

EFFECT OF TIMBD ON NEUROTOXICITY ASSOCIATED WITH HIV-1 GP120 AND

TAT

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by

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**EFFECT OF TIMBD ON NEUROTOXICITY ASSOCIATED WITH HIV-1 GP120
AND TAT**

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University of Missouri-Kansas City, 2018

ABSTRACT

Human Immunodeficiency Virus (HIV)-associated neurocognitive disorder (HAND) is one of the undermining disorders that affects majority of HIV infected patients. Patients with HAND suffer from both cognitive and motor dysfunction which is characterized by memory loss and changes in personality. HIV-1 gp120 and Tat are essential proteins that are involved in neurotoxicity. HIV-1 gp120 and Tat play a role in HAND associated oxidative stress and neuroinflammation produced in astrocytes, which is considered a major contributor for damage in CNS. HIV-1 Tat is able to affect behavior by modifying the neurotransmission genes. Natural compounds have been studied extensively as therapeutic intervention agents in neurodegenerative diseases. However, poor bioavailability and limited potency of natural compounds limited their successful use in humans. In an effort to develop a new chemical that can overcome the pitfalls associated with natural compounds, our laboratory has synthesized an analog of resveratrol 4-(E)-{(p-tolylimino)-methylbenzene-1,2-diol} (TIMBD). TIMBD is suggested to have powerful neuroprotective effects based on our preliminary findings. *The following study was based on the hypothesis that our newly synthesized compound TIMBD is able to decrease oxidative stress and neuroinflammation associated with gp120 in astrocytes and protects astrocytes from cell death. Furthermore, it is hypothesized that TIMBD improves the behavioral deficits associated with HIV-1 Tat using Tat-tg mice and the mechanism is through modulation of neurotransmitter genes.*

Additionally, TIMBD is hypothesized to decrease oxidative stress and neuroinflammation associated with HIV-1 Tat in astrocytes in vitro.

We investigated the effect of TIMBD on oxidative stress produced by gp120 in astrocytes. We measured oxidative stress markers in SVG astrocytes in response to gp120. We also measured levels of pro-oxidant and anti-oxidant enzymes in response to gp120 and TIMBD in SVG and primary astrocytes. We confirmed our results by measuring enzymes expression levels of pro- and anti-oxidant genes *in vivo* using protein extracted from mice brain tissues, which were gp120 gp120-transgenic (tg) and treated with TIMBD. We also determined the involvement of NRF2 transcription factor by measuring its expression in nuclear and cytoplasmic fractions in SVG astrocytes. We further measured cell death following TIMBD treatment on gp120- affected SVG astrocytes.

We investigated the effect of TIMBD on neuroinflammatory cytokines produced by gp120 in astrocytes. We measured expression of IL6, IL8 and CCL5 at RNA and protein levels following TIMBD or RES treatment on gp120-treated SVG astrocytes. The results were further confirmed by using immunocytochemistry technique that involved measuring inflammatory proteins in astrocytes using confocal microscopy. Next, the mechanism by which TIMBD decreased neuroinflammation associated with gp120 was determined. The expression of signaling proteins involved in AP1, STAT3 and NFκB pathways were measured at protein expression levels. The upstream signaling was further identified by measuring protein expression levels of p38MAPK, pAKT and pIKKs.

We investigated the effect of TIMBD on the behavioral deficits associated with HIV-1 Tat using Tat-transgenic (tg) mice. The anxiety-like behavior was assessed using open field test and light/dark box test following TIMBD treatment for wild type (WT)-control mice and Tat-

tg mice. The learning and spatial memory were determined using Morris water maze test which involved acquisition trials of 5 days and 1 day of probe trial. Next, the mechanism by which TIMBD improves behavioral deficits was determined by measuring protein expression levels of many neurotransmission proteins. These include neurotrophic factors BDNF and CNTF, the pre-synaptic proteins synapsin and synaptophysin, the post-synaptic proteins PSD95 and Arg3.1, and calcium signaling molecule pCAMK-II.

We studied the effect of TIMBD on HIV-1 Tat associated oxidative stress and neuroinflammation in astrocytes. We measured ROS production in SVG and primary astrocytes as response to HIV-1 Tat and TIMBD. We also measured levels of pro-oxidant and anti-oxidant enzymes in response to TIMBD and Tat in SVG astrocytes. Additionally, we measured expression of IL6 and IL8 RNA levels following TIMBD or RES treatment on Tat-transfected SVG astrocytes.

In conclusion, we demonstrated that TIMBD was able to decrease inflammation and oxidative stress associated with HIV-1 viral proteins in astrocytes. It is suggested that TIMBD provides the potential of being used as a neurotherapeutic candidate to prevent HIV-associated neurocognitive deficits.

APPROVAL PAGE

The faculty listed below, appointed by the Dean of the School of Graduate Studies have examined the dissertation titled “Effect of TIMBD on Neurotoxicity Associated with HIV-1 Gp120 and Tat” presented by Fatma Abdalla, candidate for the Doctor of Philosophy degree, and certify that in their opinion it is worthy of acceptance.

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ABBREVIATIONS

AD	Alzheimer's disease
AIDS	Acquired immunodeficiency syndrome
AKT	Protein kinase B
AL	Amyotrophic lateral sclerosis
ANI	Asymptomatic neurocognitive impairment
ANOVA	Analysis of variance
AP-1	Activator protein-1
ARE	Antioxidant Responsive-Element
Arg3.1	Activity regulated cytoskeleton-associated protein
ART	Antiretroviral therapy
ATF	Activating transcription factor
BBB	Blood-brain barrier
BDNF	Brain-derived neurotrophic factor
C	Cerebellum
CAMK-II	Calcium/calmodulin-dependent kinase II
cART	Combination ART
CCL	Chemokine ligand
cl-caspase	Cleaved-caspase
CM-H2DCFDA	5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor

CON	Control
COX2	Cyclooxygenase-2
CXCR4	α -chemokine receptor
CYP	Cytochrome P450
DAB	3'-Diaminobenzidine
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl Sulfoxide
DNPH	2,4-dinitrophenylhydrazine
Dox	Doxycyclin
DPI	Diphenyleneiodonium
FMO1	Flavin mono-oxygenase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFAP	Glial fibrillary acidic protein
GP120	Glycoprotein 120
GPX1	Glutathione peroxidase
GST	Glutathione S-transferase
H	Hippocampus
h	hour
H2DC-FDA	5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate
HAART	Highly active anti-retroviral therapy
HAD	HIV-associated dementia

HAND	HIV-associated neurocognitive disorders
HBMEC	Human brain endothelial cells
HFA	Human Fetal Astrocytes
HIV	Human Immunodeficiency Virus
HIVE	HIV encephalitis
HPRT	Hypoxanthine guanine phosphoribosyltransferase
HRP	Horse radish peroxidase
ICAM-1	Intercellular Adhesion Molecule 1
ICC	Immunocytochemistry
IKK	I κ B kinase
IL	Interleukin
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein 1
MFI	Mean fluorescence intensity
MMP	Matrix-metalloproteinases
MND	Minor neurocognitive disorder
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
MWM	Morris Water Maze
NAC	N-acetyl cysteine
NF- κ B	Nuclear factor-kappa B
NMDAR	N-methyl-D-aspartate receptor

NOS	Nitric oxide synthase
NOX	NADPH oxidase
NQO1	NAD(P)H Quinone Dehydrogenase 1
NRF2	Nuclear factor erythroid 2 (NFE2)-related factor 2
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline-Tween
PC	Parietal Cortex
PCR	Polymerase chain reaction
PD	Parkinson's disease
PFC	Prefrontal Cortex
PKC	Protein Kinase C
PNS	Peripheral nervous system
PSD95	Postsynaptic density protein-95
PVDF	Polyvinylidene difluoride
RES	Resveratrol
RIPA	Radioimmunoprecipitation assay
ROS	Reactive oxygen species
Rpm	Round per minute
RT-PCR	Reverse transcriptase-polymerase chain reaction
SEM	Standard error of mean
SIRT1	Sirtuin

SOD	Superoxide dismutase
SP1	Specificity protein 1
STAT3	Signal transducer and activator of transcription 3
TAT	Transactivator of transcription
Tg	Transgenic
TIMBD	4-(E)-{(p-tolylimino)-methylbenzene-1,2-diol}
TNF	Tumor necrosis factor
TUNEL labeling	Terminal deoxynucleotidyltransferase-mediated dUTP-nick end labeling
VCAM-1	Vascular cell adhesion molecule 1
WT	Wild type

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DEDICATION

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CHAPTER 1

GENERAL INTRODUCTION

1.1. Human Immunodeficiency Virus (HIV)

HIV infection was discovered following its first isolation from a patient with lymphadenopathy (Chiodi et al., 1988; Ho et al., 1985; John E. Bennett, 2014). Acquired immunodeficiency syndrome (AIDS) was then diagnosed as an attribution of HIV infection. HIV was then characterized as an RNA virus that belongs to a group of retroviruses (B. N. Fields, Knipe, & Howley, 1996). There are two different types of HIV viruses, HIV-1 and HIV-2. HIV-1 is considered more virulent and most common type. While HIV-1 is the common virus causing AIDS, HIV-2 is rare and infects only a smaller population due to its poor infectivity mostly found in West Africa (Faria et al., 2014; F. Gao et al., 1999; Sharp & Hahn, 2011). Worldwide, HIV-1 has extensive genetic variations, which are categorized into groups M, O and N, which are further subdivided genetically into nine categories (clades) from A to K (Osmanov et al., 2002). Among those clades B and C are considered the major circulating variants of HIV-1. Clade B represents most North America, western Europe and Australia infections while clade C is responsible for 50% of all HIV infections and represents mostly the infections in India and Africa (Atluri et al., 2015; Osmanov et al., 2002).

HIV has the capability to synthesize DNA from RNA that makes copies of itself and invades host cells/systems using reverse transcriptase. HIV attacks immune cells like CD4+ T cells and macrophages. HIV infection leads to a decrease of CD4+ T cells to low levels through a number of mechanisms, including pyroptosis of abortively infected T cells, apoptosis of uninfected bystander cells, direct viral killing of infected cells, and killing of

infected CD4+ T cells by CD8 cytotoxic lymphocytes that recognize infected cells (Cunningham, Donaghy, Harman, Kim, & Turville, 2010; Doitsh et al., 2014). As the CD4+ T cell numbers decline significantly, it results in loss of cell-mediated immunity and increase the susceptibility of the body to opportunistic infections.

1.2. HIV and global epidemic

According to recent United Nations Programme on HIV/AIDS (UNAIDS) and World Health Organization (WHO) statistics, in 2016, 36.7 million people living with HIV/AIDS worldwide (UNAIDS, 2017; UNAIDS/WHO, 2017) (**Fig. 1**). Around 1.8 million individuals became newly infected in 2016. Additionally, it is estimated that 1 million died from AIDS-related illness in 2016 (UNAIDS, 2017). Although sub-Saharan Africa still comprise the biggest number of HIV-infected population, there is a significant number of population are infected with HIV currently in the US. In the US, HIV testing has remained stable or increased in recent years. This decrease in diagnoses suggests a true decline in new infections. The decrease is considered as a result of targeted HIV prevention efforts (UNAIDS, 2017).

Summary of the global HIV epidemic

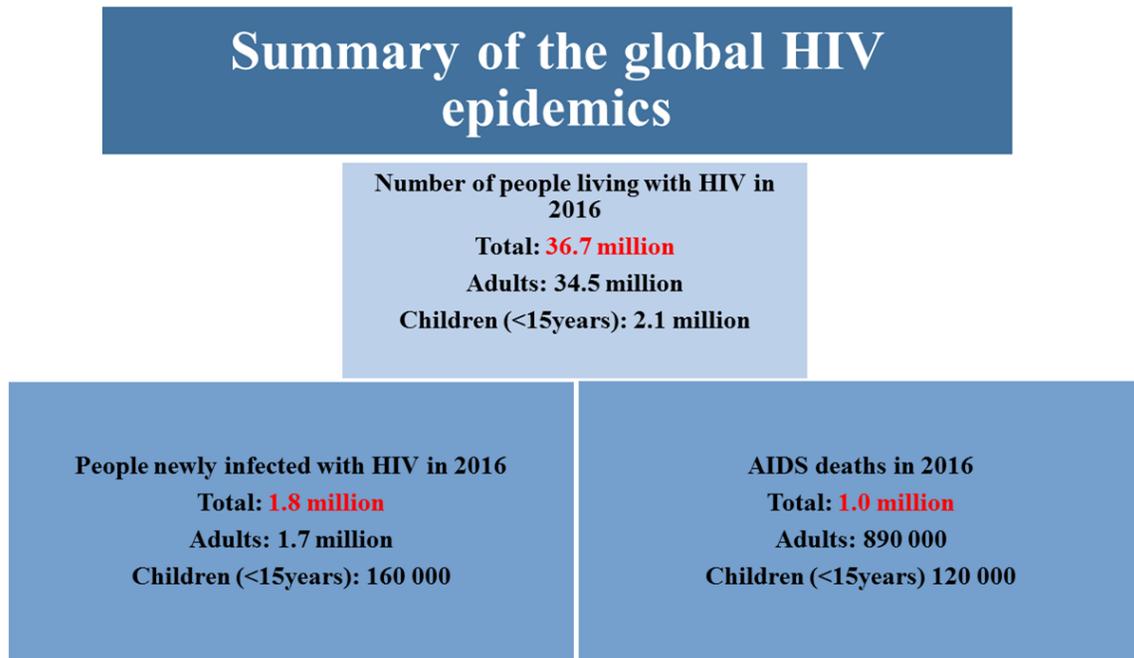


Figure 1. Statistics shows that in 2016, 36.7 million people were living with HIV/AIDS worldwide with around 1.8 million individuals became newly infected in 2016. Additionally, it is estimated that 1 million died from AIDS-related illness in 2016.

1.3. HIV infection and neuropathology

HIV infection can compromise different body systems including the central nervous system (CNS) leading to neuroAIDs which is associated with neurocognitive disorders. Although the introduction of antiretroviral therapy (ART) has limited the progression of HIV infection, the neuronal damage continues to critically affect HIV patients. HIV-neurocognitive disorders (HAND) patients suffer from cognitive, motor and behavioral impairments (Anthony & Bell, 2008; Bell, 1998). The clinical forms of HAND is classified into three categories, asymptomatic neurocognitive impairment (ANI), minor neurocognitive disorder (MND) and the most severe HIV-associated dementia (HAD) (McArthur & Brew, 2010). HIV encephalitis (HIVE) is considered the major cause of HAND and the most common neurological feature of HIV (Persidsky & Gendelman, 2003; Toborek et al., 2005). Availability of combination ART (cART) in recent years has led to a decrease in HIV RNA levels and thus in morbidity and mortality rates in HIV-infected population (Coiras, Lopez-Huertas, Perez-Olmeda, & Alcami, 2009). While cART has been successful, it does not eradicate disease reservoir cells including the resting memory CD4⁺ T cells, macrophages, and astrocytes in brain (Coiras et al., 2009; Gorantla, Poluektova, & Gendelman, 2012; Potter, Figuera-Losada, Rojas, & Slusher, 2013).

HIV initially invades the brain through the blood-brain barrier (BBB) by infected monocytes and lymphocytes. The virus then persists permanently in brain cells, mainly in microglia, macrophages, and astrocytes (Kaul, Garden, & Lipton, 2001; Tan & McArthur, 2012). HIV does not infect neurons, although neural progenitor cells are able to take up the virus (Bissel & Wiley, 2004). Neurotoxicity in patients with HIV-1 infection is thought to be mediated by HIV-1 proteins such as glycoprotein 120 (gp120) and transactivator of

transcription (Tat) protein, as well as other products released from infected cells (Banerjee, Zhang, Manda, Banks, & Ercal, 2010; Giunta et al., 2006; X. T. Hu, 2016; Nath, 2002; Ton & Xiong, 2013). The mechanisms of neurotoxicity are thought to be both direct and indirect and include glutamate excitotoxicity, oxidative stress, stimulation of inflammation, increase in apoptosis, altered calcium homeostasis, stimulation of tumor necrosis factor- α (TNF- α) and nuclear factor κ B (NF- κ B) (Nath et al., 2000; New, Maggirwar, Epstein, Dewhurst, & Gelbard, 1998), and stimulation of nitric oxide production (Raber et al., 1996).

Although the literature is not specific about how HIV infects the central nervous system (CNS), it is suggested to be via compromising the BBB integrity. Once the virus enters the damaged BBB, it enhances further viral entry. The compromise in BBB integrity is thought to be associated with neurocognitive deficits which may develop to form HAND (Annunziata, 2003; Louboutin & Strayer, 2012). It has been shown that the brain of HAND patients shows leakage of serum-protein across the BBB (Petito & Cash, 1992). Brains from HIV encephalitis (HIVE) patients have also shown abnormalities in the tight junction proteins occludin and ZO-1 (Dallasta et al., 1999). The BBB basal lamina is composed of extracellular matrix protein named laminin which acts as a substrate for matrix-metalloproteinases (MMPs). It is thought that viral proteins could trigger MMP activation which plays a role in degrading the BBB (Louboutin, Agrawal, Reyes, Van Bockstaele, & Strayer, 2010; Louboutin, Reyes, Agrawal, Van Bockstaele, & Strayer, 2011; Louboutin & Strayer, 2012). Studies have shown that HIV damage of the BBB might be mediated by a low number of infected astrocytes and exaggerated by gap junction proteins (Eugenin, Clements, Zink, & Berman, 2011).

1.4. Astrocytes in HAND

The major target cells for HIV are macrophages, microglia and astrocytes (Lassmann, Schmied, Vass, & Hickey, 1993; G. H. Li, Henderson, & Nath, 2016; McCarthy & Leblond, 1988). Astrocytes play an important role in maintaining HIV infection in the CNS. They act as a reservoir of HIV in the CNS which makes controlling HIV in the brain a challenging issue (G. H. Li et al., 2016). Astrocytes are considered the most abundant cells in the brain with a 10-fold greater abundance than neurons. This makes astrocytes very critical for HIV targeting in brain. Early studies had shown that HIV infection in the brain could occur only in macrophages and microglia. In the 1980s the first detection of HIV protein p25 in astrocytes was confirmed by glial fibrillary protein (GFAP) staining (Pumarola-Sune, Navia, Cordon-Cardo, Cho, & Price, 1987). Many *in vitro* and *in vivo* studies have demonstrated the presence of early produced viral HIV proteins although HIV infection can occur by reactivation from dormancy (G. H. Li et al., 2016; Messam & Major, 2000; Narasipura, Kim, & Al-Harhi, 2014). It is reported that astrocytes can be infected *in vivo* by HIV through two mechanisms (**Fig. 2**) (Anderson et al., 2003; Overholser et al., 2003; Tornatore, Chandra, Berger, & Major, 1994). The first mechanism is due to direct contact of HIV-infected lymphocytes with astrocytes which then leads to HIV transmission resulting in a productive infection of astrocytes. The second mechanism is suggested to be through inflammatory molecules, which are released from infected macrophages and/or microglial cells. This triggers upregulation of HIV receptors and coreceptors on astrocytes, making them highly permissive to HIV infection (G. H. Li et al., 2016). The contribution of astrocytes that are being triggered and become highly permissive to HIV infection makes them a very dangerous reservoir of the virus that can

be reactivated to produce virus to attack the peripheral organs and systems. Thus, astrocytes are considered as important target for controlling HIV infection in brain.

Astrocytes involvement in HIV mechanism

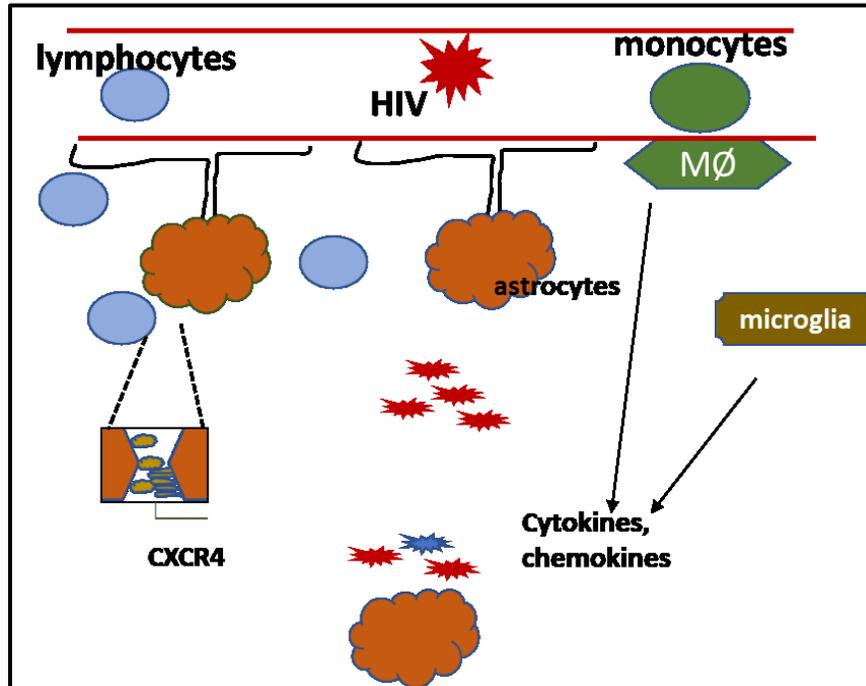


Figure 2. Astrocytes could be infected by HIV through two mechanisms. The first mechanism is due to direct contact of HIV-infected lymphocytes, which then leads to HIV transmission that can result in a productive infection of astrocytes. The second mechanism is suggested to be through inflammatory molecules, which are released from infected macrophages and/or microglial cells. This triggers upregulation of HIV receptors and coreceptors on astrocytes, making them highly permissive to HIV infection.

1.5. HIV viral proteins

HIV-1 is considered a retrovirus that comprises of an inner core with two single-stranded RNAs within an envelope. The HIV genome produces many viral proteins that play a major role in its neurotoxicity. These proteins include structural proteins such as Gag, Pol and Env and auxiliary proteins. Auxiliary proteins Tat and Rev have a regulatory function for viral replication. The other proteins Nef, Vif, Vpr, and Vpu are considered accessory proteins as they are not essential for the viral reproduction (Flint & American Society for Microbiology., 2009). The Env protein is a protein with a trimeric structure, comprised of three heterodimers of gp41 and gp120. These viral proteins localize differently within the virus according to their function. The nucleus contains the regulatory proteins which facilitate the viral mRNA production including initiation and elongation of transcription. The accessory proteins localize in the cytoplasm or the virion to assist with integration, release or viral replication. The structural proteins such as Env are responsible for virus assembly and entry of the virus into the host cell. Viral proteins are thought to be released when the rate of viral replication is high and viral particles are released. The unproductive infection of astrocytes results in production and release of Tat and other viral proteins.

1.5.1.1 HIV-1 gp120 and its role in HIV neuropathogenesis

Gp120 is one of the major HIV viral proteins that facilitates entry of the virus into the host cell (Checkley, Luttge, & Freed, 2011; Wilen, Tilton, & Doms, 2012; Yoon et al., 2010). Its interaction with the CD4 receptor and the α -chemokine receptor CXCR4 or β -chemokine coreceptors leads to conformational changes that mediate the viral entry into the host cell (Wilen et al., 2012). The virus entry into the host cell triggers the release of

inflammatory cytokines and other cellular products, which lead to neurotoxicity (Wilén et al., 2012; Y. Xu et al., 2004).

1.5.1.2. HIV-1 gp120 and oxidative stress

HIV-1 gp120 facilitates HIV viral replication and disease progression mediated by oxidative stress which subsequently affects the CNS (Samikkannu et al., 2015; Shah, Kumar, Simon, Singh, & Kumar, 2013). Oxidative stress is a process that involves formation of free radicals that are known to cause cellular damage in many diseases including HIV/AIDS (Louboutin & Strayer, 2014; Samikkannu et al., 2015). Published studies suggested that increased reactive oxygen species (ROS) levels can induce DNA damage which is correlated with cell cycle arrest (Liou & Storz, 2010; Masgras et al., 2012; Pyo, Choi, Oh, & Choi, 2013; Vafa et al., 2002). ROS are highly unstable oxygen-containing molecules that are released from cells in response to toxic damage or infection. Once ROS are released, the cell will induce anti-oxidant enzymes like superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase to overcome any oxidative stress. The imbalance between ROS production and antioxidant defense enzymes will result in an increased oxidative stress in the CNS.

Exposure to HIV-1 gp120 in animal studies causes disruption of the BBB through lesions in the microvessels, MMP activation and degradation of the basement membranes and vascular tight junction proteins leads to a loss of BBB integrity (**Fig. 3**) (Banerjee et al., 2010). The impairment of BBB by MMP increase is due to a decrease in tight junction proteins as a result of oxidative stress. This indicates gp120 might be working directly or indirectly to damage BBB. It is also reported that gp120 impairs neuron and astrocyte reuptake of glutamate process. That leads to prolonged activation of N-methyl-D-aspartate

receptor (NMDAR) with consequent disruption of cellular Ca^{2+} homeostasis. All these cascade of events that cause ROS production can lead eventually to neuronal cell death due to increase in mitochondrial permeabilization, cytochrome *c* release, and activation of caspases and endonucleases (Louboutin & Strayer, 2012; Price, Ercal, Nakaoke, & Banks, 2005).

Mechanism of BBB destruction by HIV viral proteins

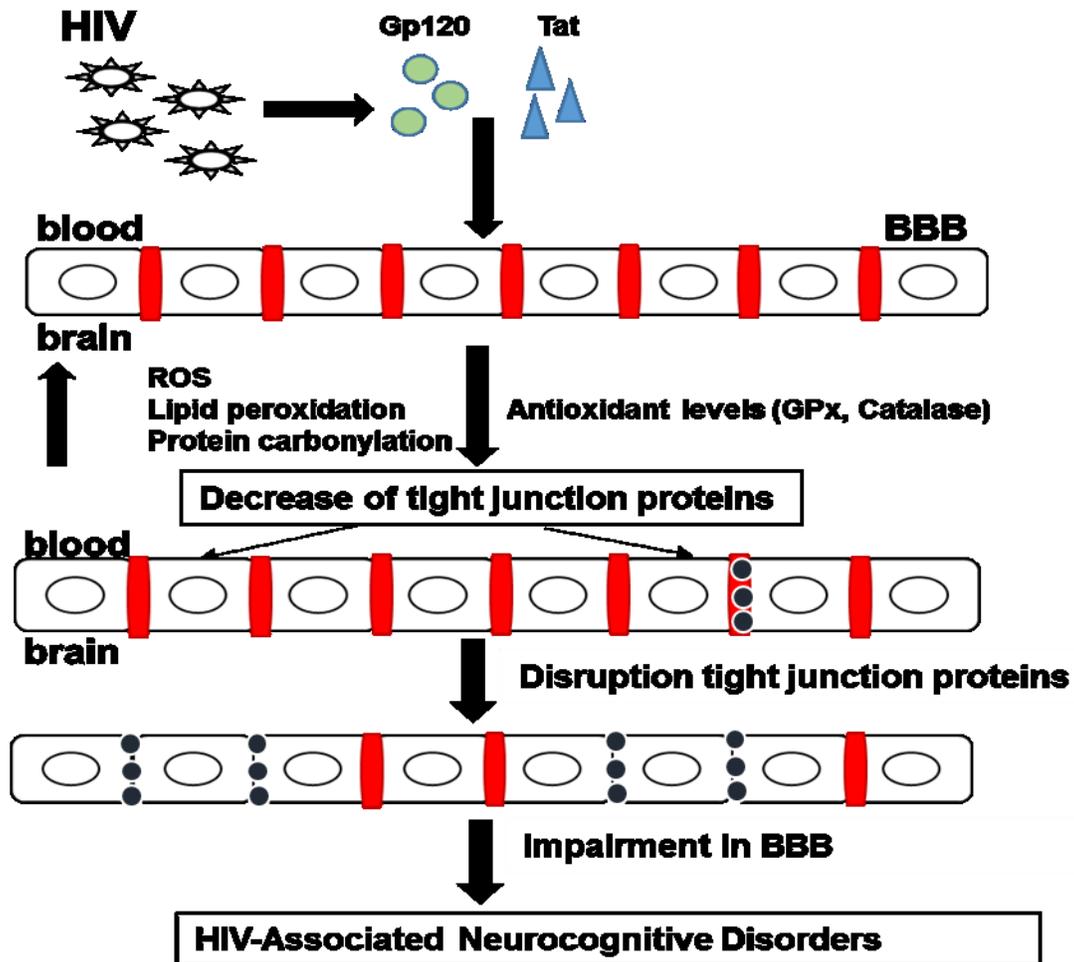


Figure 3. Suggested mechanism of HIV gp120 and Tat's mediated injury to BBB. (Figure adapted and modified from Banerjee, *et al* 2010 (Banerjee et al., 2010)).

1.5.1.3. HIV-1 gp120 and neuroinflammation

HIV-1 gp120 has been shown to induce a variety of inflammatory mediators like TNF α , IL6, IL8, and CCL5 (Ronaldson & Bendayan, 2006; Shah & Kumar, 2010; Shah, Verma, et al., 2011; M. C. Yeung, Pulliam, & Lau, 1995). Studies have shown that gp120 can induce release of IL6 in the brain endothelial cells of children (Stins et al., 2001). Gp120 can also trigger human brain endothelial cells (HBMEC) to secrete IL6 and IL8 which in turn leads to an increase in infected leucocyte adhesion and transendothelial migration through STAT pathways (A. Kim et al., 2009). Thus, STAT proteins which translocate to the nucleus are considered critical in gp120-induced BBB damage. Additionally, the crosstalk between MAPK, PI3K and PKC signaling can be involved in gp120-mediated damage to HBMEC (Kanmogne et al., 2007). Studies have shown that NF- κ B pathway is also involved in IL6 mediated secretion by gp120. Furthermore, phosphorylation of upstream regulators of NF- κ B including IKKs and I κ B α can lead consequently to induction of IL6 in astrocytes (Shah, Verma, et al., 2011). Furthermore, transcription factor AP1 is also considered a regulator of inflammation. It is composed of Fos, Jun, and ATF proteins (Herdegen & Waetzig, 2001). C-JUN is considered the most important member and has been correlated with neuronal cell death in many neurodegenerative diseases (Raivich, 2008). AP1 is reported to control IL6 mRNA transcription in human multiple myeloma cells (Xiao et al., 2004). Appropriate regulation of pro-inflammatory molecules is critical to inhibit or prevent inflammation.

1.5.2. HIV-1 Tat and its role in neuropathogenesis

HIV-1 Tat is one of the HIV viral proteins that play an important role in the expression and replication of the viral genome (Harrich, Ulich, Garcia-Martinez, &

Gaynor, 1997; Kameoka et al., 2002; Morrow, Park, & Wakefield, 1994). Many studies have reported the presence of HIV-1 Tat mRNA and protein in the brain of HIV patients (Del Valle et al., 2000; Hudson et al., 2000; Wiley, Baldwin, & Achim, 1996). Tat protein, secreted from infected cells, is able to exert its effect in uninfected cells through responsive genes including TGF β -1 promoter (Ensoli et al., 1993; Sawaya et al., 1998; Watson & Edwards, 1999). Studies have shown that HIV-1 Tat acts through modulating the expression of adhesion molecules like VCAM-1 and ICAM-1 in human endothelial cells (Dhawan, Singh, & Aggarwal, 1997). Additionally, HIV-1 Tat is suggested to impair BBB through decreasing occludin production levels (R. Xu et al., 2012). HIV-1 Tat can further increase VCAM-1 in both human vein endothelial cells and astrocytes (Song et al., 2007; Woodman, Benveniste, Nath, & Berman, 1999). In addition, studies have shown that HIV-1 Tat might play an important role in trafficking of monocytes into the CNS which is critical in the development of HIV-neurocognitive disorders (Rappaport et al., 1999).

1.5.2.1. HIV-1 Tat and oxidative stress

Previous studies have shown that HIV-1 Tat can freely penetrate neuronal cell membranes and can lead to an increase in oxidative stress markers like lipid peroxidation which resulted from the generation of ROS (Ivanov et al., 2016; Jeong et al., 2012). The oxidative stress is caused by the imbalance between production of free radicals and the ability of antioxidants defense enzymes to neutralize ROS production. Administering HIV-1 Tat protein in rat brains has been shown to cause an increase in protein oxidation which is suggested to result in oxidative damage of the neurons (Aksenov et al., 2001). HIV-1 Tat-induced oxidative stress was found to be mediated by NADPH-oxidase (NOX) enzyme in astrocytes that can increase adhesion of monocytes to astrocytes (Song et al., 2011).

NOX is a family of enzymes that are involved in the generation of ROS in many neurological disorders (Hernandes & Britto, 2012; G. H. Kim, Kim, Rhie, & Yoon, 2015; Song et al., 2011). Studies have also shown that HIV-1 Tat increases oxidative stress in brain endothelial cells (Price et al., 2005). Increase in ROS levels in brain causes an increase in MMP levels which is considered a major contributor to BBB dysfunction (Lakhan, Kirchgessner, Tepper, & Leonard, 2013; Pun, Lu, & Moochhala, 2009). Additionally, published studies have shown that HIV-1 Tat increased ROS levels in astrocytes which in turn lead to alteration in ICAM-1 and VCAM-1 adhesion molecules' levels (Song et al., 2007). The disruption of BBB by Tat may lead to transmigration of infected cells to the brain. This will lead eventually to the accumulation of inflammatory cells and cause neuropathological changes to the CNS (King, Eugenin, Buckner, & Berman, 2006).

1.5.2.2. HIV-1 Tat and neuroinflammation

HIV-1 Tat triggers neuroinflammation through perturbation of many signaling pathways including NF κ B, AP1 and SP1 signaling and subsequent upregulation of cytokines (Gonek et al., 2017; Lim & Garzino-Demo, 2000; Nath, Conant, Chen, Scott, & Major, 1999). HIV-1 Tat can induce the production of several cytokines, including IL6, IL8, IL2, TNF- α , and monocyte chemoattractant protein 1 (MCP-1) CCL2 (Buonaguro et al., 1992; Conant et al., 1998; Y. W. Lee, Hirani, Kyprianou, & Toborek, 2005; Sastry et al., 1996; Westendorp, Li-Weber, Frank, & Krammer, 1994). *In vitro* studies demonstrated that Tat treatment of mouse neurons led to activation of p38 and JNK MAPK pathways however only p38 MAPK pathway was required for Tat-mediated neuronal death (J. Cao et al., 2005; I. N. Singh et al., 2005). Tat-induced neurotoxicity was also shown to

dependent on caspase activity and many pathways are involved in Tat-associated neuronal cell death (I. N. Singh et al., 2004).

1.5.2.3. HIV-1 Tat and synaptic transmission

In the CNS, HIV-1 Tat is known to affect both glutamatergic and dopaminergic neurotransmission systems. Glutamate, which is an excitatory neurotransmitter, is critical in learning, memory, and synaptic plasticity (Rahn et al., 2012). However, an excess of extracellular glutamate may lead to excitotoxicity *in vitro* and consequently to neuronal cell death (Danbolt, 2001; Lewerenz & Maher, 2015; Nedergaard, Takano, & Hansen, 2002; Rahn et al., 2012). Glutamate excitotoxicity has been suggested as a potential mechanism that leads to neuronal injury in many neurological diseases including HAND (Sabri, Titanji, De Milito, & Chiodi, 2003). HIV-1 Tat is able to impair glutamate reuptake by astrocytes and increase the microglial glutamate release which in turn leads to elevation in the extracellular glutamate levels (Cisneros & Ghorpade, 2012; Gupta et al., 2010; Ton & Xiong, 2013). Excess glutamate levels as a result from HIV-1 Tat in the synaptic clefts, activate glutamate-gated ion channels and cause high levels of ion influx into neuronal cells leading to over-activation of downstream calcium ion-dependent effectors and signaling pathways which lead consequently to neuronal damage. The neuronal damage can then cause further release of intracellular glutamate into the extracellular space, affecting adjacent neurons as suggested in HAND (Gupta et al., 2010). Studies suggest that HIV-1 Tat can decrease presynaptic terminals by triggering NMDA receptor activity indicating that this loss is initiated by postsynaptic mechanisms [21]. Since Tat-mediated synaptic loss precedes the neuronal cell death [22, 23], targeting these steps might be a good strategy to reverse the synapse loss prior to neuronal death in HAND patients.

1.5.3. HIV-1 gp120 and Tat, and behavioral deficits

HIV-1 proteins gp120 and Tat might be responsible for cognitive and psychomotor impairment associated with HIV infection (Frankel & Young, 1998; Glowa et al., 1992; Power et al., 1998). GP120-transgenic mice showed neuropathological changes similar to human infected with HIV in both neocortex and hippocampus brain regions (Toggas, Masliah, & Mucke, 1996). Chronic glial expression of gp120 in mice resulted in open-field activity impairment and spatial reference memory deficits which is related to the neuromotor deficits associated with HAND (D'Hooge, Franck, Mucke, & De Deyn, 1999). HIV-1 Tat has also been reported to be involved in HIV associated cognitive impairments (Carey, Sypek, Singh, Kaufman, & McLaughlin, 2012; Rappaport et al., 1999). Studies revealed that clade-specific variation between HIV-1 viral strains might be attributed to *tat* gene mutations indicating its critical role in HAND development and variations within patients (Ranga et al., 2004).

1.6. Natural Compounds

1.6.1. Natural compounds and neurodegeneration

Natural compounds like polyphenols have been considered for therapeutic intervention for decades (Mileo & Miccadei, 2016; Pandey & Rizvi, 2009). The most studied effect of polyphenols is their antioxidant activity. It is well known that oxidative stress has a critical role in the pathophysiology of neurodegenerative diseases and many other types of human illnesses (Aluise et al., 2010; Athar, Back, Kopelovich, Bickers, & Kim, 2009; Bagatini et al., 2016; Gotz, Freyberger, & Riederer, 1990; Huang et al., 2010; Joshi et al., 2010; Niranjana, 2014; Reddy, Agudelo, Atluri, & Nair, 2012; B. Singh et al., 2014; Vilar-Pereira et al., 2016). The decrease in cellular reserve of the antioxidant pool

and the neuronal damage due to oxidative stress are major pathological aspects of neurodegenerative disorders including Parkinson's disease (PD), Alzheimer's disease (AD) or Amyotrophic lateral sclerosis (ALS) (Campese et al., 2004; Capani et al., 2003; Gutowicz, 2011; Halliwell, 1992; Radwanska-Wala, Buszman, & Druzba, 2008; Weinreb, Mandel, Amit, & Youdim, 2004). Polyphenols produce their antioxidant effects through different mechanisms for example inducing the expression of protective genes against oxidative stress, regulation of ROS by interacting with oxidative pathways and scavenging metal ions (Hussain et al., 2016; Kelsey, Wilkins, & Linseman, 2010; Mao, Gu, Chen, Yu, & He, 2017; Nagpal & Abraham, 2017; Qi et al., 2017). Many studies have been performed to test natural compounds like curcumin, flavonoids, and Resveratrol (RES) (Mohammadi-Bardbori, Bengtsson, Rannug, Rannug, & Wincent, 2012; Niedzwiecki, Roomi, Kalinovsky, & Rath, 2016; Panda, Chakraborty, Sarkar, Khan, & Sa, 2017)

1.6.2. Resveratrol

1.6.2.1. Resveratrol and neurodegeneration

Resveratrol has been shown to have a neuroprotective activity in many neurological disease models. For example, RES can increase β -amyloid clearance that is related to Alzheimer's disease (F. Li, Gong, Dong, & Shi, 2012; Marambaud, Zhao, & Davies, 2005). *In vitro* studies have revealed that RES protects neuroblastoma cell line against dopamine (M. K. Lee, Kang, Poncz, Song, & Park, 2007). Additionally, RES protects the cortical and hippocampal neurons due to glutamate toxicity (Fukui, Choi, & Zhu, 2010). Resveratrol has also been tested for other neurological diseases like, Huntington's disease, PD, epilepsy, and ALS (Rocha-Gonzalez, Ambriz-Tututi, & Granados-Soto, 2008; Wang et al., 2002; Wang et al., 2004). Resveratrol's neuroprotective effect against HAND and

neurodegeneration is not clear (Al Dera, 2017; Bonsack, Alleyne, & Sukumari-Ramesh, 2017; Koronowski et al., 2017; N. Singh, Agrawal, & Dore, 2013). Resveratrol has been shown to have both anti-inflammatory and anti-oxidant effects under different conditions (Upadhyay, Singh, Kumar, Prakash, & Singh, 2008). In an *in vitro* model of AD, RES protected PC12 cells from amyloid β peptide-induced toxicity through decreasing accumulation of ROS (Jang & Surh, 2003). Resveratrol was also found to protect hippocampal neurons from amyloid β peptide related cell death through PKC modulation (Han, 2003). Resveratrol has been also reported to result in proteosomal degradation of amyloid β peptide in AD *in vitro* model leading to more protective effect (Marambaud et al., 2005).

