2022

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A THESIS

Submitted to the graduate faculty of The University of Alabama at Birmingham,
in partial fulfillment of the requirements for the degree of
Master of Science

BIRMINGHAM, ALABAMA

2021

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MULTIDISCIPLINARY BIOMEDICAL SCIENCE

ABSTRACT

HIV-associated neurocognitive disorders (HAND) produces serious neurological dysfunction for > 50% of people living with HIV (PLWH). As antiretroviral therapy (ART) has transformed HIV into a chronic disease, and since most ART does not effectively cross the blood brain barrier, HAND is predicted to further increase in prevalence in PLWH. The HIV-Trans-Activator of Transcription (Tat) is known to persist in the central nervous system (CNS) of chronically infected HIV+ individuals. In several preclinical studies, HIV-Tat has been shown to be neurotoxic and important for HIV pathogenesis. Translocator protein (TSPO), an 18 kDa mitochondrial protein, has been shown to be increased via PET imaging studies in HIV+ patients, in association with cognitive dysfunction. The question is, how Tat affects TSPO is still unclear and requires further investigation. To better understand the effects of Tat on TSPO, we utilized an *in vitro* model of mixed glial cell cultures, together with an *in vivo* transgenic mice model, in which Tat is expressed at low-levels from the "leaky" Tet-on promoter. In the present study, both *in vitro* and *in vivo* models of Tat indicate an increase in TSPO levels, suggesting the potential for TSPO as a novel functional mediator of HIV-Tat-induced neuroinflammation. This result would justify further evaluation TSPO as a candidate therapeutic target for HAND and possibly other age-related neurodegenerative diseases.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

Human Immunodeficiency virus (HIV) and Neurocognitive Dysfunction: Overview

In 2019, there were approximately 38 million people across the globe with HIV/AIDS. Today, approximately 1.2 million people in the U.S. are living with HIV[1]. Nearly 50% of people living with HIV (PLWH) are over the age of 50, and are expected to live near normal life spans in the era of successful antiretroviral therapy (ART)[2]. However, ART-treated, HIV+ individuals are experiencing a greater prevalence of comorbidities that compromise long-term health, including neurological problems such as increased brain aging and HIV-associated neurocognitive disorders (HAND). Moreover, latent HIV can persist in the brain even when systemic virological control is achieved with ART, thereby hampering efforts to eradicate HIV[3]. HAND is a syndrome of progressive deterioration of memory, cognition, behavior, and motor function in HIV-infected individuals[3]. HAND is stratified into three main groups depending on the severity of neurocognitive impairment: 1) asymptomatic neurocognitive impairment (ANI); 2) minor neurocognitive disorder (MND); and 3) HIV Associated Dementia (HAD)[4]. However, the prevalence of HAD has declined with the implementation and success of ART[4].

HAND encompasses a specific group of neurological conditions that emerge from the continued exposure of central nervous system (CNS) tissue to HIV-1, HIV-1 viral proteins (Tat, Vpr, gp120, and Nef), and the combination of ART[5]. Those conditions are

associated with a metabolic encephalopathy induced by HIV infection and fueled by immune activation of macrophages, microglia and astrocytes. These cells are actively infected with HIV and secrete neurotoxic mediators of both host and viral origin[5]. Areas of the brain most vulnerable to those damaging toxins include but are not limited to the basal ganglia, subcortical white matter, and frontal cortex[6]. According to the "Global prevalence and burden of HIV-associated neurocognitive disorder" report, in 2020, the incidence and prevalence of HAND have not decreased[6]. Worldwide, it is estimated there were roughly 16 million cases of HAND in HIV-infected adults. The prevalence of asymptomatic neurocognitive impairment, mild neurocognitive disorder, and HIV-associated dementia were 23.5% (20.3-26.8), 13.3% (10.6-16.3), and 5.0% (3.5-6.8), respectively. Thus, HAND continues to remain a burden to people living with HIV even in the era of successful ART[7], since ART poorly penetrates the CNS during the early stage of infection.

HIV has been shown to enter the CNS before diagnosis and likely persists there[8]. The CNS acts as a reservoir for HIV, due to the fact that the most commonly prescribed ART does not effectively cross the blood-brain barrier (BBB)[8]. Disruption of the BBB by HIV likely contributes to the progression of neurological dysfunction. This leads to persistent cytotoxic stress and neuroinflammation that in turn compromise tissue integrity, all of which contribute to HAND development and progression[8]. At the cellular level, the primary cell targets for HIV infection in the CNS are CD4+ T cells, macrophages, and microglia. Several mechanisms have been proposed for HAND pathogenesis and can be grouped into two general pathways[9]:

A) the direct effect of the HIV-1 infection, including HIV-1 proteins.

B) the indirect consequence of infection comprising the secretion of cytokines and neurotoxins. Infected macrophages and microglia participate actively in the neurodegenerative process[9].

HIV-Tat: HIV-Trans-Activator of Transcription

HIV-Trans-Activator of Transcription (Tat) is one of the first HIV proteins required for initiating transcription of the HIV genome, and is also the earliest protein to be produced by the proviral DNA of infected cells[10]. Tat stimulates HIV-1 gene expression during transcription initiation and elongation. The protein not only drives the regulatory regions of HIV-1 but may also be actively released from infected cells and then interact with the cell surface receptors of other uninfected cells in the brain, leading to cellular dysfunction[11]. Tat that is taken up by uninfected cells may induce activation of a number of host genes. Importantly, the production of Tat is not impacted by the use of antiretroviral drugs once the proviral DNA has been formed[11].

Recent evidence indicates Tat may persist in the CNS of PLWH, even if their viremia is effectively controlled by ART, suggesting Tat may play a role in the pathogenesis of HAND. Tat is secreted by infected microglia and astrocytes, and results of preclinical studies suggest Tat regulates the neuroinflammatory response, and neurotoxicity in the progression of HAND[12].

Two main theories emerge from the literature about the mechanism of Tat toxicity[13]:

1) Tat is able to induce changes in neuronal cell homeostasis through perturbation of extracellular signaling mechanisms (e.g. changes in membrane receptor permeability and composition); or

2) Internalization of Tat protein leads to direct interaction with intracellular factors involved with Ca^{2+} regulation, transcription and translation.

HIV-1 Tat and Microglia

In the CNS, productive replication of HIV-1 can be supported by two cell types: microglia and astrocytes. Microglia are a subtype of CNS immune cells that unlike neurons and astrocytes, which have neuro-ectoderm embryonic lineage, share the same origin as macrophages and other hematopoietic cells. Microglia carry a specific role in the progression of HAND and Tat is shown to be cytotoxic and pro-inflammatory in the context of HAND pathogenesis[14].

Pathological hallmarks in advanced stages of HAND include microgial activation and formation of multinuclear giant cells. These can lead to changes in their immune effector functions, phagocytosis and pro-inflammatory signaling pathways including but not limited to TNF-alpha and chemokines[15].

HIV-1 Tat and Astrocytes

Unlike microglia, astrocytes arise from the same neuro-ectoderm embryonic lineage as neurons. They are in direct contact with neurons and play a critical supportive role in maintaining their homeostasis. Astrocytes support productive HIV-1 infection in

the CNS because they remain inaccessible to almost all known anti-retroviral treatments available[14].

