standard laboratory conditions of a 12-hour light-dark cycle (lights on at 06:00), 25 °C room temperature, humidity of 70%, with free access to food and water. The University of KwaZulu-Natal Ethics Committee approved all experimental procedures and care of animals was in accordance with Institutional guidelines.

**Reagents**

Biologically active recombinant HIV-1 clade B Tat (1-86 amino acids) was purchased from Diatheva (Milan, Italy). Rabbit polyclonal phosphor-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) antibody and β-Actin Rabbit monoclonal antibody were purchased from Cell Signaling Technology (USA). Rabbit polyclonal cleaved-Caspase-3, Active antibody, Mouse monoclonal anti-synaptophysin antibody and Rat Tumor Necrosis Factor-α (TNF-α) ELISA Kit were purchased from Sigma (St Louis, USA). The OxiSelect HNE Adduct Competitive ELISA Kit was purchased from Bio Cell (USA) and antiretroviral tablets Zidovudine (1-[(2R,4S,5S)-4-Azido-5-(hydroxymethyl)oxolan-2-yl]-5-methylpyrimidine-2,4-dione)Lamivudine (2’,3’-dideoxy-3’-thiacytidine) and Efavirenz (4S)-6-chloro-4-(2-cyclopropylethynyl)-4-(trifluoromethyl)-2,4-dihydro-1H-3,1-benzoxazin-2-one) were purchased from Pharmed (South Africa).
Treatment of animals

HIV-Tat was reconstituted in phosphate-buffered saline at 5 µg/10 µl concentration just prior to administration. Zidovudine, Lamivudine and Efavirenz were dissolved under sterile conditions in physiological saline and served as our highly active anti-retroviral therapy (HAART). The final doses of the respective drugs were 12 mg/kg Zidovudine, 6 mg/kg Lamivudine and 24 mg/kg Efavirenz and were similar to that presently prescribed to people living with HIV/AIDS in South Africa.

Animals were divided into four groups (n=10 per group). Group 1 received bilateral intrahippocampal injections of saline (10 µl per injection) as well as an intraperitoneal injection of saline (1 ml/250 g). This group served as the control. Group 2 received bilateral intrahippocampal injections of Tat (5 µg per injection) dose was shown to cause neurodegeneration (Aksenov et al., 2001) and an intraperitoneal injection of saline. Group 3 received intrahippocampal injections of Tat and an intraperitoneal injection of HAART (3 mg/ml Zidovudine, 1.5 mg/ml Lamivudine, and 6 mg/ml Efavirenz). Group 4 received intrahippocampal injections of saline and an intraperitoneal injection of HAART.

Stereotaxic surgery

The animals were anaesthetized with sodium pentobarbital (60 mg/kg, intraperitoneally (i.p.)), then placed in a stereotaxic frame (David Kopf Instruments, Tujunga, USA). The skin covering the skull was removed to expose the calvarium. Bregma and lambda were used to level the horizontal plane of the skull. The stereotaxic rat atlas of Paxinos and Watson 5th Edition (2004) was used to obtain the coordinates to ensure that the injections were into the hippocampus. The following co-ordinates were adopted AP= -3.8 mm, ML= ± 2.6 mm and
DV= 3.2 mm. When the exact positions were located on the skull, small burr holes were drilled for the lowering of the injecting device into the brain. Tat protein (5 µg/10 µl) or saline (10 µl) were administered using a Hamilton syringe which had been pretreated with dimethyldichlorosilane to prevent the drug from adhering to the inner surface of the syringe. The injection rate was 1 µl/minute. The needle was left in place for a further 5 minutes to ensure optimal diffusion of the solution. Thereafter the needle was slowly retracted from the brain over a period of 1 minute. The hole in the skull was filled with sterile cellulose (Ethicon, UK) and the incision sutured, the wound disinfected with iodine solution and cellulose also used for the dressing of the wound (Purdue Frederic, USA). Animals were then placed on a heating blanket to prevent hypothermia. They were returned to their home cages after full recovery from surgery.