1.6.2.2. Resveratrol and oxidative stress

Resveratrol has been studied extensively as antioxidant and has been shown to scavenge ROS production through stimulating the activity of many antioxidant enzymes (de la Lastra & Villegas, 2007). The antioxidant property of resevratrol is attributed to the phenolic hydroxyl group that plays an important role in electron delocalization within its chemical structure (Aggarwal & Shishodia, 2006). Zini *et al* have suggested that RES exters its antioxidant effect through three different mechanisms (Zini, Morin, Bertelli, Bertelli, & Tillement, 1999). These mechansims include competing with coenzyme Q and thus decreasing the oxidative chain complex, scavenging superoxide radicals in mitochondria and inhibiting lipid peroxidation occurred due to Fenton raction (Z. Cao & Li, 2004; Fang et al., 2002; Zini et al., 1999). It is believed that resevratrol can maintain levels of antixodiant enzymes intracellularly (Losa, 2003; N. Singh et al., 2013). Resveratrol can also increase levels of glutathione in human lymphocytes. Additionally, RES has also been

shown to induce an increase in GPx, glutathione S-transferase (GST) and glutathione reductase enzymes (Losa, 2003; Sadi, Bozan, & Yildiz, 2014). Some studies showed an opposite finding for resveratrol for example, no effect on xanthine oxidase system or superoxide levels in rat macrophages (Orallo et al., 2002).

1.6.2.3. Resveratrol and inflammation

Resveratrol have been reported to control many inflammatory diseases. Resveratrol is reported to decrease pro-inflammatory mediators' synthesis and release, eicosanoid synthesis, active immune cells, and inflammatory enzyme cyclooxygenase-2 (COX2) levels (de la Lastra & Villegas, 2005). Resveratrol-mediated anti-inflammatory activity is correlated with NF κ B and AP1 transcription factor inhibition (Adhami, Afaq, & Ahmad, 2003; de la Lastra & Villegas, 2005; Ma, Wang, Dong, Li, & Cai, 2015; Ren et al., 2013; Zhu et al., 2011). Resveratrol mediated inhibition of NF κ B plays a role in its effect in preventing protein degradation (Dirks Naylor, 2009). Resveratrol might be inhibiting NF κ B nuclear translocation through suppression of I κ B-kinase (IKK) (Holmes-McNary & Baldwin, 2000) thus leading to inhibition of β amyloid peptide induced-NF κ B-p65 nuclear translocation (Jang & Surh, 2003). Resveratrol has been shown to suppress NF κ B activity in many diseases (Adhami et al., 2003; Ma et al., 2015; Ren et al., 2013; Zhu et al., 2011) through increasing sirtuin (SIRT1) catalytic activity (Albani, Polito, Signorini, & Forloni, 2010). RES has the ability to bind the N-terminal of SIRT1 which leads to a significant increase in its activity (Borra, Smith, & Denu, 2005).

1.6.5. Pitfalls of natural compounds

Natural compounds have shown success in various model systems including rat, mouse and human cell lines. However, clinical translation has been always a big issue. The

translational limitations of natural compounds are due to a variety of reasons, mainly the lack of specificity and bioavailability. Studies demonstrated controversial results using natural compounds including resveratrol in neurodegenerative diseases experimentally (Sato et al., 2013). In a study conducted to study the anti-aging properties of RES in animals, RES showed same effects as calorie-restricted diets in improving aging indicating it was not effective. Despite all the findings on natural compounds, there is still lack of evidence about their therapeutic effects in humans (Kjaer et al., 2015; Tome-Carneiro et al., 2013). Poor bioavailability and limited potency have been identified as the main issue related to natural products (Ginsburg & Deharo, 2011; Walle, 2011). Moreover, pharmacokinetic studies in healthy human subjects have revealed that RES is extensively metabolized in the liver with an oral bioavailability of less than 1% (Walle, 2011). Clinical trials failed to prove RES' efficacy in a disease setting (Nguyen et al., 2009; Subramanian et al., 2010). In a systematic review for RES' therapeutic effects, it is concluded that the published data regarding RES is not sufficient to justify its use in humans (Vang et al., 2011). Although animal data has presented some promising results, RES' benefits and toxicity in humans remains uncertain (Soleas, Diamandis, & Goldberg, 1997; Vang et al., 2011).

1.6.6. Resveratrol Analog TIMBD

Although RES has multi-target functioning in different disease models, its use as therapeutic drug candidate has not been useful. Recent studies have aimed on modifying the structure of RES to improve its potency, efficacy, target specificity, metabolism and its serum concentrations. Our laboratory has synthesized RES analogs in an effort to improve its therapeutic properties (Siddiqui et al., 2013). Resveratrol was modified by introducing

an aza-linkage and adding a methyl group to improve its potency and lipid solubility. The analogs were initially tested for their chemotherapeutic effects against breast cancer. Two analogs were found to be effective in decreasing breast cancer cells growth *in vitro* (Siddiqui et al., 2013) **Fig. 4**.

Our laboratory has previously reported the effectiveness of TIMBD in decreasing proliferation of breast cancer cells (Chatterjee et al., 2017; Ronghe, Chatterjee, Singh, et al., 2016; Siddiqui et al., 2013). The mechanisms suggested included interference with estrogen receptors and modulation of oxidative stress (Chatterjee et al., 2017; Ronghe, Chatterjee, Singh, et al., 2016; Siddiqui et al., 2013). TIMBD has been shown to be effective in decreasing the cellular growth of breast cancer cells through differential regulation of estrogen receptors α and β and consequently cell cycle molecules (Ronghe, Chatterjee, Singh, et al., 2016). The laboratory further found that TIMBD exerts an antioxidant effect that protects normal cells more than cancer cells. The antioxidant effect is thought to be through modulation of the NRF2 transcription factor and its downstream antioxidant enzymes (Chatterjee et al., 2017). Our laboratory further aimed to study the effectiveness of the analog TIMBD as a neuroprotective agent with its potential use in HAND. Since our target for treatment in HAND is the brain, we conducted a preliminary study to test whether TIMBD could penetrate BBB using *in vivo* mouse model. Our preliminary studies showed that TIMBD can cross the BBB freely using experimental mice. This indicates that TIMBD can enter the brain easily and can potentially produce its desired neuroprotection/neurotherapeutic effects.

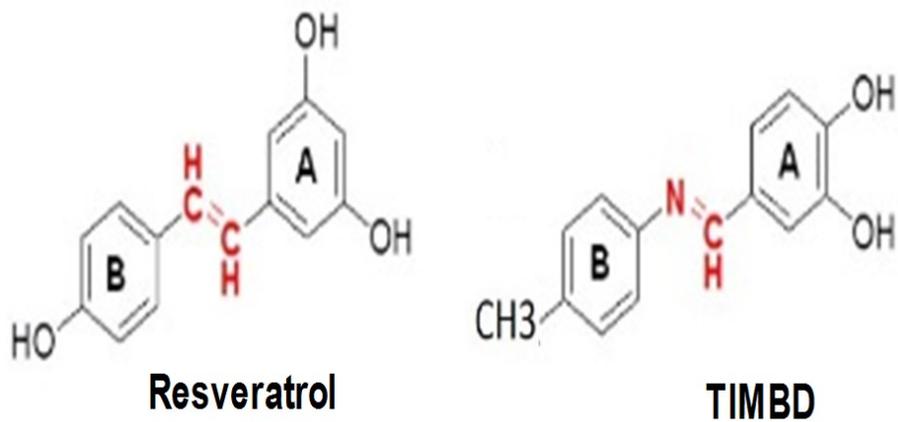


Figure 4. Chemical structure of RES and TIMBD.

CHAPTER 2

HYPOTHESIS AND SPECIFIC AIMS

2.1. Hypothesis

This dissertation study was based on the hypothesis that TIMBD can decrease the neurotoxicity associated with HIV-1 gp120 and HIV-1 Tat. TIMBD is hypothesized to achieve this effect by modulation oxidative stress, inflammatory cytokines' production and neurotransmission genes expression. The goal of this study is to elucidate the therapeutic effects of TIMBD in suppressing inflammation and oxidative stress and recovering lost neurotransmission signals due to HIV. As a result, TIMBD may improve the neurocognitive symptoms associated with HIV and control progression of HAND.

2.2. Specific Aims

In this study, we aimed to identify the effect of TIMBD on alleviating neurotoxicity associated with HIV-1 gp120 and Tat through the following aims:

- To determine the effect of TIMBD in modifying oxidative stress-mediated damage and consequent cell death induced by gp120 using astrocytes. We will also study the anti-oxidant effect of TIMBD using gp120-transgenic mice treated with either TIMBD or RES.
- To determine the effect of TIMBD on modulating the inflammatory mediators induced by gp120 in astrocytes. In addition, we will study the effect of TIMBD on the upstream mechanisms involved in gp120-associated neuroinflammation.
- To determine the effect of TIMBD in modulating the HIV-Tat-associated behavioral deficits associated with Tat and the involvement of neuroplasticity genes using TAT-transgenic mice.

- To determine the effect of TIMBD on modulating neuroinflammation and oxidative stress associated with HIV-gp120 and -Tat in astrocytes. In addition, we will study the effect of TIMBD on upstream mechanisms associated with the neuroinflammation and oxidative stress.

The findings of these studies will have clinical importance in introducing TIMBD as a potential candidate that can be used to control HAND through multiple mechanisms including, decreasing oxidative stress and inflammation, and recovering neurotransmission loss due to HIV. Altogether, TIMBD's suggested aims could lead to its use as interventional therapy in HAND patients.

CHAPTER 3

GENERAL MATERIALS AND METHODS

3.1. Cell culture and chemicals

All experiments were performed using SVG astrocytes (a clone of human astrocytes SVG) (Major et al., 1985) unless stated otherwise. Human Fetal Primary astrocytes (HFA) were obtained from aborted fetal brain tissues (obtained from BDRL, Seattle, WA, USA). The cells were cultured in Dulbecco's modified Eagle's Medium (DMEM) that was supplemented with 10% fetal bovine serum and 1% non-essential amino acids, 1% sodium bicarbonate, 1% L-glutamine and 0.1% gentamicin. The cells in culture were maintained at 37°C with 5% CO₂. HIV1-gp120 (pSyn-gp120 JR-FL) plasmid used for transfection was obtained as described (Catalog # 4598, NIH AIDS research) (Shah, Verma, et al., 2011). Recombinant HIV-1 IIIB gp120 was obtained from the NIH AIDS reagents program as described in previous publications (Shah, Verma, et al., 2011). Resveratrol (RES) was purchased from Sigma–Aldrich (St. Louis, MO). Resveratrol analog, 4-(E)-{(p-tolylimino)-methylbenzene-1,2-diol} (TIMBD) was synthesized and purified by our laboratory as reported previously (Siddiqui et al., 2013). Resveratrol and TIMBD were dissolved in dimethyl sulfoxide (DMSO) prior to treatments. The concentration of DMSO in all experiments was always 1/1000th (vol/vol) of the final medium volume. All antibodies used have been described in detail in Table.1.

3.2. Transient transfection

SVG astrocytes were transiently transfected with 2 µg HIV1-gp120 or 0.3 µg HIV1-Tat plasmid using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA) as previously described (Abdalla, Nookala, Padhye, Kumar, & Bhat, 2017). Briefly,

SVG astrocytes were plated either in 0.5×10^6 cells or 0.25×10^6 cells per well in 6 or 12-well plates one day prior to treatments and allowed to adhere overnight. On the day of the experiment, cells were washed twice with 1 ml phosphate-buffered saline (PBS), 1 ml of serum-free DMEM were added. For treatments, cells were treated 1 h prior to transfection with either TIMBD or RES. The transfection mix containing Lipofectamine and Optimem (reduced serum medium obtained from Thermo Fisher Scientific, Waltham, MA) mixture with or without the plasmid were added per well to 6-well plate. Cells were washed with 1 ml PBS twice after 5 h of transfection, and media was replaced with 1 ml serum-supplemented DMEM.

3.3. Reverse transcription and real-time PCR

Real-time polymerase chain reaction (PCR) was used to quantify mRNA expression levels of various genes as indicated. Cultured cells were lysed using the IBI-RNA kit (Midwest Scientific, St Louis, MO) as suggested by the manufacturer. Briefly, cells were lysed using 350 μ l of IBI lysis buffer for 5 minutes at room temperature. The lysed cells were then transferred to a 1.5 ml centrifuge tube and centrifuged for 1 minute at 14,000 rpm. The isolated RNA was then precipitated using 350 μ l absolute ethanol, then transferred to RB column tube placed on 2 ml collection tube. The columns full of RNA were pre-washed by adding 400 μ l pre-wash buffer followed by spinning for 30 seconds at 10,000 rpm. The isolated RNA was then washed twice using 600 μ l wash buffer and centrifuged for 30 seconds at 10,000 rpm after each wash. After drying the columns for 3 minutes at room temperature, 50 μ l RNAase-free water was added to dissolve the RNA. The RNA was then collected by centrifuging the columns over pre-labelled collection tubes for 1 minute at 10,000 rpm. Concentration of RNA was measured using Nanodrop ND-

1000 (Thermo Fisher Scientific, Nanodrop instrument, Waltham, MA) and 100 ng was used to measure the mRNA expression levels of various genes using real-time reverse transcription (RT-PCR). Primer sequences and conditions for RT-PCR have been described in Table 1 reported previously in the following publications (Shah & Kumar, 2010; Shah, Verma, et al., 2011). The expression of hypoxanthine phosphoribosyl transferase (HPRT), a housekeeping gene, was used for quantification and standardization purposes, as reported previously (Shah & Kumar, 2010; Shah, Verma, et al., 2011). The expression levels of IL6, IL8 and CCL5 relative to HPRT were determined by using the $2^{-\Delta\Delta Ct}$ method.

3.4. Western Blotting analysis

Total protein was extracted from whole cell lysates using radioimmunoprecipitation assay (RIPA) buffer (Boston Bioproducts, Ashland, MA). Following the addition of 200 μ l RIPA buffer to the cells, the lysate was homogenized and then spun at 10,000 rpm for 10 minutes and supernatant was collected. For nuclear and cytoplasmic extractions, the cells were trypsinized using 250 μ l of 0.25% trypsin for 2 minutes at 37°C. The cells were then collected and centrifuged at 10,000 rpm for 5 minutes. The cell pellet was suspended in 300 μ l for cytoplasmic extraction buffer (10 mM HEPES, 50 mM NaCl, 0.5 M sucrose, 0.1 mM EDTA and 0.5% TritonX-100), followed by incubation on ice for 10 minutes and washing with 500 μ l PBS. For nuclear extraction, 200 μ l nuclear extraction buffer (10mM HEPES, 500 mM NaCl, 0.1mM EDTA, 0.1 mM EGTA and 0.1% IGEPAL) was added to the cell pellet and incubated on ice for 15 minutes. Nuclear protein was collected after spinning for 10 minutes at 14,000 rpm. Protein concentration was determined using BCA kit (Pierce Biotechnology, Rockford, IL).

Twenty to forty micrograms of total protein (amounts may differ by different protein to be analyzed) were size fractionated on 12% SDS-polyacrylamide gel at 90V for 3 hours, and transferred using transfer buffer to Polyvinylidene difluoride (PVDF) membranes at 100V for 90 minutes. Membranes were blocked in 5% dry non-fat milk/PBS/0.05% Tween-20 (PBST) at room temperature for 1 h. The membranes were incubated with primary antibodies diluted as described in Table 2 in PBST overnight at 4°C followed by washing with PBST for three times 7 minutes each time. Following that, membranes were incubated with the corresponding secondary antibody, diluted as described in Table 2 in PBST at room temperature for 1.5 h. After incubation, membranes were washed and chemiluminescent detection was performed using the BM Chemiluminescence Detection kit (Roche, Indianapolis, IN) and the FluorChem HD2 Imaging system (Alpha Innotech Corporation, San Leandro, CA), with AlphaEaseFC Image Analysis software (Alpha Innotech Corporation, San Leandro, CA). Membranes were probed for GAPDH and Lamin-B for normalization of protein expression levels.

Table 1: List of primers

No.	Gene	Primer Sequence	PCR Conditions			
			Reverse Transcription	Denaturation	Annealing	Extension
1	IL6	Fwd:5'-GGT ACA TCC TCG ACG GCA TC-3'	50°C for 30min 95°C for 15min	95°C for 15 Sec	57.5°C for 1 min	
		Rev: 5'-CCA GTG CCT CTT TGC TGC TT-3'				
		Probe: 5'-FAM-CAG CCC TGA GAA AGG AGA CAT GTA ACA GGA GGT AA-3'-BHQ1				
2	IL8	Fwd: 5'-CTC TTG GCA GCC TTC CTG ATT-3'	50°C for 30min 95°C for 15min	95°C for 30 Sec	61°C for 30 sec	
		Rev: 5'-TAT GCA CTG ACA TCT AAG TTC TTT AGC A- 3'				
		Probe: 5'-FAM-CTT GGC AAA ACT GCA CCT TCA CAC AGA-3'BHQ1				
3	CCL5	Fwd: 5' ACC AGT GGC AAG TGC TCC A-3'	50°C for 30min 95°C for 15min	95°C for 15 Sec	54°C for 30 sec	76°C for 30 sec
		Rev: 5' ACC CAT TTC TTC TCT GGG TTG GCA -3'				
4	HPRT	Fwd: 5'-GCT TTC CTT GGT CAG GCA GTA-3'	50°C for 30min 95°C for 15min	95°C for 15 Sec	55°C for 30 sec	76°C for 30 sec
		Rev: 5'-CCA ACA CTT CGT GGC GTC CTT T-3'				

Table 2: List of antibodies

Number	Name	Cat.No.	Company	Dilution
1	GAPDH Rabbit mAb	2118L	Cell Signaling	(1:2000)
2	gp91-phox (NOX2) goat pAb	sc-5827	Santacruz	(1:1000)
3	Lamin B goat pAb	sc-6216	Santacruz	(1:2000)
4	p-CaMKII (T286) Rabbit Ab	3361S	Cell Signaling	(1:2000)
5	Synaptophysin Rabbit Ab	5461S	Cell Signaling	(1:2000)
6	SHANK2	12218S	Cell Signaling	(1:2000)
7	Synapsin-1	5297S	Cell Signaling	(1:4000)
8	P-c-Jun S73	3270S	Cell Signaling	(1:2000)
9	PSD95	3409S	Cell Signaling	(1:2000)
10	BDNF rabbit mAb	Ab108319	Abcam	(1:2000)
11	Arg3.1 rabbit pAb	Ab23382	Abcam	(1:2000)
12	CNTF rabbit pAb	Ab46172	Abcam	(1:2000)
13	NQO1 mouse mAb	3187S	Cell Signaling	(1:2000)
14	GPX1 Rabbit mAb	3286S	Cell Signaling	(1:1500)
15	Catalase Rabbit mAb	12980S	Cell Signaling	(1:1500)
16	Cleaved Caspase-3 Rabbit mAb	9664L	Cell Signaling	(1:1500)
17	c-Jun Rabbit mAb	9165S	Cell Signaling	(1:2000)
18	c-Fos Rabbit mAb	2250S	Cell Signaling	(1:2000)
19	Phospho-c-Fos Ser32 Rabbit mAb	5348S	Cell Signaling	(1:2000)
20	NF-KappaB p65 Rabbit mAb	8242S	Cell Signaling	(1:2000)
21	P-AKT S473 Rabbit mAb	4060S	Cell Signaling	(1:2000)
22	AKT1 Rabbit Mab	2938s	Cell Signaling	(1:2000)
23	p-IKK-alpha S176 - Rabbit mAb	2078S	Cell Signaling	(1:2000)
24	pSTAT3 Y705 Rabbit mAb	9145S	Cell Signaling	(1:2000)
25	STAT3 Rabbit mAb	4904S	Cell Signaling	(1:2000)
26	P38-gamma MAPK Rabbit Ab	2307S	Cell Signaling	(1:2000)
27	IkB-alpha Rabbit Mab	9242s	Cell Signaling	(1:2000)
28	pP38 MAPK Mouse Mab	SC-7973	Santacruz	(1:1000)
29	SOD1 Rabbit pAb	SC-11407	Santacruz	(1:1000)
30	FMO1 Rabbit pAb	Sc-98502	Santacruz	(1:1000)
31	MMP9 Mouse Mab	Sc-21733	Santacruz	(1:1000)
32	SOD3 Rabbit Pab	SC-67089	Santacruz	(1:1000)
33	anti-rabbit IgG	7074S	Cell Signaling	(1:2000)
34	Donkey anti-goat IgG	Sc-2020	Santacruz	(1:2000)
35	Goat anti-mouse IgG	Sc-2005	Santacruz	(1:2000)
36	rabbit anti-IL-6	Ab6672	Abcam	(1:500)
37	rabbit anti-IL-8	Sc-7922	Santacruz	(1:500)
38	rabbit anti-CCL5	PA1-84182	Thermo Fisher	(1:500)
39	GFAP (GA5) Mouse mAb	3760S	Cell Signaling	(1:500)
40	rabbit IgG (conjugated with Alexa Fluor 488)	#4340	Cell Signaling	(1:500)
41	mouse IgG (conjugated with Alexa Fluor 555)	#4409	Cell Signaling	(1:500)

3.5. Measurement of reactive oxygen species (ROS) production

To measure oxidative stress produced in astrocytes, 0.25×10^6 SVG astrocytes were seeded per well in 12-well plate and allowed to adhere overnight. Next day, cells were treated with either TIMBD or RES followed by transient transfection with HIV1-gp120 plasmid as described in the previous section. Subsequently, the cells were washed twice with PBS and incubated with $5 \mu\text{M}$ 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, (CM-H₂DCFDA) (Molecular Probes, Carlsbad, CA) for 30 minutes in PBS at 37°C. Following that, the cells were washed twice with PBS to remove any unloaded dye. The fluorescence intensity was measured using FITC wavelengths in a FACSCanto II Flow Cytometer (BD Biosciences, San Jose, CA). Unstained cells were used as negative controls and cells treated with $500 \mu\text{M}$ H₂O₂ prepared in PBS as positive controls. The mean fluorescence intensities (MFI) proportional to ROS generation were compared between different treatments and with HIV1-gp120 group.

3.6. Protein carbonylation assay

The measurement of carbonylated protein was performed using OxiSelect™ Protein Carbonyl ELISA Kit (Cell Biolabs, San Diego, CA) as per manufacturer's protocol. Briefly, SVGA cells were treated with either TIMBD or RES and lysed with RIPA lysis buffer followed by homogenization. The protein samples were verified to be free of nucleic acid contamination by measuring the absorbance at 260 nm and 280 nm. The ratio of 280/260 greater than 1 indicates pure protein sample. 100 μl of 10 $\mu\text{g}/\text{mL}$ proteins prepared in PBS were used for each well for the assay. The lysates were mixed with 100 μl of 0.04 mg/mL of 2,4-Dinitrophenylhydrazine (DNPH) prepared in DNPH diluent provided by the manufacturer. The mixture was then incubated in dark for 45 minutes at room temperature.

The wells were washed 5 times with 250 μ L of PBS/Ethanol (1:1, v/v) for 4 minutes each. After removing excess solution by pipetting, wells were washed 2 times with 250 μ L PBS. The surface of each well, was then blocked using 200 μ L of Blocking Solution provided by manufacturer per well for 2 h at room temperature followed by 3 times washing with 250 μ L of wash buffer. Next, the wells were incubated with 100 μ L of 1:1000 of anti-DNP antibody for 1 hour at room temperature followed by incubation with horseradish peroxidase (HRP) conjugated (1:1000) antibody for 1 h at room temperature. Finally, 100 μ L substrate solution was added to each well and incubated for 20 minutes. The Stop solution (100 μ L) is added to each well and the plate was measured at 450 nm as the primary wavelength. The carbonylated protein concentration was calculated as described by manufacturer by plotting protein carbonyl BSA standard curve SD. The unknown samples were then determined using the equation obtained from the SD curve as nmol/mg of protein carbonyl.

3.7. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) Assay

DNA damage was determined using TUNEL assay according to the manufacturer's protocol (GenScript, Piscataway, NJ) and as described in Shah, *et al* (Shah et al., 2013). Briefly, 2×10^5 SVG astrocytes were seeded in each well of 12-well per plate and the cells were treated with either TIMBD or RES followed by transfection with HIV1-gp120 plasmid as described above. At the end of the experiment, cells were air-dried for 5 minutes and fixed with 4% paraformaldehyde in PBS for 30 minutes at room temperature. Following fixation, the cells were incubated overnight at 4 °C and permeabilized using 1% Triton X-100. The cells were blocked in 3% H₂O₂ in methanol and labeled with the TUNEL reaction mixture (Biotin-11-dUTP and TdT) and streptavidin-FITC. The cells were washed

with PBS to remove excess of labeling reagents and incubated with POD-conjugated Anti-FITC substrate. Finally, 3,3'-diaminobenzidine (DAB) reagent was added and the cells were observed under the light microscope. Images were captured using Leica DMI 300B (Leica Microsystems Inc., Buffalo Grove, IL).

3.8. Multiplex Protein Analysis

SVGA culture supernatants collected after 48 h of transfection with HIV-gp120 plasmid as described above were centrifuged for 5 minutes and stored at -80 °C. Bio-Plex system (Life Science Research, Hercules, CA) and the associated Bio-Plex Manager 5.0 software were used to measure the protein expression levels of IL6, IL8 and CCL5 in supernatants as reported previously (Nookala & Kumar, 2014; Shah & Kumar, 2010; Shah, Verma, et al., 2011). Briefly, standards (prepared as described by manufacturer in complete medium) or supernatant samples were added to a prepared BioPlex plate containing BioPlex bead stock. The plate was incubated for 30 minutes at room temperature with detection antibody provided by the manufacturer. After washing with wash buffer, phycoerythrin (PE)-conjugated streptavidin was added. The plate was incubated for 10 minutes and then washed. The concentration of IL6, IL8 and CCL5 were determined and analyzed using Bio-Plex 5 software.

3.9. Immunocytochemistry

Immunolocalization of IL6, IL8 and CCL5 in SVG astrocytes was determined as described previously (Abdalla et al., 2017; Nookala & Kumar, 2014). SVG astrocytes were cultured on coverslips in 6-well plates to 80% confluency. The cells were transfected as described for 24 h. Golgi Stop (BD Biosciences, San Jose, CA) 1 mg/ml was added 6 h prior to termination of the experiment to prevent IL6, IL8 and CCL5 release from

astrocytes into the cytosol. The cells were washed in PBS and fixed for 20 minutes in 1:1 (v/v) ice-cold acetone/methanol followed by air drying for 5 minutes. The cells were washed with PBST and non-specific binding was blocked by incubation with 1% BSA in PBST for 30 minutes. Cells were incubated overnight with a mixture of antibodies (mouse anti-GFAP (Glial fibrillary acidic protein) and rabbit anti-IL6 or anti-IL8 or anti-CCL5 at dilution described in Table 2) overnight. The cells were washed with PBST twice and incubated with secondary antibodies containing Alexflour 555 labeled anti-rabbit IgG and Alexflour 555 labeled anti-mouse IgG in a dark room for 1 h. Cells on coverslips were washed with PBST three times and were mounted on a glass slide containing DAPI (Vector Laboratories, Burlingame, CA, USA). The images were taken using confocal microscope, (Leica TCS SP5 II, Leica Microsystems, Wetzlar, Germany). GFAP was used as a housekeeping gene reference for analysis of the data.

3.10. Animals

The doxycycline (Dox) inducible Tat transgenic mice (C57/BL6J) were obtained from Dr. Kurt Haucer at Virginia Commonwealth University. In these mice, HIV-1 Tat in the brain is expressed using a tet-on inducible system and GFAP promoter. The mice were maintained in our Laboratory Animal Research Core (LARC) facility at UMKC with 12-hour light and dark cycles. Access to water and food was provided ad libitum. The animal procedures performed were approved by the UMKC Institutional Animal Institutional Biosafety Committee (IACUC). In all experiments, unless stated otherwise, comparisons were made between non-transgenic wild-type mice and Tat-transgenic mice treated with Dox. Mice (2 – 4 months of age were) given Dox food as 6 g/kg body weight as formulated chow until the end of the experiment. For gp120 mice, SJL mice were used

which described in Shah, et al (Shah, Vaidya, Bhat, & Kumar, 2016) and originally generated as described previously (Toggas et al., 1994). The SJL mice were obtained from Dr. Marcus Kaul, Sanford Burnham Medical Research Institute and bred in the UMKC-LARC facility. The SJL wild type mice served as control mice. The SJL/gp120 mice expressing gp120 under the regulatory control of modified GFAP. Control and treated (as described in results section) animals were sacrificed after the last day of behavioral testing. Brains were removed, and brain regions were separated. One brain half (hemibrain) was processed for RNA or protein isolation.

3.11. TIMBD treatment

TIMBD or RES were prepared in pellet form in our pellet press. Pellets containing 10 mg of TIMBD or RES were mixed with Cholesterol as a binder to make 30 mg pellets. The pellets were inserted subcutaneously at day 0 and mice were treated for 3 months prior to starting the behavioral experiments.

3.12. Genotyping

The genotyping was confirmed using cut and digested mouse tail. Briefly, cut tail samples were digested using 400 μ l lysis buffer and 2.5 μ l proteinase-K overnight in a water bath at 55 °C. Next day, 6M sodium chloride was added to the tail solution and the solution was centrifuged at 10,000 rpm for 5 minutes. Next, 300 μ l of supernatant was transferred to a different tube, then 350 μ l of isopropanol was added and incubated at room temperature for 10 minutes. Following that, the solution was centrifuged at 10,000 rpm for 10 minutes. After that, the supernatant was discarded and the tube was air dried. Next, the pellets were washed with 70% ethanol followed by centrifugation at 10,000 rpm for 10 minutes. The supernatant was discarded, and the pellets were allowed to air dry completely.

Thirty five μ l of TE buffer was added to each pellet to dissolve the DNA and the concentration was measured using Nanodrop device. TAT-gene in the DNA was then amplified using PCR. The amplified product DNA (8 μ l) mixed with 2 μ l 5x loading dye was separated in 2% agarose gel using electrophoresis. TAT-positive mice showed a product band at 200 bp which is the predicted size of the TAT PCR product. The same procedure was performed to confirm genotyping for gp120 mice by PCR to amplify the gp120 gene.

3.13. Behavioral assays

3.13.1. Y-maze

The Y-maze assay was used to assess short-term memory. The maze apparatus has three arms (represented by different letter) which are 12 inches in length. The animal was placed in one of the arms and allowed to move freely in the maze exploring for 5 minutes. An overhead camera was used to record the movement of the animals. The arms were cleaned using 70% ethanol between measurements to remove any mice residue. Each arm entry is considered a scoring. The number of times an arm was entered by mouse were counted and considered as marker for locomotor activity. A triad which consists of set of three arm entries and scoring all three letters is considered as alternation. Spontaneous alterations were considered as a marker for working memory. The following equation was used to calculate the spontaneous alternations:

$$\% \text{ Spontaneous alternations} = ((\text{number of triads} / (\text{total number of arm entries} - 2)) \times 100.$$

3.13.2. Morris Water Maze

Morris water maze is widely used test to assess impairment in spatial learning and memory in rodents (D'Hooge & De Deyn, 2001; Morris, 1984). Briefly, a galvanized tank

with 4 ft diameter was filled with water 25°C with to a depth of 12 inches. An escape platform of 10 cm diameter was submerged 1 cm below the water surface (**Fig. 5**). Navigation cues were placed outside around the arena. The mouse was placed in water and allowed to swim freely for 60 seconds. In the first day of trials' the mouse should learn to escape to the platform within 60 seconds. The test was performed for 5 days with 4 trials of 60 seconds each day. Each day, the location of the trials order was changed following the order in table 3.

Table 3: The various locations where the mice were placed each trial in the water maze each day.

	Trial 1	Trial 2	Trial 3	Trial 4
Day 1	N	E	SE	NW
Day 2	SE	N	NW	E
Day 3	NW	SE	E	N
Day 4	E	NW	N	SE
Day 5	N	SE	E	NW

Latency of escape to the platform was measured in time. The probe trial was performed on day 6 to assess memory retention by the mice. On this day, the escape platform was removed and the mouse was inserted at the location opposite of the platform's original place and allowed to swim freely. The time spent by each mouse in the platform quadrant was measured by use of the overhead camera.

Diagram of Morris water maze apparatus

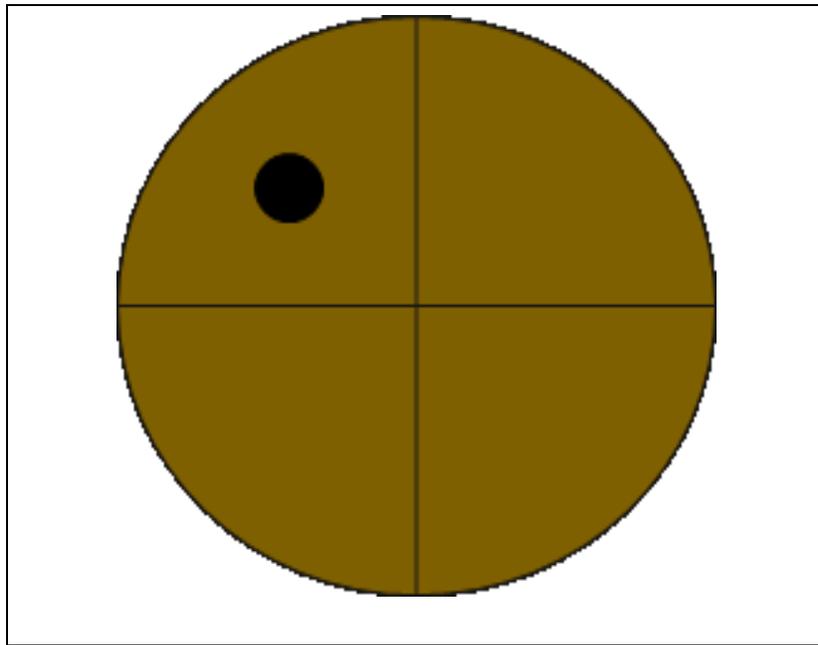


Figure 5. The diagram presents the different directions in which the mouse is placed each time. The escape island location is presented as a dark circle

3.13.3. Open field test

The open field test is used to identify locomotor activity and anxiety-like behavior. The apparatus is composed of a 40 cm X 40 cm open box made with four black opaque sides of plexi 24 glass (**Fig. 6**). The mice rearing was recorded using infra-red photo beam arrays which were placed 2 inches above the base of the box. The mice were allowed to acclimatize in the testing room for 1 h before the assay is performed. Simply, the mouse is placed in one corner randomly and given 5 minutes to explore the arena in which the activity is monitored using the overhead camera. The box was cleaned with 70% ethanol after each mouse. The distance travelled, number of entries and time spent in the center were calculated and used as anxiety parameters.

3.13.4 Light/Dark box

The Light/dark box is used as a direct measure of anxiety in mice. Briefly, the assay box is composed of two compartments, light and dark, which are separated by an opaque plexi glass wall that has an opening in the center (**Fig. 7**). As in the open field test, the infra-red beam arrays were used to record mouse rearing activity. On the day of the test, the mouse is placed in the light compartment and allowed to move freely between the two compartments for 10 minutes. The time spent in the light area versus the dark area was used as an indicator of anxiety-like behavior. The box was cleaned with 70% ethanol after each mouse to avoid interference between mice.



Figure 6. Diagram of open field test apparatus. The diagram presents the arena, which consist of center area and periphery.

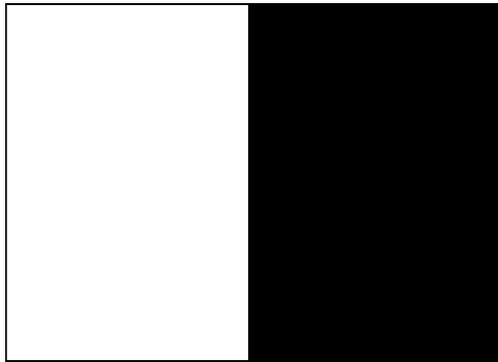


Figure 7. Diagram of Light/dark box apparatus. The diagram presents the arena, which consist of light area and dark area with opening between them.

3.14. Brain collection

At the end of the whole set of experiments, mice were anesthetized with 5% isoflurane followed by perfusion of the brain with 8 ml isotonic PBS prior to isolation of brain regions. Briefly, after separating pons and medulla, the cerebellum was dissected from both hemispheres. The brain was separated into two hemispheres. The prefrontal cortex region was separated, followed by dissecting the hippocampus. Hippocampus was separated from the rest of the brain, which was considered as the parietal cortex. Brain regions were immediately frozen in liquid nitrogen. Separated brain tissues were homogenized in 500 µl of RIPA buffer containing protease and phosphatase inhibitors and centrifuged at 14,000 rpm for 15 minutes at 4°C and the supernatant was collected. The protein concentration was determined as described in previous section. Proteins from different mice in each group were pooled together and used for electrophoresis as described previously.

3.15. Brain to body weight ratio

Mice were weighed and humanely euthanized following anaesthetizing with 2% to 5% isoflurane to collect the brain. Mice weight was recorded following the euthanization. Brain weight was measured after dissecting and the separating brain. The brain to body weight ratio was calculated.

3.16. Statistical Analysis

The Statistical analyses were performed using Statistical Package for Social Sciences (SPSS, version 23 for windows, IBM, Armonk, NY). One-way Anova with Tukey's post hoc analysis for multiple comparisons was used for all cell culture experiments and the mice experiments. Escape latency in Morris water maze was analyzed

using Two-way Anova with Tukey's post hoc analysis for multiple comparisons. $p < 0.05$ was considered as statistically significant.