The effects of Tat on glial cells are likely to contribute in part to the Tat-mediated loss of brain cells *in vitro* and *in vivo*[15]. Studies have shown that Tat promotes lysosomal exocytosis in astrocytes and contributes to astrocyte-mediated neurotoxicity in mouse primary astrocytes, through glial fibrillary acidic protein (GFAP) activation and endoplasmic reticulum stress induction in astrocytes. Moreover, Tat stimulates the production of pro-inflammatory cytokines in the brain and neurotoxins in these cells, as well as induces a milieu of cytokines and chemokines in macrophages and astrocytes, which subsequently recruit more macrophages/monocytes and lymphocytes into the CNS and alter astrocytes growth.

Several HIV-1 Tat transgenic mouse models have been developed to study the role of HIV-1 Tat protein in HIV pathogenesis. To gain a better understanding of the role of Tat in HIV and the mechanisms of Tat neurotoxicity, specifically resulting from Tat protein expression in the brain, the He lab combined the Doxycycline (Dox)-regulated (rtTA) gene expression system with a brain specific promoter, i.e., the glial fibrillary acid protein (GFAP)[17]. They characterized the phenotypic and neuropathologic features of these mice. Both in *vitro* and *in vivo* assays confirmed that Tat expression occurred exclusively in astrocytes and was Dox-dependent. Tat expression in the brain caused failure to thrive, hunched posture, tremor, ataxia, and slow cognitive and motor movement, seizures, premature death, together with extensive neuropathological changes such as loss of cerebellum and cortex, neuronal death (apoptosis), astrocytosis, degeneration of neuronal

dendrites, and the CNS infiltration of monocytes and activated T lymphocytes[17]. These results demonstrate that Tat expression in the absence of HIV-1 infection is sufficient to cause neuropathology similar to most of those noted in the brain of AIDS patients[17].

Using Tat transgenic mice, studies have shown the deleterious effects of Tat on particular brain regions including the cortex, the striatum, the dentate gyrus, and CA3 region of the hippocampus. Studies suggest the conditional expression of HIV-Tat specifically in the CNS of mice using a Tet-on driven, GFAP promoter induces behavioral deficits and increases in neuroinflammatory cytokines, as well a reduction in structural (e.g., β-III-tubulin) and synaptic (e.g., synaptophysin; postsynaptic density 95 protein or PSD-95) markers[18].

The effects of Tat on the solubility of proteins in neuronal cells

Under persistent infection of HIV, some neuropathological markers of neurodegenerative disease are found to aggregate and translocate to different organelles. For instance, Tat has been shown to directly interact with amyloid β peptide in in *vitro* studies. Under the presence of Tat, the amyloid fibrils turned into double twisted fibrils followed by populations with thick unstructured filaments and aggregated large patches in a dose-dependent manner. These data suggest the amyloid β-Tat complexes accounted for the increased damage, likely through pore formation in membranes, leading to neurotoxicity[19].

Tat has also been shown to stimulate autophagy through increasing BAG3 levels (an anti-apoptotic and pro-autophagic factor in several neoplastic and normal cells) in human glial cells[20], which might interfer the lysosomal and the proteasomal pathway of cellular protein degradation, potentially leading to or might be the reason from the accumulation of misfolded proteins, and play another role in modulating the autophagy/apoptosis balance[20] . Moreover, Tat has been shown in another study to cause cell cycle disturbance through its interaction with p53, a tumor suppressor protein. Extracellular Tat is internalized by neurons and astrocytes and this may inhibit p53 ubiquitination leading to p53 accumulation. p53 accumulates in microglia and astrocyte nuclei in a subset of AIDS patients without dementia, while increased neuronal p53 was only observed in cases of HAD. HIV-1 has been shown to eventually stimulate activation of p53, which thereby alters the phenotype of uninfected microglia, and eventually affects the cell transfromation, leads to apoptosis and neuronal loss[21].

It is unknown whether Tat interferes with the solubility, localization, or interaction of other proteins during, and if these changes in intracellular function contribute to the onset and progression of neurotoxicity in the CNS of PLWH. If Tat decreases the relative solubility of proteins, it suggests their potential to aggregate and contribute to the pathogenesis of neurological disease.

Together, these findings from pre-clinical studies suggest Tat itself may cause neuronal dysfunction in chronically infected HIV+ individuals that sensitizes them to cognitive dysfunction and premature brain aging.

Translocator protein 18kDa (TSPO) in Central Nervous System

TSPO is a mitochondrial transmembrane protein that was initially discovered in 1977 as the binding site for benzodiazepine. The translocator protein (18 kDa) (TSPO) is localized primarily in the outer mitochondrial membrane of steroid-synthesizing cells, including those in the central and peripheral nervous systems. One of TSPO's main functions is the transport of the substrate cholesterol into mitochondria, a prerequisite for steroid synthesis[19].

TSPO is enriched in the CNS, particularly in glial cells with microglia bearing higher expression than other types. TSPO has been implicated in a multitude of metabolic processes, and is known to become highly enriched in diseased brains during the process of neurodegeneration. TSPO expression may constitute a biomarker of brain inflammation and reactive gliosis that could be monitored by using radiolabeled TSPO ligands as neuroimaging agents[20].

Studies using TSPO-binding ligands have revealed that this protein participates in a variety of cellular functions, including cholesterol transport and steroid hormone synthesis, mitochondrial permeability transition pore (mPTP) opening, mitochondrial respiration, apoptosis, proliferation, tumorigenesis, and inflammation[21]. In the brain, because there is a noticeable increase in TSPO levels in activated microglia during neuroinflammation, as well as marked immunosuppressive potency of TSPO ligands, TSPO has become an attractive pharmacological target of diagnostic imaging for the treatment of neurological and neuropsychological diseases[22].

In the CNS, TSPO is highly expressed in glial cells (e.g., astrocytes, microglia). For decades, some studies have used TSPO ligands to assess levels of neuroinflammation and microglia activation and to understand the pathophysiological roles of TSPO in the brain. However, our understanding of TSPO function in the brain remains limited, partly owing to a lack of studies employing cell-type specific approaches in normal and pathological states[23].

The upregulation of TSPO in microglia and astrocytes is directly associated with the degree of damage and prolonged and robust inflammation may cause severe damage to neurons and nerve fibres. However, TSPO is also an attractive drug target for controlling neuroinflammation[23]. The neural lineage-specific TSPO knockout mice showed a decrease in astrogliosis in response to neural injury in EAE, a popular animal model of multiple sclerosis. This decline in astrogliosis was associated with lessening the severity of EAE, indicating a functional role for TSPO in activation of astrocytes[23]. A recent CRISPR-Cas9-mediated TSPO gene knockout study in human primary microglia reported that TSPO in human microglia is not related to steroidogenesis but to alterations in cytosolic Ca^{2+} and VDAC expression[24].

In response to injury, inflammation, and disease, TSPO expression is induced primarily in microglia and astrocytes in the CNS, and in macrophages and/or Schwann cells in the peripheral nervous system. Thus, glial cells probably mediate the beneficial effects of TSPO ligands on neurons, either directly or indirectly, via secreted neurosteroids, other metabolites or proteins. However, neurons can also express TSPO under certain

circumstances, such as inflammatory states, which is consistent with direct neuronal actions of TSPO ligands. Experimental studies suggest that TSPO ligands also serve as markers for the state and progression of traumatic brain injury, in which mitochondria are primary targets. Importantly, the timing of TSPO expression tracks glial cell activation that occurs not only due to injury but also during regeneration, and thus may qualify as a molecular sensor of active repair processes[24,25].