After the intrahippocampal injections, animals were given 7 days to recover from surgery and for Tat neurotoxicity to manifest prior to HAART treatment. Previous studies in our laboratory have shown that toxic effects of HV-1 Tat is present on day 7 and this include abnormalities in learning and cognitive function (Makhathini et al., 2013). This was followed by a further 7 days treatment with either HAART or Saline. These treatments were given twice a day (10:00 and 16:00) intraperitoneally. At the end of the treatment, the animals were decapitated and their brains dissected. The hippocampal tissues were collected and snap frozen in liquid nitrogen and stored at -80 °C until biochemical analyses were performed. The tissue samples of different animals were used for each assay.

**Enzyme linked immunosorbent assay (ELISA) of 4-Hydroxynonenal (HNE) and Tumor necrosis factor α (TNF-α).**

4-Hydroxynonenal (HNE) production was quantified using a competitive ELISA. Plate wells were first coated with HNE conjugate after which 50 µl of sample supernatant or HNE
standards were added to each well and incubated for 10 minutes at room temperature. After brief incubation, 50 µl of anti-HNE antibody was added and incubated at room temperature for 1 hour on an orbital shaker. Secondary antibody-HRP conjugated (100 µl) was added to each well followed by addition of substrate solution for a color change reaction.

TNF-α was quantified using a sandwich ELISA. Plate wells were pre-coated with rat TNF-α antibody. Sample supernatant or protein standard (100 µl) were added to each well and incubated for 2.5 hours at 4 °C with gentle shaking on an orbital shaker. Biotinylated anti-rat TNF-α antibody was added to each well. HRP-conjugated streptavidin was added to each well and incubated for 10 minutes followed by addition of (3, 3’, 5, 5’-tetramethylbenzidine) TMB substrate solution for color development and measurement of absorbance on a plate reader.

**Western blot analysis of caspase-3, synaptophysin and pERK1/2**

Hippocampal tissue was homogenized in 600 µl RIPA buffer (150 mM NaCl, 1000 mM Tris, 10% SDS, Triton X 100, 24 mM Sodium deoxycholate and 500 mM EDTA). Homogenates were centrifuged twice at 3578 g for 10 minutes at 4 °C. Supernatant was collected and protein concentration determined using the Bradford method (Kruger, 2009). After protein determination and standardization of protein concentration, equal amount of protein samples (50 µg) were diluted with Bio Rad sample buffer (4% Sodium dodecyl sulfate, 20% Glycerol, 125 mM Tris, 0.02% Bromophenol blue 200, 10% β-mercaptoethanol) in a ratio of 1:2. Samples were denatured for 5 minutes at 95 °C. Equal amounts of protein samples were resolved by electrophoresis on a 10% sodium dodecyl sulfate polyacrylamide gel (at 200 V for 1 hour) in running buffer (25 mM Tris base, 190 mM Glycine and 0.1% sodium dodecyl sulfate). Proteins were transferred onto nitrocellulose membrane (Bio-Rad) using transfer buffer (25 mM Tris base, 190 mM Glycine and 20% methanol) at 100 V for 1 hour.
Membrane was then blocked with 5% milk dissolved in 0.1% PBS-Tween (136.90 mM NaCl, 2.68 mM KCl, 10.14 mM NaHPO₄, 1.76 mM KH₂PO₄ 0.1% Tween) for 2 hours before adding primary antibody (1:1000 dilution in TBS-Tween) and incubating overnight. The membrane was washed three times with 0.1 M PBS-Tween for 10 minutes. The secondary antibody (1:10000 dilutions) was added and incubated with membrane for 2 hours at room temperature. The membrane was then washed three times with PBS-Tween and washed once with PBS. Chemiluminescence peroxidase substrate-3 reagents were added to the membrane and the membrane was viewed on a chemi-doc system (Bio-Rad, SA). Densitometric values were normalized to β-actin.