CHAPTER 4

EFFECT OF TIMBD ON GP120-INDUCED OXIDATIVE STRESS AND CONSEQUENT CELL DEATH

4.1. Introduction

One of the major Human Immuno-Deficiency Viral (HIV) proteins is glycoprotein 120 (gp120) (Jana & Pahan, 2004; Yoon et al., 2010). HIV-1 gp120 facilitates HIV viral replication and disease progression, which subsequently affects the central nervous system (CNS) (Samikkannu et al., 2015; Silverstein et al., 2012). This viral protein is the major protein that mediates entry of the virus into the host cell (Blumenthal, Durell, & Viard, 2012; Wilen et al., 2012). HIV-1 gp120 interaction with the CD4 receptor and the α -chemokine receptor CXCR4 or β -chemokine coreceptors leads to conformational changes that mediate the viral entry into the host cell (**Fig. 8**) (Blumenthal et al., 2012; Ray & Doms, 2006; Wilen et al., 2012). Virus entry into the host cell triggers the release of inflammatory cytokines and other cellular products, which leads to neurotoxicity (Bao, Gur, & Yarden, 2003; Rock et al., 2004). HIV-1 gp120 mediated oxidative stress produces free radicals that are known to cause cellular damage in many diseases including HIV/acquired immunodeficiency syndrome HIV/AIDS (Louboutin & Strayer, 2014; Ronaldson & Bendayan, 2008; Shah et al., 2013). Furthermore, oxidative stress produced by gp120 in astrocytes is considered a major contributor to damage in the CNS (Louboutin, Agrawal, et al., 2010; Shah et al., 2013). Reactive Oxygen Species (ROS) are highly unstable oxygen-containing molecules that are released from cells in response to toxic damage or infection (Lobo, Patil, Phatak, & Chandra, 2010; Zorov, Juhaszova, & Sollott, 2014). Once ROS are released, the cell induces anti-oxidant enzymes like superoxide dismutase (SOD),

glutathione peroxidase (GPX), and catalase (Szymonik-Lesiuk et al., 2003; Weydert & Cullen, 2010). The imbalance between ROS production and antioxidant defense enzymes will result in the CNS damage (Birben, Sahiner, Sackesen, Erzurum, & Kalayci, 2012; Halliwell, 1992; Uttara, Singh, Zamboni, & Mahajan, 2009). Studies have revealed that increased ROS-induced DNA damage is correlated with cell cycle arrest (Colin et al., 2014; Pyo et al., 2013). Exposure to HIV-1 gp120 in animal studies caused disruption of the Blood-Brain Barrier (BBB) through lesions in the micro-vessels, matrix metalloproteinase (MMP) activation, and degradation of the basement membranes and vascular tight junction proteins which lead to a loss of BBB integrity (Louboutin, Agrawal, et al., 2010; Louboutin et al., 2011; Louboutin & Strayer, 2012). Activated MMPs due to oxidative stress decrease tight junction proteins through proteolysis and leads to BBB impairment (Louboutin, Agrawal, et al., 2010; Louboutin et al., 2011; Louboutin & Strayer, 2012). This cascade of events induces ROS production and leads to neuronal cell death after mitochondrial permeabilization, cytochrome *c* release, and activation of caspases and endonucleases (Louboutin & Strayer, 2012; Price et al., 2005).

Plant-derived phytoestrogens are suggested to have wide therapeutic effects in different pathological conditions (Koehn & Carter, 2005; Nobili et al., 2009). Resveratrol (RES) is one of those known phytoestrogens with potentially wide therapeutic activities (F. Li et al., 2012; Marambaud et al., 2005). It is suggested that RES could suppress oxidative stress by modulating anti-oxidant defense mechanisms (Mizutani, Ikeda, Kawai, & Yamori, 2001; Tung et al., 2014). Although RES has shown therapeutic effects in many studies, poor specificity and bioavailability have presented a challenge for its clinical use (Ginsburg & Deharo, 2011; Walle, 2011). To overcome these problems associated with

RES, our group has synthesized many RES analogs including 4-(E)-{(p-tolylimino)-methylbenzene-1,2-diol} (TIMBD) (**Fig. 9**). Previous studies have shown that TIMBD has potent antioxidant effects in breast cancer (Chatterjee et al., 2017). Therefore, we hypothesize that TIMBD may potentially be used clinically to inhibit the development of HIV-1 gp120-induced oxidative stress.

HIV-1 gp120 interaction with the CD4 receptor and coreceptors

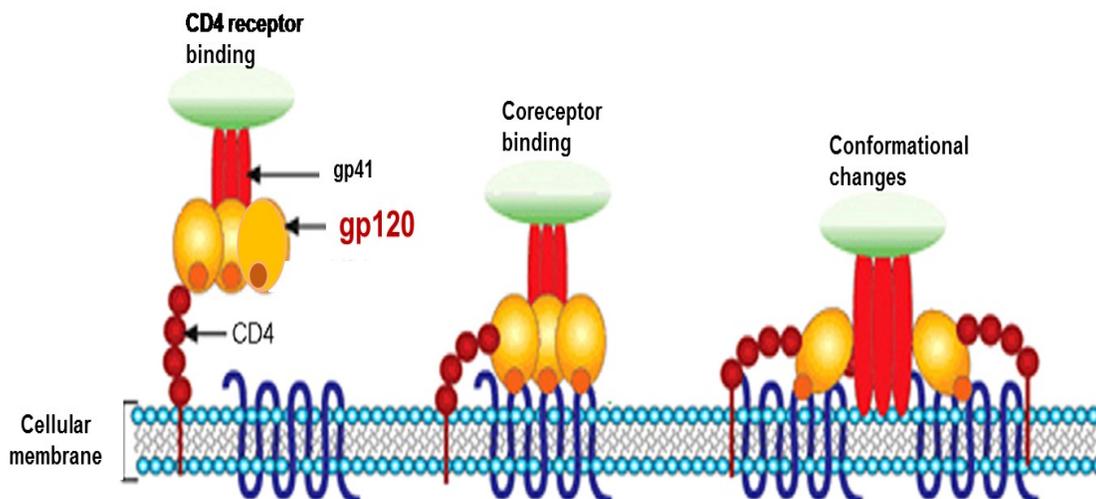
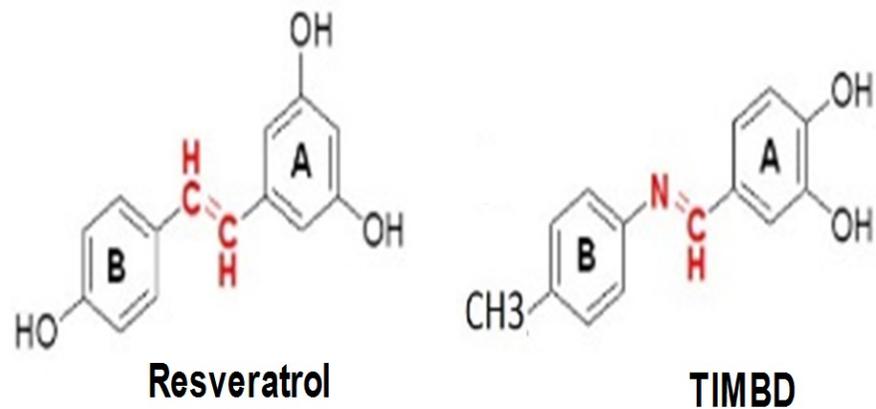


Figure 8. Figure adapted and modified from Ray *et al* 2006 (Ray & Doms, 2006)

Resveratrol and TIMBD

(A)



(B)

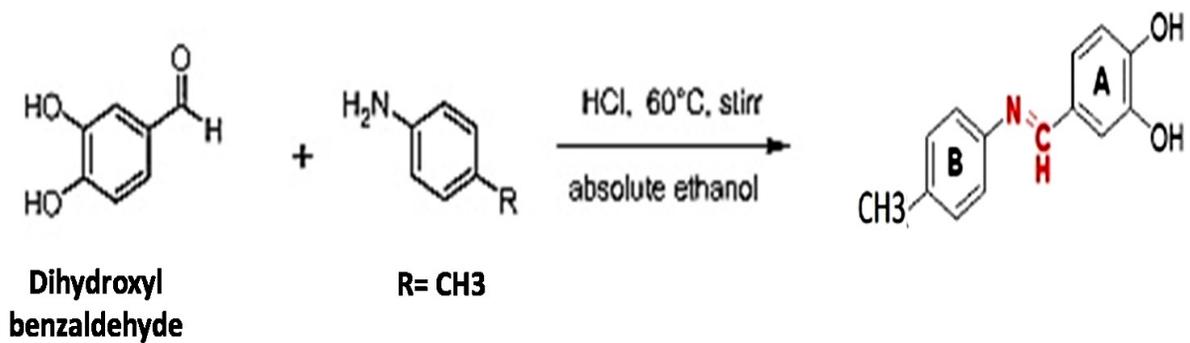


Figure 9. Chemical structure of Resveratrol and TIMBD (A). Synthesis of TIMBD from two parent compounds (B).

4.2. Results

In the present study, we aimed to study the effect of TIMBD AND compared to that of RES against HIV-1 gp120-induced oxidative stress using SVG and primary astrocytes

4.2.1 TIMBD decreases HIV-1 gp120-mediated oxidative stress in SVG astrocytes

Previous studies have shown that gp120 induces ROS in astrocytes (Shah et al., 2013). This is suggested to be attributed due to an imbalance between the oxidative stress and the anti-oxidant defense system. It has been shown that gp120 induces maximal ROS levels using CM-H2DCFDA-FACS assay at 24 h (Shah et al., 2013). In order to determine which RES analog has the most powerful antioxidant effect in decreasing ROS levels, SVG astrocytic cells were treated with 50 μ M of either RES or RES analogs (names TIMBD analogs/compounds, E, A, D, and F) 1 h prior to transfection with gp120 plasmid using serum-free medium. The cells were then transiently transfected with 2 μ g plasmid encoding HIV-1 gp120 for 5 h, then was replenished by fresh medium, followed by incubation for 24 h. Mean fluorescence intensity (MFI) was measured to indicate the levels of ROS in SVG astrocytes in response to gp120 and different treatments. HIV-1 gp120 increased ROS levels, and among RES analogs, TIMBD showed the most powerful effect of limiting the increase of ROS levels due to gp120 treatment (**Fig. 10**). Based on these results, only TIMBD was used for future studies as a potential neuroprotective drug.

To confirm the effect of TIMBD and RES on cell viability, a cell viability test using MTT assay was conducted in which the SVGA cells were treated with a wide range of doses of RES and TIMBD for 72 h or 96 h. TIMBD showed no significant effect on cell viability at all doses tested while the highest dose of RES decreased cell viability to 60% (**Fig. 11 panels A and B**). The CM-H2DCFDA-FACS assay was repeated using a 25 μ M

dose of either TIMBD or RES. TIMBD decreased ROS levels compared to RES at lower doses (**Fig. 12**). To confirm that TIMBD reduces oxidative stress, oxidative stress damage markers like protein carbonyl content was measured. TIMBD was able to decrease the gp120-associated increase in protein carbonylation significantly compared to gp120 only (**Fig. 13**) confirming its effect as an antioxidant. The effect of TIMBD on the ROS producing enzyme NADPH-Oxidase (NOX2) was also measured. It has been reported that gp120 increases the expression levels of NOX2 in astrocytes (Shah et al., 2013). We confirmed that gp120 increases protein expression levels of NOX2 and that TIMBD reduces the gp120-mediated increase of NOX2 levels (**Fig. 14**). Thus, our results indicate that TIMBD, and not RES, decreases gp120-associated ROS and the enzyme involved in the production of ROS.

TIMBD decreases HIV-1 gp120 induced oxidative stress compared to RES or RES analogs

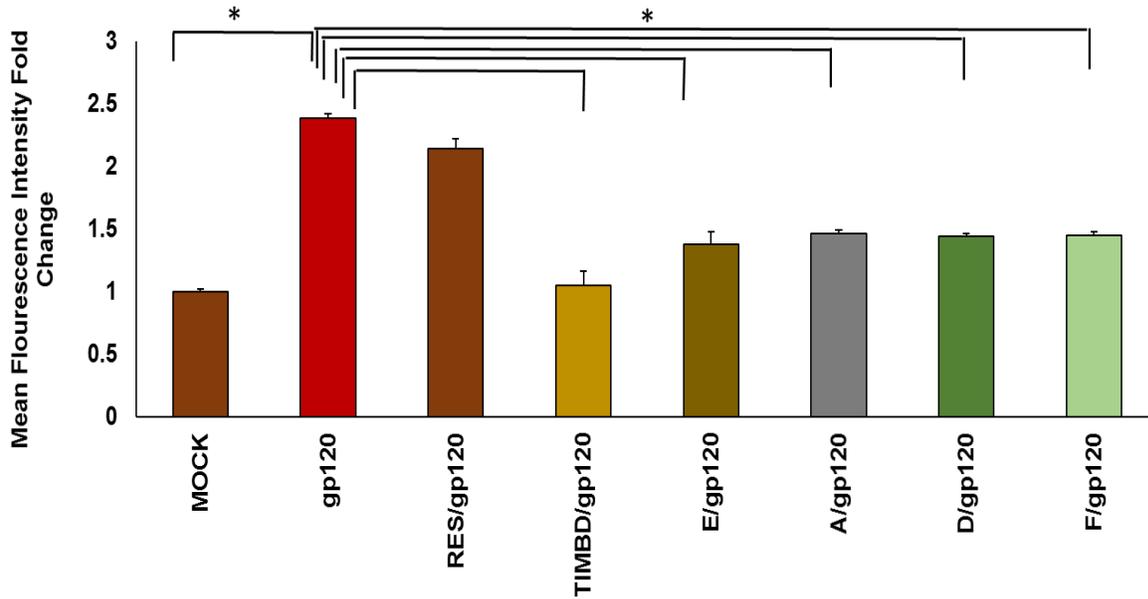


Figure 10. SVG astrocytes were treated with 50 μ M TIMBD, or RES or RES analogs E, A, D or F for 1 h prior to transfection with 4 μ g plasmid encoding HIV-1 gp120 for 24 h. The cells were incubated with CM-H2DCFDA and fluorescence intensity was measured using flow cytometry. Each bar represents mean \pm SEM from at least three independent experiments with three replicates in each experiment. One-way Anova was used for statistical analysis and statistical significance is denoted as * (p-value \leq 0.05).

TIMBD is safer than RES on astrocytic cells viability

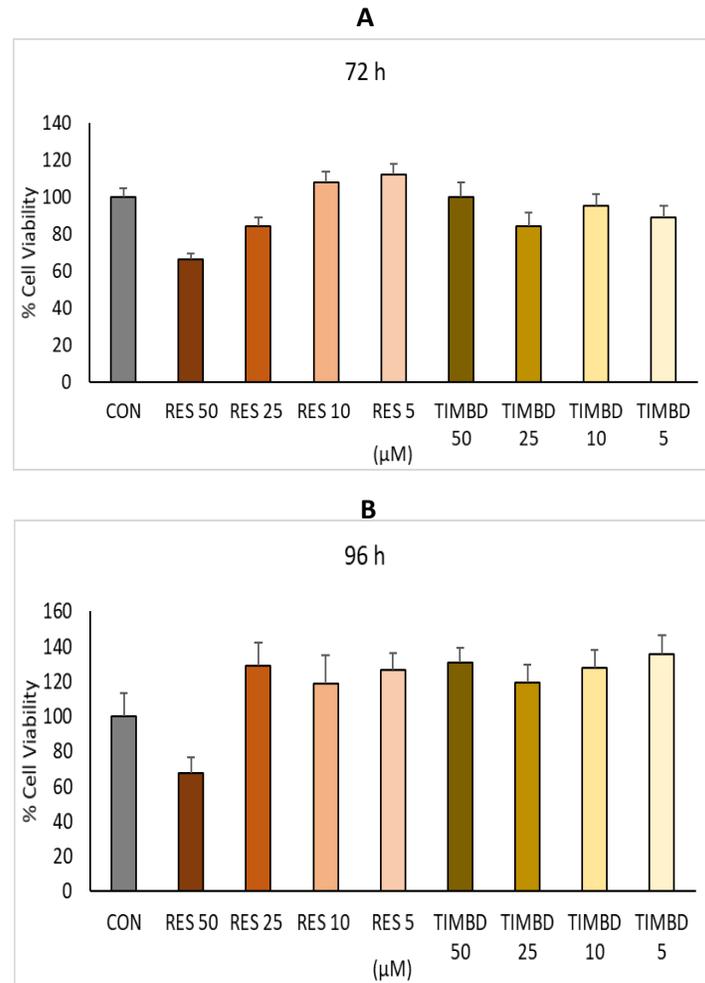


Figure 11. SVG astrocytes were treated with different doses (50 to 5 μM) of TIMBD or RES for 72 h (A) and 96 h (B). The cells were then incubated with MTT and absorbance was measured using microplate reader to calculate the percentage of cell viability. Each bar represents mean \pm SEM from at least three independent experiments with three replicates in each experiment. One-way Anova was used for statistical analysis and statistical significance is denoted as * (p-value \leq 0.05) and ** (p-value \leq 0.01).

TIMBD decreases HIV-1 gp120 induced oxidative stress

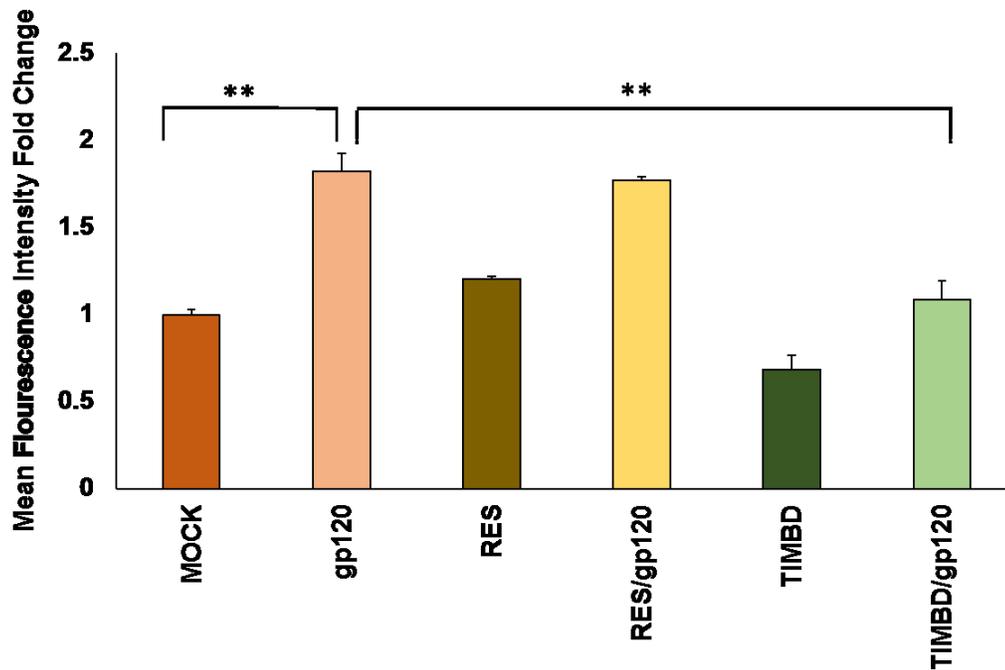


Figure 12. SVG astrocytes were treated with 25 μ M TIMBD or RES 1 h prior to transfection with plasmid encoding HIV-1 gp120 for 24 h. The cells were incubated with CM-H2DCFDA and fluorescence intensity was measured using flow cytometry. Each bar represents mean \pm SEM for at least 3 independent experiments, with each experiment done in triplicate. One-way Anova was used for statistical analysis and statistical significance is denoted as ** (p-value \leq 0.01).

TIMBD decreases HIV-1 gp120 induced protein carbonylation

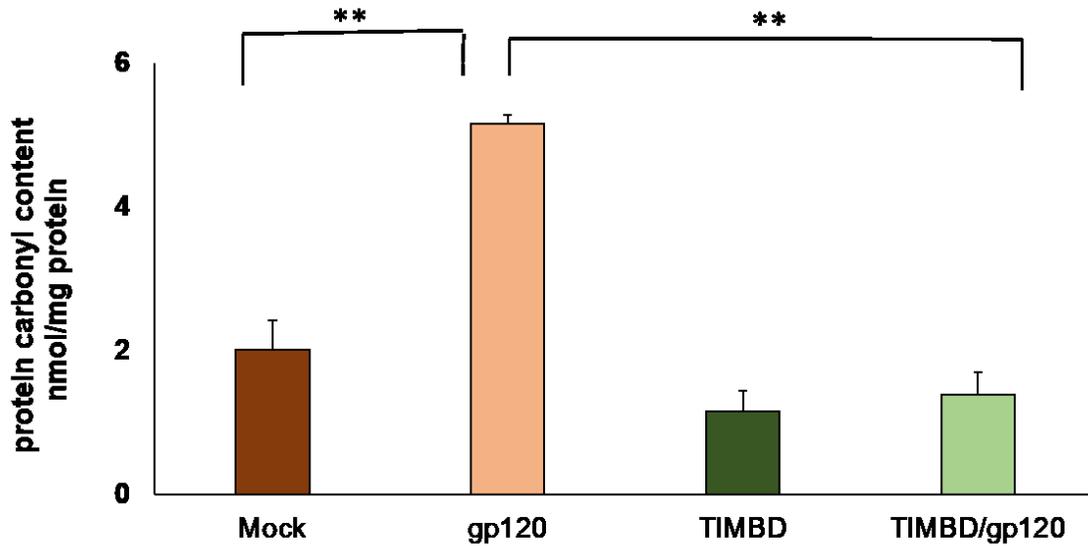


Figure 13. SVG astrocytes were treated with 25 μ M TIMBD 1 h prior to transfection with plasmid encoding HIV-1 gp120 for 24 h. Protein carbonyl concentration (nmol/mg) was determined following TIMBD treatment. Each bar represents mean \pm SEM for at least 3 independent experiments, with each experiment done in triplicate. One-way Anova was used for statistical analysis and statistical significance is denoted as ** (p -value \leq 0.01).

TIMBD decreases HIV-1 gp120 associated increase in pro-oxidant enzyme NOX2 in SVG astrocytes

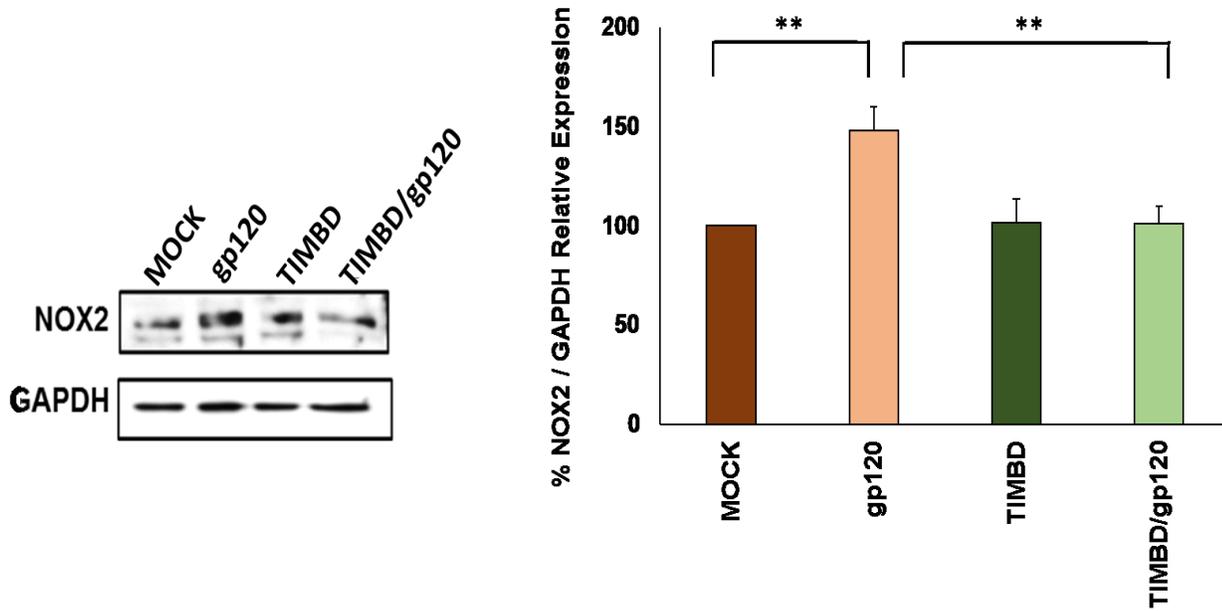


Figure 14. SVG astrocytes were treated with 50 μ M TIMBD 1 h prior to transfection with plasmid encoding HIV-1 gp120 for 24 h. Expression levels of pro-oxidant enzyme NOX2 were determined using western blotting following 24 h HIV-1 gp120 transfection and TIMBD treatment. Each bar represents mean \pm SEM for at least 3 independent experiments, with each experiment done in triplicate. One-way Anova was used for statistical analysis and statistical significance is denoted as ** (p-value \leq 0.01).

4.2.2. TIMBD decreases gp120-associated oxidative stress in SVG astrocytes by modulating protein expression levels of antioxidant enzymes

It is known that the antioxidant defense system in the cell is responsible for neutralizing ROS levels produced in response to various insults (Szymonik-Lesiuk et al., 2003; Weydert & Cullen, 2010). However, compromising this defense system can lead to oxidative stress-mediated damage. The antioxidant system known to be involved includes glutathione peroxidase (GPx1), catalase and superoxide dismutase (SOD) which are the major enzymes that can neutralize ROS molecules. Other antioxidant enzymes have also been reported to be involved in balancing ROS levels including NAD(P)H dehydrogenase [quinone] 1 (NQO1) and Flavine Mono-oxygenase (FMO1). To identify the mechanism by which TIMBD decreases gp120-induced ROS levels, SVG astrocytic cells were treated with 50 μ M of TIMBD one hour prior to transfection with gp120 plasmid using serum-free medium. The cells were transiently transfected with 2 μ g plasmid encoding HIV-1 gp120 for 5 h and after a medium change, were incubated for 24 h. Protein was isolated using established protocols as described for western blotting analysis in the Methods section. HIV-1 gp120 decreased the protein expression levels of antioxidant enzymes GPx1, catalase, SOD1, NQO1 and FMO1 and TIMBD treatment recovered their expression levels significantly, compared to gp120 (**Fig. 15, 16 and 17**). These results suggest that TIMBD can recover the antioxidant enzyme levels and thus lead to a decrease in ROS production and the consequent oxidative-stress damage.

TIMBD restores gp120-mediated decrease in anti-oxidant enzymes (GPX1 and Catalase) levels in SVG astrocyte

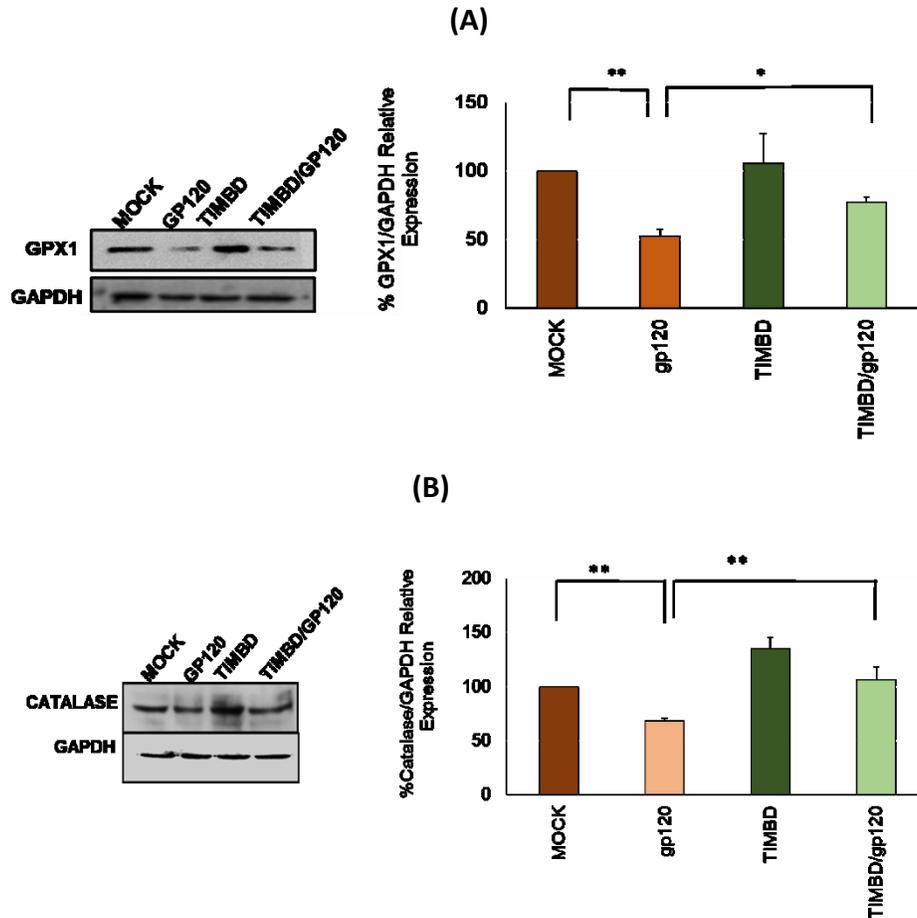


Figure 15. SVG astrocytes were treated with 50 μ M TIMBD 1h prior to transfection with plasmid encoding HIV-1 gp120 for 24 h. Protein expression levels of GPX1 (A) and Catalase (B) were measured per well with GAPDH used as loading control. Each bar represents mean \pm SEM for 3 independent experiments, with each experiment done in triplicate and the western blots are representative images. One-way Anova was used for statistical analysis and statistical significance is denoted as * (p-value \leq 0.05) and ** (p-value \leq 0.01).

TIMBD restores gp120-mediated decrease in anti-oxidant enzymes (SOD1 and NQO1) levels in SVG astrocyte

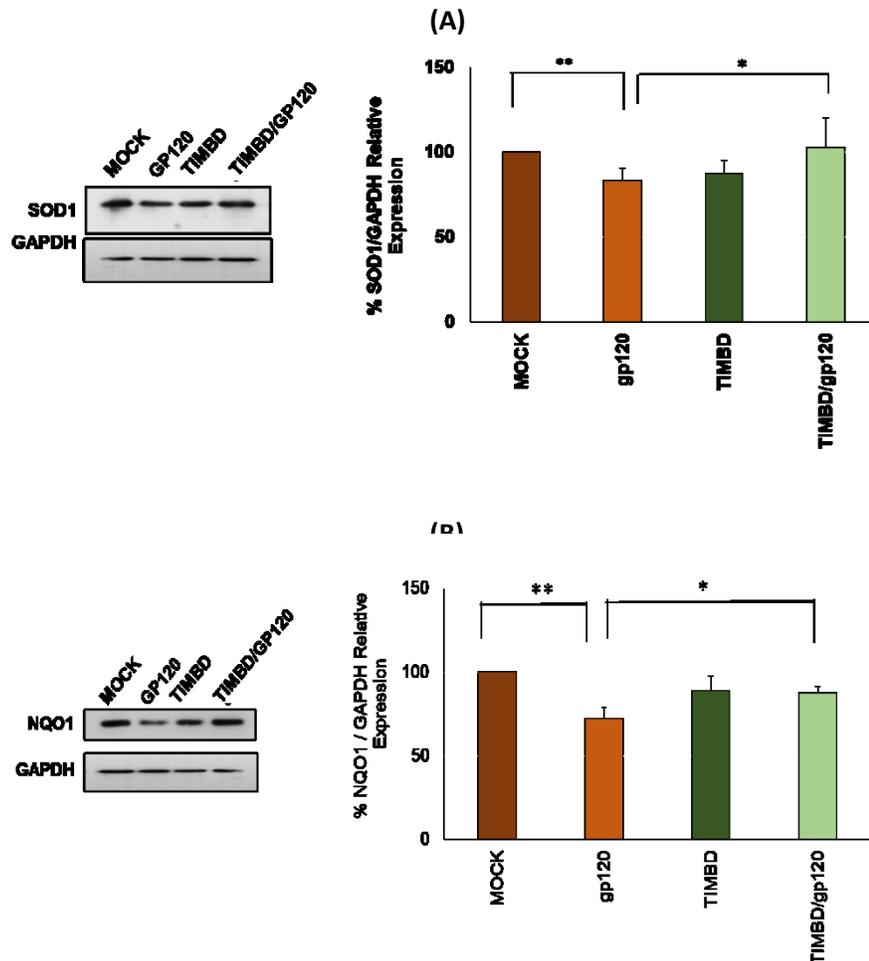


Figure 16. SVG astrocytes were treated with 50 μ M TIMBD 1h prior to transfection with plasmid encoding HIV-1 gp120 for 24 h. Protein expression levels of SOD1 (A) and NQO1 (B) were measured per well with GAPDH used as loading control. Each bar represents mean \pm SEM for 3 independent experiments, with each experiment done in triplicate and the western blots are representative images. One-way Anova was used for statistical analysis and statistical significance is denoted as * (p-value \leq 0.05) and ** (p-value \leq 0.01).

TIMBD restores gp120-mediated decrease in anti-oxidant enzyme (FMO1) levels in SVG astrocyte

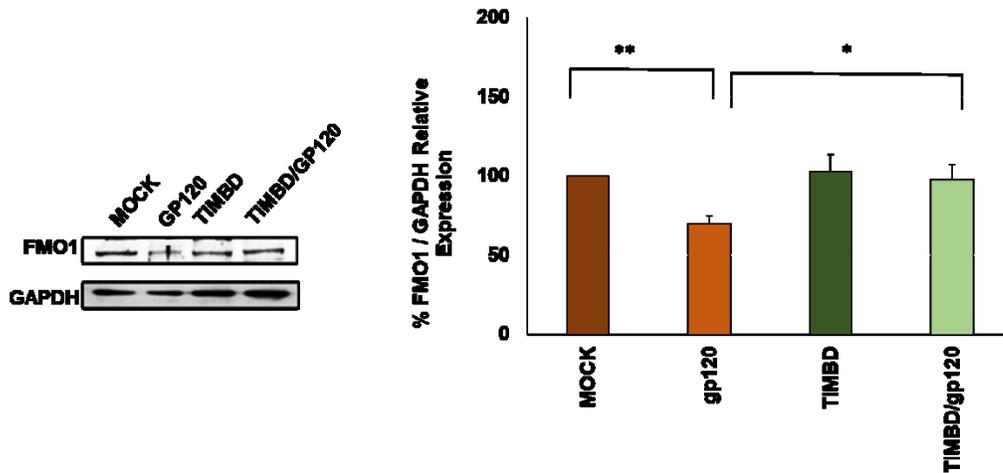


Figure 17. SVG astrocytes were treated with 50 μ M TIMBD 1h prior to transfection with plasmid encoding HIV-1 gp120 for 24 h. Protein expression levels of FMO1 was measured per well with GAPDH used as loading control. Each bar represents mean \pm SEM for 3 independent experiments, with each experiment done in triplicate and the western blots are representative images. One-way Anova was used for statistical analysis and statistical significance is denoted as * (p-value \leq 0.05) and ** (p-value \leq 0.01).

4.2.3. TIMBD decreases gp120-associated oxidative stress in primary astrocytes

We further aimed to confirm TIMBD's observed antioxidant effect in SVG astrocytes using primary astrocytes. Primary astrocytes were treated with 50 μM TIMBD for 1 h followed by treatment with 50 ng/ μl of HIV-1 gp120-IIIb recombinant protein for 6 h. At the end of the experiment, the protein was collected and used to measure oxidative stress-involved enzymes. The effect of TIMBD on NOX2, catalase and SOD3 enzymes was examined. Pre-treatment with 50 μM TIMBD decreased the gp120-induced NOX2 levels significantly, compared to control, while TIMBD decreased NOX2 levels significantly below control levels (**Fig. 18**). In addition, gp120 decreased protein expression levels of antioxidant enzymes catalase and SOD3 and TIMBD treatment recovered both catalase and SOD3 protein expression levels significantly, compared to gp120 (**Fig. 19 panels A and B**). These results suggest that TIMBD is capable of decreasing HIV-1 gp120-associated oxidative stress in both SVG astrocytic cells and in primary astrocytes.

TIMBD decreases protein expression levels of pro-oxidant enzyme NOX2 in primary astrocytes

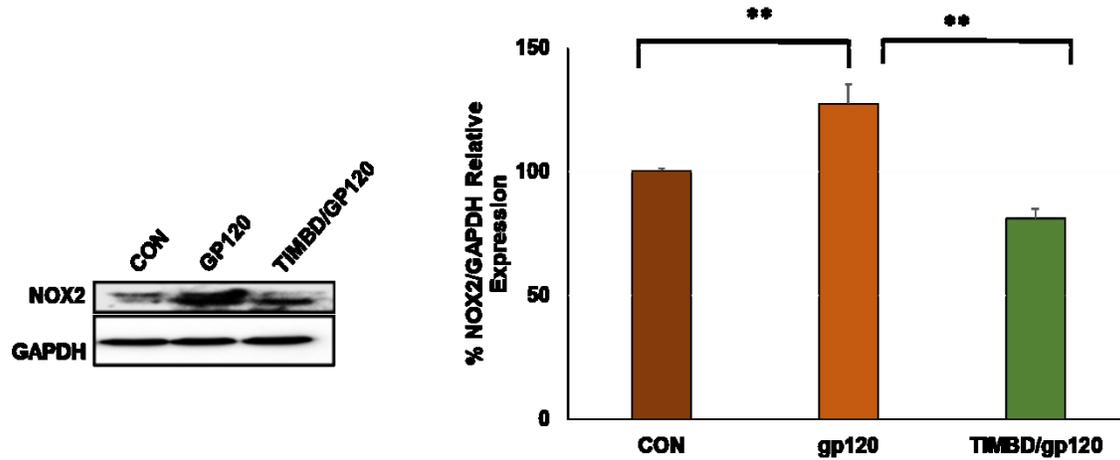


Figure 18. Primary astrocytes from one donor were treated with 50 μ M TIMBD 1 h prior to treatment with 50 ng of gp120-IIIb recombinant protein for 6 h. Protein expression levels of NOX2 were measured per well with GAPDH as loading control. Each bar represents mean \pm SEM for 3 independent replicates, and the western blots images are representative images. One-way Anova was used for statistical analysis and statistical significance is denoted as ** (p-value \leq 0.01).