Recent results of positron emission tomography (PET) imaging studies indicate upregulation of TSPO in the CNS of PLWH in association with neuroinflammation and cognitive decline[28]. These studies suggest that TSPO either contributes to or is in response to neuroinflammation in HIV and may be a promising new target for monitoring the progression of HAND[28] . Another point concerning the role of TSPO is that its altered expression not only is associated with the inflammatory response, but may also signify an aberration in cellular metabolism, energy homeostasis, or oxidative stress during inflammation[28]. Therefore, correlation of altered TSPO levels with microglia and/or astrocytes may either contribute to the neuroinflammatory response, and/or may be a direct response to neuroinflammation in these cells.

As both Tat and TSPO are clinically relevant to the neurological manifestations of HIV, this project may establish TSPO as a novel functional mediator of HIV-Tat-induced neuroinflammation and neurotoxicity. This would also justify further evaluation of TSPO as a candidate therapeutic target for HAND and possibly other age-related neurodegenerative diseases.

GAPS IN KNOWLEDGE

From previous studies, we know Tat persists in the virally surpressed CNS of PLWH and correlates to neurodegeneration.[29] We also know TSPO is upregulated in the CNS of PLWH, and is widely used for PET imaging of neuroinflammation in human disease. However, whether a relationship exists between Tat and TSPO, i.e., does Tat affect levels of TSPO, has not been studied and require further investigation. In addition, Tat is known to interact with and affect the relative solubility of other proteins that contribute to the onset and progression of neurodegenerative disease. Whether these effects of Tat are related to TSPO are also unknown and also require further investigation.

HYPOTHESIS

Our lab is interested in investigating the function of HIV-Tat in *in vitro* and *in vivo* models of HAND. Knowing the molecular mechanism of HIV-Tat- associated neurotoxicity could improve our understanding of HAND pathogenesis and lead to novel therapies. In this study, we focused on TSPO, a neuroinflammatory that is upregulated in the CNS of PLWH, as a potential mediator of Tat-induced neurotoxicity. We are interested in determining not only if Tat regulates levels of TSPO, but also if Tat regulates its relative solubility.

We hypothesize that HIV-Tat increases levels of TSPO. If our hypothesis is correct, it will warrant further investigation of TSPO as a promising new target for monitoring the progression of HAND. A successful outcome would also provide a novel finding of the potential association TSPO in the regulation of Tat in HAND, where Tat may alter the properties of TSPO that accumulate in age-related neurodegenerative diseases, where its protein property might become less soluble under the presence of Tat.

In future studies, we would propose more mechanistic analyses to determine the role of TSPO in regulating HAND pathogenesis and would validate the need to further investigate mechanisms that minimize Tat-associated neurotoxicity in chronic HIV infection.

CHAPTER 2

METHODS

In vitro model of HAND: treatment of glial cell cultures with recombinant Tat

Dissociated, mixed glial cell cultures were prepared from the cortex of P2-4 day old mouse pups (wild type) and grown to confluency in flasks with periodic media change. Mixed glial cells were maintained in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 1% Penicillin/Streptomycin; 7.5% $NaHCO_3$ and 50% glucose. Once confluent, glial cells were plated in six well plates or 100 mm dishes. Upon reaching confluency, typically after 48 hours, cells were treated with either vehicle (VEH) control, endotoxin lipopolysaccharide (LPS, 1 ug/ml) as the inflammatory positive control or recombinant Tat_{1-86} (100 nM), a generous gift of Dr. Jamil Saad (UAB Department of Microbiology). Cells were treated for 48 hrs prior to collecting lysates for western blot analysis.

Preparation of whole cell lysates

Media was transferred from wells/dishes to numbered 15 ml conical tubes. Plates/dishes were then rinsed with 1X Phosphate-buffered saline. Accutase (AT104 Innovative Cell Technologies) was added and plates/dishes were incubated for 5 min to detach cells. FBS-containing media was then used to neutralize the cells. Wells were scraped thoroughly (back and forth, side to side, around the edges) to detach any remaining

cells that still presented on wells/dishes. Collected cells were transferred to tubes and 1X PBS was then used to rinse wells to collect remaining cells. 1X PBS was then transferred to tubes, and tubes were centrifuged at 1500 rpm, 4^{o}C for 5 min. Supernatants were aspirated without disrupting pellets. Ice cold 1X PBS was added and tubes were centrifuged again at 1500 rpm for 5 min. Supernatants were aspirated again without disrupting the pellets. Lysis buffer (50-70 ul) containing 25 mM HEPES, 5 mM EDTA, 5 mM $MgCl_2$, 1% SDS, 1% Triton X, 1% protease (Sigma P8340) and 1% phosphatase inhibitors (Sigma P5726 and Sigma P2850) was then added each tube. Pellets were resuspended in lysis buffer by pipetting up and down 10 times. Samples were stored at -20^{O}C at least overnight, until they were sonicated. Samples were sonicated on ice for 10s, 50% power for three-five seconds until pellets were broken up. This step is to completely lyse the cells and shear the DNA (no longer viscous). Samples were then spun in 15 mL tubes (3000 rpm, 2 min, 4°C) to collect lysates that sprayed up the tube wall during sonication, and lysates were transfered to 1.5 mL microcentrifuge tubes. Lysates were then spun again for 10 min, 10,000 rpm, 4°C. Supernatants were transferred to fresh microcentrifuge tubes and stored at -20°C until further use.

Preparation of cell lysates - Detergent soluble versus insoluble protein fractions

Triton X-soluble and insoluble fractions of cell lysates were collected, using 1% Triton X for soluble fractions and 2% SDS for insoluble fractions (pellets that did not solubilize following incubation with lysis buffer containing Triton X). Cells were lysed first in buffer containing Tris/HCl 50mM, pH 7.4; NaCl 175 mM; EDTA 5 mM; 1% protease (Sigma P8340) and 1% phosphatase inhibitors (Sigma P5726 and Sigma P2850),

and in the absence of detergent. After that, samples were sonicated for 10s, 30% power on ice, then quickly spun at 2000 rpm for 1 minute at 4°C. Triton X (10%) was then added to lysates make a final concentration of 1%. Lysates were incubated on ice for 30 minutes. A small aliquot was taken from each sample and designated as the "whole fraction". Samples were then spun at 15,000 x *g* for 1h at 4°C, and supernatants were carefully transfered to fresh tubes and designated as the "Triton X-soluble fractions". Pellets were reuspended in lysis buffer containing 2% SDS. Samples were then sonicated on ice for 10s, 30% power, and were labeled as "Triton-X insoluble fractions".

In vivo model of HAND: Tat Transgenic Mice

Mice that conditionally express the HIV-Tat transgene in a CNS-targeted manner via a brain specific promoter, the glial fibrillary acid protein GFAP-driven, Tet-on promoter (rtTA-Tat) were used[18]. rtTA-Tat mice did not receive doxycycline (DOX) treatment (no induction of Tat expression), to take advantage of low-level Tat expression in these mice that results from the "leaky" Tet-on promoter, which mimics levels of Tat in the CNS of chronically infected individuals[18]. Mice expressing the Tet-on GFAP promoter (rtTA) without the Tat transgene were used as controls for our study. We proposed two groups of mice: rtTA promoter control mice, shown previously not to produce neurological deficits ($n = 4$) and rtTA-Tat mice that did not receive DOX ($n = 4$). Mice were euthanized at 17-18 month-old since the effects of Tat in the aging brain were targeted.