**Statistical analysis**

The data were analyzed using GraphPad Prism (version 5, San Diego, California, USA) and are expressed as mean ± SEM. The data were subjected to one-way analysis of variance (ANOVA) followed by Tukey’s post-hoc multiple comparison tests. Differences were considered significant when p< 0.05.
Results

Cleaved -Caspase-3 levels in the hippocampal tissue

Results of the ANOVA showed that there was a significant difference in cleaved caspase-3 levels between the different groups \([F(3, 15)=19.76, \ p< 0.0001]\). Tukey’s post-hoc test revealed that Tat+Saline and Saline+HAART treatment caused a significant increase in cleaved caspase-3 when compared to the Saline+Saline and Tat+HAART treated groups (\(p < 0.05\), Fig. 1b).

Lipid peroxidation through quantification of 4-hydroxynonenal (HNE) in hippocampal tissue

Results of the ANOVA showed that there was a significant difference in hippocampal HNE levels between the groups \([F(3, 15)= 11.23, \ p< 0.0001]\). Tukey’s post hoc test revealed that HNE levels were increased in all Tat and/or HAART treated groups when compared to the Saline+Saline group (\(p < 0.05\), Fig. 2).

TNF-α expression after Tat and HAART treatment

ANOVA revealed a significant difference in hippocampal TNF-α levels \([F(3, 15)= 11.94, \ p< 0.0001]\). Tukey’s post hoc test showed that Tat+HAART and Saline+HAART treated animals had a significantly higher level of TNF-α when they were compared to the Saline+Saline group of animals Figure 3. The TNF-α level in the Tat+HAART group of rats was significantly higher than that of the Tat+Saline rats (\(p < 0.05\), Fig. 3).
Activation of extracellular signal-regulated kinase (pERK 1/2) pathway

Results of the ANOVA showed that pERK levels differed significantly between groups \[F(3, 15)=13.91, \ p< 0.0001\]. Tukey’s post hoc test revealed that Tat+HAART and Saline+HAART treated animals showed a significant increase in pERK1/2 protein levels when they were compared to the Saline+Saline group \(P < 0.05\), Figure 4). Saline+HAART treatment caused a significant increase in pERK 1/2 when it was also compared to the Tat+Saline treated group \(P < 0.05\), Fig. 4b).

Synaptophysin expression after Tat and HAART treatment

ANOVA revealed a significant difference in synaptophysin levels of the different groups \[F(3, 15)=4.200, \ p< 0.01\]. Tukey’s post-host test showed that Tat+Saline and Tat+HAART caused a significant loss of Synaptophysin when they were compared to the Saline+Saline treated group \(p < 0.05\), Fig. 5b).
Discussion

Our results show that Tat protein caused an increase in cleaved caspase-3 in rat hippocampal tissue. This concurs with previous studies which demonstrated that Tat increased cleaved caspase-3 in cell culture (Ramautar et al., 2012). Tat was suggested to induce an increase in cytoplasmic free Ca\(^{2+}\) concentration in rat hippocampal cell culture which activated gene transcription, leading to an increase in cleaved caspase-3 expression (Krumen et al., 1998). HAART treatment reduced the Tat-induced increase in cleaved caspase-3 protein expression in the rat hippocampus. This effect of HAART has not been shown before and the mechanism involved in this HAART effect is not known.

Animals that received HAART treatment after saline injection into the hippocampus had a high expression of cleaved caspase-3 in the hippocampus. Previous studies have shown that at least one of the antiretroviral drugs used in the present study, Efavirenz cause apoptosis of neurons through cleave caspase-3 formation (Opie et al., 2007, Bumpus, 2011). Treatment with HAART could therefore be detrimental.