TIMBD increases protein expression levels of anti-oxidant enzymes in primary astrocytes

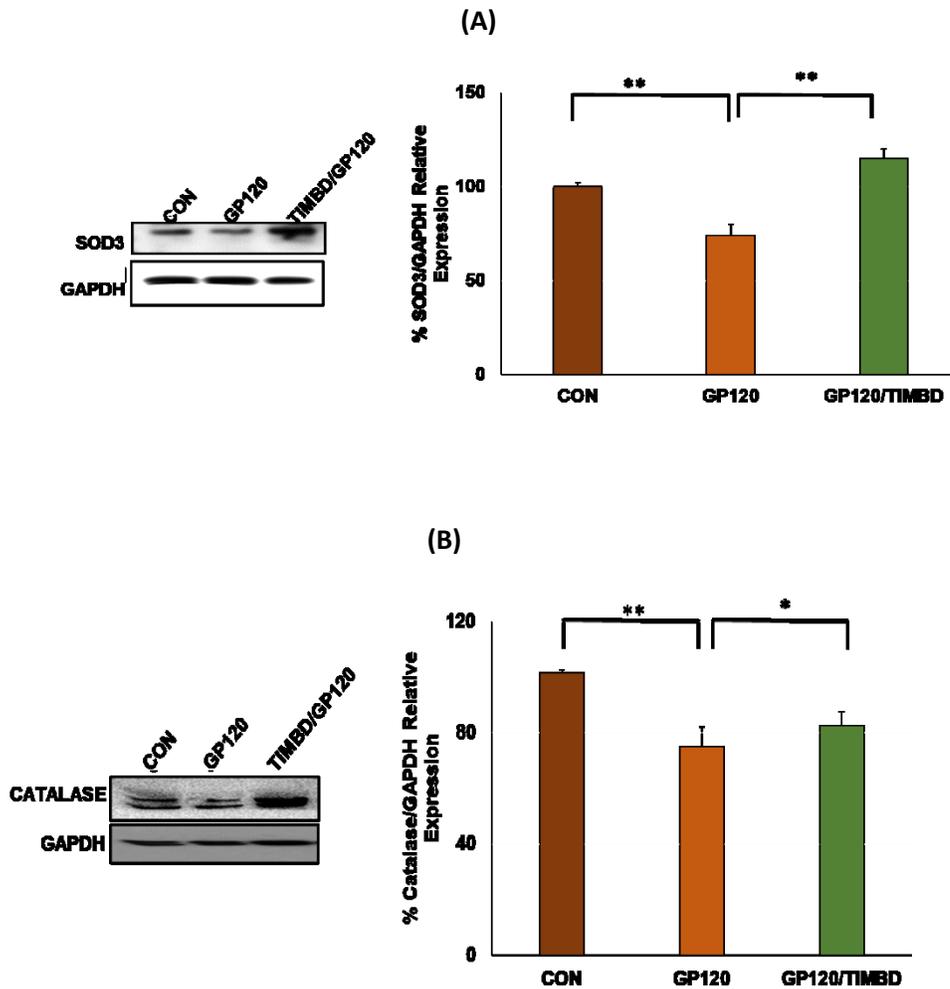


Figure 19. Primary astrocytes from one donor were treated with 50 μ M TIMBD 1 h prior to treatment with 50 ng of gp120-IIIb recombinant protein for 6 h. Protein expression levels of SOD3 (A) and Catalase (B) were measured per well with GAPDH used as loading control. Each bar represents mean \pm SEM for 3 independent replicates, and the western blots are representative images. One-way Anova was used for statistical analysis and statistical significance is denoted as * (p-value \leq 0.05) and ** (p-value \leq 0.01).

4.2.4. TIMBD increases nuclear translocation of NRF2

The antioxidant enzyme expression is controlled by transcription factor NRF2. Once activated, NRF2 translocates from the cytoplasm to the nucleus where it binds to antioxidant response elements (ARE), which stimulate expression of antioxidant enzyme genes. In our study we found that gp120 decreased the nuclear expression levels of NRF2 in SVG astrocytes. Treatment with TIMBD caused an increase in the translocation level of NRF2, compared to gp120 (Fig. 20). These results suggest that TIMBD might be controlling gp120-induced oxidative stress through activation of NRF2 transcription factor.

TIMBD restores gp120-mediated decrease in NRF2 nuclear translocation

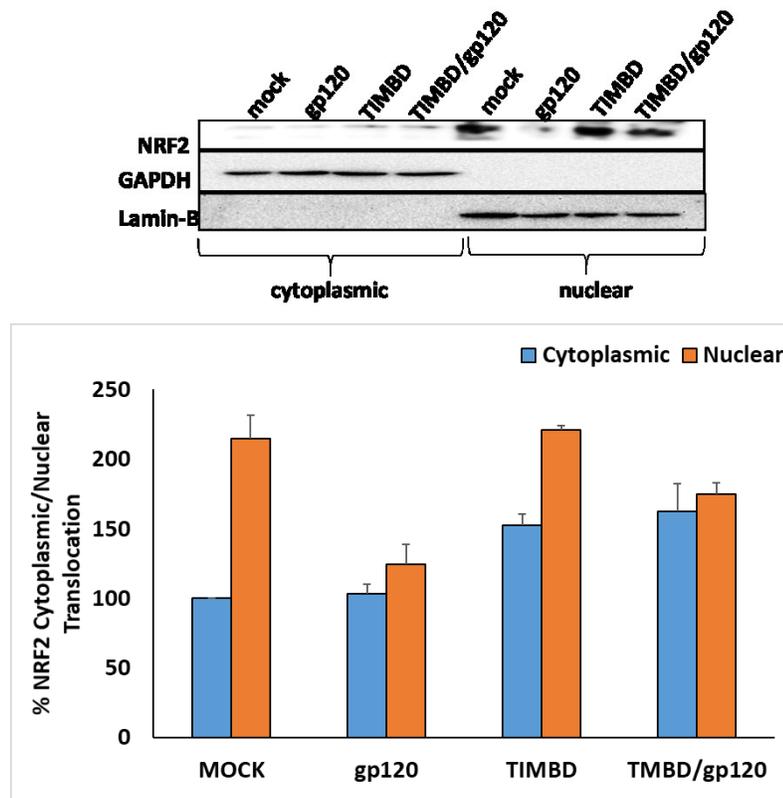


Figure 20. SVG astrocytes were treated with 50 μ M TIMBD 1 h prior to transfection with plasmid encoding HIV-1 gp120 for 12 h. Protein expression levels of NRF2 in cytoplasmic and nuclear extracts were measured with GAPDH and Lamin-B as loading controls. Each bar represents mean \pm SEM for 3 independent replicates, and western blots images are representative images. One-way Anova was used for statistical analysis and statistical significance is denoted as * (p-value \leq 0.05).

4.2.5. TIMBD modulates oxidative stress *in vivo* using gp120-tg mice

To confirm TIMBD's antioxidant effect *in vivo*, gp120-transgenic mice (-tg) were used. GP120-tg mice have been previously reported to have high oxidative stress damage in their brains (Louboutin, Agrawal, et al., 2010). NOX is reported to mediate ROS increase in HIV transgenic rats (Cho, Lee, & Song, 2017). NOX is suggested to be a mediator for oxidative stress formation which may further cause neuronal cell death and behavioral impairment. SJL control and gp120-tg mice of 8 weeks old were treated with control, RES or TIMBD pellets (**Fig. 21**). Following 12 weeks, protein were collected from mice brain as described in Methods section. In tg-mice, gp120 was able to increase expression levels of NOX2 in hippocampus (H), prefrontal cortex (PFC) (**Fig. 22**) and parietal cortex (PC) brain regions (**Fig. 23**), and TIMBD treatment significantly decreased the expression levels of NOX2, with a larger observed effect than RES. Similarly, SOD3 expression levels were also decreased by gp120 in brain regions (**Fig. 24 and 25**). TIMBD treatment increased SOD3 expression levels significantly only in the PFC region while RES had less or no effect (**Fig. 24 panel B**). Little or no effect of gp120 on SOD1 and SOD2 protein expression levels were observed in different brain regions and treatments with TIMBD or RES did not reveal any significant changes for SOD1 (**Fig. 26 and 27**) and SOD2 (**Fig. 28 and 29**). Collectively, these results suggest that TIMBD might be able to decrease oxidative stress *in vivo* by decreasing prooxidant and increasing antioxidant enzyme expression levels.

Schematic overview of the experimental design

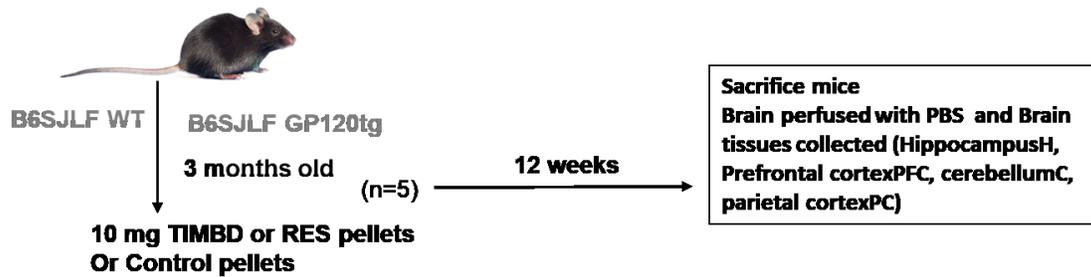


Figure 21. Eight-week old control mice and HIV-1 gp120-tg mice were used for the experiment. Control or TIMBD pellets were implanted for 12 weeks. Animals were then sacrificed on week 12. At the end of the experiment, mice were euthanized for collection of different brain regions including PFC, H, PC and C to identify the expression of various proteins involved in oxidative stress.

TIMBD reduces HIV-1 gp120 associated increase in NOX2 expression levels in mice

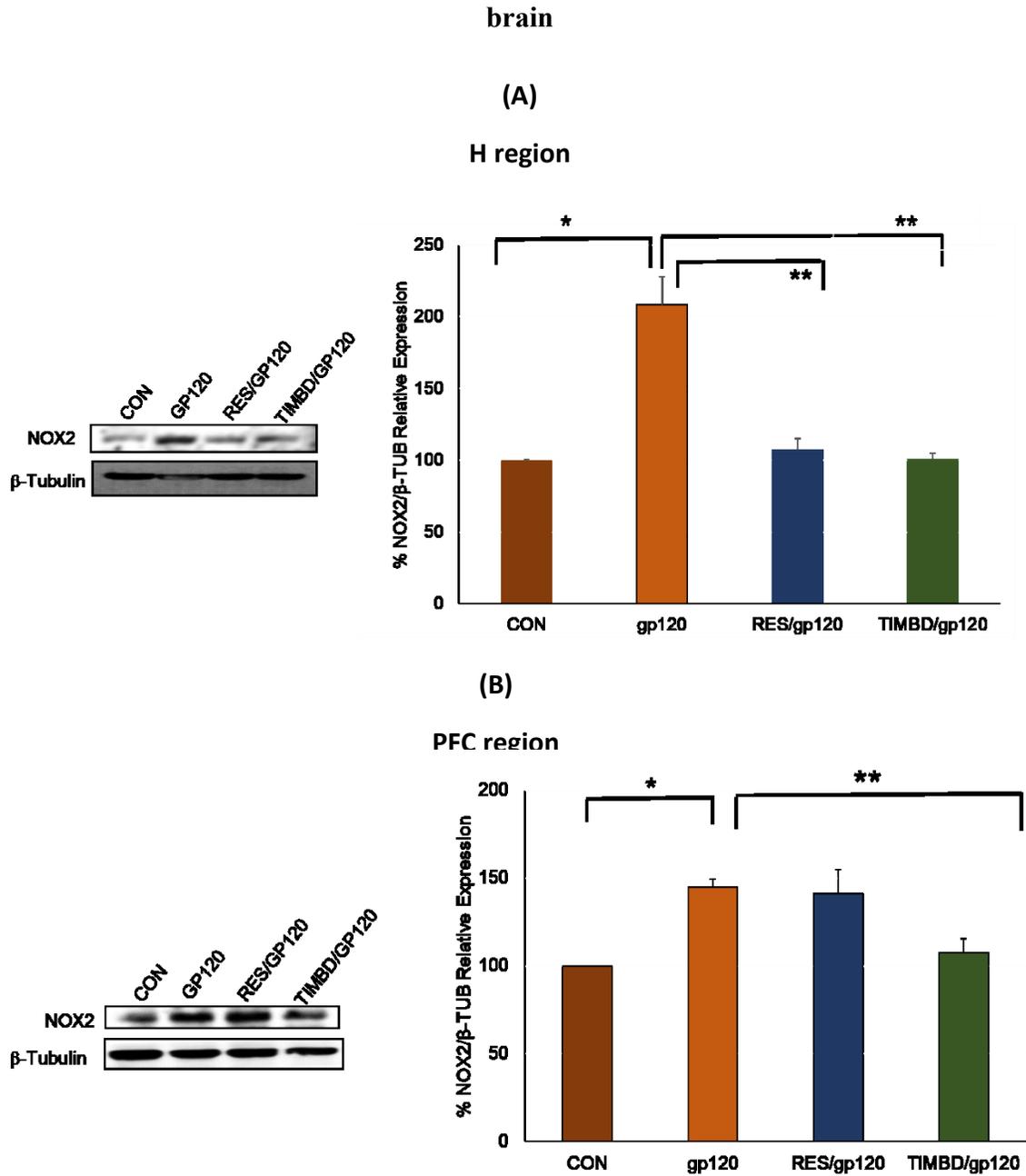


Figure 22. Brain regions were collected from HIV-1 gp120-tg and control mice following 12 weeks of treatment with TIMBD, or RES, or CON pellets. Expression levels of NOX2 were determined in H (A) and PFC (B) brain regions using western blotting. Each bar represents mean \pm SE for at least 3 independent westerns. One-way Anova was used for statistical analysis and statistical significance is denoted as * (p-value \leq 0.05) and ** (p-value \leq 0.01).

**TIMBD reduces HIV-1 gp120 associated increase in NOX2 expression levels in mice
brain**

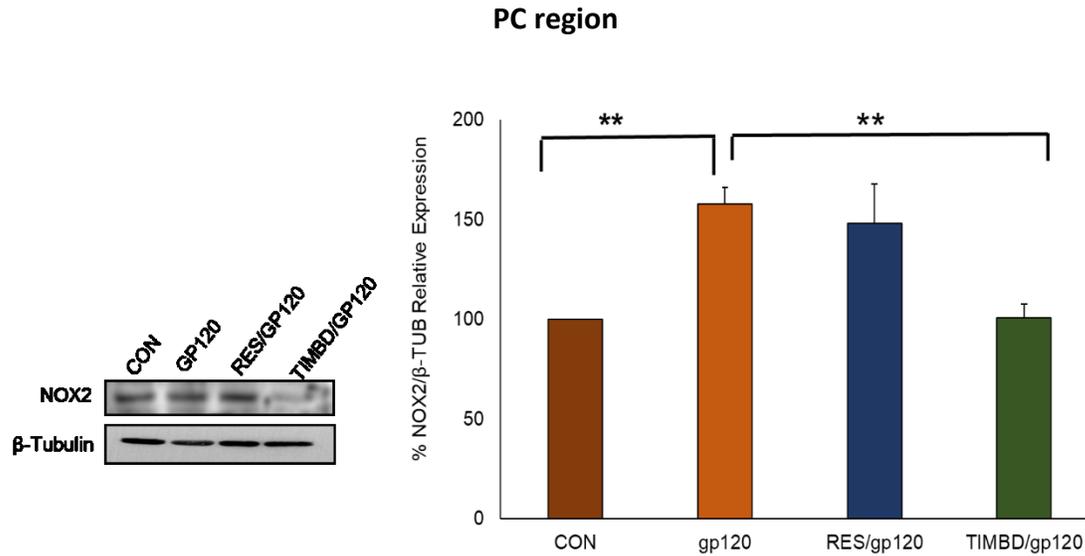


Figure 23. Brain regions were collected from HIV-1 gp120-tg and control mice following 12 weeks of treatment with TIMBD, or RES, or CON pellets. Expression levels of NOX2 were determined in PC brain regions using western blotting. Each bar represents mean \pm SE for at least 3 independent westerns. One-way Anova was used for statistical analysis and statistical significance is denoted as ** (p-value \leq 0.01).

TIMBD increases HIV-1 gp120-mediated decrease in SOD3 expression levels mice

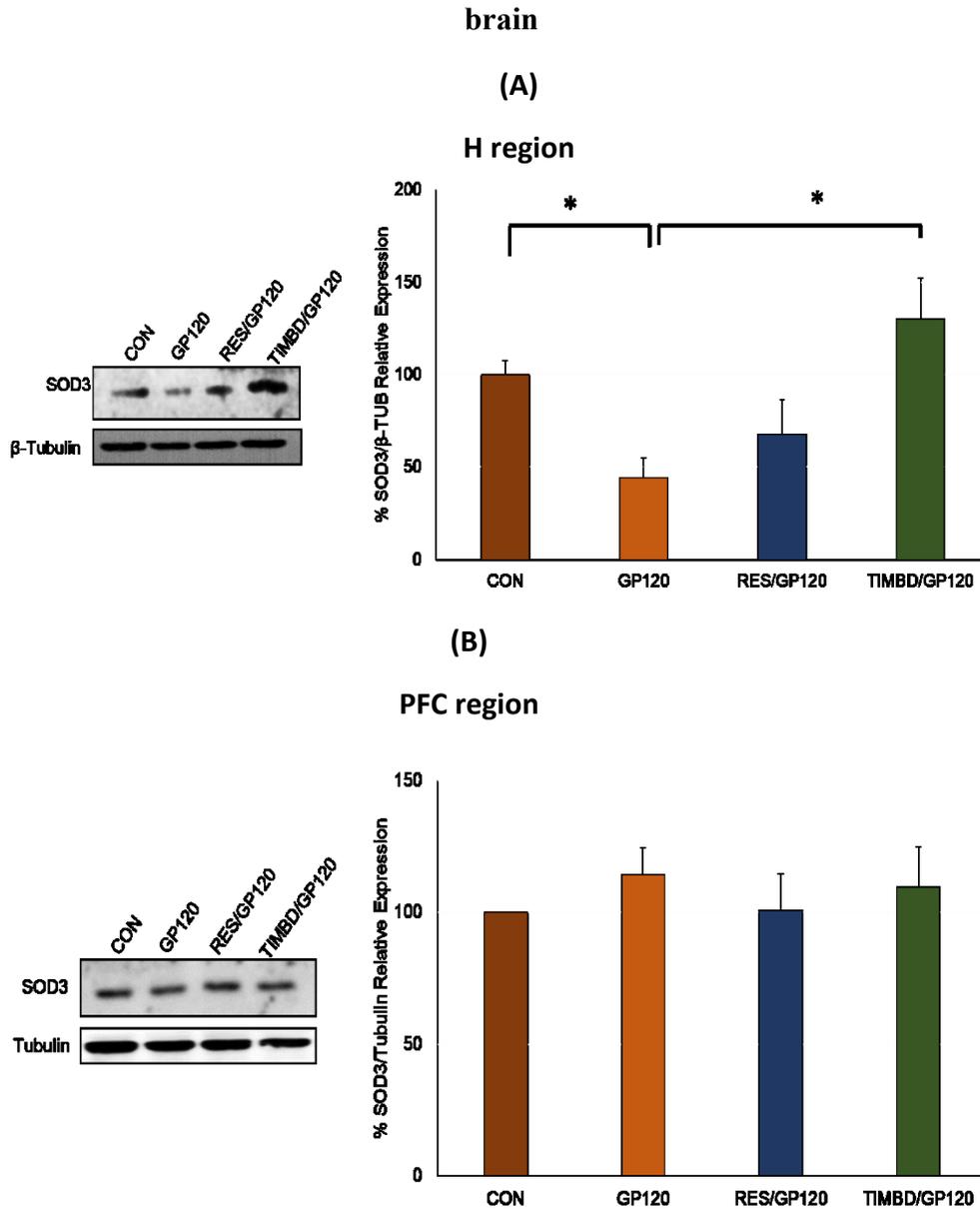


Figure 24. Brain regions were collected from HIV-1 gp120-tg mice and WT controls following 12 weeks of treatment with TIMBD, or RES, or CON pellets. Expression levels of SOD3 were determined in H (A) and PFC brain regions using western blotting. Each bar represents mean \pm SEM for at least 3 independent westerns. One-way Anova was used for statistical analysis and statistical significance is denoted as * (p -value \leq 0.05).

TIMBD has no effect on SOD3 expression levels mice brain region (PC)

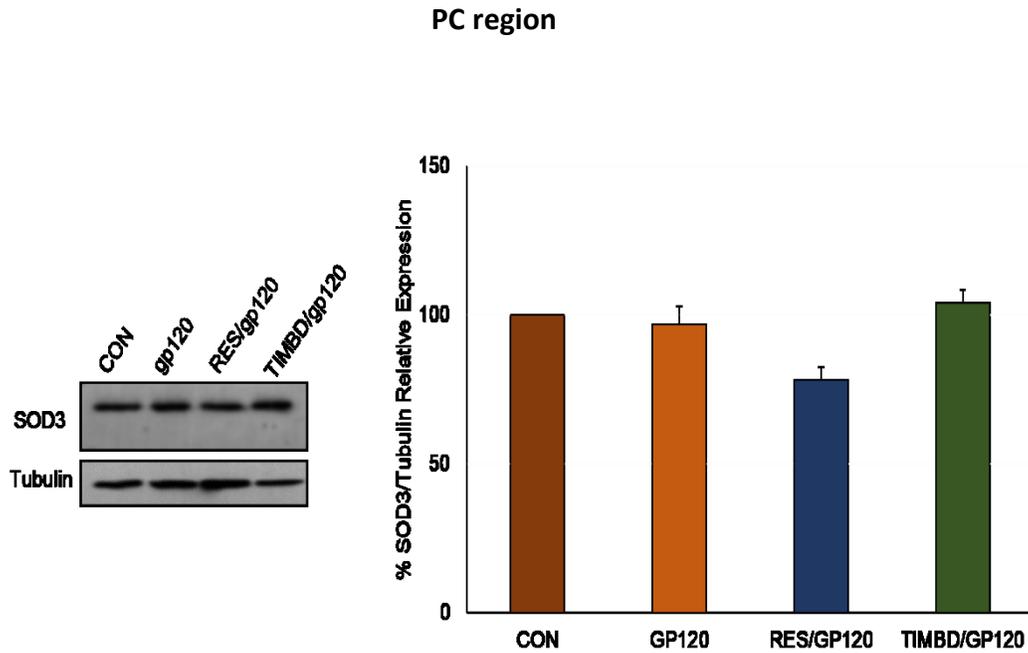


Figure 25. Brain regions were collected from HIV-1 gp120-tg mice and WT controls following 12 weeks of treatment with TIMBD, or RES, or CON pellets. Expression levels of SOD3 were determined in PC brain regions using western blotting. Each bar represents mean \pm SEM for at least 3 independent westerns. One-way Anova was used for statistical analysis and statistical significance is denoted as * (p-value \leq 0.05) and ** (p-value \leq 0.01).

TIMBD has no effect on SOD1 expression levels in mice brain

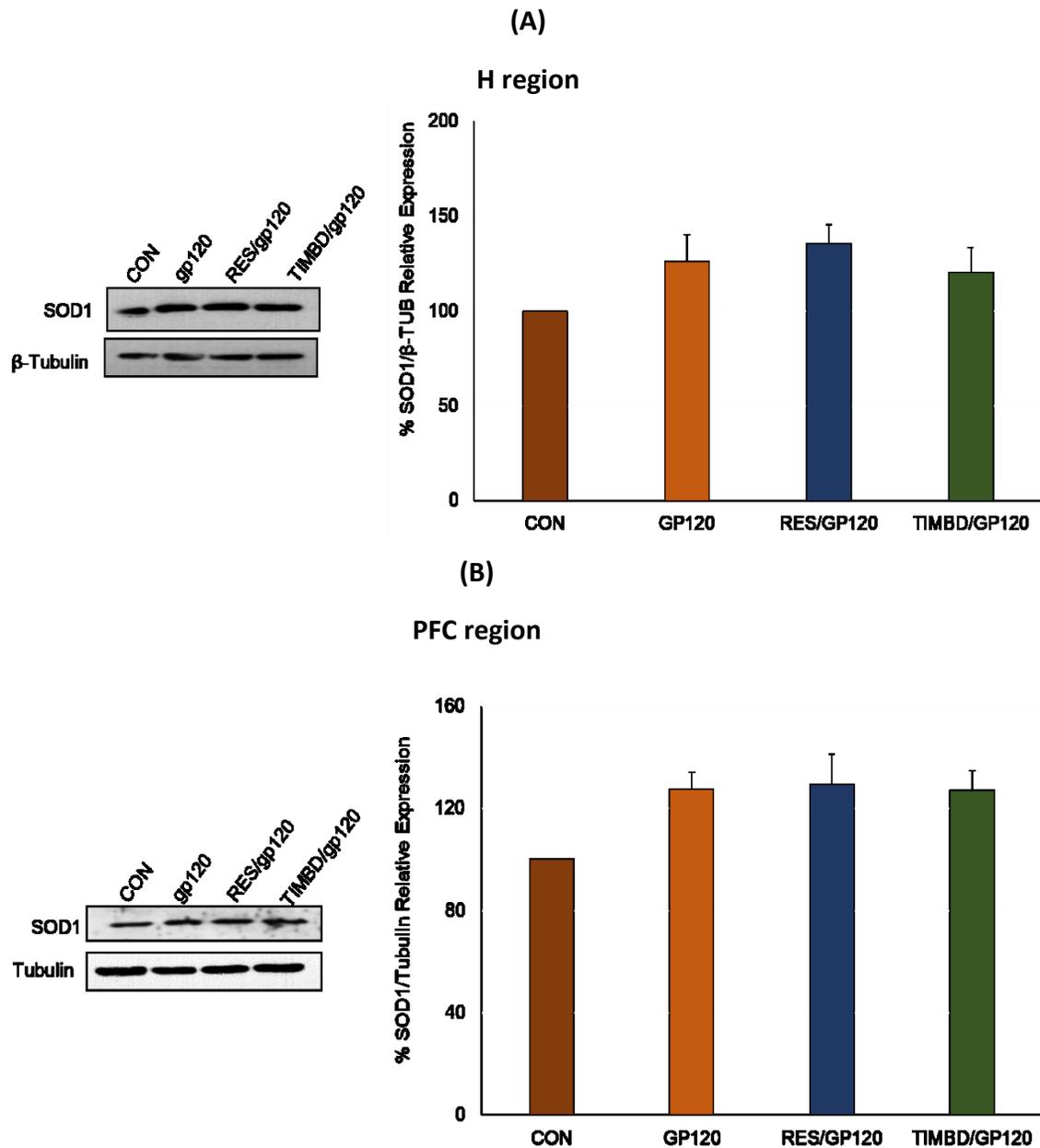


Figure 26. Brain regions were collected from HIV-1 gp120-tg mice and WT controls following 12 weeks of treatment with TIMBD, or RES, or CON pellets. Expression levels of SOD1 were determined in H (A) and PFC (B) brain regions using western blotting. Each bar represents mean \pm SEM for at least 3 independent westerns. One-way Anova was used for statistical analysis and statistical significance is denoted as * (p-value \leq 0.05) and ** (p-value \leq 0.01).

TIMBD has no effect on SOD1 expression levels in mice brain

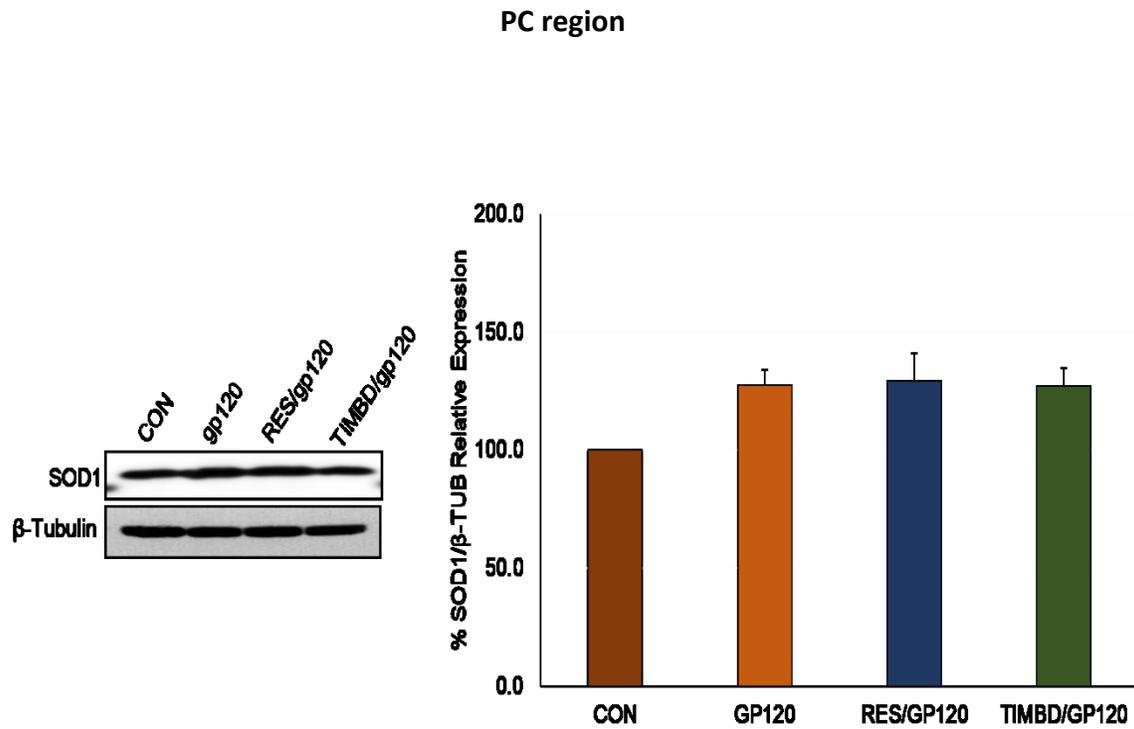


Figure 27. Brain regions were collected from HIV-1 gp120-tg mice and WT controls following 12 weeks of treatment with TIMBD, or RES, or CON pellets. Expression levels of SOD1 were determined in PC brain region using western blotting. Each bar represents mean \pm SEM for at least 3 independent westerns. One-way Anova was used for statistical analysis and statistical significance is denoted as * (p-value \leq 0.05) and ** (p-value \leq 0.01).

TIMBD and HIV-1 gp120 have no effects on SOD2 expression levels in mice brain

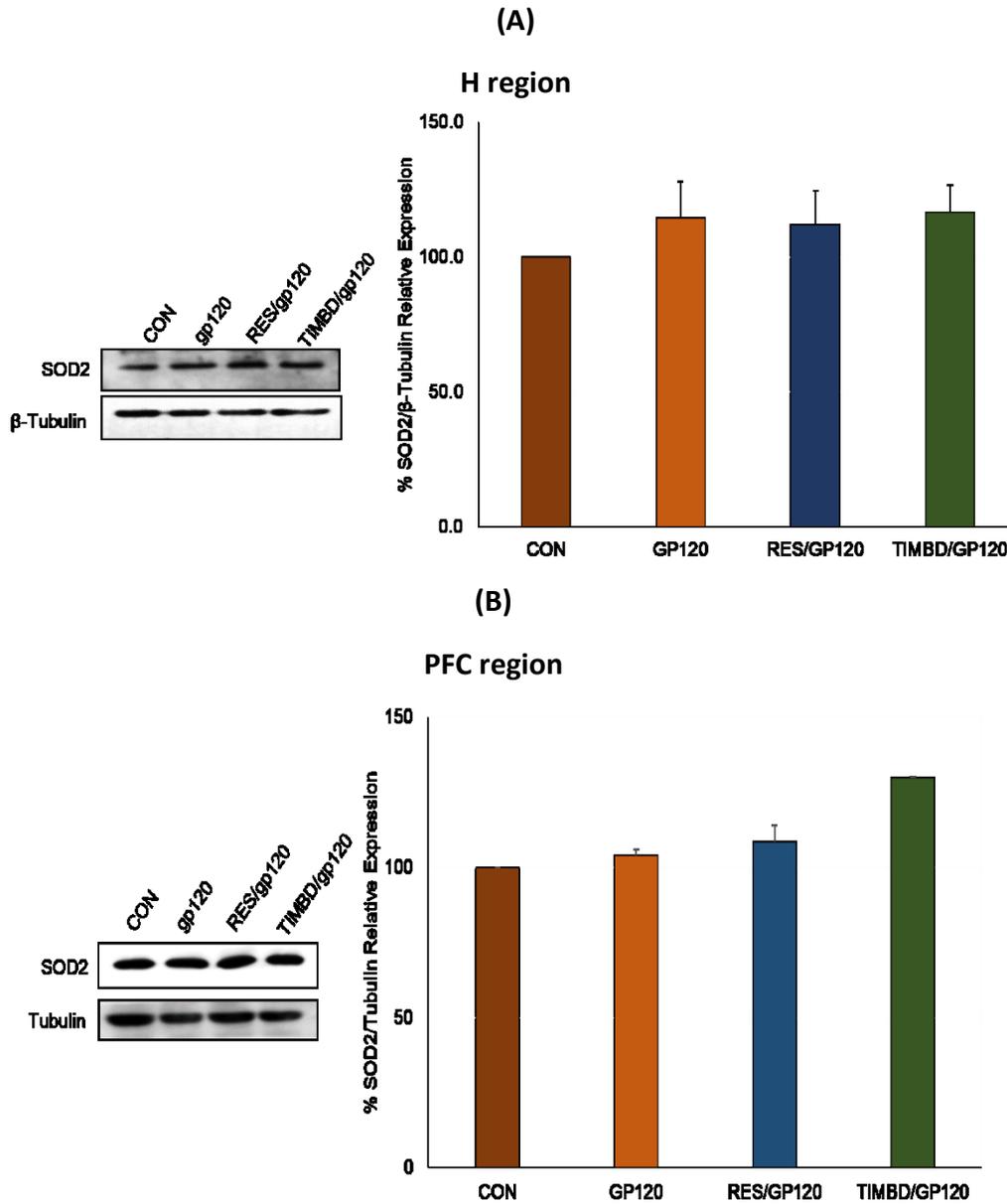


Figure 28. Brain regions were collected from HIV-1 gp120-tg mice and WT controls following 12 weeks of treatment with TIMBD, or RES, or CON pellets. Expression levels of SOD2 were determined in H (A) and PFC (B) brain regions using western blotting. Each bar represents mean \pm SEM for at least 3 independent westerns. One-way Anova was used for statistical analysis and statistical significance is denoted as * (p -value \leq 0.05) and ** (p -value \leq 0.01).

TIMBD and HIV-1 gp120 have no effects on SOD2 expression levels in mice brain

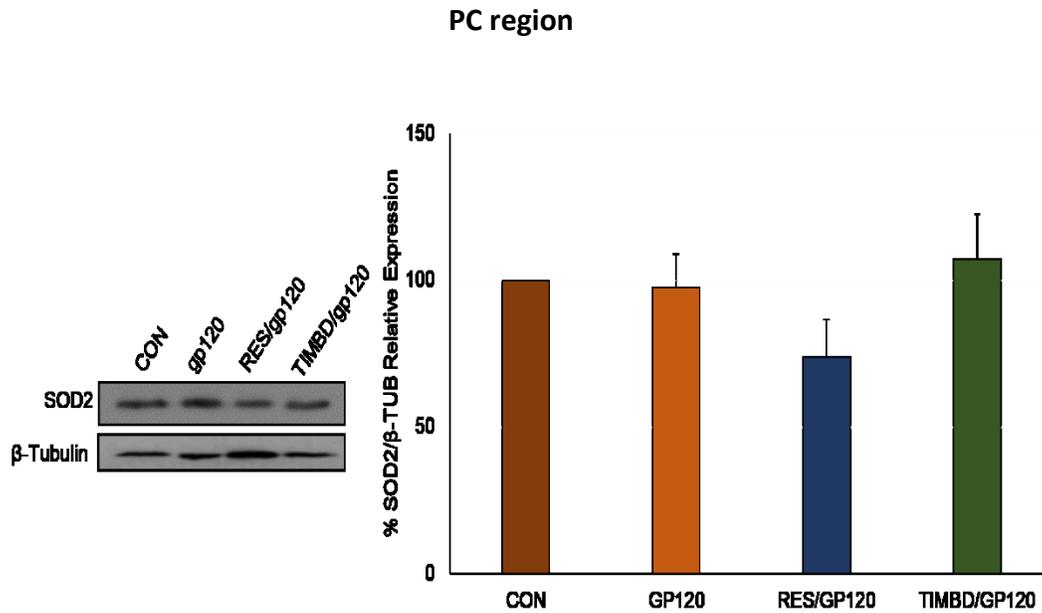


Figure 29. Brain regions were collected from HIV-1 gp120-tg mice and WT controls following 12 weeks of treatment with TIMBD, or RES, or CON pellets. Expression levels of SOD2 were determined in PC brain region using western blotting. Each bar represents mean \pm SEM for at least 3 independent westerns. One-way Anova was used for statistical analysis and statistical significance is denoted as * (p-value \leq 0.05) and ** (p-value \leq 0.01).

4.2.6. TIMBD decreases expression levels of MMP9 *in vitro* and *in vivo*

Neurodegeneration has been correlated with an increase in protein expression of matrix-metalloproteinase-9(MMP9). MMP9 plays an important role in breaking the integrity of the BBB leading to leakage through the CNS. Gp120 has been shown to increase MMP9 expression which is associated with the neuronal damage caused by gp120 (Louboutin, Agrawal, et al., 2010; Louboutin et al., 2011; Louboutin & Strayer, 2012). To determine the effects of gp120 and TIMBD treatment on MMP9 expression levels, SVG astrocytic cells were treated with 50 μ M of TIMBD or RES one hour prior to transfection with gp120 plasmid using serum-free medium. Cells were transiently transfected with 2 μ g plasmid encoding HIV-1 gp120 for 5 h and after medium change, cells were incubated for 24 h. Protein was isolated and western blotting analysis was performed. HIV-1 gp120 increased protein expression levels of MMP9 while TIMBD treatment but not RES treatment was able to decrease the expression levels of MMP9 significantly, compared to gp120 (**Fig. 30**). To confirm our finding *in vivo*, gp120-tg mice were used, which have been shown to have high brain MMP9 levels (Louboutin, Agrawal, et al., 2010). Our results indicates that gp120 increased expression levels of MMP9 in PFC and H brain regions (**Fig. 31 panels A and B**). TIMBD treatment significantly decreased the expression levels of MMP9, compared to RES (**Fig. 31 panels A and B**). These results suggest that TIMBD might be able to preserve the BBB integrity *in vivo* by decreasing MMP9 expression levels.