Mouse euthanasia and tissue processing

Mice were euthanized by transcardial perfusion with saline. Different brain regions including the cortex, hippocampus, midbrain, cerebellum, pons, medulla and the remaining "rest of brain" were rapidly dissected and snap frozen over dry ice for subsequent preparation of brain homogenates and western blot analysis.

Preparation of brain homogenates

Brain homogenates were prepared from cortical or hippocampal tissue in buffer containing Tris/HCl 50mM, pH 7.4; NaCl 175 mM; EDTA 5 mM; 1% protease and 1% phosphatase inhibitors, using a glass tissue homogenizer. Buffer plus inhibitors were added proportionally to the weight of tissue (300ul of buffer plus inhibitors/ 10mg of tissue). Brain tissues were homogenized by rotating a glass rod in glass tube in a circular motion for 30-50 strokes, then up and down 20 strokes to ensure tissues were completely disrupted. After that, homogenates were transfered to 1.5mL microfuge tubes. 10% Triton X was added to make the final concentration of brain homogenates to 1%, and the homogenized samples were left on ice for 30 minutes. A small aliquot (~50ul) was taken from each sample and designated as the "whole fraction". Samples were then spun at 15,000 g for one hour at 4^{o}C. Supernatants were aliquoted as the "Triton-X soluble" fraction. The pellet was resuspended in lysis buffer containing 2% SDS and designated as the "Triton X-insoluble" fraction. Insoluble samples were then sonicated on ice for 10s, with 30% power.

Western blot analysis[29]

Bicinhoninic Acid Protein Assays (BCA) were ran for each sample to determine protein concentrations and equal loading of protein into gels. Equal amounts (5-15 µg) of protein were separated by size and charge, using 15% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and subsequently transfered onto polyvinylidene (PVDF) membranes. Following transfer, membranes were blocked for 30 minutes in 5% milk, followed by the addition of TSPO primary antibody (rabbit-anti-TSPO; Abcam ab 109497 anti-PBR antibody [EPR5384]) overnight at 4ºC on a rotary shaker. After primary incubation, membranes were washed with Tris-buffered saline with 0.1% Tween® 20 Detergent (TBST) 3 times for 5 minutes each. Next, blots were incubated with rabbit secondary [Bio-rad #170-6515 Goat Anti-Rabbit IgG] for one hour. Membranes were washed 3 times for 10 minutes with TBST. Bands were detected using detection reagent Pierce ECL (Thermo-Fisher 32106) and imaged with a Bio-Rad ChemiDoc Imager, using fluorescent dectection at 700 and 800 nm channels. Membranes were then stripped and re-probed for Actin loading control (42 kDa) [Mouse anti-Actin antibody; Sigma A1978-200] to normalize for gel loading, followed by incubation with HRP-linked, anti-mouse secondary antibody [Cell Signaling #7076]. Protein bands for TSPO (18 kDa) and Actin (42 kDa) were quantified using UN-SCAN-IT (Silk Software) graph digitizer software by comparing relative density and concentration of bands to total protein, using Actin to normalize for the loaded protein. Results were then analyzed using GraphPad Prism 8 software.

Statistical Analysis

Statistical analysis was performed using Prism 7 (GraphPad). All graphs were created in Prism 7. One-way repeated measures ANOVA was used to test the statistical differences among the means of three or more groups, followed by Bonferroni's multiple comparison post hoc test. Unpaired t-test was used to compare the averages/means of two independent or unrelated groups. Data was expressed as mean ± SD. For all tests, statistical significance was set a priori at $p^* < 0.05$. Replicates within experiments were performed and averaged together. Power calculations were performed using G*power software[19] with 80% power, to determine sample size needed in future experiments to detect meaningful differences with an 80% probability.

CHAPTER 3

RESULTS

To determine if Tat affected TSPO levels, *in vitro* and *in vivo* models were used. TSPO levels were determined using western blot. In our *in vitro* model, mixed glial cell cultures were treated with recombinant Tat 100nM. Cell lysates were then collected and fractionated into three different preparations: whole cell lysates, Triton X-soluble fractions, and Triton-X insoluble fractions. With our *in vivo* model, mice that conditionally express the HIV-Tat transgene in a CNS-targeted manner via a brain specific promoter, the glial fibrillary acid protein GFAP-driven, Tet-on promoter (rtTA-Tat) were used. Cortical and hippocampal regions from each mouse were collected and homogenized, then fractionated into either rwhole homogenates, Triton-X soluble fractions, or Triton-X insoluble fractions. For westerns, proteins were normalized to Actin and then divided by their individual control values.

HIV-Tat increases TSPO levels in mixed glial cultures

Quantification of TSPO via western blot analysis revealed an effect of Tat on TSPO levels, where HIV-Tat increased TSPO in whole cortical mixed glial cell lysates (**Figure 1**). To determine dose responsiveness of Tat on TSPO, cultured glial cells were treated for 48 hours with negative control vehicle (VEH) or different concentration of Tat (0.3-100nM). Actin (42 kDa) served as loading control. Westen blot data indicated significant increases in 18kDa TSPO at [Tat] ≥ 0.3 nM in the whole cell lysates.

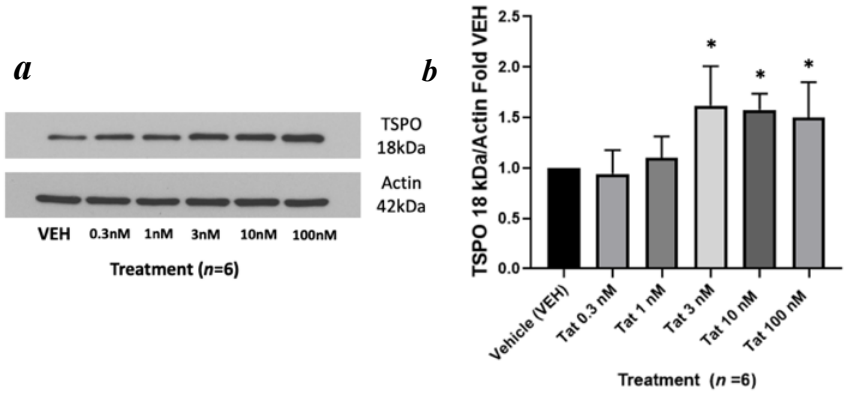

Figure 1: HIV-Tat increased TSPO in glial cell culture lysates in a concentration-dependent manner.

a) Representative western blot of TSPO (18 kDa) in whole cortical cell lysates following treatment for 48h with Tat (0.3-100 nM). with ≥ 3 nM Tat. Actin (42 kDa) served as the loading control.
b) Bar graph indicates significant increases (1-way ANOVA, *$p<0.05$ ($p= 0.02$ with Tat 3nM; 0.02 with Tat 10nM and 0.03 with Tat 100nM, respectively; Bonferroni post hoc test) in 18 kDa TSPO in lysates from cells treated with ≥ 3 nM Tat. Data are expressed as mean ± SD fold change of TSPO levels in vehicle-treated cells.

To validate data from our dose response experiment, we treated cortical glial cells with 100nM Tat and chose this concentration for future experiments. At this concentration, Tat significantly increased TSPO levels in whole cortical cell lysates (**Figure 2**), corroborating results obtained in **Figure 1**. Mixed cortical glial cultures were treated for 48h with negative control labled as vehicle (VEH), positive control LPS, or Tat 100 nM.