An increase of ROS in cells causes oxidative stress which leads to lipid peroxidation and other cell injuries (Berlett and Stadtman, 1997). One of the by-products of lipid peroxidation is HNE (Williams et al., 2006). Our results show that Tat caused an increase in HNE. This finding concurs with previous studies. Pocernich et al. (2005) showed that Tat increased HNE in rat cerebral cortical neurons. Tat protein likely caused ROS production leading to increased lipid peroxidation and increased HNE (Haughey et al., 2004, Banerjee et al., 2010). Treatment with HAART did not reduce the Tat-induced increase in HNE levels. Animals that received only HAART also had high levels of HNE. This can be attributed to the fact that both nucleoside and non-nucleoside reverse transcriptase inhibitors have also been shown to cause accumulation of ROS in primary cortical neuroglia, pure neurons and astrocytes.

HAART treatment increased TNF-α levels in both saline- and Tat-injected rat hippocampus while Tat had no effect. Since TNF-α has been shown to have anti-apoptotic effects through activation of NF-κB which increases expression of anti-apoptotic factors (Tamatani et al., 1999), this may be the mechanism of its beneficial effects.

Similarly, HAART treatment increased pERK levels in both the hippocampi of saline- and Tat-injected rats. The mechanism that leads to the increase of pERK is not known, but we suggest that it may be linked to the increase in TNF-α. Pulliam et al. (2001) showed that TNF-α increased pERK protein expression in brain cell cultures. TNF-α has been shown to exert neuroprotective effects by activation of anti-apoptotic factors (Bcl-2 and Bcl-x) in rat primary hippocampal neurons (Tamatani et al., 1999). A study conducted by Seo and Webster (2002) showed that TNF-α inhibits influenza viral replication in cultured porcine lung epithelial cells. TNF-α has also been shown to inhibit HIV entry by down regulating CD4 expression in tissue culture-differentiated macrophages (Herbein et al., 1996). HAART induced TNF-α production could be an additional beneficial effect of HAART, since TNF-α has anti-apoptotic and anti-viral replication effects. The absence of effect of Tat on TNF-α and pERK levels is contrary to expectation (Leghmari et al., 2008, Lee et al., 2011). This could be due to the fact that Tat stimulation of TNF-α expression is time-dependent and its effect may decrease over time (Leghmari et al., 2008). In this study the effects induced by Tat protein were observed after 14 days. The study conducted by Agrawal et al. (2007) showed
that Tat induced apoptosis in primary neuron cultures in a dose- and time-dependent manner.

Other studies showed that the initial effect of Tat such as astrocyte activation and oxidative stress are observed on day 7 after intracerebral injection of Tat (Bansal et al., 2000, Aksenov et al., 2003). Some of the effects of HAART on the brain may also result indirectly from its effects on peripheral tissue. For instance previous studies have shown that HAART increases in mRNA expression of IL-6 and TNF-α in adipose tissue (Lagathu et al., 2004). It is therefore possible that these proinflammatory cytokines can be produced peripherally, cross the BBB and mediate drug-induced negative effects on the brain.

As shown in previous studies, Tat caused a loss of synaptophysin, consistent with a loss of presynaptic terminals in hippocampal neurons in culture (Shin and Thayer, 2013). Treatment with HAART did not reverse synaptophysin loss, suggesting that it was unable to rescue synaptosomal loss.

In conclusion, HAART treatment does have beneficial effects but it cannot reverse some of the toxic effects of Tat protein such as lipid peroxidation and decrease in synapse density. Our findings affirm the need for supplementary treatment in addition to antiretroviral therapy in patients with HIV. Also this study provides novel insight into a possible mechanism of HAART action against some of the neurotoxic effects of HIV Tat protein.

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Fig. 1

Active caspase-3 levels in hippocampal tissue of rats that received bilateral intrahippocampal injections of Tat or saline, followed by treatment with HAART or saline for 7 days, 14 days after Tat injection. a The western blot representation of cleaved caspase-3 and β-actin. b Graph representing the cleaved caspase-3 measurements after normalization against β-actin. *Significantly different from Saline+Saline group. # Significantly different from the Tat+HAART treated rats
Fig. 2 HNE levels in hippocampal tissue of rats that received bilateral intrahippocampal injections of Tat or saline, followed by treatment with HAART or saline for 7 days, 14 days after Tat injection. * Significantly different from Saline+Saline group
Fig. 3 TNF-α levels in hippocampal tissue of rats that received bilateral intrahippocampal injections of Tat or saline, followed by treatment with HAART or saline for 7 days, 14 days after Tat injection. *Significantly different from Saline+Saline group, # significantly different from Tat+Saline treated group.
Fig. 4 Phosphorylated ERK 1/2 levels in hippocampal tissue of rats that received bilateral intrahippocampal injections of Tat or saline, followed by treatment with HAART or saline for 7 days, 14 days after Tat injection. 