TIMBD decreases protein expression levels of MMP9 in SVG astrocytes

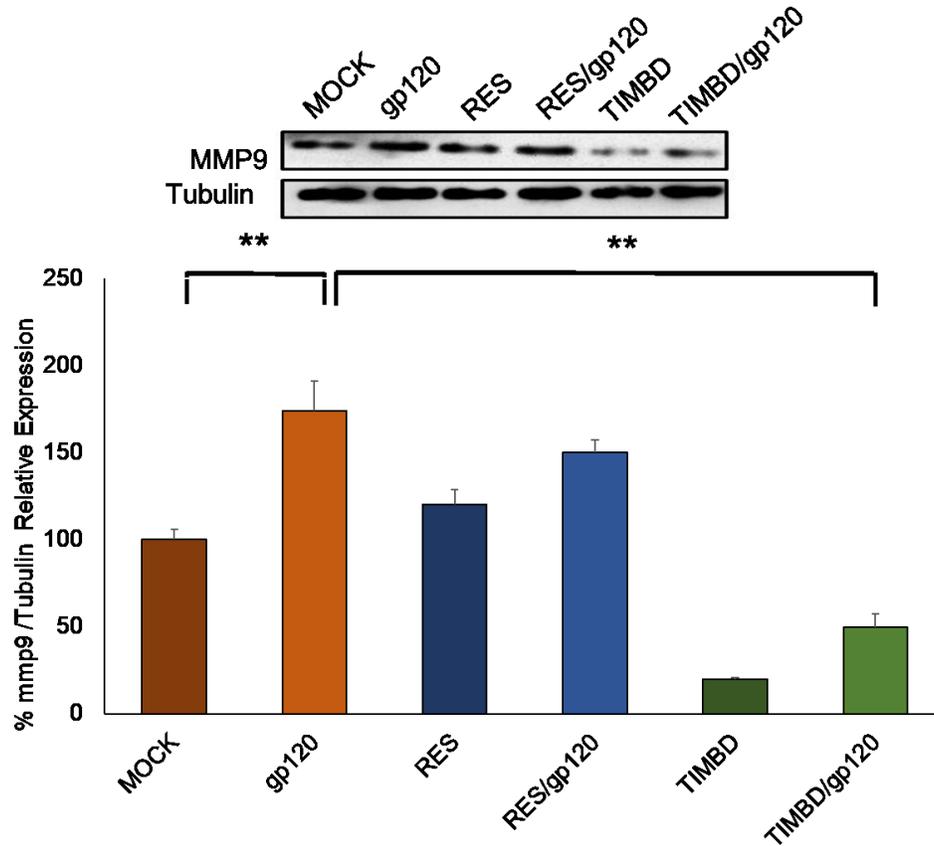


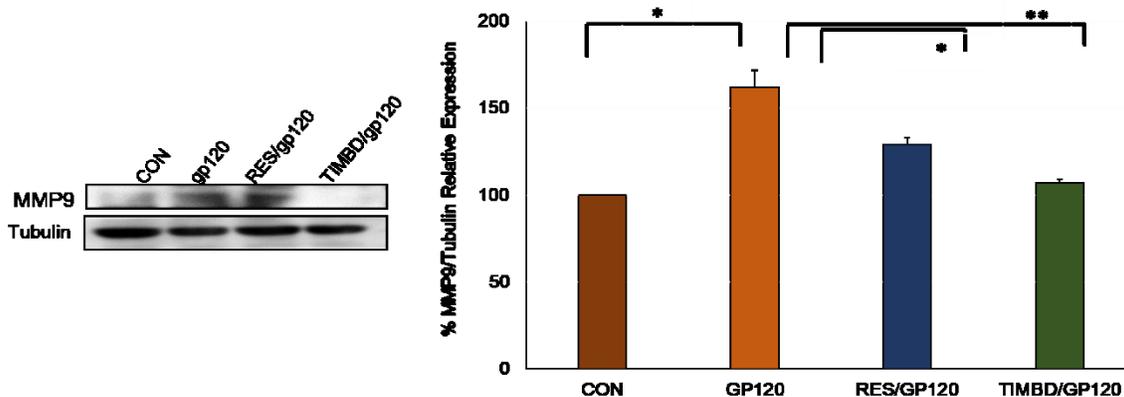
Figure 30. SVG astrocytes were treated with 50 μ M TIMBD or RES 1 h prior to transfection with plasmid encoding HIV-1 gp120 for 24 h. Protein expression levels of MMP9 were measured in wells using β -Tubulin as loading control. Each bar represents mean \pm SEM for 3 independent replicates, and the western blots are representative images. One-way Anova was used for statistical analysis and statistical significance is denoted as ** (p-value \leq 0.01).

TIMBD reduces HIV-1 gp120 associated increase in MMP9 expression levels in

mice brain

(A)

PFC region



(B)

H region

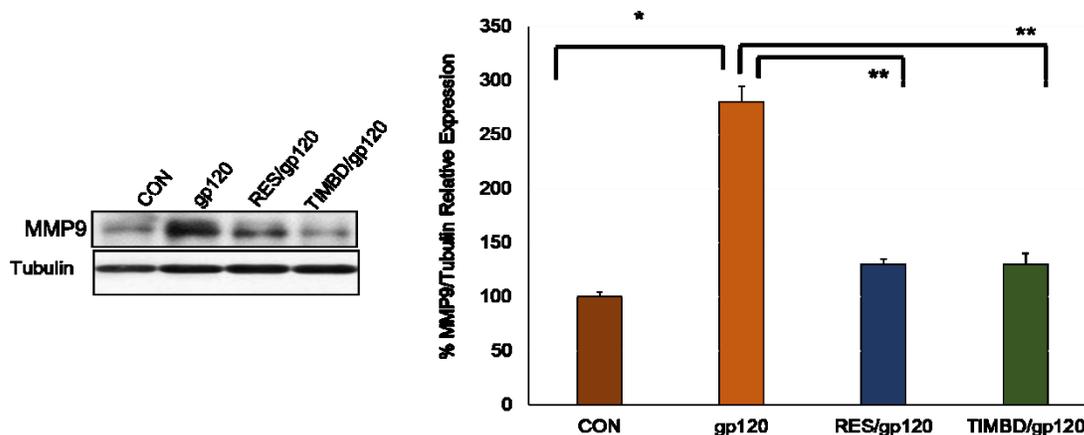


Figure 31. Brain regions were collected from HIV-1 gp120-tg mice and WT controls following 12 weeks of treatment with TIMBD, or RES, or CON pellets. Expression levels of MMP9 were determined in PFC (A) and H (B) brain regions using western blotting. Each bar represents mean \pm SE for at least 3 independent westerns. One-way Anova was used for statistical analysis and statistical significance is denoted as * (p-value \leq 0.05) and ** (p-value \leq 0.01).

4.2.7. TIMBD decreases gp120-associated cell death in SVG astrocytes

Published literature suggests that oxidative stress affects cell proliferation and leads subsequently to cell death (Kapur et al., 2016; Klein & Ackerman, 2003). Therefore, effect of TIMBD and RES on gp120-associated cell death was investigated. Gp120 significantly increased levels of terminal deoxynucleotidyltransferase-mediated dUTP-nick end labeling (TUNEL)-positive astrocytes, compared to mock (**Fig. 32 panel A**). Treatment with TIMBD decreased levels of TUNEL-positive cells, compared to gp120 (**Fig. 32 panel A**). However, there was no effect of treatment with RES (**Fig. 32 panel A**). Hoechst staining showed that TIMBD but not RES decreased the number of stained cells, indicating less damage, compared to gp120 or RES (**Fig. 32 panel B**). Studies have shown that programmed cell death apoptosis is involved in gp120-mediated cell death in astrocytes (Shah et al., 2013). To investigate whether TIMBD can modulate apoptosis, the activity of apoptotic marker caspase-3 was determined. Treatment with TIMBD decreased the cleaved- (cl-) caspase-3 levels significantly, compared to gp120, indicating that TIMBD indeed decreased gp120-associated apoptosis (**Fig. 32 panel C**). Taken together, these results indicated that TIMBD is capable of decreasing cell death associated with gp120. In contrast, RES is not able to do this in SVG astrocytes.

TIMBD decreases HIV-1 gp120-associated cell death in SVG astrocytes

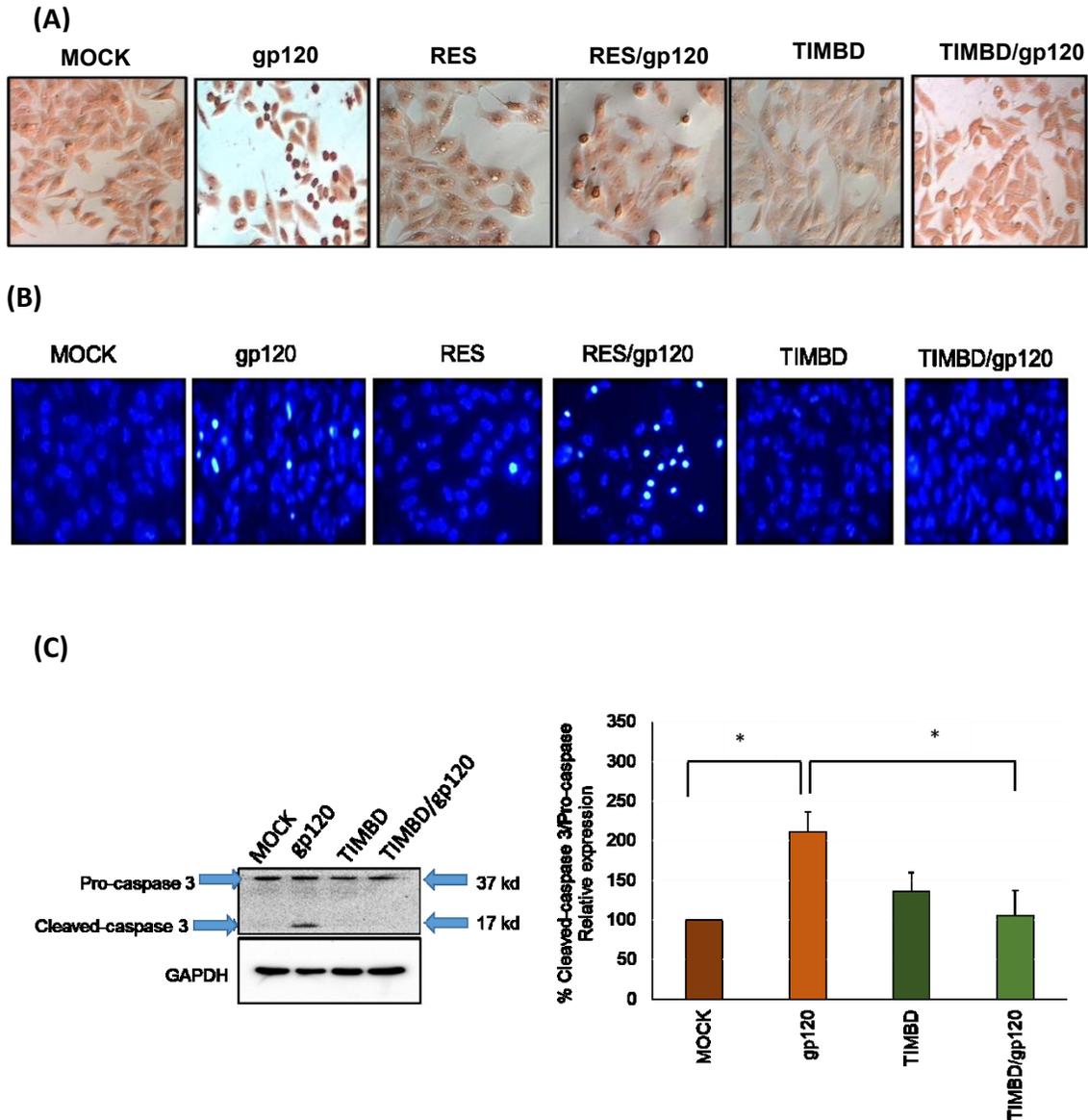


Figure 32. SVG astrocytes were treated with 50 μ M TIMBD 1 h prior to transfection with plasmid encoding HIV-1 gp120 for 24 h. (A) The effect of TIMBD on TUNEL labelling were determined. (B) The effect of TIMBD on DNA-damage using Hoechst staining were determined. The images were taken using light microscopy. The presented data are representative images. (C) Protein expression levels of phosphorylated caspase-3 were measured with GAPDH as loading control. Each bar represents mean \pm SEM for 3 independent experiments, with each experiment done in triplicate and the western blots are representative images. One-way Anova was used for statistical analysis and statistical significance is denoted as * (p -value \leq 0.05).

4.3. Discussion

Following the development of ART, HIV patient survival and immune outcome have improved significantly (Ellis, Langford, & Masliah, 2007; McArthur & Brew, 2010; Woods, Moore, Weber, & Grant, 2009). However, neurological abnormalities persist in these patients. HAND is manifested by increases in neuroinflammation, ROS production, BBB damage and neuronal cell death (Kanmogne et al., 2007; Mollace et al., 2001; Toborek et al., 2005). The severity of HAND is classified into three categories: asymptomatic neurocognitive impairment, minor neurocognitive disorder and, most severe, HIV-associated dementia (McArthur & Brew, 2010). Thus, it remains critical to develop drugs that can improve the quality of life of HAND patients.

Oxidative stress has been considered a major mechanism for HAND pathogenesis in CNS (Uttara et al., 2009). HIV viral proteins for example gp120 triggers formation of ROS (Ran et al., 2017; Samikkannu et al., 2015; Shah et al., 2013). HIV-1 gp120 has been reported previously to induce ROS formation in astrocytes (Reddy, Gandhi, et al., 2012; Shah et al., 2013). In general, high ROS levels cause a redox imbalance within the cell. To protect cells from oxidative stress, cells stimulate the production of antioxidant genes. NRF2 transcription factor regulates antioxidant defense enzymes by binding to antioxidant response element (ARE) in the promoter region of antioxidant genes. Upregulating antioxidant enzymes through NRF2 activation has been linked to suppression of oxidative damage in neurological disease models (B. Gao, Doan, & Hybertson, 2014; Reddy, Gandhi, et al., 2012; Scapagnini et al., 2011; Zhang et al., 2013). NADPH oxidase isotype NOX2 is one of the critical enzymes involved in ROS production (Bedard & Krause, 2007; Maghzal, Krause, Stocker, & Jaquet, 2012). It is mainly expressed in brain (Dugan et al.,

2009; Schiavone, Riezzo, Turillazzi, & Trabace, 2016). NOX2 upregulation has been reported in many neurological diseases (Bedard & Krause, 2007; Fischer et al., 2012; Segal, 2008; Youn, Cho, Kim, Choi, & Park, 2017). Recent studies revealed that NOX2 is involved in gp120-mediated oxidative stress in astrocytes (Reddy, Agudelo, et al., 2012; Shah et al., 2013). Antioxidant enzymes like catalase and GPX1 are dysregulated by HIV viral proteins Tat and gp120 (Price et al., 2005). The extracellular isoform of SOD enzymes (SOD3) has been reported to be critical for brain physiology and pathology in mice (Oury, Card, & Klann, 1999). SOD3 plays an important role in the pathogenesis of many diseases including cardiovascular disease and breast cancer (Fukai, Folz, Landmesser, & Harrison, 2002; Qin, Reszka, Fukai, & Weintraub, 2008; B. Singh & Bhat, 2012; Teoh, Fitzgerald, Oberley, & Domann, 2009). However, until now, no information has been published related to SOD3's involvement in HIV neuropathogenesis. In our study, we report for the first time that gp120-tg mice have lower expression levels of SOD3 in their brain, compared to WT control mice. Opposite of that, NOX2 enzyme was elevated in gp120-tg mice brain. TIMBD treatment increased expression levels of SOD3 while it decreased expression levels of NOX2 in gp120-tg mice. Our *in vitro* results demonstrated that TIMBD might be decreasing oxidative stress, produced by gp120, through activating NRF2 transcription factor which induces upregulation of antioxidant enzymes.

Overall, our study demonstrates that activation of NRF2 by TIMBD in astrocytes decreases NOX2 levels and increases antioxidant enzymes, compared to gp120-treated cells (**Fig. 33**). Our results suggest that the increase in NOX2 expression and decrease in SOD3 expression levels *in vivo* may lead to increased susceptibility to HIV1-induced oxidative damage. TIMBD reversed the gp120 effects, both *in vitro* and *in vivo*, indicating

its capability in controlling oxidative stress associated with gp120. These findings suggest that TIMBD may be beneficial in preventing oxidative stress increased in HAND patients and may be a potential therapy against HAND.

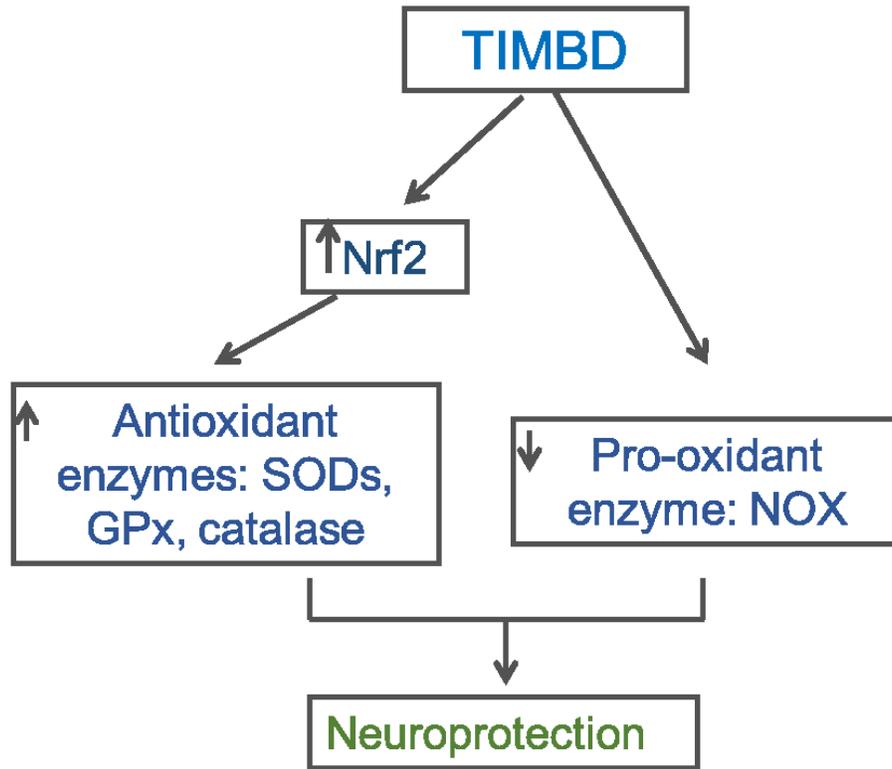


Figure 33. Schematic illustration of the suggested mechanism for inhibition of gp120-induced oxidative stress by TIMBD.

CHAPTER 5

EFFECT OF TIMBD ON GP120-INDUCED INFLAMMATORY CYTOKINES/CHEMOKINES IN ASTROCYTES

5.1. Introduction

Many of the HIV-associated diseases, for example neurodegeneration, are considered potential health concerns globally (Anzala et al., 1995; Morgan, Mahe, Mayanja, & Whitworth, 2002). HIV is known to infiltrate the brain with a potential to infect different cells of the brain. In the years following HIV infection, patients show signs of neurocognitive problems named HIV-associated neurocognitive disorders (HAND) (Alfahad & Nath, 2013; Atashili et al., 2013; Barber et al., 2014). Following infection of the central nervous system (CNS), viral proteins like gp120, Tat and vpr are released from the infected brain cells, causing injury to the brain and potentially leading to cognitive and motor dysfunction in the infected individuals (Clifford & Ances, 2013; Toborek et al., 2005). Patients suffer from memory loss, personality changes, diminished mental capacity and inability to use acquired knowledge (Fellows, Byrd, Morgello, & Manhattan, 2013; Letendre, Ellis, Ances, & McCutchan, 2010; Letendre et al., 2009). Complete understanding of HIV-associated neurotoxicity has been difficult. HIV viral proteins released from infected microglia and monocytes have been generally considered as direct and indirect contributors to HAND (Al-Harhi et al., 2014; Markowitz, White, Kolson, & Jordan-Sciutto, 2007). Additionally, astrocytes can also be infected with HIV leading to activation of astrocytes and subsequent release of inflammatory mediators which in turn lead to neuronal death directly or indirectly (Gorry et al., 2003; Gray et al., 2014).

One of the major HIV viral proteins is glycoprotein 120 (gp120) that has been extensively studied for its neurotoxic contribution to the CNS (Louboutin & Strayer, 2012). Furthermore, HIV-1 gp120 has been shown to induce a variety of inflammatory mediators like TNF α , IL6, IL8 and CCL5 (Shah & Kumar, 2010; Shah, Singh, Buch, & Kumar, 2011; Shah, Verma, et al., 2011). Appropriate regulation of pro-inflammatory molecules is critical to inhibit or prevent inflammation. Cellular signal transduction pathways have been shown to be perturbed by HIV-1 gp120 (Shah & Kumar, 2010; Shah, Singh, et al., 2011; Shah, Verma, et al., 2011). Although chemokine receptors and their ligands play a key role in both HIV infection and immune response, how to modify these signaling processes by therapeutic intervention is still debated and under active investigation. Reduction of cytokine production is considered an efficient therapeutic target. Cytokine expression levels are known to be controlled by their regulating transcription factors AP1, STAT3 and NF κ B (Ronaldson, Ashraf, & Bendayan, 2010; Squarize, Castilho, Sriuranpong, Pinto, & Gutkind, 2006). Suppression of the activation of the above transcription factors or their upstream regulatory molecules can be significant effective ways to decrease the expression levels of cytokines.

Resveratrol (RES) is a polyphenolic phytoalexin natural compound found in many natural fruits like berries and nuts (Sharma, Anjaneyulu, Kulkarni, & Chopra, 2006; Wallerath et al., 2002). Resveratrol has been studied extensively in several types of pathological disorders and found to be effective in study models (Athar et al., 2009; Bagatini et al., 2016; B. Singh et al., 2014; Vilar-Pereira et al., 2016). For example, RES has been studied for its role against oxidative stress, inflammation, cancers and many other pathological conditions (Athar et al., 2009; Bagatini et al., 2016; B. Singh et al., 2014;

Vilar-Pereira et al., 2016). It has been shown to produce its antioxidant effect through upregulation of the Nrf2 transcription factor and consequently through the transcriptional activation of antioxidant enzymes (Reddy, Agudelo, et al., 2012). Resveratrol has also been studied for its anti-inflammatory role and recently its effect has been suggested to be regulated by the modulation of the NFκB transcription factor and its upstream molecule Sirt1 (Buhrmann, Shayan, Popper, Goel, & Shakibaei, 2016; F. Yeung et al., 2004; Zhu et al., 2011). The low potency of RES has however limited its use in clinical settings. Moreover, pharmacokinetic studies in healthy human subjects has revealed that RES is extensively metabolized in the liver with an oral bioavailability of less than 1% which further limited its success in clinical trials (Walle, 2011). To improve the biological efficacy and potency of RES, we have synthesized several RES-analogs (Siddiqui et al., 2013). One of the synthesized RES-analogs, 4-(E)-{(p-tolylimino)-methylbenzene-1,2-diol} (TIMBD), has been found to be effective in decreasing the proliferation of breast cancer cells as well as decreasing oxidative stress (Chatterjee et al., 2017; Ronghe, Chatterjee, Bhat, Padhye, & Bhat, 2016; Ronghe et al., 2014; Siddiqui et al., 2013).

In the present study, we tested the effect of TIMBD against HIV-1 gp120-associated neuroinflammation. Our study suggests that TIMBD has a higher potency than RES in inhibiting gp120-induced inflammatory cytokine expression in SVG astrocytes. Our study further indicates that STAT3, AP1, and NFκB are involved in the inhibition of gp120-induced cytokine expressions by TIMBD. These results indicate that TIMBD might be a promising therapeutic candidate for decreasing HIV-associated neuroinflammation and other neurological diseases that are mediated by HIV.

5.2. Results

In the present study, we investigated the effect of TIMBD against HIV-1 gp120-induced inflammation using SVG astrocytes and compared its efficacy with that of RES.

5.2.1. TIMBD inhibits HIV-1 gp120-induced IL6 mRNA and protein expression levels

It has been previously reported that gp120 increases the mRNA and protein expression levels of proinflammatory cytokines IL6, IL8, and CCL5 in SVG astrocytes (Shah & Kumar, 2010; Shah, Singh, et al., 2011; Shah, Verma, et al., 2011). It is also shown that gp120 increases maximal mRNA expression of IL6 at 6 h and protein at 48 h post-transfection (Shah, Verma, et al., 2011). To determine the effects of TIMBD on the expression levels of IL6 mRNA and protein, SVG astrocytic cells were treated with doses ranging from 6 to 50 μ M of either TIMBD or RES one hour prior to transfection with gp120 plasmid in the serum-free medium. Then the cells were transiently transfected with 2 μ g plasmid encoding HIV-1 gp120 for 5 h, after a medium change, cells were incubated for 6 h or 48 h. RNA and protein were isolated using established protocols (Shah & Kumar, 2010; Shah, Singh, et al., 2011; Shah, Verma, et al., 2011). HIV-1 gp120 induced IL6 mRNA expression levels to 28.5 ± 4.1 fold increase while treatment with TIMBD decreased expression levels of IL6 mRNA significantly in a dose-dependent manner (**Fig. 34 panel A**). RES decreased gp120-induced IL6 mRNA expression levels only at the 50 μ M dose (**Fig. 34 panel B**). RES at the 50 μ M dose was somewhat cytotoxic to SVG astrocytes (**Fig. 11 in chapter 4**).

The effect of TIMBD on gp120-induced protein expression levels was further investigated by measuring levels of secreted IL6 protein in supernatant, 48 h following transfection of SVG cells with HIV-1 gp120. A significant increase in IL6 protein

expression levels was observed in gp120-transfected cells (18.3 ± 2.8 ng/ml) compared to control, while TIMBD decreased IL6 protein expression levels in a dose-dependent manner (**Fig. 35**). Immunocytochemical (ICC) analysis was performed to measure the effect of TIMBD or RES on IL6 protein expression levels. As shown in **Fig. 36**, gp120 increased the IL6 protein expression while TIMBD, but not RES, decreased IL6 protein expression levels in SVG astrocytic cells. The intensity of IL6 relative to GFAP in HIV-1 gp120 transfection was found to be 2.2 ± 0.1 fold (**Fig. 36**). TIMBD treatment decreased gp120-induced IL6 intensity significantly to 1.12 ± 0.2 fold, while RES' effect is less potent than that of TIMBD. Thus, our results indicate that TIMBD but not RES is able to decrease IL6 expression significantly.

TIMBD but not RES decreases IL6 mRNA expression levels in a dose-dependent manner

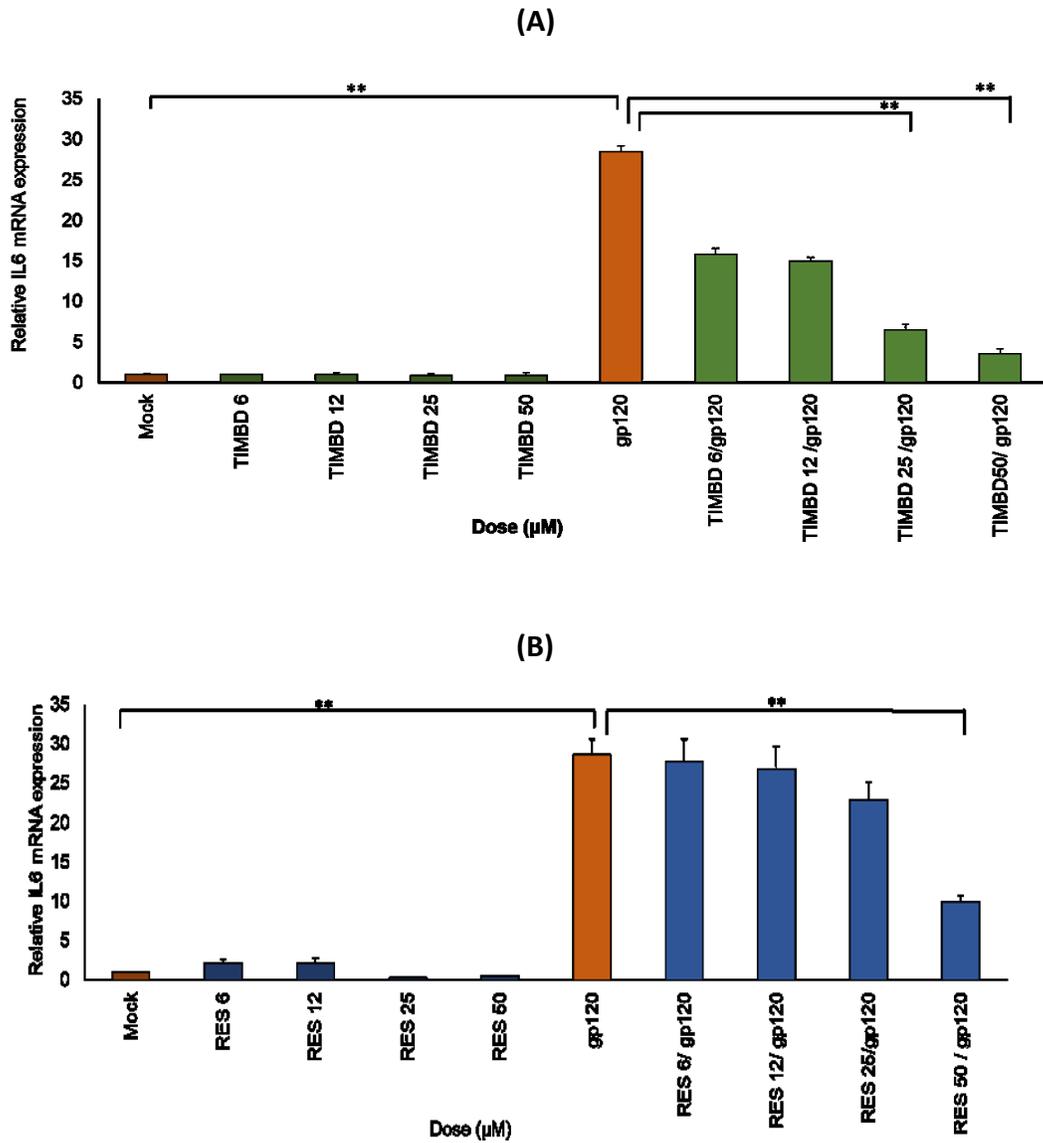


Figure 34. SVG astrocytes were treated with 6 to 50 μ M TIMBD (A) or RES (B), 1 h prior to transfection with plasmid encoding HIV-1 gp120, for 5 h. The cells were harvested at 6 h and expression levels of IL6 mRNA were determined by RT-PCR. Each bar represents mean \pm SEM for at least 3 independent experiments, with each experiment done in triplicate. One-way Anova was used for statistical analysis and statistical significance is denoted as ** (p-value \leq 0.01).

TIMBD decreases IL6 protein expression levels in a dose-dependent manner

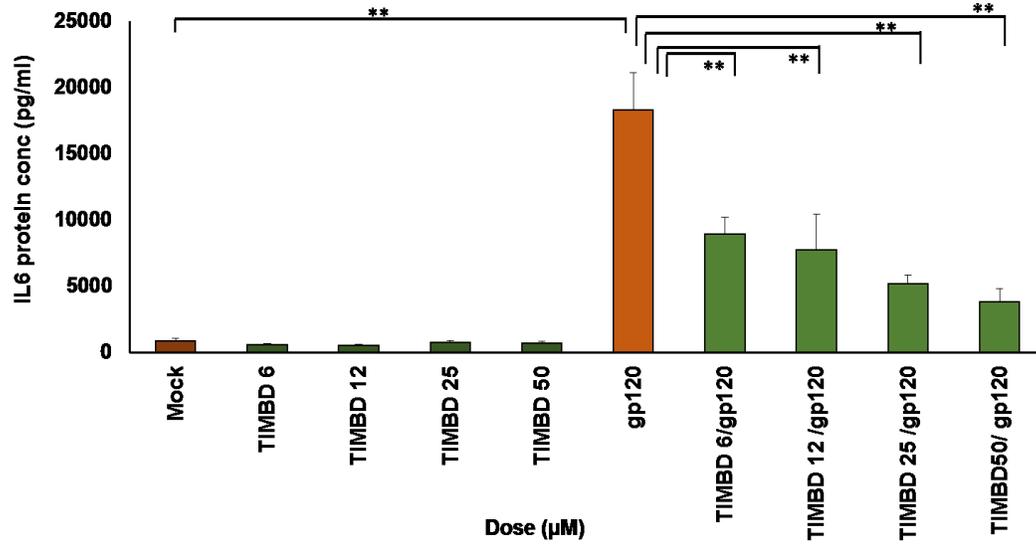


Figure 35. SVG astrocytes were treated with 6 to 50 μ M TIMBD, 1 h prior to transfection with plasmid encoding HIV-1 gp120 for 5 h. The supernatant was collected at 48 h post-transfection and IL6 protein concentrations were measured by multiplex cytokine assay. Each bar represents mean \pm SEM for at least 3 independent experiments, with each experiment done in triplicate. One-way Anova was used for statistical analysis and statistical significance is denoted as ** (p-value \leq 0.01).

TIMBD decreases IL6 protein expression levels significantly than that of RES

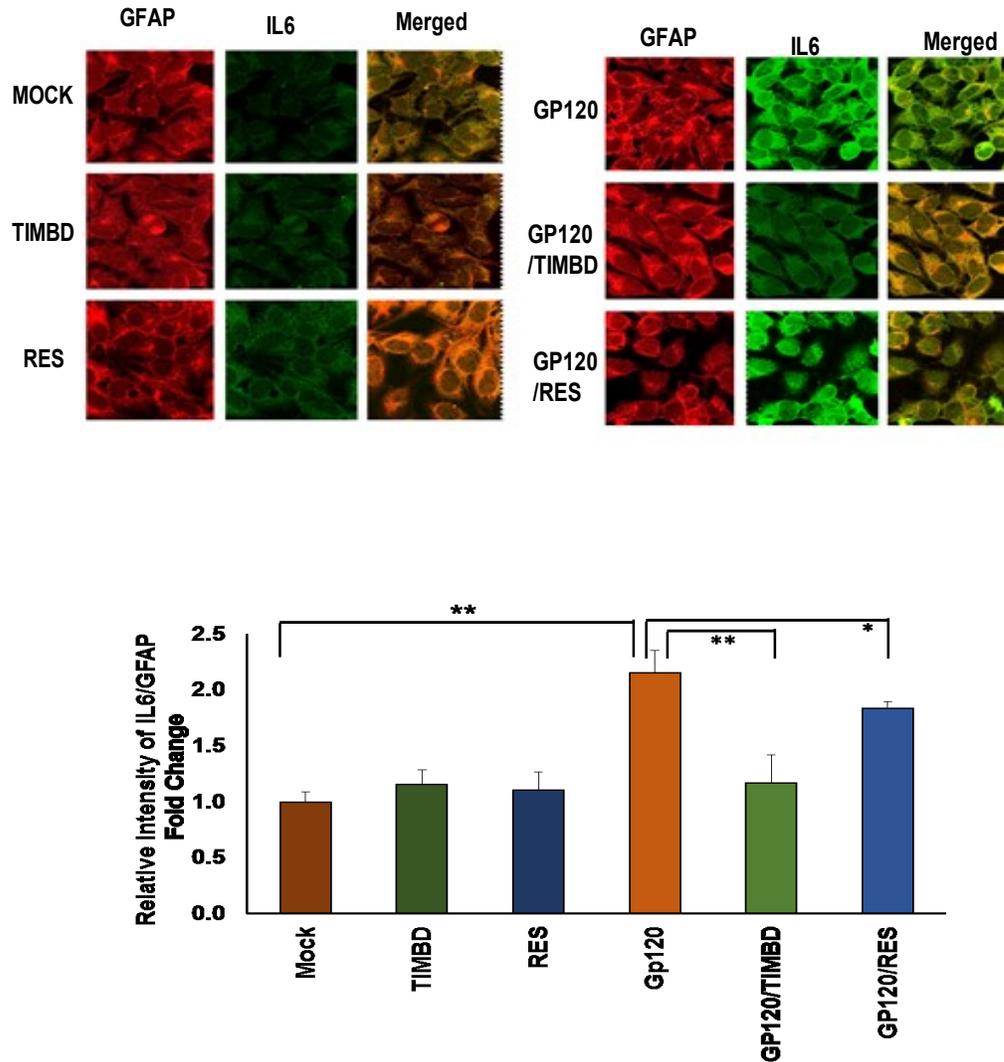


Figure 36. SVG astrocytes were treated with TIMBD or RES, 1 h prior to transfection with plasmid encoding HIV-1 gp120, for 5 h. Expression levels of IL6 and GFAP proteins were assayed by immunocytochemical methods and analyzed using confocal microscopy. The intensity of IL8/GFAP was calculated using imageJ software. Each bar represents mean \pm SEM for at least 3 independent experiments, with each experiment done in triplicate. One-way Anova was used for statistical analysis and statistical significance is denoted as * (p-value \leq 0.05) and ** (p-value \leq 0.01).

5.2.2. TIMBD inhibits HIV-1 gp120-induced IL8 mRNA and protein expression levels

The effect of TIMBD on IL8 mRNA and protein expression levels was investigated. It has previously been shown that gp120 induces maximal mRNA expression at 6 h and protein at 48 h post-transfection (Shah & Kumar, 2010). To determine the effect of TIMBD on expression levels of IL8 mRNA and protein, SVG astrocytic cells were treated with doses ranging from 6 to 50 μ M of either TIMBD or RES one hour prior to transfection with gp120 plasmid in serum-free medium. Then the cells were transiently transfected as described previously for 6 h or 48 h. RNA and protein were isolated using established protocols as mentioned earlier. HIV-1 gp120 increased IL8 mRNA expression levels 11.9 ± 0.8 fold compared to control while treatment with TIMBD decreased expression levels of IL8 mRNA significantly in a dose-dependent manner (**Fig. 37 panel A**), while RES did not show any effect on gp120-induced IL8 mRNA expression levels at any dose (**Fig. 37 panel B**).

The effect of TIMBD on gp120-induced protein expression levels was investigated further. Expression levels of secreted IL8 protein were measured in supernatant 48 h following transfection of SVG cells with HIV-1 gp120. A significant increase in IL8 protein expression levels observed in gp120 transfected cells to 490 ± 50 pg/ml, while TIMBD decreased IL8 protein expression levels in a dose-dependent manner compared to control (**Fig. 38**). Immunocytochemical analysis was performed to study the effect of TIMBD or RES on IL8 protein expression levels. As shown in (**Fig. 39**), gp120 induced the IL8 protein expression while TIMBD, but not RES, decreased IL8 protein expression levels in SVG astrocytic cells. The intensity of IL8 relative to GFAP with HIV-1 gp120 transfection was found to be 2.60 ± 0.02 fold (**Fig. 39**). TIMBD treatment decreased gp120-

induced IL8 intensity to 1.50 ± 0.02 fold while RES showed no effect on IL8 intensity of 2.40 ± 0.01 fold. Thus, our results indicate that TIMBD and not RES inhibits proinflammatory cytokine IL8 at both mRNA and protein expression levels.

TIMBD but not RES decreases IL8 mRNA expression levels in a dose-dependent manner

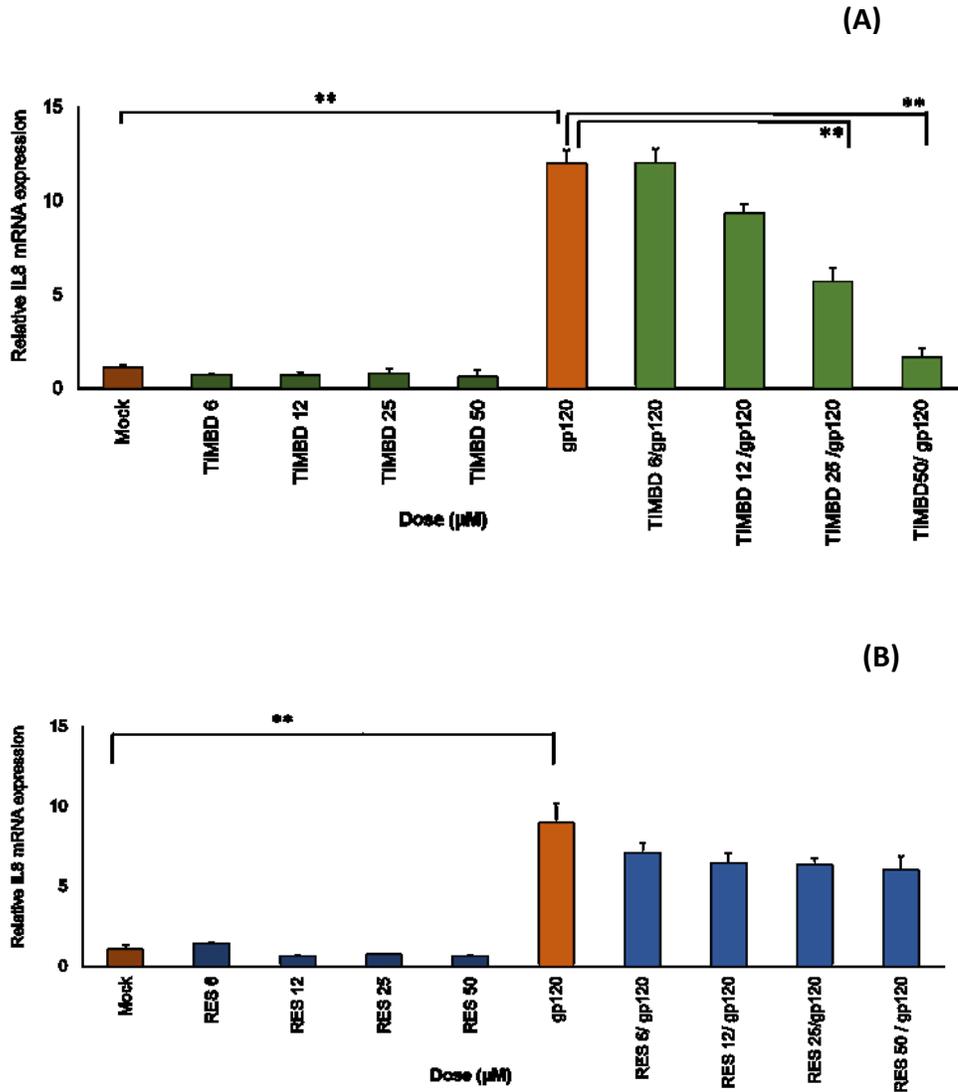


Figure 37. SVG astrocytes were treated with 6 to 50 μM TIMBD (A) or RES (B), 1 h prior to transfection with plasmid encoding HIV-1 gp120 for 5 h. The cells were harvested at 6h and the expression levels of IL8 mRNA were determined by RT-PCR. Each bar represents mean ± SE for at least 3 independent experiments, with each experiment done in triplicate. One-way Anova was used for statistical analysis and statistical significance is denoted as * (p-value ≤ 0.05) and ** (p-value ≤ 0.01).