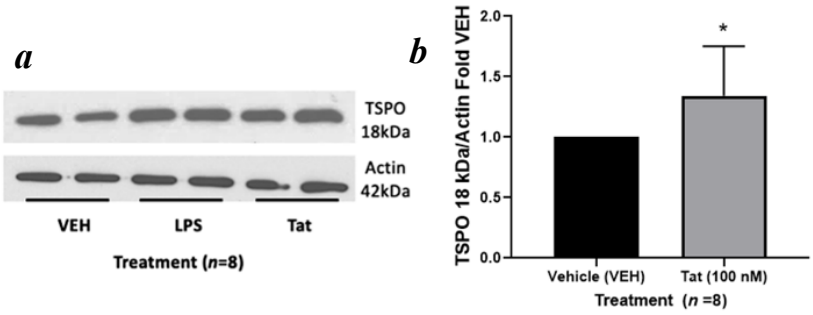

Figure 2: HIV-Tat increases TSPO levels in whole cell lysates of glial cell cultures
a) Representative western blot data of glial cell lysates treated with either vehicle (VEH), LPS (1μg/ml) or 100 nM Tat. Lysates were probed for TSPO (18 kDa) or Actin (42 kDa) loading control.
b) Bar graph indicates a significant increase (unpaired t-test, *$p<0.05$ ($p = 0.02$)) in TSPO levels, normalized to Actin levels. Data are expressed as mean ± SD fold change of TSPO levels in vehicle-treated cells.

Tat did not alter Triton X-soluble or Triton X-insoluble levels of TSPO in cortical glial cell lysates

Tat has been shown to alter and interact with other proteins that contribute to neuropathology, for example, interacts with amyloid precursor protein (APP) both *in vitro* and *in vivo*[19] and increases the level of amyloid beta, which commonly accumulates in the senile plaques of Alzheimer's disease patients. To determine if Tat alters TSPO by affecting its solubility, we performed fractionation experiments of cell lysates, first using the mild detergent Triton X to generate a "Triton X-soluble" fraction. The resulting insoluble pellet was further solubilized with 2% SDS, and supernatants from these preparations were designated as "Triton X-insoluble" fractions[19].

There were no significant differences observed in TSPO levels in Triton-X soluble fractions and Triton-X insoluble/SDS soluble fractions **(Figure 3)**. However, there did appear to be a general trend of increase in TSPO following treatment with Tat, in both Triton X-soluble and Triton X-insoluble fractions (**Figure 3**). Since standard deviations

were high from these experiments, increasing the sample size in prospective experiments may provide sufficient power to test for significant differences. Sufficient sample sizes and power analysis for this experiment are shown in **Table 1**.

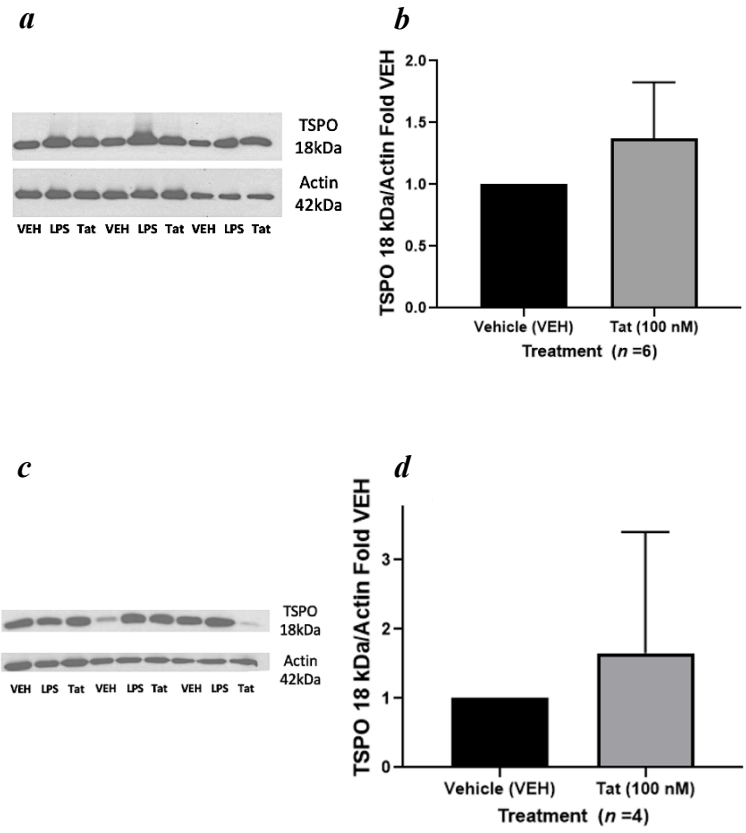

Figure 3: Tat did not alter Triton X-soluble or Triton X-insoluble levels of TSPO in glial cell lysates.

a) Representative western blot analysis for levels of TSPO (18 kDa) and Actin loading control (42 kDa) in Triton X-soluble fractions of cortical glial cell lysates collected following treatment for 48 hours treated with either vehicle (VEH), LPS (1µg/ml) or 100 nM Tat.

b) Bar graph indicates quantification of 18 kDa TSPO levels, normalized to levels of Actin (42 kDa) loading control, in Triton X-soluble lysate fractions. Data are expressed as mean ± SD fold change of TSPO levels in vehicle-treated cells (unpaired t-test, $p = 0.1056$). All values were normalized to the loading control Actin.

c) Representative western blot on levels of TSPO (18 kDa) and Actin loading control (42 kDa) in Triton X-insoluble fractions of cortical glial cell lysates collected following treatment for 48 hours treated with either vehicle (VEH), LPS (1µg/ml) or 100 nM Tat.

d) Bar graph indicates quantification of 18 kDa TSPO levels, normalized to levels of Actin (42 kDa) loading control, in Triton X-insoluble lysate fractions. Data are expressed as mean

± SD fold change of TSPO levels in vehicle-treated cells (p = 0.5110). All values were normalized to the loading control Actin.

No significant change of TSPO levels in cortical whole homogenates from Tat transgenic mice

Mice that conditionally expressed HIV-Tat transgene in a CNS-targeted manner via GFAP-driven, Tet-on promoter (rtTA-Tat) were used, to validate the results from our *in vitro* analysis of TSPO. However, mice did not receive doxycycline treatment, thus Tat gene expression was not induced, to take advantage of low-level Tat expression in these mice, resulting from the "leaky" Tet-on promoter as previously discussed[7].

Quantification of TSPO levels using western blot analysis revealed no effect of Tat in cortical brain whole homogenates, since data shows no significant change of TSPO levels with low-level Tat expression in transgenic mice model **(Figure 4)**.