a Western blot representation of pERK. b Graph showing pERK measurements after normalization against β-actin. *Significantly different from Saline+Saline group, # significantly different from Tat+Saline treated rats.
Fig. 5 Synaptophysin levels in hippocampal tissue of rats that received bilateral intrahippocampal injections of Tat or saline, followed by treatment with HAART or saline for 7 days, 14 days after Tat injection. a Western blot representation of synaptophysin. b Graph showing synaptophysin measurements after normalization against β-actin. *Significantly different from Saline+Saline group.
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Chapter 3
Conclusions and Future considerations

The introduction of highly active antiretroviral therapy (HAART) has contributed positively to the life expectancy of people living with HIV by adding an 11.3 years gain from 49.2 to 60.5 years (Bor et al., 2013). But as patients with HIV live longer, complications associated with long-term HAART begin to emerge (Schambelan et al., 2002). Despite extensive research done on the pathophysiology of HIV, investigations into HAART have been limited. The present study was subsequently conducted to improve the current understanding of the effects of HAART in the central nervous system and to assess its efficacy in protecting the central nervous system against the toxic effects of HIV Tat protein.

Our first aim was to confirm that Tat protein is neurotoxic and indeed leads to cell death. By injecting Tat protein directly into rat’s hippocampus, we could show evidence of oxidative stress, apoptosis and decreased synapse formations. These findings clearly demonstrated the toxic effects of Tat protein and indicated the mechanism by which Tat protein could cause cell death. For the second aim we determined the potential of HAART to protect against Tat protein toxicity, rats that received bilateral injections of Tat protein into their dorsal hippocampus, were subsequently treated with a clinically-relevant combination of antiretroviral drugs. In these experiments we could show that the antiretroviral treatment was only partially effective in reversing the toxic effects of HIV Tat. Our third aim was to investigate whether HAART has any toxic effects on the central nervous system. Our findings revealed that HAART activated signaling pathways and stimulated the release of pro-inflammatory cytokines. These observations suggested that HAART might be toxic on its own and therefore the administration of these drugs to patients should be done with caution.
In conclusion, the findings of our study did not support our hypothesis entirely in that HAART was unable to reverse the toxic effects of HIV Tat protein completely. The data showed that HAART may have some beneficial effect but it is not efficient in protecting hippocampal cells against Tat protein induced damage. Since our experiments also showed that HAART may have deleterious effects itself, and therefore patients on HAART should be monitored carefully for signs of abnormal function of their central nervous system.

The present study focused on the effects of Tat and HAART on certain proteins in the hippocampus. While informative, the proteins served only as a window into the consequences of Tat and HAART exposure. A more comprehensive proteomic approach should be considered in future studies as this may provide a broader understanding of how Tat and HAART influence the proteome of the hippocampus. A further benefit of such an approach would be the possible identification of alternative targets for intervention. Another aspect worthy of further investigation is the period of HAART treatment. Since we only treated our animals for 7 days while humans are on HAART for much longer, it would be useful to investigate the effects of a much longer period of drug administration. Of particular interest would be the effects of long term HAART on the epigenome and transcriptome of brain areas associated with cognitive function. And finally the current study only measured biochemical parameters. Inclusion of cognitive behavioural assessments for animals treated with Tat and HAART may provide additional insights into the molecular mechanisms underlying the development of cognitive impairments as seen in HIV-associated neurocognitive disorder (HAND).
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