TIMBD decreases IL8 protein expression levels in a dose-dependent manner

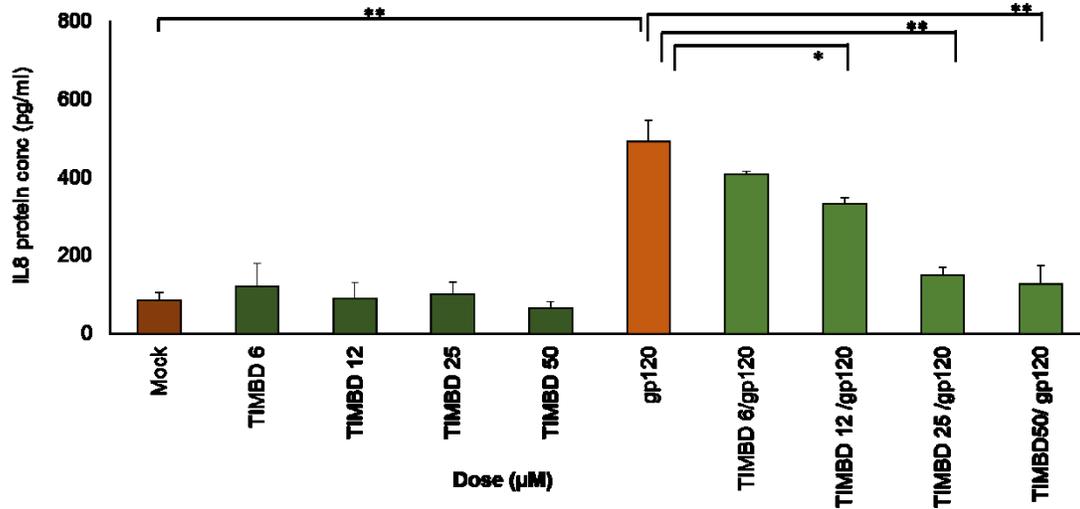


Figure 38. SVG astrocytes were treated with 6 to 50 μ M TIMBD, 1 h prior to transfection with plasmid encoding HIV-1 gp120, for 5 h. The supernatant was collected at 48 h and IL8 protein concentrations were measured by multiplex cytokine assay. Each bar represents mean \pm SE for at least 3 independent experiments, with each experiment done in triplicate. One-way Anova was used for statistical analysis and statistical significance is denoted as * (p-value \leq 0.05) and ** (p-value \leq 0.01).

TIMBD but not RES decreases IL8 protein expression levels

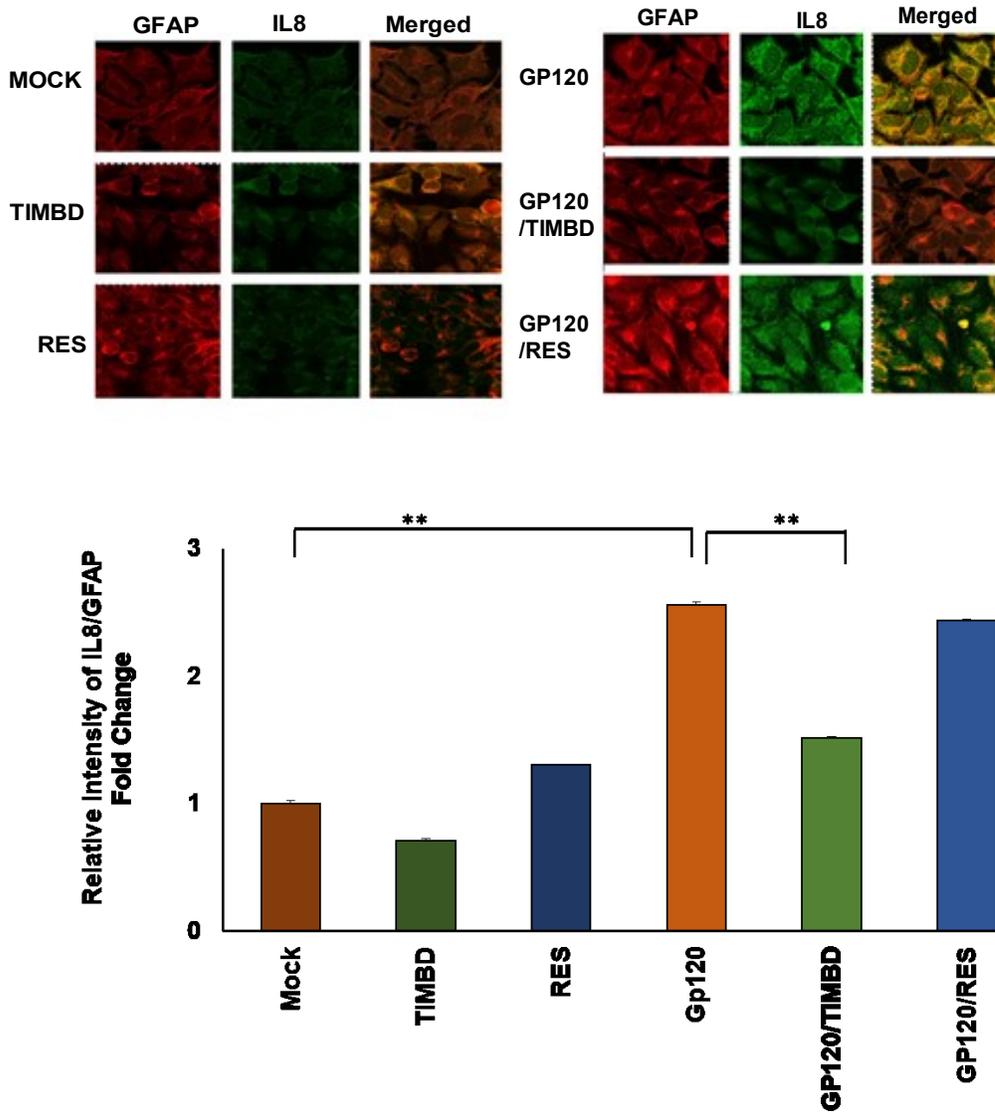


Figure 39. SVG astrocytes were treated with TIMBD or RES, 1 h prior to transfection with plasmid encoding HIV-1 gp120 for 5 h. Expression levels of IL8 and GFAP proteins were assayed by immunocytochemical methods and analyzed using confocal microscopy. The intensity of IL8/GFAP was calculated using imageJ software. Each bar represents mean \pm SEM for at least 3 independent experiments, with each experiment done in triplicate. One-way Anova was used for statistical analysis and statistical significance is denoted as ** (p-value \leq 0.01).

5.2.3. TIMBD does not affect CCL5 expression

The effect of TIMBD on gp120-induced CCL5 mRNA and protein expression levels was also investigated. Neither TIMBD (**Fig. 40 panel A** and **Fig. 41**) nor RES (**Fig. 40 panel B**) show any inhibitory effect at any dose tested on gp120-induced CCL5 expression at either mRNA or protein expression levels. Confocal imaging also showed the same trend with no effect of TIMBD or RES on CCL5 protein expression levels (**Fig. 42**).

Neither TIMBD nor RES affects CCL5 mRNA expression levels

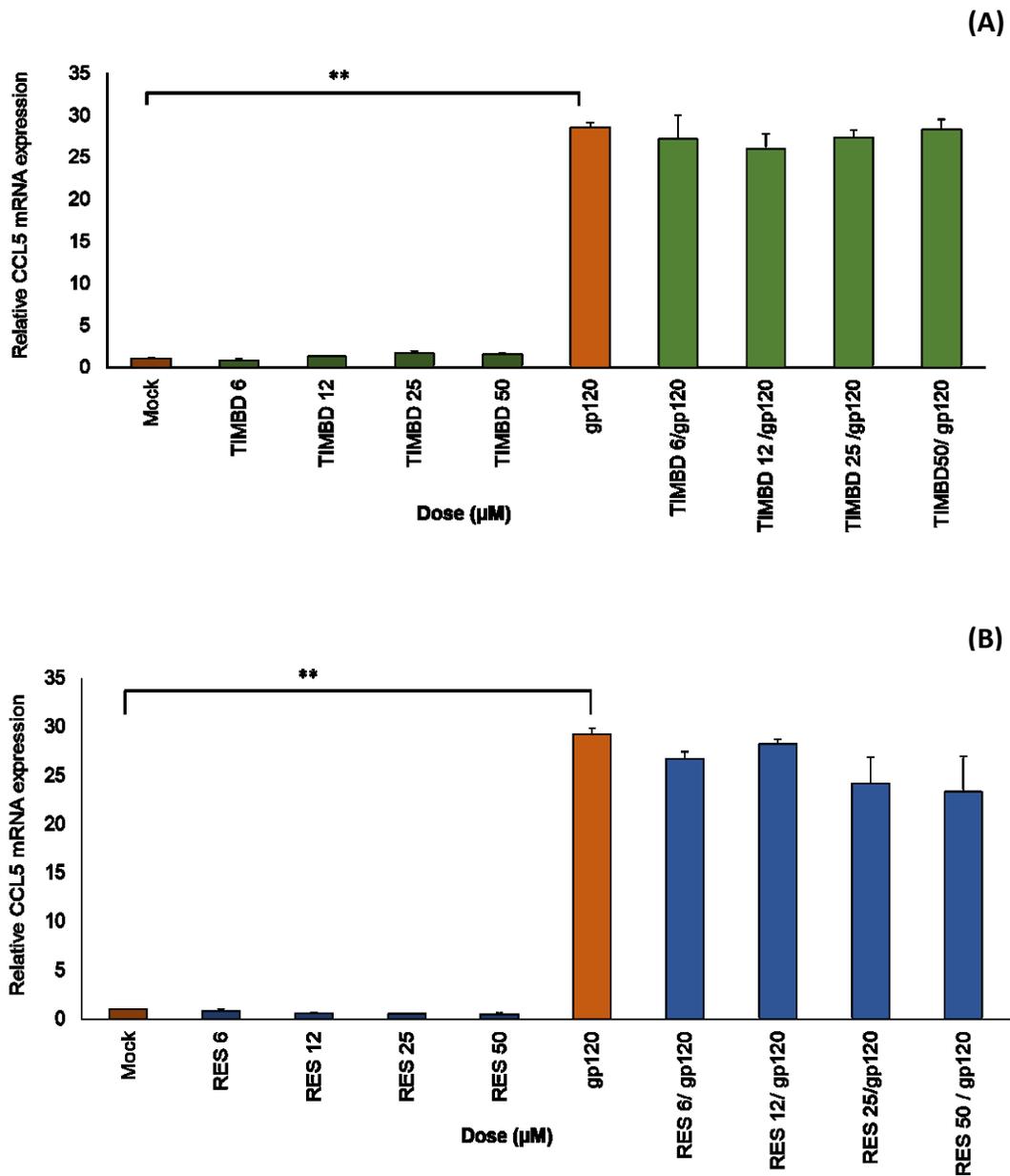


Figure 40. SVG astrocytes were treated with 6 to 50 μM TIMBD (A) or RES (B), 1 h prior to transfection with plasmid encoding HIV-1 gp120, for 5 h. The cells were harvested at 6 h and the expression levels of CCL5 mRNA were determined by RT-PCR. Each bar represents mean \pm SEM for at least 3 independent experiments, with each experiment done in triplicate. One-way Anova was used for statistical analysis and statistical significance is denoted as ** (p-value \leq 0.01).

TIMBD has no effect on CCL5 protein expression levels

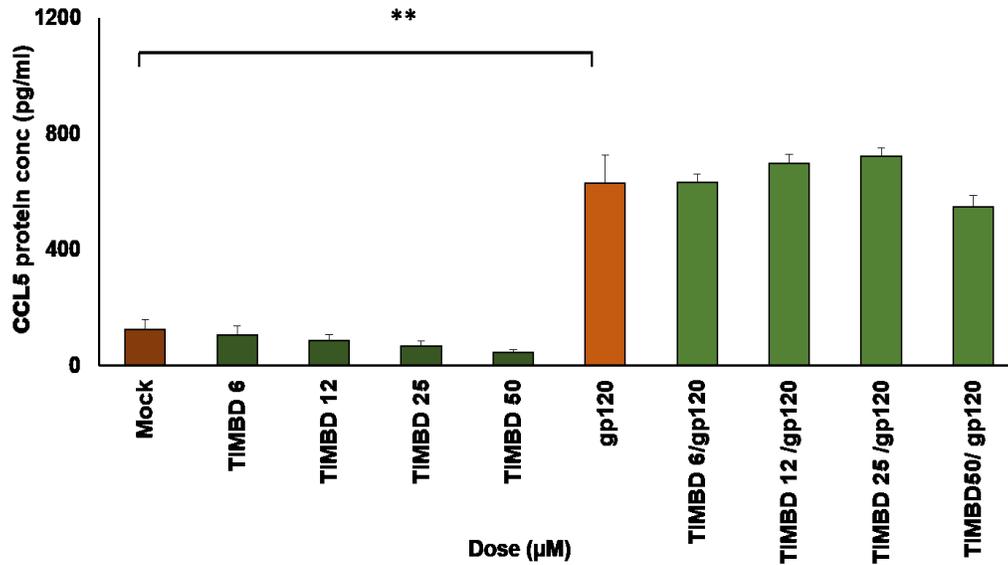


Figure 41. SVG astrocytes were treated with 6 to 50 μ M TIMBD, 1 h prior to transfection with plasmid encoding HIV-1 gp120, for 5 h. The supernatant was collected at 48 h and CCL5 protein concentrations were measured by multiplex cytokine assay. Each bar represents mean \pm SEM for at least 3 independent experiments, with each experiment done in triplicate. One-way Anova was used for statistical analysis and statistical significance is denoted as ** (p-value \leq 0.01).

Neither TIMBD nor RES has any effect on CCL5 protein expression levels

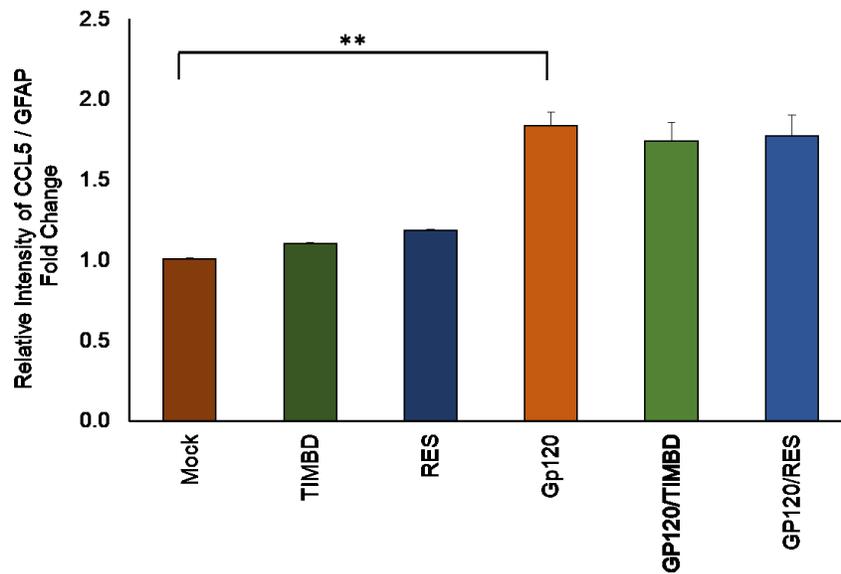
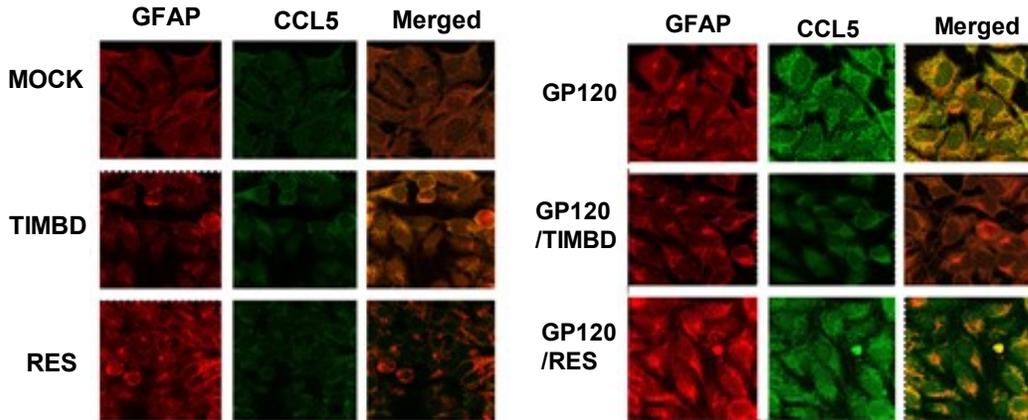


Figure 42. SVG astrocytes were treated with TIMBD or RES, 1 h prior to transfection with plasmid encoding HIV-1 gp120, for 5 h. Expression levels of CCL5 and GFAP proteins were assayed by immunocytochemical methods and analyzed using confocal microscopy. The intensity of CCL5/GFAP was calculated using imageJ software. Each bar represents mean \pm SEM for at least 3 independent experiments, with each experiment done in triplicate. One-way Anova was used for statistical analysis and statistical significance is denoted as ** (p-value \leq 0.01).

5.2.4. TIMBD inhibits phosphorylation of STAT3, AP1 and p38MAPK in SVG astrocytes

Phosphorylation of STAT3 and AP1 dimer (cJUN and cFOS) is known to be induced by HIV-1 gp120 that consequently leads to increases in IL6 and IL8 mRNA and protein expression levels (Del Corno et al., 2014; Shah et al., 2016). Therefore, the effect of TIMBD and RES on HIV-1 gp120-induced phosphorylation of STAT3 and AP1 transcription factors was examined using western blotting, at 6 h post-transfection, with gp120 plasmid. Pre-treatment with 50 μ M TIMBD decreased HIV-1 gp120-induced phosphorylation of STAT3, which was comparable to mock, while RES did not show any inhibitory effect (**Fig. 43**).

TIMBD's effects on the phosphorylation of cJUN or cFOS protein units which comprise the AP1 dimer was also assessed. TIMBD decreased both p-cJUN and cFOS protein expression levels significantly while RES did not affect their expression (**Fig. 44**). Additionally, the effect of TIMBD on the upstream signaling molecules regulating proinflammatory cytokines was investigated. By 1 h post-transfection of SVG astrocytes with HIV-1 gp120 the phosphorylation of p38MAPK increased to 160 ± 10 % relative to GAPDH. TIMBD showed a stronger inhibitory effect, compared to RES, on p-p38MAPK protein expression levels (**Fig. 45**). Additionally, total protein expression levels for AP1 (cJUN and cFOS) were analyzed and no change in their levels in gp120-transfected SVG astrocytes was observed compared to mock. These results suggest that TIMBD can affect the signaling proteins better than RES and thus may lead to decreases in IL6 and IL8 production.

TIMBD decreases protein expression levels of phosphorylated STAT3

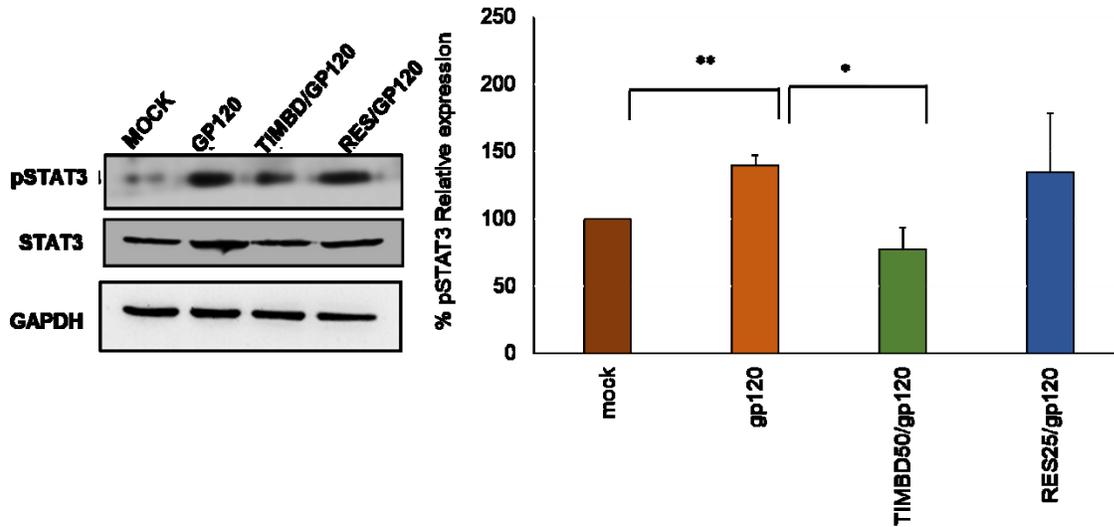


Figure 43. SVG astrocytes were treated with TIMBD or RES, 1 h prior to transfection with plasmid encoding HIV-1 gp120, for 6 h. Expression levels of phosphorylated and total STAT3 were identified using western blotting. Each bar represents mean \pm SEM for at least 3 independent experiments, with each experiment done in triplicate. One-way Anova was used for statistical analysis and statistical significance is denoted as * (p-value \leq 0.05) and ** (p-value \leq 0.01).

TIMBD decreases the expression levels of AP1 subunits p-cJUN and p-cFOS

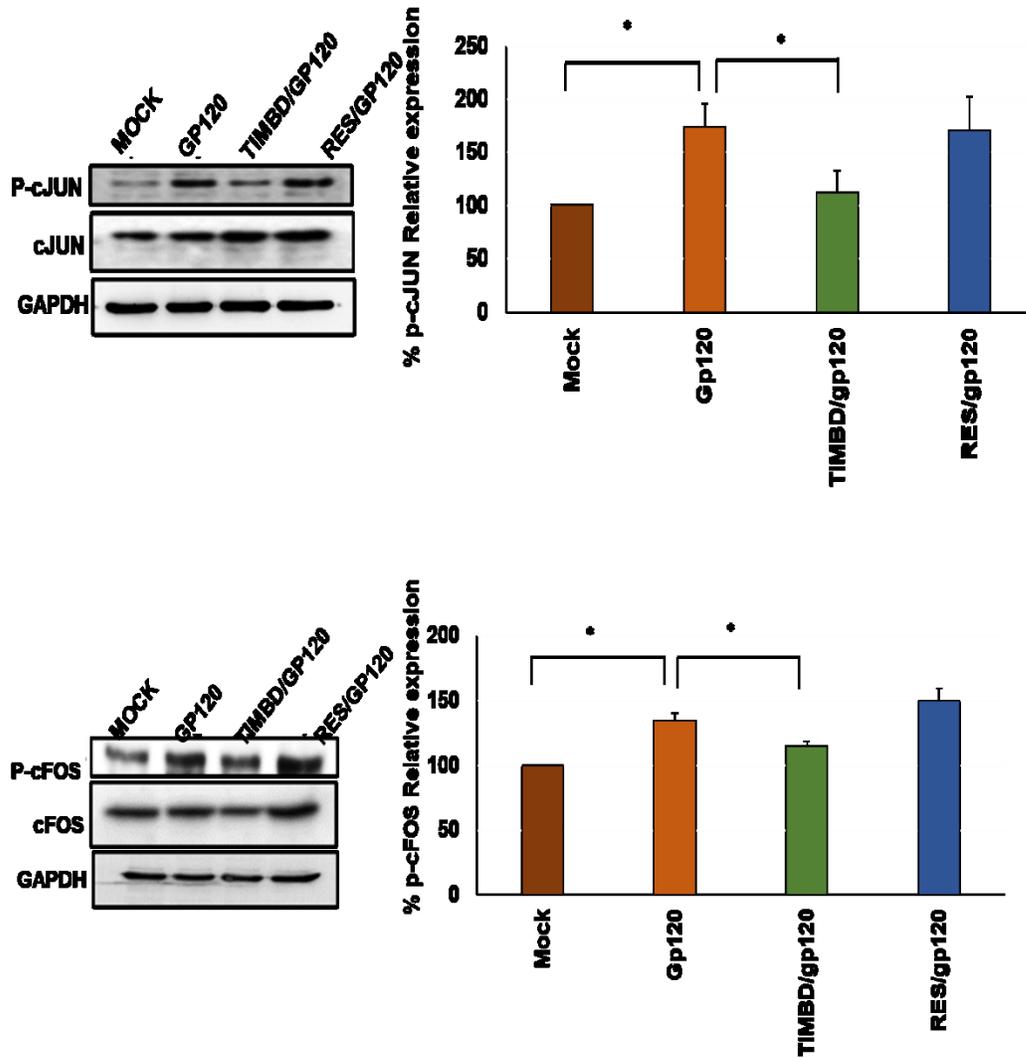


Figure 44. SVG astrocytes were treated with TIMBD or RES, 1 h prior to transfection with plasmid encoding HIV-1 gp120, for 6 h. Expression levels phosphorylated and total cJUN (A) and cFOS (B) were determined using western blotting. Each bar represents mean \pm SEM for at least 3 independent experiments, with each experiment done in triplicate. One-way Anova was used for statistical analysis and statistical significance is denoted as * (p-value \leq 0.05).

TIMBD decreases protein expression levels of phosphorylated p38MAPK

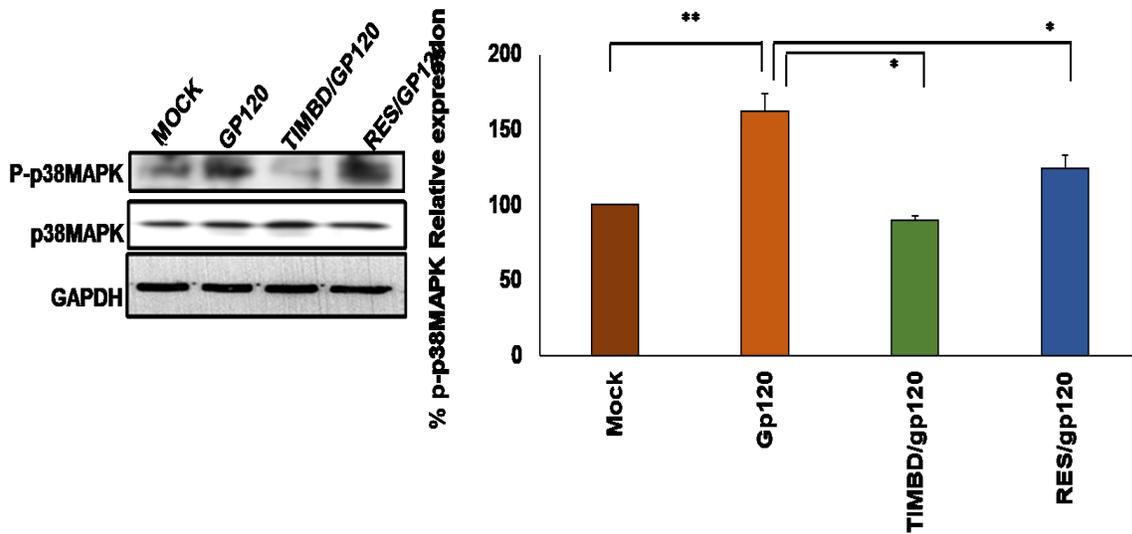


Figure 45. SVG astrocytes were treated with TIMBD or RES, 1 h prior to transfection with plasmid encoding HIV-1 gp120, for 1 h. Expression levels phosphorylated and total p38 MAPK were determined using western blotting. Each bar represents mean \pm SEM for at least 3 independent experiments, with each experiment done in triplicate. One-way Anova was used for statistical analysis and statistical significance is denoted as * (p-value \leq 0.05) and ** (p-value \leq 0.01).

5.2.5. TIMBD decreases HIV-1 gp120-induced NFκB activity in SVG astrocytes

Transcription factor NFκB is considered a major player in the regulation of transcription of many proinflammatory cytokines (Brasier, 2006; Gilmore, 1999, 2006; Monaco et al., 2004). Previous studies have shown that RES works through the NFκB pathway to suppress inflammation in many pathological conditions (Bagul, Deepthi, Sultana, & Banerjee, 2015; Gonzales & Orlando, 2008; Ren et al., 2013; Robich et al., 2010). In our study, the effect of both TIMBD and RES on NFκB translocation was investigated. Gp120 increased nuclear translocation of NFκB p65 200.1 ± 20.0 percent and both RES and TIMBD suppressed the NFκB p65 subunit translocation to the nucleus (**Fig. 46**). AKT and IKKs phosphorylation is involved in the upstream regulation of NFκB activity. Treatment of SVGA with recombinant gp120 protein for 20 min following TIMBD pretreatment lead to a significant suppression of phosphorylation of both AKT (**Fig. 47 panel A**) and IKKs (**Fig. 47 panel B**). When total protein expression levels for AKT and IKKs were analyzed, no change in their levels in gp120-treated SVG astrocytes was observed compared to controls or TIMBD treated cells. Taken together, these results indicate that RES might work only through NFκB, whereas TIMBD exerts its effect on multiple pathways that inhibit IL6 and IL8 mRNA and protein expression in SVGA cells.

TIMBD decreases nuclear translocation of NFκB-p65 in SVG astrocytes

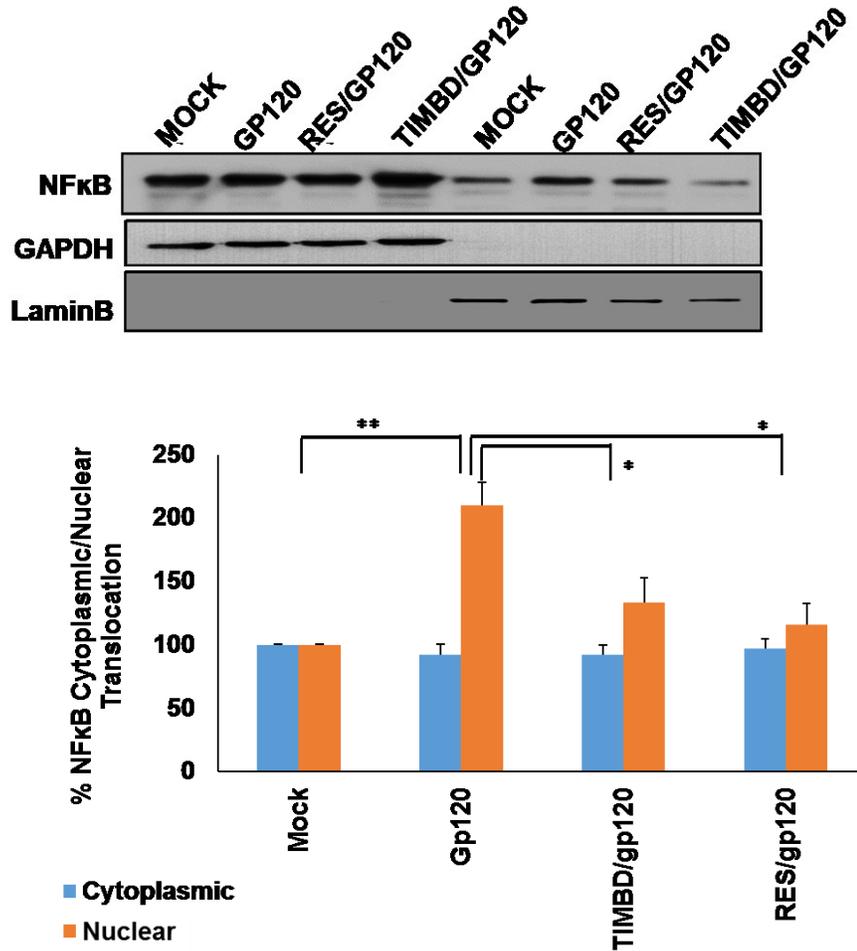


Figure 46. SVG astrocytes were treated with TIMBD or RES, 1 h prior to transfection with plasmid encoding HIV-1 gp120, for 6 h. Protein expression levels of NFκB p65 in cytoplasmic and nuclear extracts were measured with GAPDH and LaminB as loading controls. Each bar represents mean \pm SEM for 3 independent replicates, and the western blots are representative images. One-way Anova was used for statistical analysis and statistical significance is denoted as * (p-value \leq 0.05) and ** (p-value \leq 0.01).

TIMBD decreases protein expression levels of phosphorylated AKT and IKKs

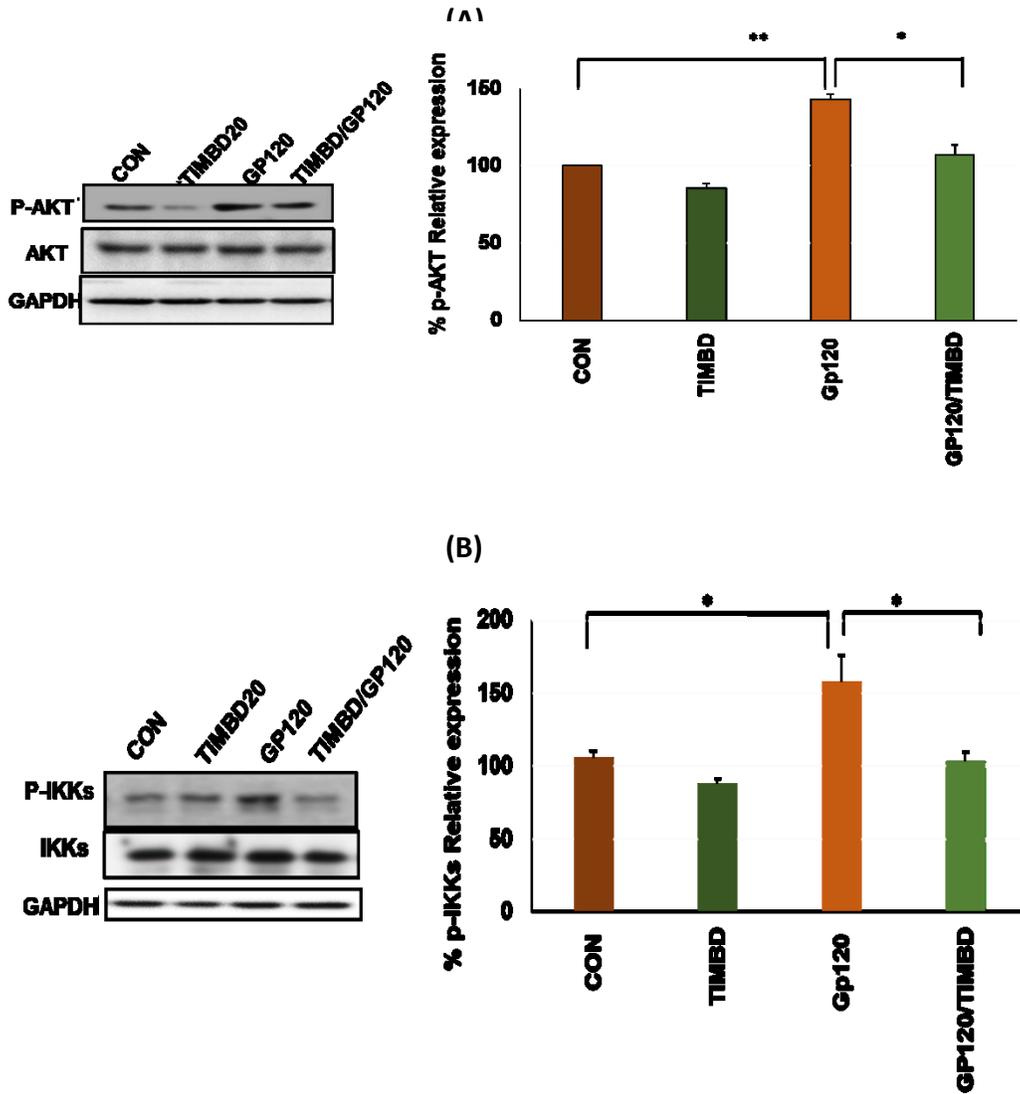


Figure 47. SVG astrocytes were treated with TIMBD or RES, 1 h prior to treatment with gp120-IIIb recombinant protein, for 10 minutes. Expression levels of phosphorylated and total AKT (A) and IKKs (B) were identified using western blotting. Each bar represents mean \pm SEM for at least 3 independent experiments, with each experiment done in triplicate. One-way Anova was used for statistical analysis and statistical significance is denoted as * (p-value \leq 0.05) and ** (p-value \leq 0.01).

5.3. Discussion

Astrocytes are considered the most abundant non-neuronal cells in the brain which play a supportive role in the survival and functioning of neurons and maintenance of neuronal integrity (Garman, 2011; Tsai et al., 2012). Activation of astrocytes by HIV leads to oxidative stress and neuroinflammation and thus may play an important role in HIV-associated neuropathogenesis (J. Li, Bentsman, Potash, & Volsky, 2007). Previous studies have indicated that HIV gp120 protein associated inflammation plays a key role in neurodegeneration occurring in HIV patients. Thus, efforts are being made to develop investigative molecules to inhibit HIV gp120-associated inflammation. Although neuronal death is considered the major cause of the neurodegeneration occurring in HIV patients, astrocytes are considered vital because they are involved directly and indirectly in the neuronal death. HIV gp120 has been shown to induce oxidative stress and to lead to the induction of proinflammatory cytokines in astrocytes (Louboutin, Reyes, Agrawal, Van Bockstaele, & Strayer, 2010; Rao, Eugenin, & Prasad, 2016; Reddy, Agudelo, et al., 2012; Shah & Kumar, 2010).