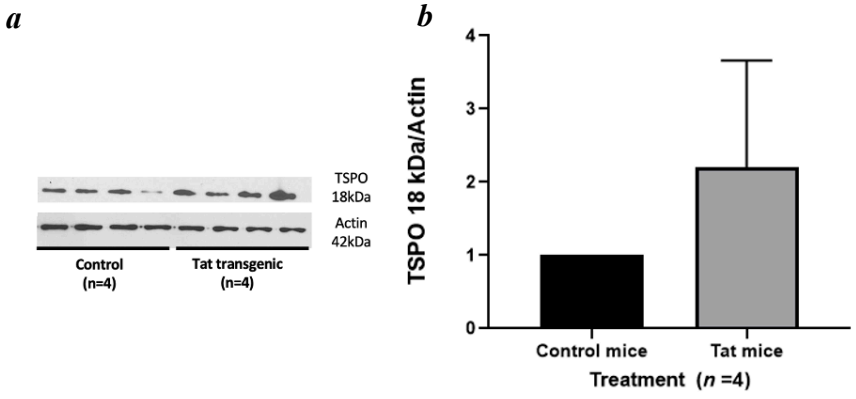

Figure 4: Effects of chronic low-level expression of Tat in transgenice mice on levels of TSPO in whole cortical homogenates

a) Representative western blot of TSPO (18 kDa) levels and Actin loading control (42 kDa) in whole cortical homogenates obtained from control or Tat transgenic mice (*n* = 4 mice/group).

b) Bar graph indicates 18 kDa TSPO levels, normalized to 42 kDa Actin levels, in control mouse and Tat transgenic mouse cortical whole homogenates (*n* = 4 mice/group). Data are expressed as mean ± SD fold change of TSPO levels (unpaired t-test, p = 0.1947). All values were expressed relative to values obtained in control homogenates.

Chronic, low-level expression of HIV-Tat in mice increases TSPO levels in Triton X-soluble cortical homogenates

To determine if Tat alters TSPO's relative solubility, brain fractionations were performed to investigate the TSPO levels in Triton-X soluble and Triton-X insoluble/SDS soluble fractions of cortical homogenates **(Figure 5)**. We observed a significant increase in levels of TSPO in Triton X-soluble fractions of cortical homogenates, but not in Triton X-insoluble fractions.This finding reveals the potential effect of Tat on the solubility of TSPO levels in the cortex.

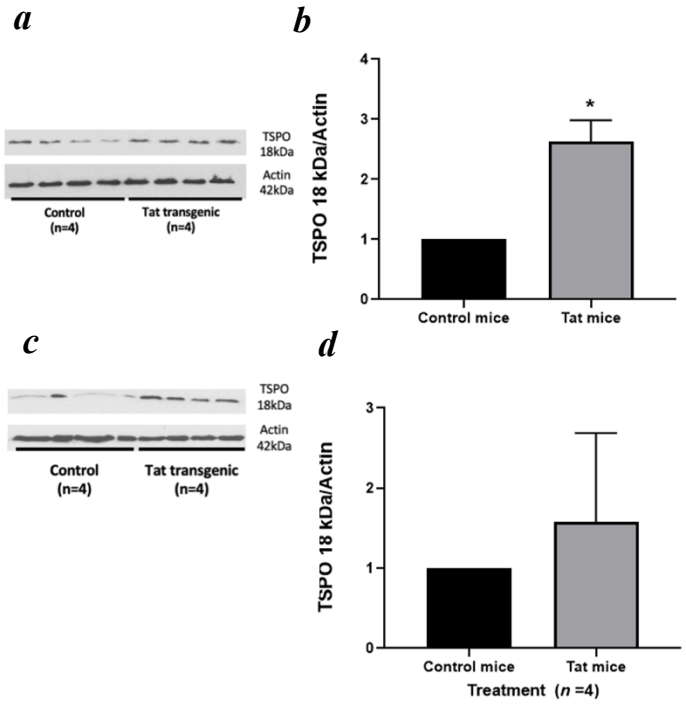

Figure 5: TSPO levels in Triton X-soluble and Triton X-insoluble fractions of Tat transgenic cortical brain homogenates.
a) Representative western blot of TSPO (18 kDa) levels and Actin loading control (42 kDa) in Triton X-soluble cortical homogenates obtained from control or Tat transgenic mice (*n* = 4 mice/group).
b) Bar graph indicates 18 kDa TSPO levels, normalized to 42 kDa Actin levels, in control mice and Tat transgenic mouse Triton X-soluble cortical homogenates (*n* = 4 mice/group). Data are expressed as mean ± SD fold change of TSPO levels (unpaired t-test, p= 0.02, *p< 0.05). All values were expressed relative to values obtained in control homogenates.

c) Representative western blot of TSPO (18 kDa) levels and Actin loading control (42 kDa) in Triton X-insoluble cortical homogenates obtained from control or Tat transgenic mice (n = 4 mice/group).
d) Bar graph indicates 18 kDa TSPO levels, normalized to 42 kDa Actin levels, in control and Tat transgenic mouse Triton X-insoluble cortical homogenates (n = 4 mice/group). Data are expressed as mean ± SD fold change of TSPO levels. Significant differences in TSPO levels were not observed (unpaired t-test, p = 0.3773). All values were expressed relative to values obtained in control homogenates.

Chronic, low-level expression of HIV-Tat did not change levels of TSPO in mouse hippocampal homogenates

We also assessed levels of TSPO in hippocampal homogenates from control and Tat transgenic mice, to assess TSPO levels in a different brain region that is also affected in HAND[7]. Quantification of TSPO levels indicated no effects of chronic, low levels of Tat in whole homogenates, although there appeared to be a trend suggesting Tat increased levels of TSPO, though not statistically significant **(Figure 6)**. Similarly, significant differences were also not observed by Tat in Triton X-soluble or Triton X-insoluble homogenates processed from mouse hippocampus **(Figure 7)**.

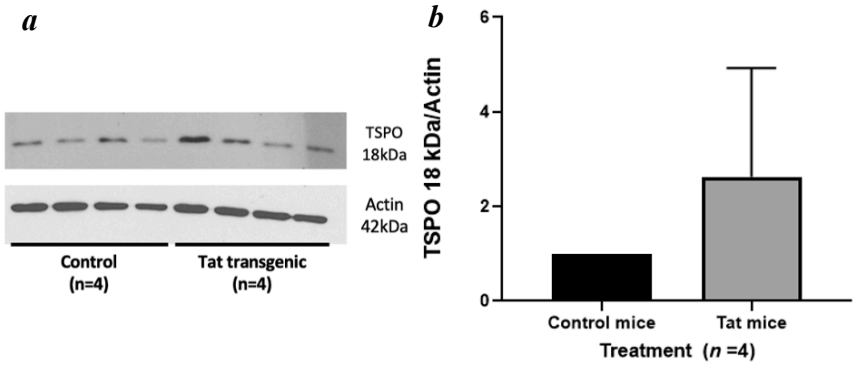

Figure 6: Effects of chronic low-level expression of Tat transgenice mice on levels of TSPO in whole hippocampal homogenates

a) Representative western blot analysis of levels of TSPO (18 kDa) and Actin loading control (42 kDa) in whole hippocampal homogenates obtained from control or Tat transgenic mice (n = 4 mice/group).
b) Bar graph indicates 18 kDa TSPO levels, normalized to 42 kDa Actin levels, in control and Tat transgenic mouse hippocampal whole homogenates (n = 4 mice/group). Data are expressed as mean ± SD fold change of TSPO levels (unpaired t-test, p = 0.2527). All values were expressed relative to values obtained in control homogenates.