Resveratrol is a naturally occurring polyphenol that is studied extensively for its potential to prevent many diseases (Athar et al., 2009; Bagatini et al., 2016; B. Singh et al., 2014; Vilar-Pereira et al., 2016). Resveratrol has been shown to play an important role in oxidative stress, inflammation, neurodegeneration and cancers (Athar et al., 2009; Bagatini et al., 2016; B. Singh et al., 2014; Vilar-Pereira et al., 2016). Studies also have shown that RES is able to reduce inflammation and oxidative stress associated with HIV gp120 in brain cells (Reddy, Agudelo, et al., 2012). However, RES has failed to exert its chemotherapeutic effects in many clinical trials (Walle, 2011). This lack of efficacy has

been attributed to many factors including stability or rapid metabolism of RES. To improve the efficacy of RES, we synthesized several RES analog including 4-(E)-{(p-tolylimino)-methylbenzene-1,2-diol} (TIMBD) (Siddiqui et al., 2013). In the present study, we have shown that TIMBD has a higher potency towards inhibiting production of gp120-induced inflammatory cytokines. Since inflammation plays a vital role in neuropathogenesis, availability of TIMBD to prevent a neurodegenerative disease that affects individuals infected with HIV is critical in prevention of this debilitating disorder. TIMBD has CH₃ substituent at a C-4 position on the B ring instead of the hydroxyl group in RES which makes it more lipid-soluble and an aza-linkage (C=N) bond which gives it higher bond dissociation energy (615 kJ/mole) compared to C=C bond (265 kJ/mole) of RES (Siddiqui et al., 2013). Our studies show that TIMBD is more effective than RES in inhibiting gp120-induced IL6 and IL8 production. The potential difference between RES and TIMBD effects may be because of the structural modifications introduced in TIMBD. Further studies will need to be conducted to determine the stability and bioavailability of TIMBD.

Our results indicate that TIMBD can decrease gp120-mediated increase in mRNA and protein expression levels of IL6 and IL8 in a dose-dependent manner. On the contrary, we found that RES can result in a decrease in IL6 mRNA expression levels only at 50 μ M. This dose was found to be cytotoxic to SVG astrocytes (**Fig. 11**). To dissect the mechanism by which TIMBD might potentially affect IL6 and IL8 expression, we determined that TIMBD but not RES, affected protein expression levels of phosphorylated STAT3 and phosphorylated dimer of AP1 (cJUN and cFOS), with expression levels of both proteins being decreased. We further studied the role of MAP kinase in TIMBD-mediated inhibition of gp120-induced inflammation. We found that TIMBD decreases expression levels of

phosphorylated p38MAPK significantly compared to RES. We focused only on p38MAPK in this study as it is reported as most important in IL6/STAT3 signaling (Del Corno et al., 2014). We also determined the effect of TIMBD on nuclear translocation of NFκB which plays a key role in promoting transcription of IL6 and IL8 (Shah & Kumar, 2010; Shah, Verma, et al., 2011). Previous studies have shown that RES produces its anti-inflammatory effects through decreasing the NFκB translocation from cytoplasm to nucleus and could be attributed to increasing SIRT1 expression that is known as an inhibitor for NFκB translocation. We found that both TIMBD and RES decrease NFκB expression levels in the nucleus. Our results indicate that TIMBD can decrease IL6 and IL8 expression through multiple pathways rather than only one pathway as in RES. That may explain why TIMBD decreases IL6 and IL8 expression levels more than RES which showed effect only at a higher dose. In our study, we did not determine the effect of TIMBD on different isoforms of p38 MAPK, AKT or IKKs which may explain why TIMBD could regulate only IL6 and IL8 but not CCL5. Further studies will need to be conducted to analyze the differential effects of different isoforms of these enzymes to figure out why TIMBD regulates only IL6 and IL8 but not CCL5. It is also possible that other pathways are involved in CCL5 production, which remain unaffected by TIMBD treatment.

In summary, our results show that TIMBD decreases HIV-1 gp120-associated production of IL6 and IL8 cytokines in astrocytes. Our studies further suggest that TIMBD might be working through decreasing the expression levels of phosphorylated p38MAPK, which results in decreasing the phosphorylation of AP1 and STAT3 transcription factors and thus their translocation to the nucleus. TIMBD also decreases protein expression levels of phosphorylated AKT and IKKs which are major players for NFκB translocation from

cytoplasm to nucleus leading to decrease in the expression levels of NFκB in the nucleus. These findings suggest that TIMBD might be working through multiple signaling pathways to decrease HIV-1 gp120 associated cytokine production in astrocytes (**Fig. 48**).

Schematic illustration of the suggested mechanism for inhibition of gp120-induced IL6 and IL8 by TIMBD in SVG astrocytes

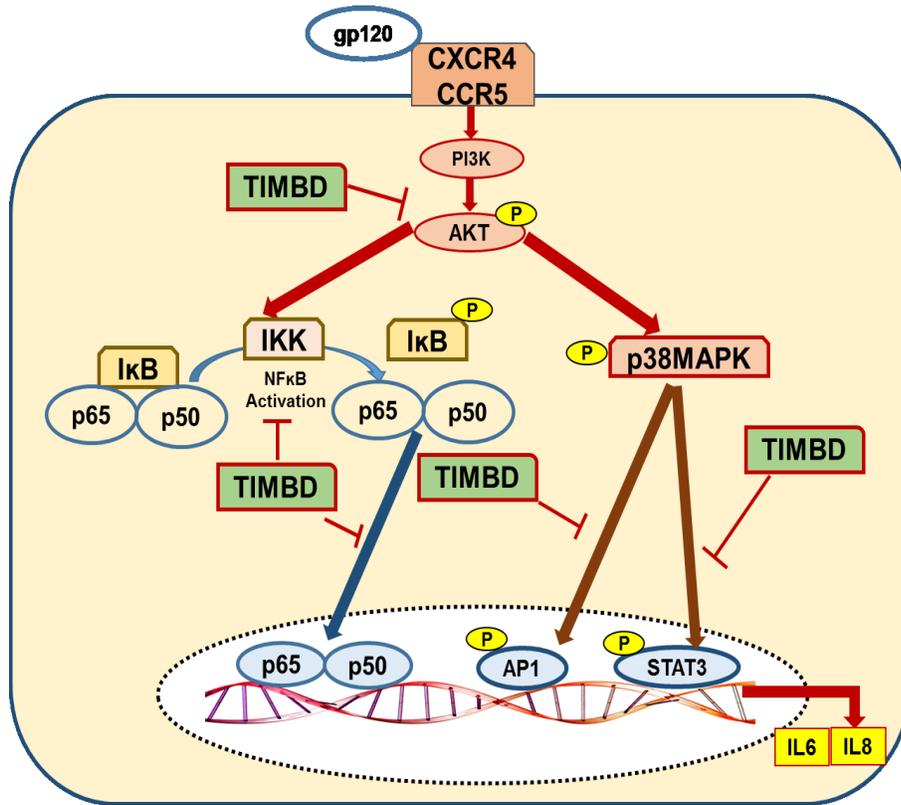


Figure 48. Schematic illustration of the suggested mechanism for inhibition of gp120-induced IL6 and IL8 by TIMBD in SVG astrocytes.

CHAPTER 6

EFFECT OF TIMBD ON HIV TAT-ASSOCIATED BEHAVIORAL DEFICITS AND NEUROTRANSMISSION GENES

6.1. Introduction

There are more than one million people in the US who are infected with HIV (Dahal, Chitti, Nair, & Saxena, 2015; Dailey et al., 2017). HIV infection can lead to neuronal damage, named neuroAIDs (Ghafouri, Amini, Khalili, & Sawaya, 2006; Hazleton, Berman, & Eugenin, 2010; Kovalevich & Langford, 2012). Although the introduction of antiretroviral therapy has controlled HIV infection progression, neuronal damage still critically affects many HIV patients (Kranick & Nath, 2012). Patients with HIV suffer from cognitive deficits and memory loss which is called HIV-associated neurocognitive disorder (HAND) (Clifford & Ances, 2013; Watkins & Treisman, 2015; Woods et al., 2009).

HIV-1 Tat is one of the HIV viral proteins that play an important role in the expression and replication of the viral genome (Morrow et al., 1994). Studies have detected the presence of HIV-1 Tat mRNA and protein in the brain of HIV patients (Hudson et al., 2000; Wiley et al., 1996). Tat protein secreted from infected cells is able to exert its activity in uninfected cells through responsive genes (Ensoli et al., 1993; Watson & Edwards, 1999). Previous studies have demonstrated that HIV-1 Tat can freely penetrate neuronal cell membranes and can lead to an increase of oxidative stress markers like lipid peroxidation as well as the release of inflammatory cytokines which leads to neuronal death (Decrion, Dichamp, Varin, & Herbein, 2005; S. H. Kim, Smith, Tan, Shytle, & Giunta, 2015; Louboutin & Strayer, 2014; Pu et al., 2003; Toborek et al., 2003).

In the central nervous system (CNS), HIV-1 Tat is able to affect central neurotransmission by either modifying the release of neurotransmitters or by altering the functions of their corresponding receptors (Neri, Musante, & Pittaluga, 2007). Studies demonstrated that HIV-1 Tat could decrease presynaptic terminals by triggering NMDA receptor activity, indicating that this loss is initiated by postsynaptic mechanisms (Shin & Thayer, 2013). Since Tat-mediated synaptic loss precedes neuronal cell death (H. J. Kim, Martemyanov, & Thayer, 2008; Masliah & Terry, 1993), targeting these steps might be a good strategy to reverse the synapse loss prior to neuronal death in HAND patients.

HIV-1 Tat-transgenic (Tat-tg) mice models have been used recently to study the pathogenesis of Tat in the CNS and other body parts (Bruce-Keller et al., 2008; Carey et al., 2012; Fitting et al., 2012; Hauser et al., 2009). The GFAP-driven doxycycline (Dox)-inducible Tat₁₋₈₆ transgenic mice model developed to study Tat involvement in mice brain (Bruce-Keller et al., 2008; Carey et al., 2012; Fitting et al., 2012; Hauser et al., 2009; Shah, Verma, et al., 2011). This model has been extensively used for mechanistic studies of the intrinsic effects of Tat in neuroAIDs pathogenesis (Bruce-Keller et al., 2008; Carey et al., 2012; Fitting et al., 2012; Hauser et al., 2009).

Natural compounds have been studied extensively in chemotherapeutic intervention in many diseases (Koehn & Carter, 2005; Nobili et al., 2009). Many natural compounds like curcumin, flavonoids, and resveratrol (RES) have been tested for their effects against neurodegeneration (Andrade, Grosso, Valentao, & Bernardo, 2016; Anekonda, 2006; Costa et al., 2016; S. Hu et al., 2015; F. Li et al., 2012; Marambaud et al., 2005; Monroy, Lithgow, & Alavez, 2013; Schroeter et al., 2002; Sun, Wang, Simonyi, & Sun, 2010; Tellone, Galtieri, Russo, Giardina, & Ficarra, 2015). However, experimental

studies have yielded controversial results for natural compounds including RES in neurodegenerative disease (Sato et al., 2013). Despite the many observations for various natural compounds, there is a lack of evidence for their therapeutic effects in humans (Kjaer et al., 2015; Tome-Carneiro et al., 2013). Poor bioavailability and limited potency have been considered as the main issues related to these natural products bioactivity in humans (Ginsburg & Deharo, 2011; Walle, 2011).

In an effort to develop new chemical compounds that can overcome the pitfalls associated with the natural compounds, our laboratory has synthesized an analog of RES 4-(E)-{(p-tolylimino)-methylbenzene-1,2-diol} (TIMBD) (Siddiqui et al., 2013). In this study, the effect of TIMBD was investigated on modulating the behavioral deficits associated with HIV-1 Tat using Tat-tg mice and the involved modulation of neurotransmission.

6.2. Results

HIV-associated neurocognitive disorder is one of the undermining health disorders that affects majority of HIV-infected patients (Clifford & Ances, 2013; Saylor et al., 2016). Patients with HAND experience many behavioral deficits (Clifford & Ances, 2013; Saylor et al., 2016). Thus, the need to develop therapies to improve the life quality of HIV patients has been critical. In this study, the effect of TIMBD on the behavioral deficits and the related synaptic proteins, associated with HIV-1 Tat using Tat-tg mice, were determined.

6.2.1. Effect of TIMBD in HIV-1 Tat ambulation

To study the effect of TIMBD on the behavioral deficits associated with HIV-1 Tat, HIV-1 Tat-tg mice were used in all the experiments. Both male and female mice were used in all groups to exclude gender differences. Wild-type (WT) mice which do not express HIV-1 Tat and transgenic mice carrying reverse tetracycline transactivator (rtTA) genes were used as controls. Vehicle or 10 mg TIMBD pellets were implanted subcutaneously in the designated groups for specific treatment for 12 weeks. Doxycycline diet was also started at the time of treatment implantation of pellets (**Fig. 49**). The expression of HIV-1 Tat was determined using RT-PCR in four brain regions, hippocampus (H), parietal cortex (PC), prefrontal cortex (PFC) and cerebellum (C) (**Fig. 50**). Mice weights were recorded weekly until the end of the experiment. No change in relative mouse weights was observed between treatment groups in female (**Fig. 51 panel A**) or male mice (**Fig. 51 panel B**) with respect to control treatments. Comparing the ratio of brain weight to whole-mouse weight, no differences were observed between treatment groups in female and male mice (**Fig. 52**).

Schematic overview of the experimental design with Tat-transgenic mice

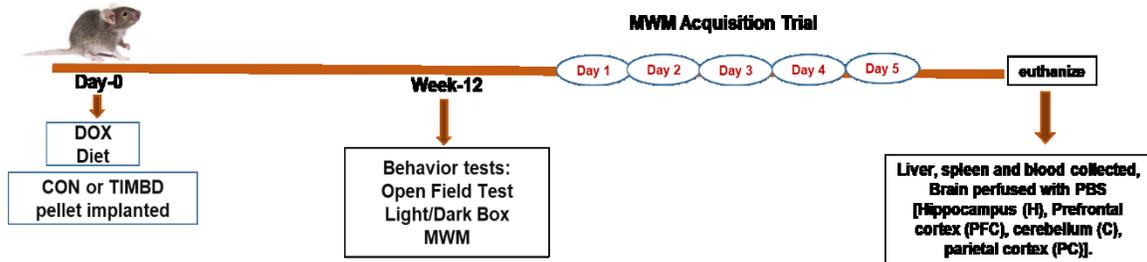


Figure 49. Eight-week old control mice and HIV-1 Tat-tg mice were used for the experiment. Control (CON) or TIMBD pellets were implanted as described in Methods section and DOX diet started for 12 weeks. Animals then underwent behavioral assessment using the Open Field Test, the Light/Dark Box test and the Morris Water Maze (MWM) during week 12. At the end of the experiment, mice were euthanized for collection of different brain regions, including PFC, H, PC and C, to identify the expression of various synaptic proteins.

HIV-Tat expression confirmed in brain regions H, PFC, PC and C

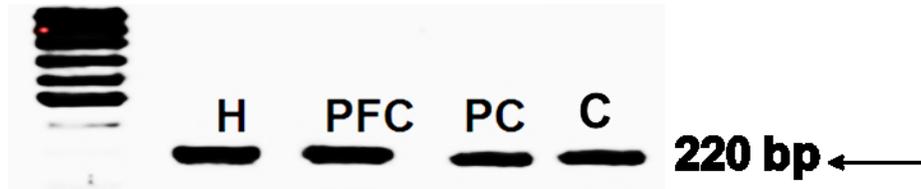
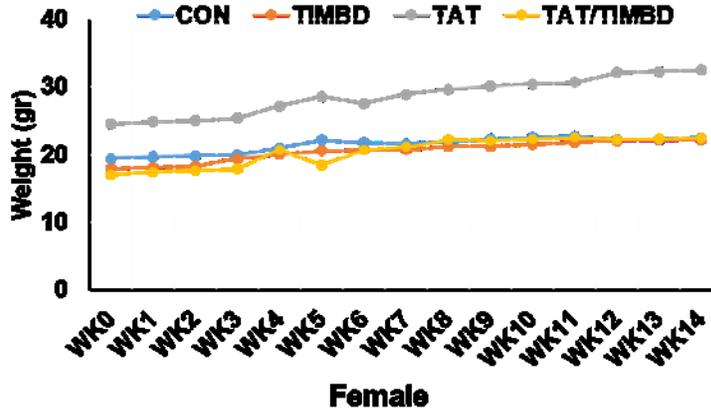


Figure 50. HIV-Tat expression was confirmed in brain regions of H, PFC, PC and C. Brain regions were collected from HIV-1 Tat-tg mice and WT controls following 12 weeks of treatment with TIMBD or CON pellets. Brain regions were isolated from mice at the end of the experiment. Total RNA was extracted from different brain regions and 100 ng RNA was used for RT-PCR. The amplified PCR product was run on 2% agarose gel. HIV-1 Tat expression was confirmed by the presence of a band at 220 bp in H, PFC, PC and C. The figure demonstrates a representative image of an agarose gel showing Tat expression in one mouse.

Weight record of mice during the experiment

(A)



(B)

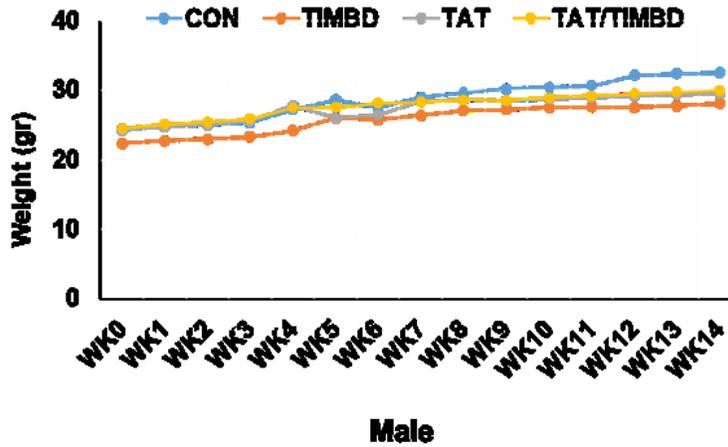
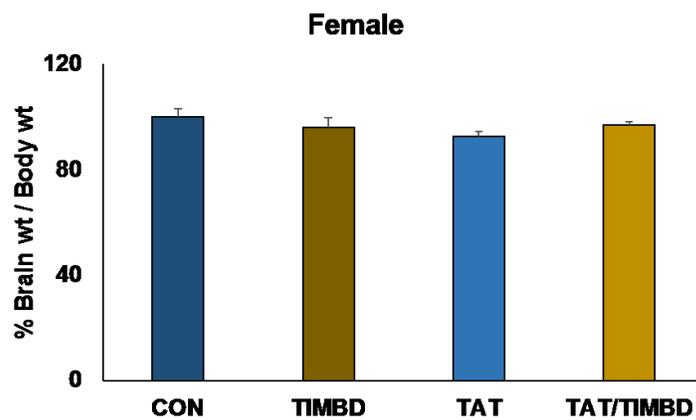


Figure 51. Weight record of mice: Relative mouse weights did not change between treatment groups over time. Eight-week old control mice and HIV-1 Tat-tg mice were treated with CON or TIMBD pellets for 12 weeks. Body weight for mice were recorded weekly for both female (A) and male (B) mice until the end of the experiment.

No difference in brain weight over body weight for mice from different groups

(A)



(B)

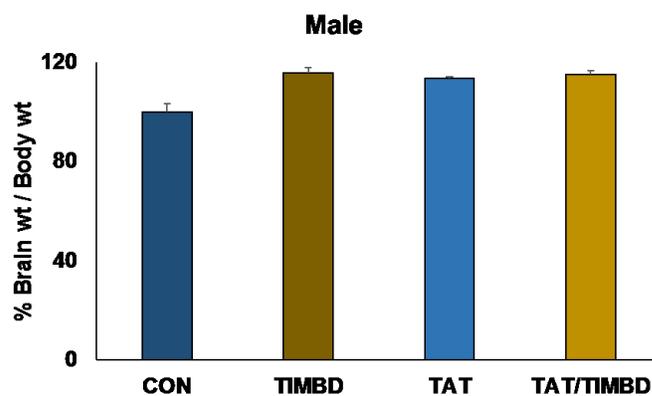


Figure 52. The mouse brain weight/body weight ratio was plotted for both female (A) and male mice (B) \pm SEM. No difference was observed between treatment groups (n= 9 to 11 mice).

6.2.2. Effect of TIMBD on HIV-1-Tat associated anxiety-like behavior using Open Field Test

The effect of TIMBD and HIV-1 Tat on anxiety-like behavior of mice was studied. Mice were treated as stated in the Methods section. At the time of testing, mice were placed in an open field box and allowed 30 seconds to habituate and then given 5 min to explore the arena. The distance traveled, number of entries and the time spent in the center area were measured. These parameters are commonly used to determine anxiety-like behavior (Hahn et al., 2015). We identified that the time spent in the center area was significantly decreased in female Tat-tg mice compared to WT (11.5 ± 3.9), and that TIMBD treatment of these female Tat-tg mice increased the time significantly (20 ± 7.1) (**Fig. 53 panel A**). The distance travelled in center area was decreased in female Tat-tg mice (0.7 ± 0.2). TIMBD treatment of these female Tat-tg mice increased the distance travelled (4.2 ± 0.8) significantly to control levels (**Fig. 53 panel B**). The number of entries were also decreased in female Tat-tg mice (4 ± 1) and TIMBD treatment of these female Tat-tg mice increased the number of entries almost to control levels (20 ± 3) (**Fig. 54 panel A**). In contrast, the same phenomena of an anxiety-like behavior was not observed in male Tat-tg mice. It has been suggested that gender differences might play a role in the sensitivity of mice in this assay. However, the total distance traveled by Tat-tg mice was significantly less than that of WT controls, for both female and male mice (8.0 ± 0.6 and 5.4 ± 1.4 , respectively) (**Fig. 54 panel B**). TIMBD treatment of Tat-tg mice showed a significant improvement in the total distance traveled in the whole arena for both female and male mice (**Fig. 54 panel B**). In conclusion, the open field test results demonstrated that TIMBD was able to increase the time spent in the center area, distance traveled in the center area and the number of

entries into center area, suggesting that it can improve the anxiety-like behavior in Tat-tg mice.

TIMBD decreases the anxiety-like behavior using open field test

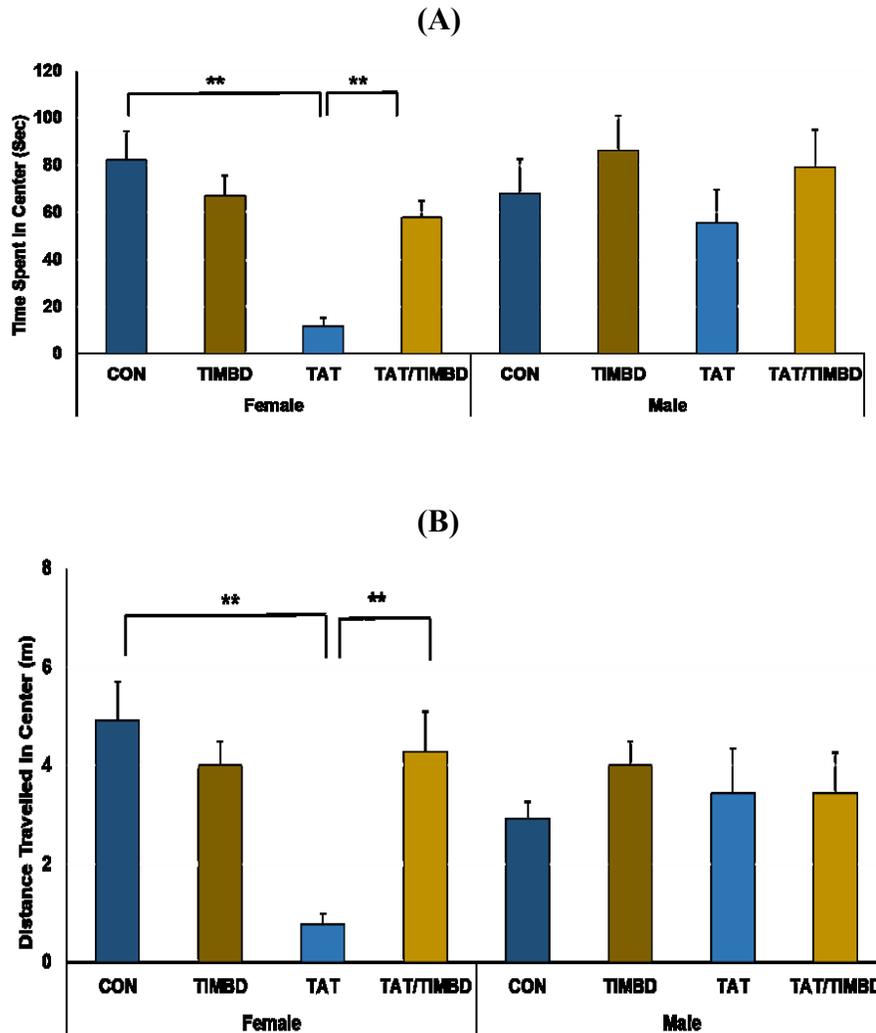
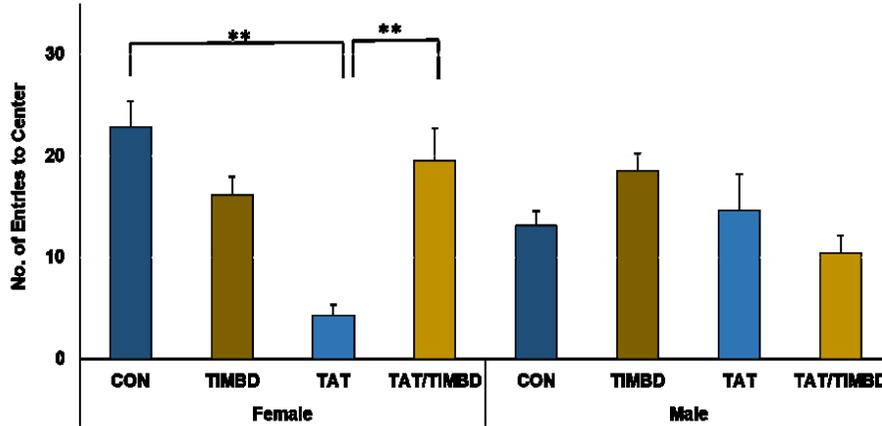


Figure 53. Mice were given 5 minutes to explore the open field arena. Data from the time spent in the center area (A) and the traveled distance in the center area (B) are presented. All four parameters measured were significantly lower in HIV-1 Tat female mice compared to WT controls ($p < 0.0001$). TIMBD-treated HIV-1 Tat female mice showed recovery in those parameters ($p < 0.01$). While in HIV-1 Tat male mice there is also a decrease, it is not significant compared to WT mice and to TIMBD-treated mice (significant recovery: $p \leq 0.05$). ** indicates p -value < 0.01 compared to WT controls; TIMBD-treated HIV-1 Tat mice were compared to HIV-1 Tat mice.

TIMBD decreases the anxiety-like behavior using open field test

(A)



(B)

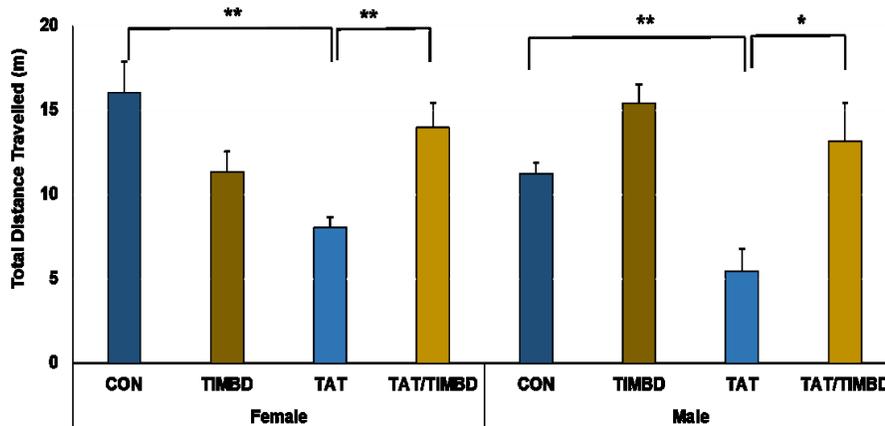


Figure 54. Mice were given 5 minutes to explore the open field arena. Data from the number of entries to the center area (A) and the total distance travelled in the arena (B) are presented. All four parameters measured were significantly lower in HIV-1 Tat female mice compared to WT controls ($p < 0.0001$). TIMBD-treated HIV-1 Tat female mice showed recovery in those parameters ($p < 0.01$). While in HIV-1 Tat male mice there is also a decrease, it is not significant compared to WT mice and to TIMBD-treated mice (significant recovery: $p \leq 0.05$). * indicates p -value < 0.05 and * < 0.01 compared to WT controls; TIMBD-treated HIV-1 Tat mice were compared to HIV-1 Tat mice.

6.2.3. Effect of TIMBD HIV-1 Tat-associated anxiety-like behavior

To further assess the effect of TIMBD on anxiety-like behavior associated with HIV-1 Tat, a light/dark box test was conducted. This test is considered a direct measure of anxiety-like behavior (Bourin & Hascoet, 2003; Takao & Miyakawa, 2006). The test was performed by placing the mice into the light/dark box in the light area and then allowed to explore for 10 minutes. The time spent in the light area was measured and used as an indicator of the anxiety-like behavior. As observed, the time spent in the light zone was significantly decreased in female Tat-tg mice (86.4 ± 10.2) and in male Tat-tg mice (58.6 ± 12.8) compared to WT control mice. TIMBD treatment of Tat-tg mice reversed the decrease in time spent in the light zone in female (131.3 ± 8.5) and male mice (128 ± 9.8) (**Fig. 55**). This assay was able to detect differences more clearly than the previous assay in both genders. In conclusion, TIMBD might be able to improve the anxiety-like behavior associated with HIV-1 Tat in both female and male mice.

TIMBD reduces anxiety-like behavior in Light/Dark box test

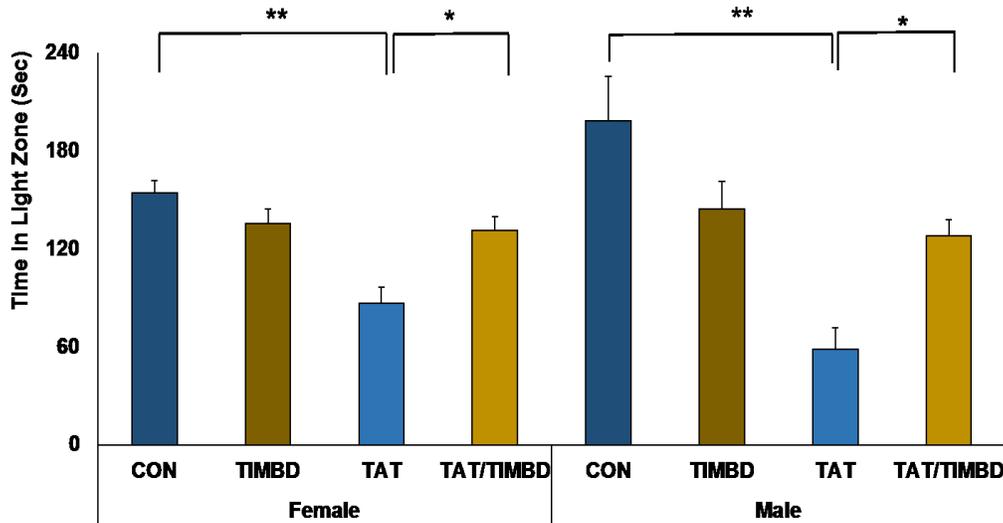


Figure 55. Mice were given 5 minutes to explore the light/dark arena. Data from time spent in the light zone are presented. The time spent in the light zone by HIV-1 tat female mice ($p=0.0006$) and male mice ($p=0.002$) was significantly lower than WT controls. TIMBD-treated HIV-1 Tat female mice showed recovery in time spent ($p=0.016$) as did HIV-1 tat male mice ($p=0.05$). There was no statistical difference between control sexes (female vs. male) as calculated through ANOVA. * indicates p -value < 0.05 and ** < 0.01 compared to WT controls; TIMBD-treated HIV-1 Tat mice were compared to HIV-1 Tat mice.

6.2.4. Effect of TIMBD HIV-1-Tat associated deficits in spatial learning and memory

Spatial learning and memory deficits have been reported previously in HIV-1 Tat-tg mice (Carey et al., 2012). In this study, Morris Water Maze (MWM) test, a well-known test to identify any impairments in spatial learning or memory in mice, was performed (Brandeis, Brandys, & Yehuda, 1989; D'Hooge & De Deyn, 2001; Morris, 1984; Vorhees & Williams, 2006). In this study, the effect of TIMBD on spatial learning and memory in Tat-tg mice was determined using MWM. Spatial learning was evaluated by quantifying the time spent by each group to escape the water and reach the platform. Measurements were collected over 5 days of 4 trials each day. On the 6th day, a probe trial was performed to determine the reference memory by removing the platform used for escape and by quantifying the time spent by each group in the quadrant in which the platform was originally placed. As seen in results from (**Fig. 56, panels A and B**), both female and male WT control mice learned gradually to escape to the platform. The escape latency decreased from (41.7 and 50.6 sec) on day1 to (13.2 and 11.0 sec) on day5 in female and male WT control mice, respectfully. Tat-tg mice demonstrated a decrease in escape latency time for both female and male mice (29.6 and 21.1 sec) on day5, which differed significantly from control WT mice. TIMBD treatment of Tat-tg mice improved the escape latency time significantly on day3, day4 and day5 for female (34.5, 20.5, 16.1 sec) and male mice (23.5, 15.8, 16.5 sec) compared to Tat-tg mice (**Fig. 56, panels A and B**). Probe trial results showed that Tat-tg mice spent less time in the platform quadrant than WT control mice (4.0 ± 1.4 and 12.2 ± 0.7 sec), for female and male mice, respectively (**Fig. 57**). TIMBD treatment of Tat-tg mice improved the reference memory, as indicated by the increase in the time spent in the platform quadrant compared to Tat-tg mice (12 ± 1 and 22.1 ± 2.1 sec)

for female and male mice, respectively (Fig. 57). TIMBD treatment of WT control mice caused no differences compared to vehicle-treated mice and no gender differences were observed.

TIMBD treated HIV-1 Tat mice show more recovery in impaired spatial learning

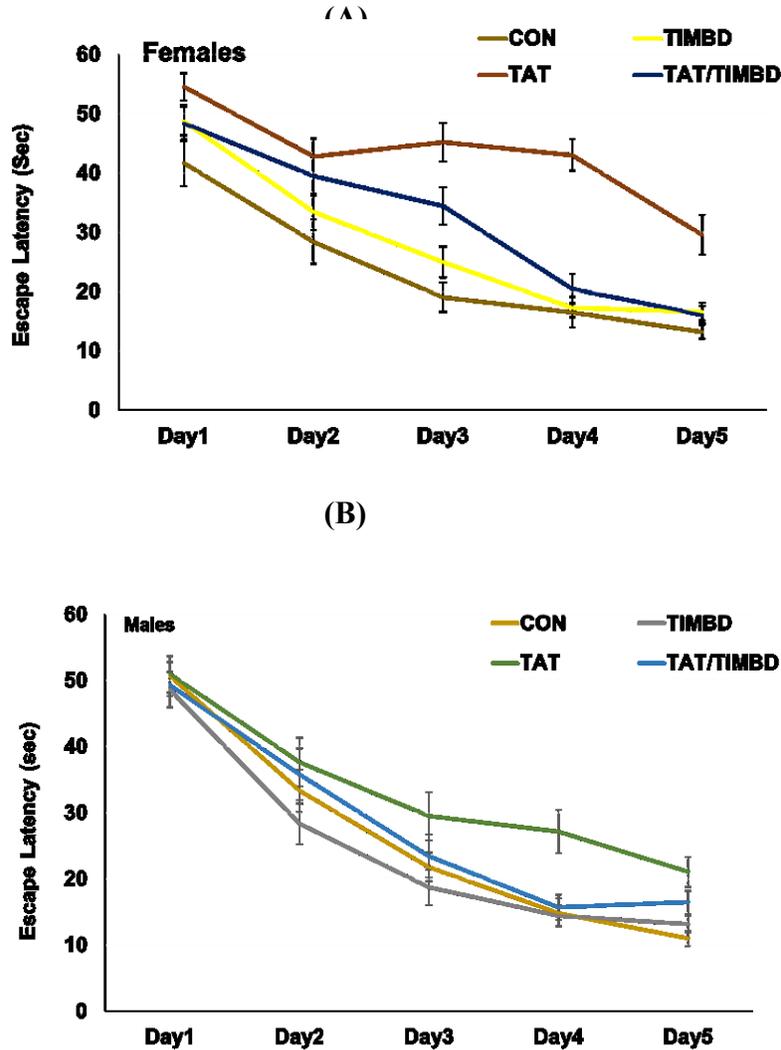


Figure 56. Eight-weeks old control WT-mice and HIV-1 Tat-tg mice were implanted with either vehicle or TIMBD for 12 weeks and analyzed using the Morris water maze test. The experiment was conducted to measure the escape latency (sec) over five days of acquisition trials. Values shown are mean \pm SEM of all trials in all the mice of each group of female (A) and male (B) mice. There was significant increase in the escape latency of HIV-Tat female and male mice relative to controls. TIMBD-treated mice showed no difference from vehicle-treated mice. TIMBD-treated HIV-1 Tat female and male mice showed a significant decrease in escape latency ($p < 0.001$).

TIMBD-treated HIV-1 Tat mice show more recovery of impaired memory

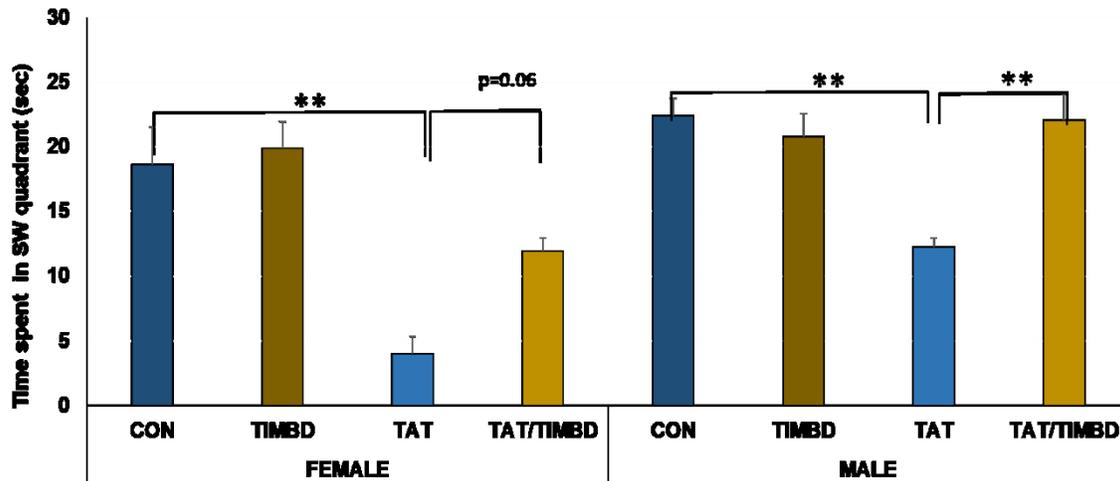


Figure 57. Each mouse was given 60 sec probe trial and the time spent in the target quadrant was calculated. The time spent by HIV-1 tat male and female mice were significantly higher than WT controls. TIMBD-treated HIV-1 tat female mice showed recovery in time spent ($p=0.06$) while males shows significant recovery ($p \leq 0.01$). There was no statistical difference between the sexes (female vs. male) as calculated through ANOVA. ** < 0.01 compared to WT controls; TIMBD-treated HIV-1 tat mice were compared to HIV-1 Tat mice.