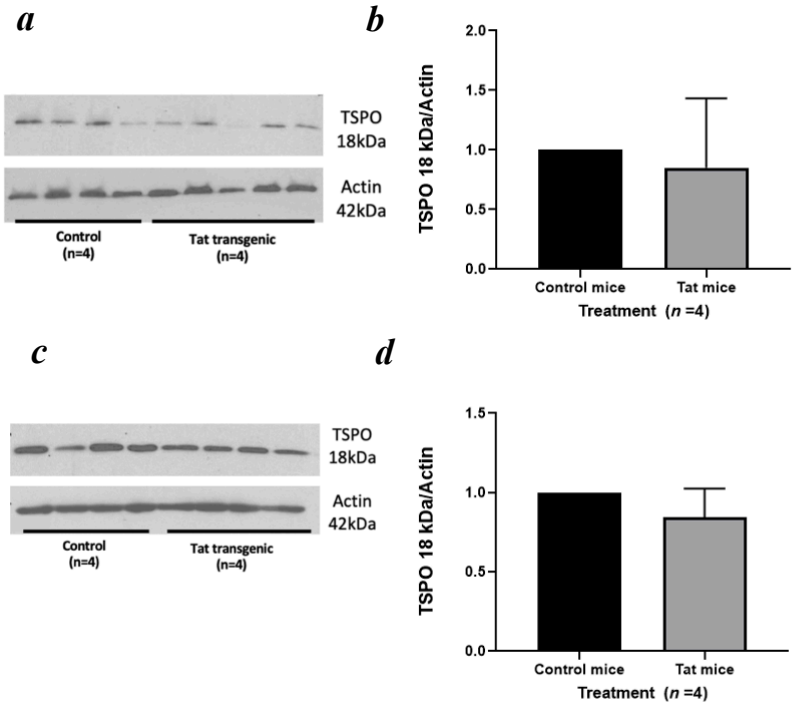

Figure 7: TSPO levels in Triton X-soluble and Triton X-insoluble fractions of Tat transgenic hippocampal brain homogenates.
a) Representative western blot of TSPO (18 kDa) levels and Actin loading control (42 kDa) in Triton X-soluble hippocampal homogenates obtained from control or Tat transgenic mice (n = 4 mice/group).
b) Bar graph indicates 18 kDa TSPO levels, normalized to 42 kDa Actin levels, in control and Tat transgenic mouse Triton X-soluble hippocampal homogenates (n = 4 mice/group). Data are expressed as mean ± SD fold change of TSPO levels (unpaired t-test, p = 0.6445). All values were expressed relative to values obtained in control homogenates.
c) Representative western blot of TSPO (18 kDa) levels and Actin loading control (42 kDa) in Triton X-insoluble hippocampal homogenates obtained from control or Tat transgenic mice (n = 4 mice/group).
d) Bar graph indicates 18 kDa TSPO levels, normalized to 42 kDa Actin levels, in control and Tat transgenic mouse Triton X-insoluble hippocampal homogenates (n = 4 mice/group). Data are expressed as mean ± SD fold change of TSPO levels. Significant

differences in TSPO levels were not observed (unpaired t-test, $p = 0.183$). All values were expressed relative to values obtained in control homogenates.

CHAPTER 4

DISCUSSION

To understand how Tat affects TSPO in pre-clinical models of HAND, we used western blot analysis to quantify TSPO levels in both *in vitro* and *in vivo* Tat models. Our results indicated recombinant Tat significantly increased TSPO in mixed glial whole cell lysates. This finding suggests TSPO is a reliable *in vitro* marker of the neuroinflammatory response that is induced by Tat and suggests the mechanism of Tat-induced neuroinflammation may involve regulation. Furthermore, this finding validates the potential for TSPO to serve as neuroinflammatory marker for HAND.

However, effects of Tat were not observed in Triton X-soluble or Triton X-insoluble lysate fractions of glial cell lysates. It is possible that had we assessed different (e.g., later than 48 hours) time points, we may have observed a difference of TSPO with respect to its relative solubilty. It is also possible that increasing the sample size in these experiments could have provided the power to detect significant differences, as the variability (as indicated by standard deviation) was high in these fractionation experiments.

To further validate our findings of our *in vitro* model, we took advantage of mice expressing low-level Tat results from the "leaky" Tet-on promoter, which more closely mimics low levels of Tat found in the CNS of PLWH[7]. We studied the cortex and hippocampus, brain regions that have been shown to be more susceptible to Tat from previous studies[37]. Results generated from the *in vivo* model once again confirmed Tat

increased TSPO levels, there was only an increase in the cortical Triton X-soluble fraction. Significant differences were not observed in the whole cortical homogenates or Triton-insoluble fractions from the cortex. Furthermore, no effects of Tat were observed on levels of TSPO in hippocampal homogenates. It is very possible that our low sample size (n = 4 mice/group) limited our power to detect meaningful differences between groups.

Our findings may suggest the effects of Tat on TSPO are brain region-specific, whereby cortical neuronal cells are more sensitive than those from the hippocampus. Moreover, it is possible the effects of Tat on TSPO were diluted by the many different cell types that could be present in the brains of control vs. Tat transgenic mice, as TSPO has been shown to release mostly in glial cells[19]. The future study of different brain regions besides the cortex and hippocampus (e.g., the striatum or other brain regions harvested in this study) would validate if there is brain region specificity regarding the effects of Tat on levels of TSPO.

For better experimental design in the future, we performed power analyses to determine the sample size that would be required to obtain a meaningful test for significance difference in TSPO levels, in both cell lysates and brain homogenates **(Table 1)**. Based on G*power analysis[17], to test for meaningful significant differences, we would need a minimum of 11 and 17 wells/treatment for future analysis of Triton X-soluble and Triton X-insoluble levels of TSPO in glial cell lysates, respectively. We would need a minimum of n = 9-11 mice/group for *in vivo* analysis of TSPO in cortical brain homogenates, depending on the desired endpoint. Note the number of mice required to detect meaningful differences in the hippocampus are more variable, ranging from 4-35 mice/group depending on the endpoint.

Samples	Suggested sample size from G*power analysis (*n*)
MIXED GLIAL CELL LYSATES	
Cortical glial cell lysates - Triton X-soluble fractions	11
Cortical glial cell lysates - Triton X- insoluble fractions	17
BRAIN HOMOGENATES	
Whole cortical brain homogenates from Tat transgenic mice	11
Cortical brain homogenates from Tat transgenic mice - Triton X-insoluble fractions	9
Hippocampal whole brain homogenates	15
Hippocampal brain homogenates from Tat transgenic mice - Triton X-soluble fractions	35
Hippocampal brain homogenates from Tat transgenic mice - Triton X-insoluble fractions	4

Table 1: Suggested sample size for *in vitro* and *in vivo* data from G*power analysis to detect significant differences in TSPO levels.

In this study, our data did not support or validate the alteration of Tat on TSPO's solubility between soluble and insoluble fractions. However, if the suggested sample size is utilized in prospective experiments, we would have a more definitive insight on the relationship between Tat and TSPO. Mitochondrial membrane proteins, like TSPO, are important regulators of mitochondrial homeostasis of ion transport, ATP/ADP transport, and mitochondria fusion/fission. Therefore, defects in these proteins are associated with numerous diseases. If Tat alters TSPO's function on the mitochondria membrane, it can potentially lead to mitochondrial damage, which likely contributes to the onset and progression of neurodegenerative disease. One possible scenario is Tat might trigger the translocation of other proteins that interact with Tat. Thus, effects of Tat on the ability of TSPO to interact with other proteins is another area worthy of future exploration.

TSPO has been widely used as a marker of neuroinflammation, but the relationship between TSPO and HIV-Tat, and their underlying mechanism has not been strongly understood. In this study, we established a general relationship between TSPO and HIV-Tat, in the context of in *vitro* and *in vivo* models. Even though our results did not indicate robust significant differences between soluble and insoluble fractions, they confirm the role glial cells play in response to HIV-Tat. Further experiments are needed to better understand if TSPO regulates the functional response of HIV-Tat in cultured astrocytes, microglia, or neurons, or if Tat interacts with different cell cycle-related proteins, which may also relate to TSPO. These data also demonstrate that even very low-level expression of Tat (without induction by doxycycline in the CNS of these mice) may result in chronic glial cell activation.

The use of TSPO as a clinical diagnostic marker of brain injury and neuroinflammation has increased exponentially in the last decade, and to find the relationship between TSPO and HIV-Tat would further suggest its utility as a potential marker in the progression of HIV in HAND. Collectively, results of this study suggest the potential for TSPO to serve as a therapeutic target in HAND, but additional experiments would be required to validate this claim.

CHAPTER 5

FUTURE DIRECTIONS

Time course for effects of Tat on TSPO

In our study, we only measured levels of TSPO with Tat treatment after a fixed time point, 48 hours. Since Tat increases TSPO in glial cell lysates, different time points should be investigated to determine if TSPO appears to increase/decrease at other time points, especially in insoluble fractions when cells are treated with Tat in a shorter or longer time. These additional experiments may further elucidate the effects of Tat on the relative solubility, and in turn functional properties of TSPO.

Larger sample size in cell cultures and in Tat transgenic mice

As evidenced from *in vivo* data and our power analysis, a larger sample size could help allow us to test for meaningful differences regarding the effects of Tat on TSPO levels in our *in vitro* and *in vivo* models of HAND. A reasonable increase in sample size, as suggested by power analysis, would allow a meaningful test for significance. Moreover, aging may have also impacted TSPO levels in control and transgenic mice used in our study, as the age of mice used were 17-18 months old. Therefore, younger groups of mice should be considered in the future, to avoid the potential effects of aging that may have confounded our results.

Analysis of TSPO levels in different brain regions

In this project, we only focused on cortical and hippocampal homogenates. However, by performing future biochemical assays on different brain regions, we could determine if increasing TSPO or other biomarkers are brain region-specific, as a function of Tat expression. Furthermore, it will be prudent in future studies to also quantify level of Tat expressed in mouse brain tissue, as there may also be brain region-specific differences in the expression of Tat in these mice that may in turn cause differential effects on TSPO.

Doxycycline-inducible astrocyte-specific HIV-1 Tat transgenic mice (iTat)

In the present study, we focused on mice that were not treated with doxycycline, to take advantage of the known "leaky" promoter that induces low-level expression of Tat in these mice[7]. However, future experiments using doxycycline could determine if a more robust expression of Tat had a more profound influence on levels of TSPO in these mice. Doxycycline-inducible astrocyte-specific HIV-1 Tat transgenic mice will also allow us to define the direct effects of Tat on astrocytes and the molecular mechanism of Tat-induced GFAP expression/astrocytosis, astrocyte-mediated Tat neurotoxicity, Tat-impaired neurogenesis, Tat-induced loss of neuronal integrity, and exosome-associated Tat release and uptake[31]. Thus, the doxycycline-induced expression of Tat in these mice could provide additional proof of concept that Tat affects TSPO, suggesting a relationship between secretion of Tat in chronic HIV infection and alterations in TSPO, and their potential relationship in the pathogenesis of HAND.

Effects of Tat on other inflammatory markers and synaptic/cytoskeletal markers

Future studies should also quantify potential changes in other inflammatory markers like glial fibrillary acidic protein (GFAP) positive astrocytes or Iba-1 positive microglia, which we would expect to increase as per previous studies[7]. In addition, neuronal markers such as the cytoskeletal protein βIII-Tubulin, or the presynaptic vesicle protein p38 (Synaptophysin) could be analyzed, as they have been shown previously to decrease as a function of Tat expression in this mouse model[7].

Regulation of TSPO function using TSPO knock down mice and mice that over-express TSPO

Another possibility for a future study would be to employ lentiviral knock down of TSPO mice, or lentiviral-mediated over-expression TSPO mice, and access the mitochondria membrane potential together with oxidative stress analysis in those mice. This finding will provide more complete information, e.g., if stimulating TSPO in glial cells attenuates Tat-induced mitochondrial dysfunction, oxidative stress and secretion of pro-inflammatory cytokines. Conversely, with TSPO knockdown, we would expect to see an exacerbation of these neuronal stress endpoints.

Increasing TSPO levels/function in neurons affects their sensitivity to Tat

Our project focused on mixed glial cell cultures. However, by performing experiments on neurons, we could also determine their relative sensitivity to TSPO agonists or upregulation, and might suggest a direct, neuroprotective role of TSPO in CNS disease. In a model of multiple sclerosis, TSPO deficiency exacerbates astrocyte inflammatory

response, even though other studies using TSPO ligands suggest reduced inflammatory response[38].This may suggest off-target effects of TSPO ligands, and/or model-specific effects of TSPO that would require further investigation. We focused on glial cells in general, but not astrocytes or microglia specifically. Astrocytes, microglial or neuronal TSPO maybe important in regulating Tat function in HAND, thus a more careful analysis of each neuronal cell type would provide useful information regarding cell-type differences in response to Tat on TSPO in these cells.

TSPO localization and interaction with other proteins

There could be a possible change in the solubility of TSPO under Tat regulation, in particular when the CNS is responding to persistent neuroinflammation. Performing immunocytochemistry, or double-labeled immunofluorescence will provide an opportunity to observe if TSPO, under the presence of Tat, will localize and interact with Tat or different mitochondrial proteins in diseased states[38], for example, the voltage-dependent anion channel protein (VDAC), a protein that mediates the flux of ions, including $Ca^{2+,}$ nucleotides, and metabolites across the outer mitochondrial membrane. On the other hand, studying the interaction of TSPO with amyloid beta or alpha synuclein[40], may give us better understanding on how these putative interactions contribute to the mechanism of Tat regulating CNS dysfunction in PLWH.

SUMMARY

Among HIV-1 proteins, Tat is highly involved in regulating host cell response to viral infection and persists in the CNS of PLWH. In several preclinical studies, HIV Tat produces a marked neuroinflammatory response and is highly neurotoxic. Therefore, Tat may directly regulate the onset and progression of neurotoxicity seen in PLWH. To understand how Tat changes TSPO levels, a common biomarker for neuroinflammation, we utilized an *in vitro* mixed glial cell cultures together with an *in vivo* model of Tat transgenic mice. Our data indicates Tat increased TSPO levels in whole glial cell lysates; Significant effects of Tat on TSPO were also observed in Triton X-soluble fractions of cortical brain homogenates. While the relative effects of Tat on TSPO were limited, it is possible that many of our studies were under-powered to detect meaningful differences between groups. Our study has established a relationship between Tat and TSPO in preclinical models of HAND that may be bolstered by further experiments, and in turn a potential role of TSPO in regulating the neuropathogenesis of Tat. Future studies are warranted to explore the relative relationship between HIV-Tat and TSPO in preclinical models of HAND. These important preclinical studies may validate TSPO as a suitable marker to monitor the onset and progression of HAND, and as a possible therapeutic target for treating HAND.

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