6.2.5. Effect of TIMBD on HIV-1 Tat-associated decrease in neurotrophic factors

Neurotrophic factors such as brain-derived neurotrophic factor (BDNF) and ciliary neurotrophic factor (CNTF) are very critical for neuronal survival, growth, and differentiation in the CNS, and peripheral nervous system (PNS) (Chao, 2003; Chao, Rajagopal, & Lee, 2006). Studies have revealed that down-regulation of BDNF is involved in neurodegeneration associated with HIV infection (Bachis, Major, & Mocchetti, 2003). The neurotrophic factors are able to bind to their corresponding receptors to initiate a variety of signaling cascades that promote survival of neurons (Crowder & Freeman, 1998; Hetman, Kanning, Cavanaugh, & Xia, 1999; Ramirez et al., 2001; Yao & Cooper, 1995). Thus, it is critical to determine the effect of TIMBD on the expression levels of neurotrophic factors BDNF and CNTF. In this study, the effect of TIMBD was determined on the protein expression levels of BDNF and CNTF in mice brains. HIV-1 Tat decreased protein expression levels of BDNF in PFC, H, and PC in both female and male mice, compared to WT control mice. TIMBD treatment of Tat-tg mice restored the expression levels of BDNF which were decreased in both female and male mice in PFC, H, and PC regions as shown in **Fig. 58, 59 and 60**. CNTF protein levels were also decreased by HIV-1 Tat in both female and male mice in PFC, H, and PC brain regions. However, TIMBD treatment was able to restore the decreased levels of CNTF closer to WT control levels in PFC, H, and PC in both female and male mice (**Fig. 62, 63 and 64**). HIV-1 Tat in C region did not affect protein expression levels of BDNF and CNTF for both female and male mice (**Fig. 61 and 65**) TIMBD treatment of WT control mice, as well as gender differences, showed no effect on expression levels of BDNF and CNTF. These results demonstrated

that TIMBD was able to restore decreased levels of neurotrophic factors in PFC, H, and PC associated with HIV-1 Tat.

TIMBD restores HIV-1 Tat associated decrease in BDNF expression levels

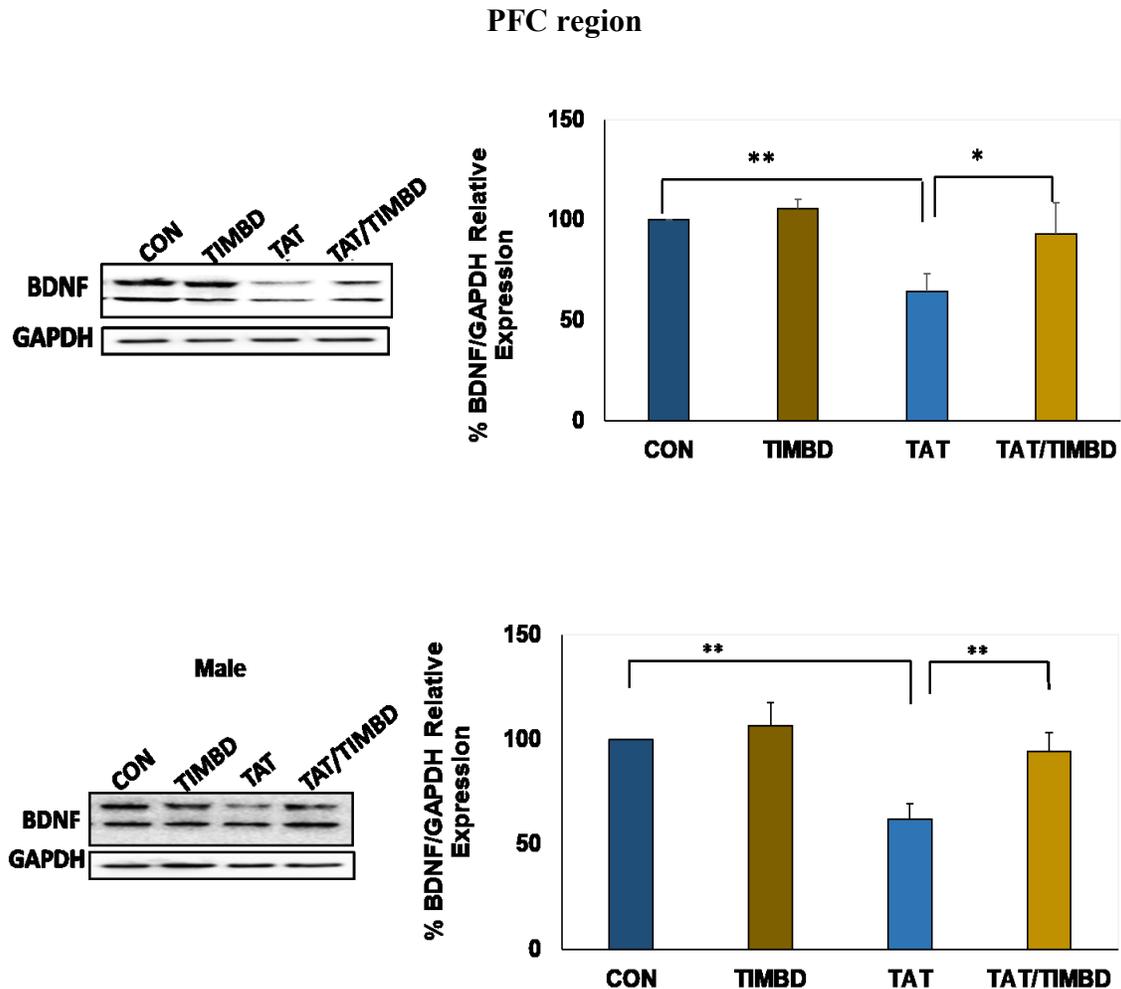


Figure 58. Brain regions were collected from HIV-1 Tat-tg mice and WT controls following 12 weeks of treatment with TIMBD or CON pellets. Expression levels of BDNF were identified in PFC region as presented using western blotting for both female and male mice. Each bar represents mean \pm SEM values for at least 3 independent westerns. One-way Anova was used for statistical analysis and statistical significance is denoted as * (p-value \leq 0.05) and ** (p-value \leq 0.01).

TIMBD restores HIV-1 Tat associated decrease in BDNF expression levels

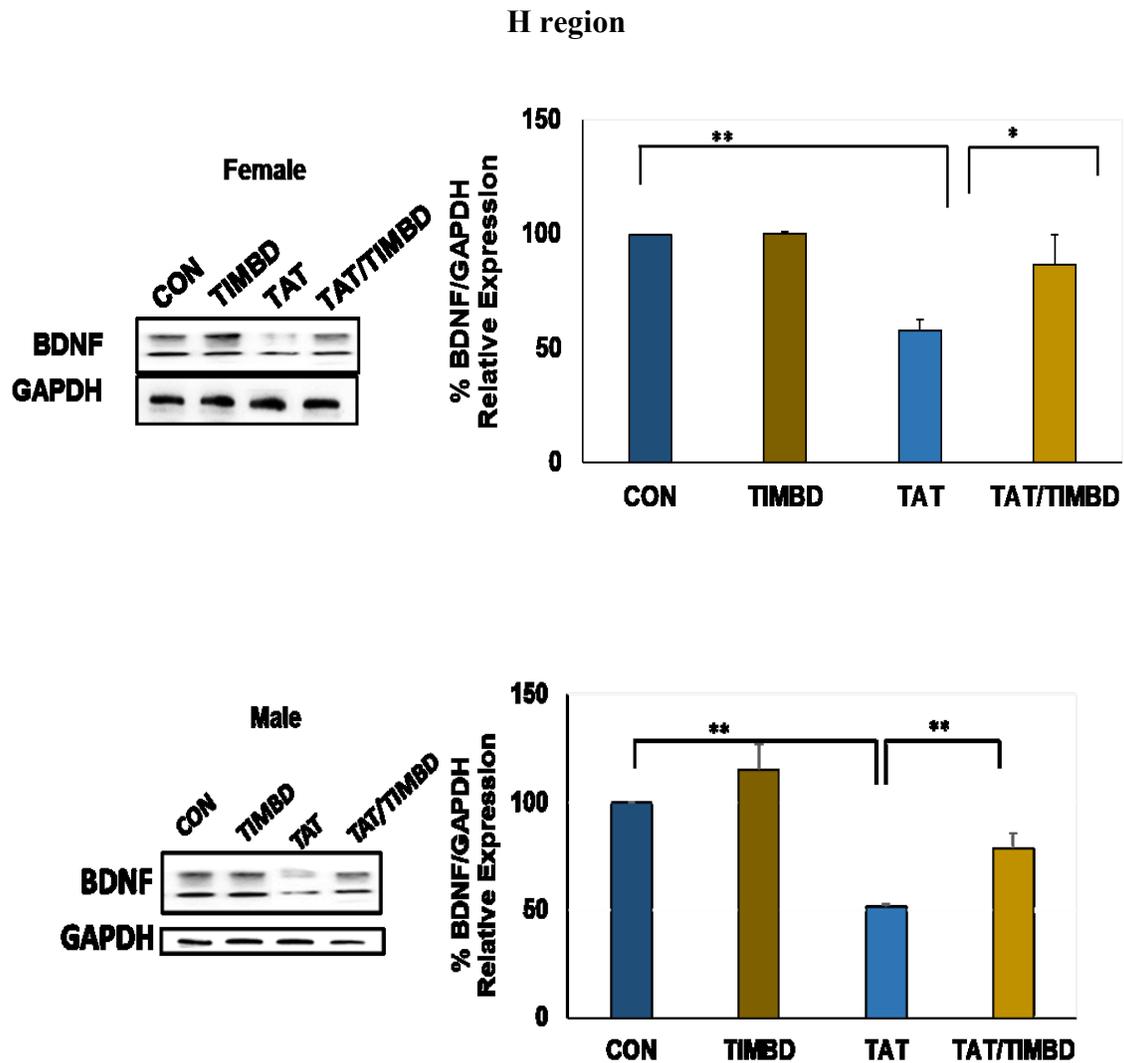


Figure 59. Brain regions were collected from HIV-1 Tat-tg mice and WT controls following 12 weeks of treatment with TIMBD or CON pellets. Expression levels of BDNF were identified in H region as presented using western blotting for both female and male mice. Each bar represents mean \pm SEM values for at least 3 independent westerns. One-way Anova was used for statistical analysis and statistical significance is denoted as * (p-value \leq 0.05) and ** (p-value \leq 0.01).

TIMBD restores HIV-1 tat associated decrease in BDNF expression levels

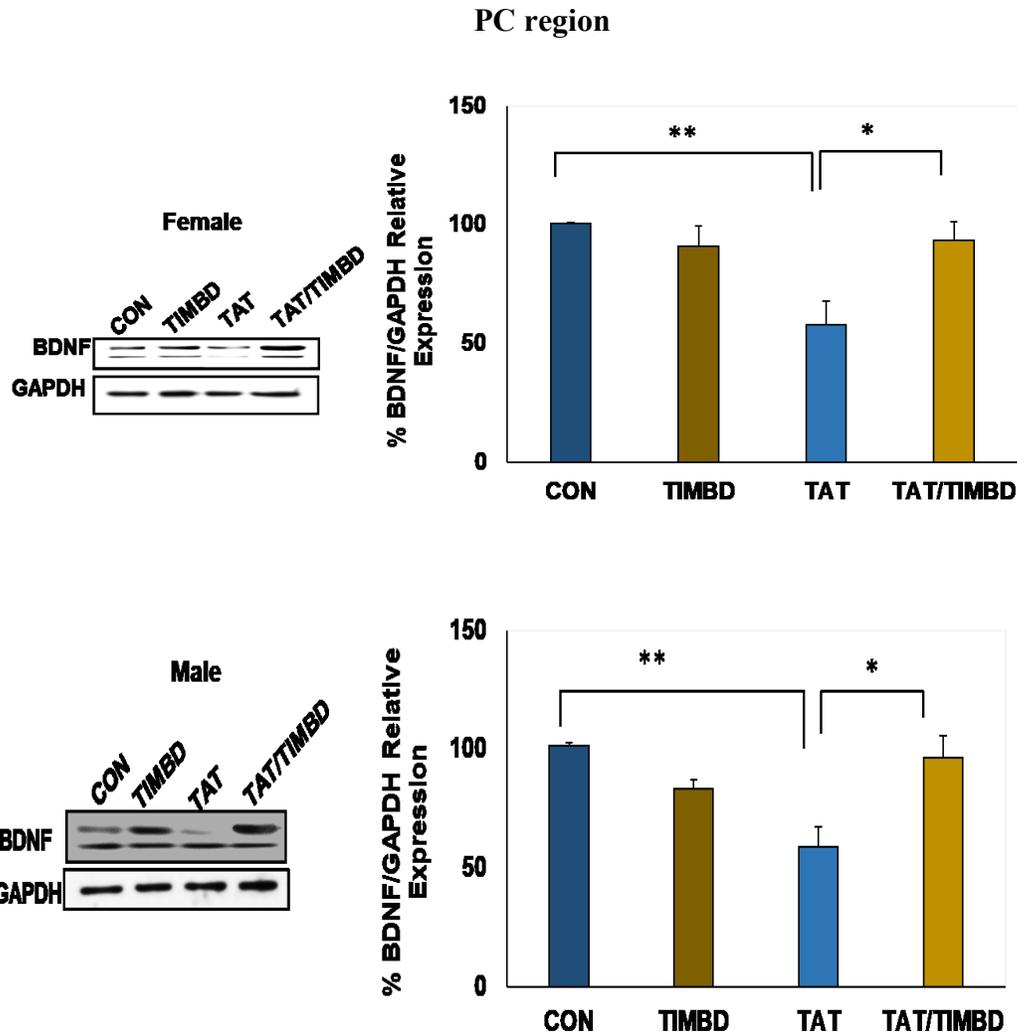


Figure 60. Brain regions were collected from HIV-1 Tat-tg mice and WT controls following 12 weeks of treatment with TIMBD or CON pellets. Expression levels of BDNF were identified in PC region as presented using western blotting for both female and male mice. Each bar represents mean \pm SEM values for at least 3 independent westerns. One-way Anova was used for statistical analysis and statistical significance is denoted as * (p-value \leq 0.05) and ** (p-value \leq 0.01).

TIMBD restores HIV-1 Tat associated decrease in BDNF expression levels

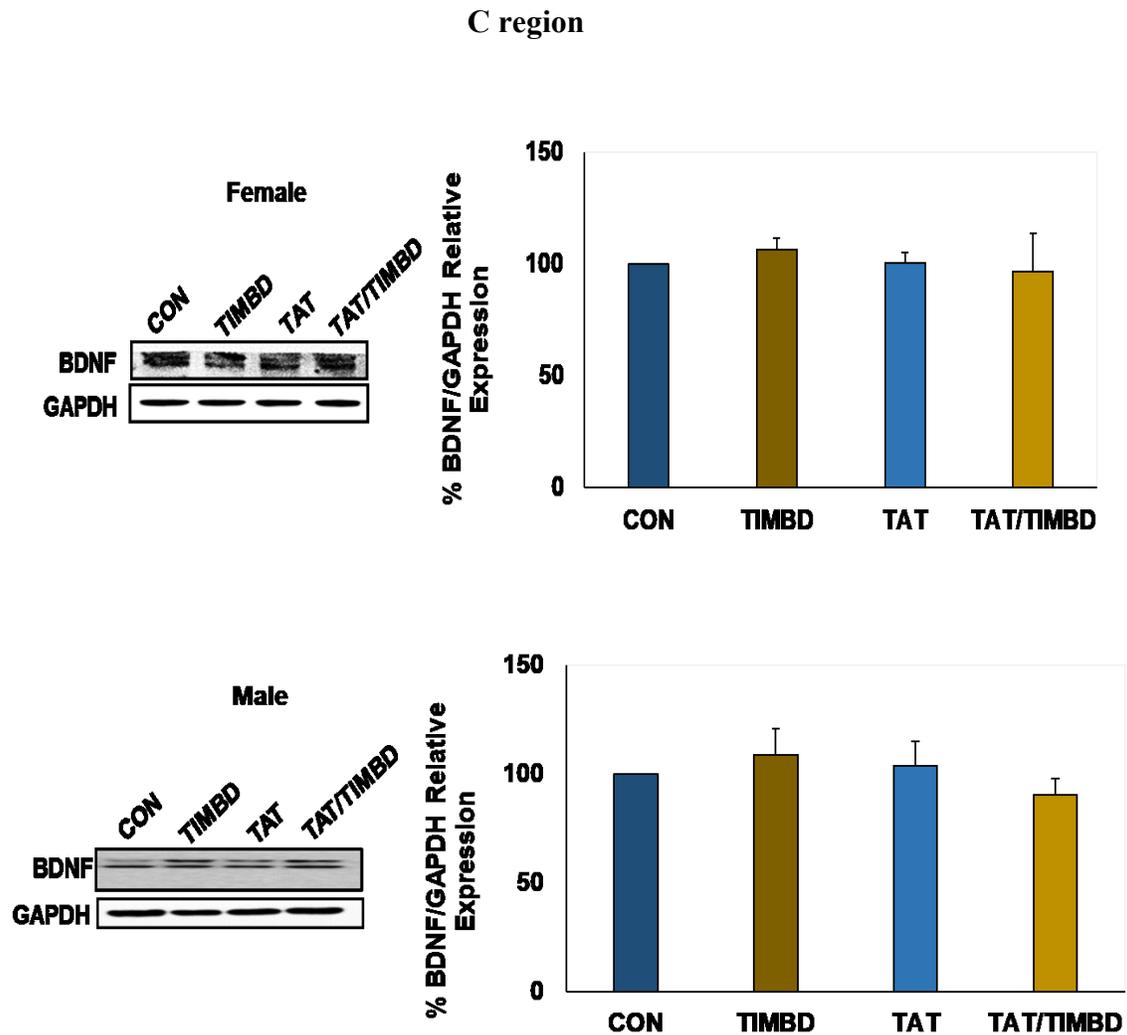


Figure 61. Brain regions were collected from HIV-1 Tat-tg mice and WT controls following 12 weeks of treatment with TIMBD or CON pellets. Expression levels of BDNF were identified in C region as presented using western blotting for both female and male mice. Each bar represents mean \pm SEM values for at least 3 independent westerns. One-way Anova was used for statistical analysis and statistical significance is denoted as * (p-value \leq 0.05) and ** (p-value \leq 0.01).

TIMBD restores HIV-1 Tat associated decrease in CNTF expression levels
PFC region

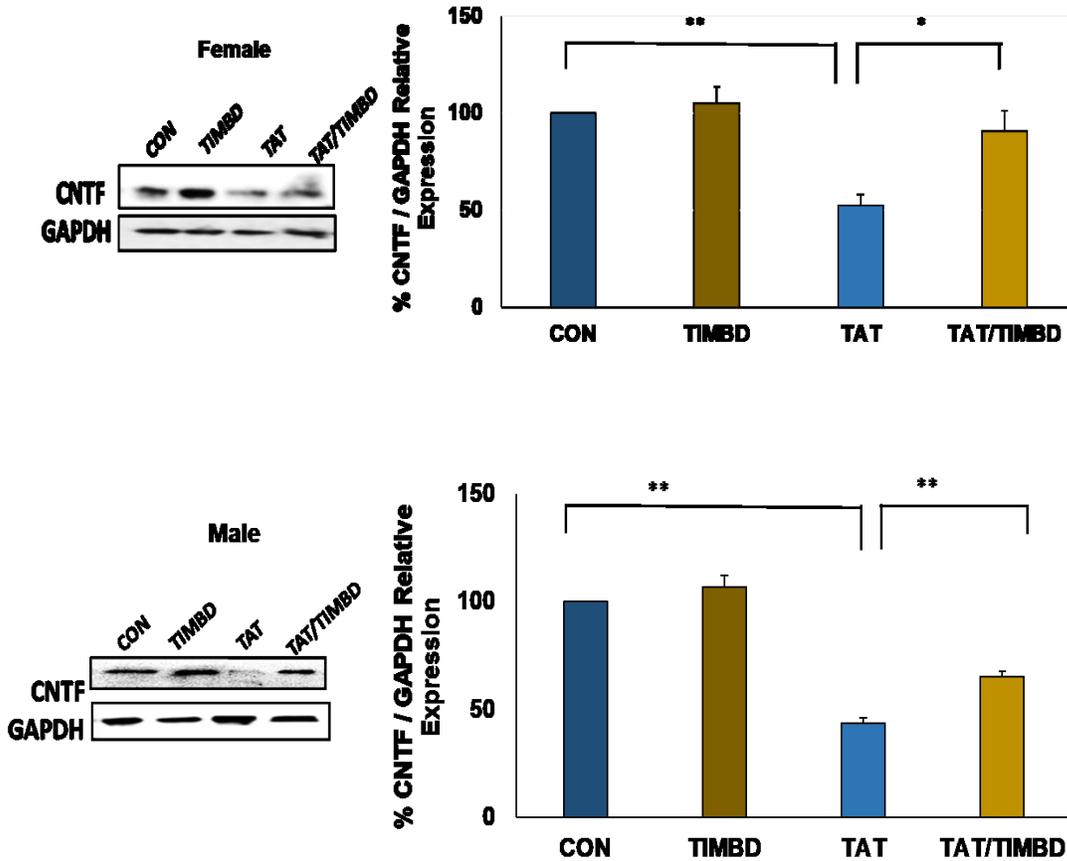


Figure 62. Brain regions were collected from HIV-1 Tat-tg mice and WT controls following 12 weeks of treatment with TIMBD or CON pellets. Expression levels of CNTF were identified in PFC region as presented using western blotting for both female and male mice. Each bar represents mean \pm SEM values for at least 3 independent westerns. One-way Anova was used for statistical analysis and statistical significance is denoted as * (p-value \leq 0.05) and ** (p-value \leq 0.01).

TIMBD restores HIV-1 Tat associated decrease in CNTF expression levels

H region

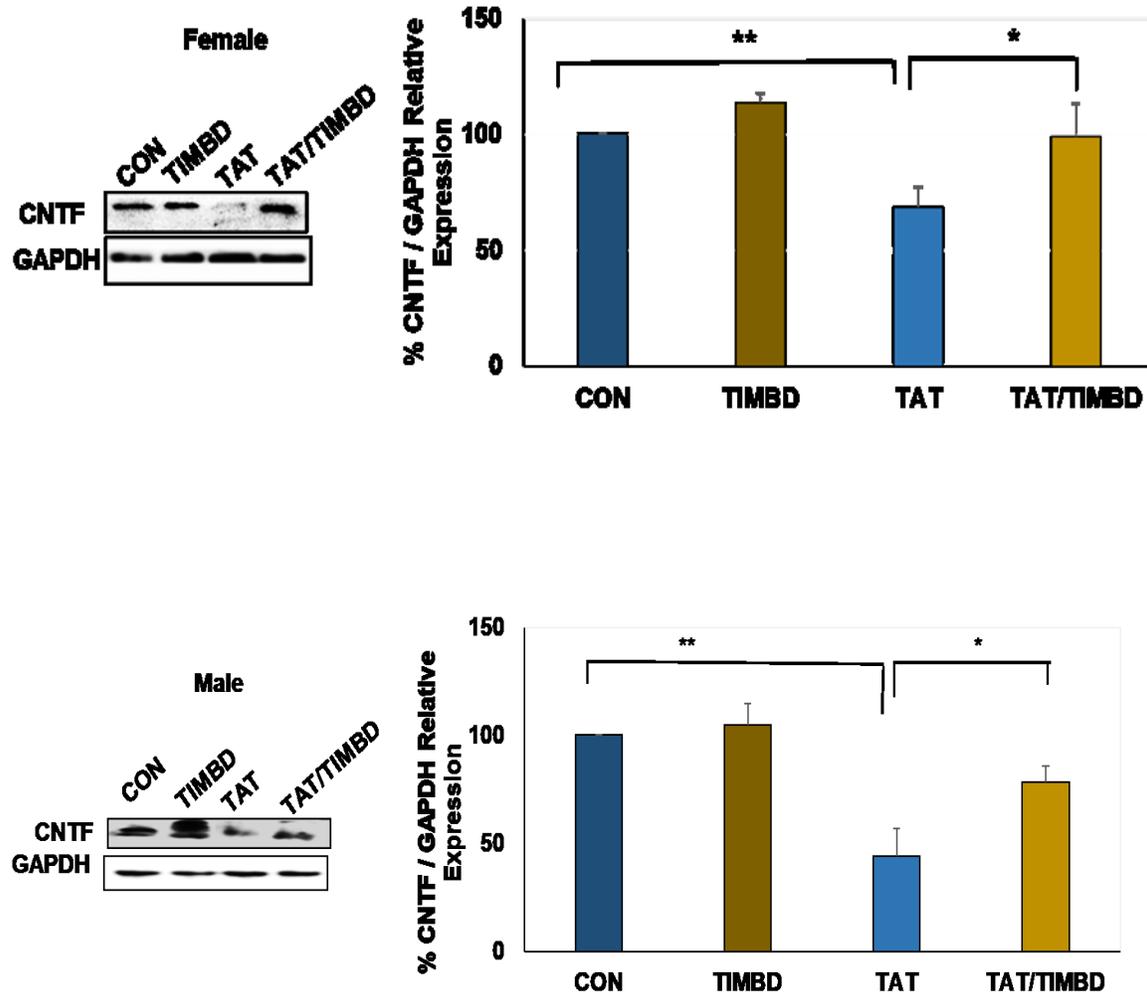


Figure 63. Brain regions were collected from HIV-1 Tat-tg mice and WT controls following 12 weeks of treatment with TIMBD or CON pellets. Expression levels of CNTF were identified in H region as presented using western blotting for both female and male mice. Each bar represents mean \pm SEM values for at least 3 independent westerns. One-way Anova was used for statistical analysis and statistical significance is denoted as * (p-value \leq 0.05) and ** (p-value \leq 0.01).

TIMBD restores HIV-1 Tat associated decrease in CNTF expression levels

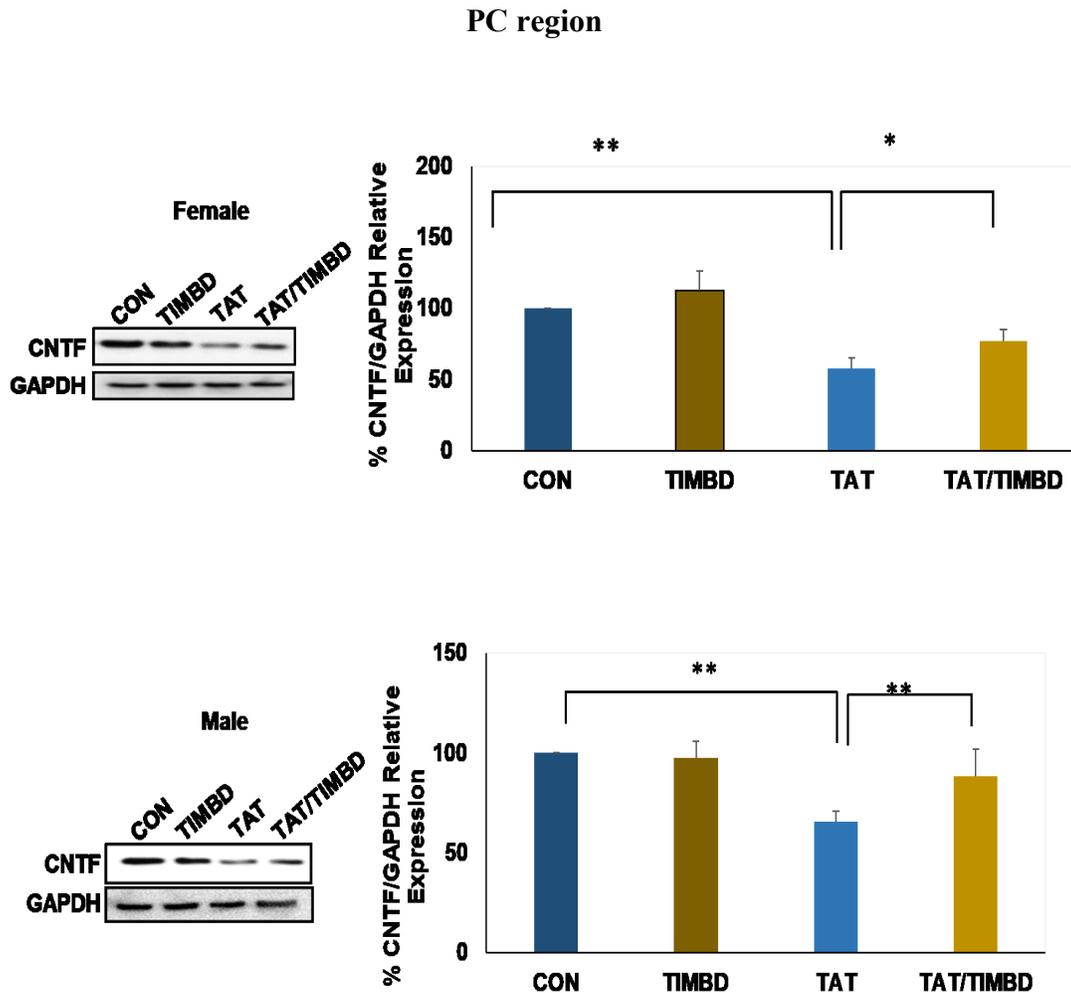


Figure 64. Brain regions were collected from HIV-1 Tat-tg mice and WT controls following 12 weeks of treatment with TIMBD or CON pellets. Expression levels of CNTF were identified in PC region as presented using western blotting for both female and male mice. Each bar represents mean \pm SEM values for at least 3 independent westerns. One-way Anova was used for statistical analysis and statistical significance is denoted as * (p-value \leq 0.05) and ** (p-value \leq 0.01).

TIMBD restores HIV-1 Tat associated decrease in CNTF expression levels

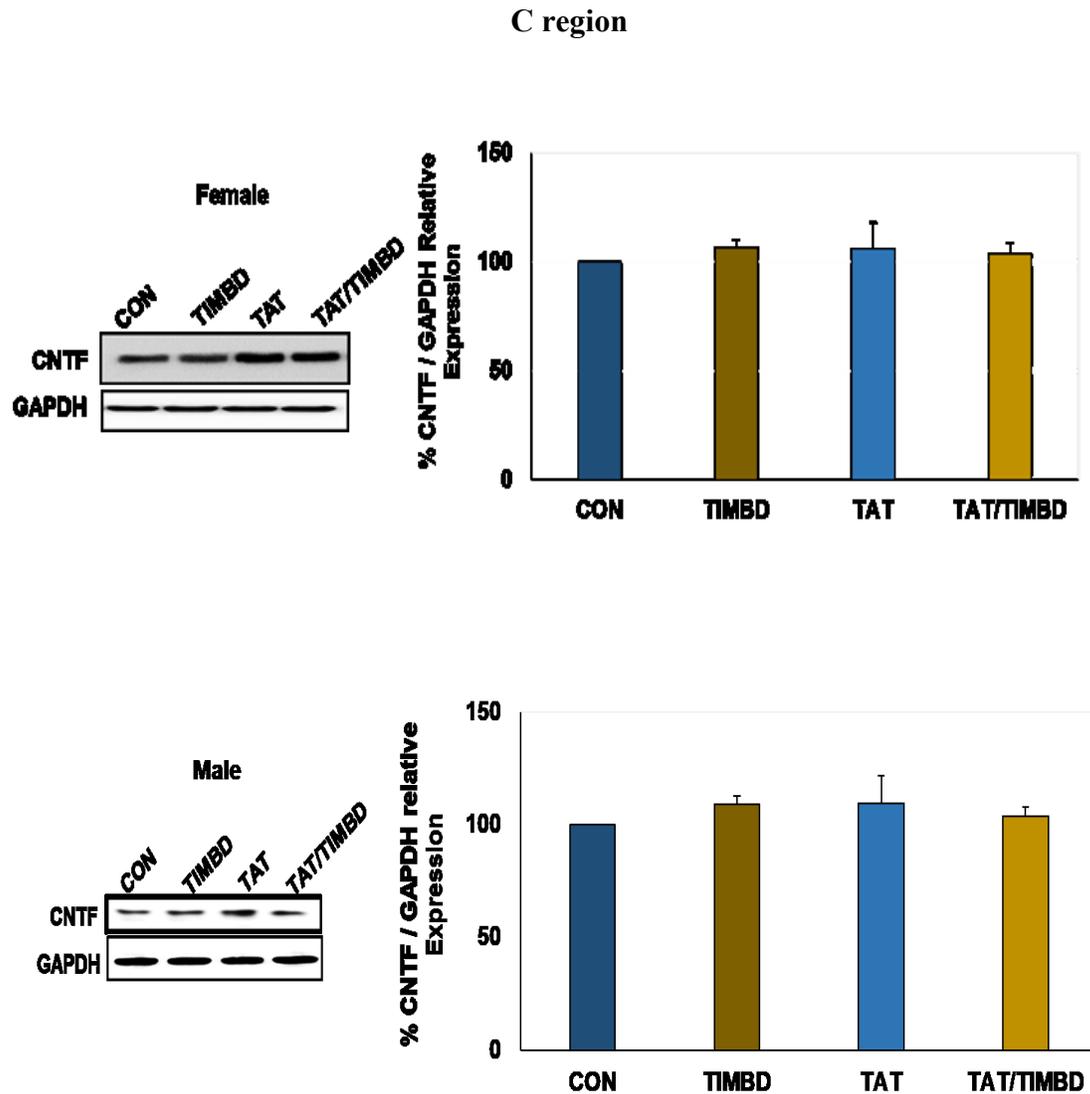


Figure 65. Brain regions were collected from HIV-1 Tat-tg mice and WT controls following 12 weeks of treatment with TIMBD or CON pellets. Expression levels of CNTF were identified in C region as presented using western blotting for both female and male mice. Each bar represents mean \pm SEM values for at least 3 independent westerns. One-way Anova was used for statistical analysis and statistical significance is denoted as * (p-value \leq 0.05) and ** (p-value \leq 0.01).

6.2.6. Effect of TIMBD and HIV-1 Tat on the expression levels of presynaptic proteins

The effect of TIMBD on HIV-1 Tat-associated effects on presynaptic proteins synapsin1 and synaptophysin were determined. Synaptophysin is a presynaptic calcium-binding glycoprotein that is considered a major component for synaptic vesicles (Johnston & Sudhof, 1990; Rehm, Wiedenmann, & Betz, 1986; Wiedenmann & Franke, 1985). HIV-1 Tat has been reported to induce loss of presynaptic terminals in hippocampal cultures (Shin & Thayer, 2013). In our study, protein expression levels of synaptophysin in HIV-1 Tat was decreased significantly in both female and male mice in PFC, H, and PC brain regions (**Fig. 66, 67 and 68**). Synaptophysin levels were not changed in C region in Tat-tg or TIMBD-treated Tat-tg mice (**Fig. 69**). TIMBD treatment restored synaptophysin expression levels that decreased with HIV-1 Tat in PFC, H, and PC brain regions. Moreover, HIV-1 Tat decreased synapsin1 expression levels in PFC, H, and PC brain regions in both female and male mice (**Fig. 70, 71 and 72**). TIMBD treatment of Tat-tg mice increased the expression levels of synapsin1 significantly compared to Tat-tg mice group (**Fig. 70, 71 and 72**). Synapsin1 expression levels in C brain region were not changed in either female or male mice (**Fig. 73**). TIMBD treatment of WT control mice did not show any effect.

TIMBD restores HIV-1 Tat-associated decrease in synaptophysin expression levels

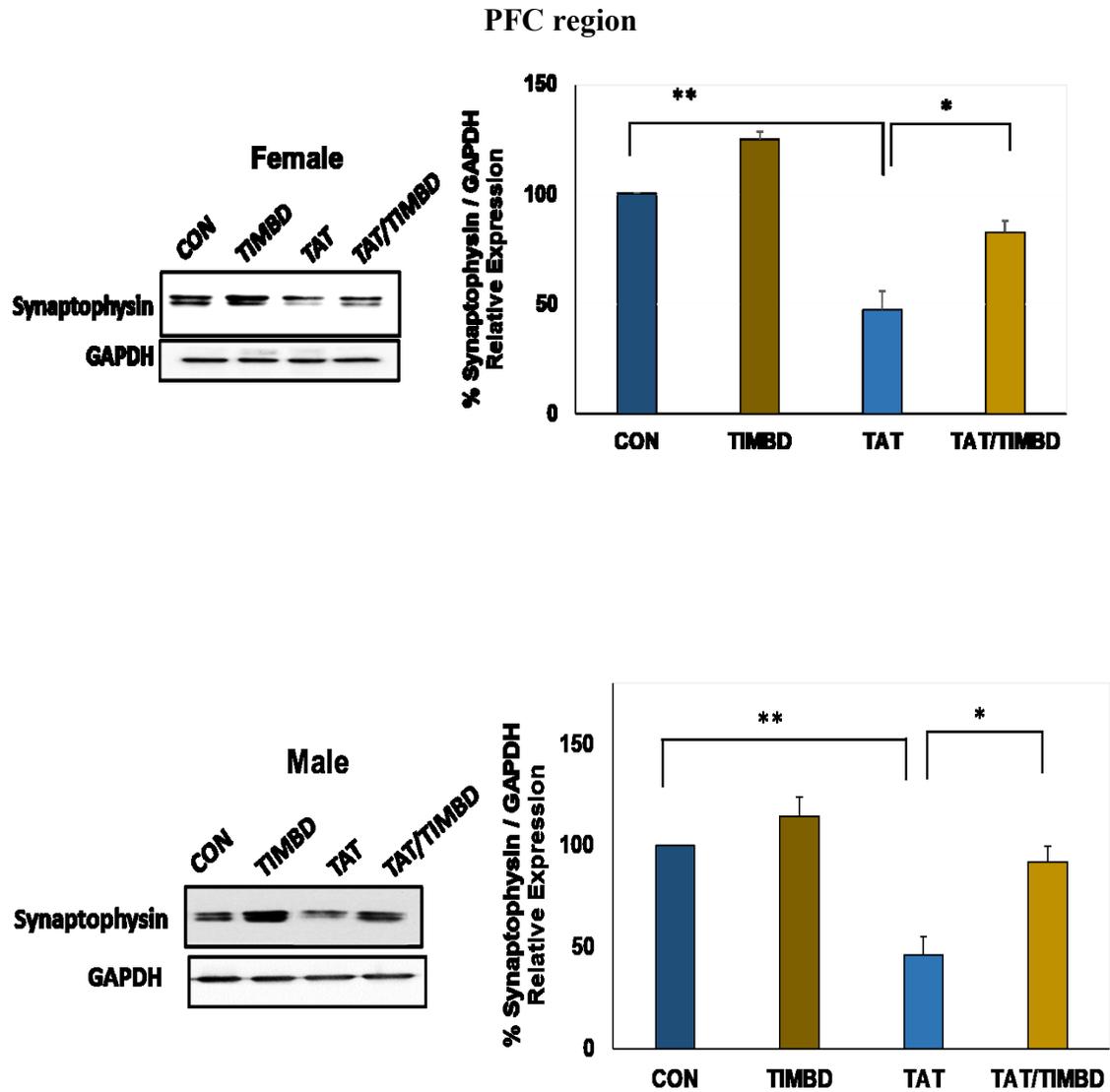


Figure 66. Brain regions were collected from HIV-1 Tat-tg mice and WT controls following 12 weeks of treatment with TIMBD or CON pellets. Expression levels of Synaptophysin were identified in PFC region as presented using western blotting for both female and male mice. Each bar represents mean \pm SEM values for at least 3 independent westerns. One-way Anova was used for statistical analysis and statistical significance is denoted as * (p-value \leq 0.05) and ** (p-value \leq 0.01).

TIMBD restores HIV-1 Tat-associated decrease in synaptophysin expression levels

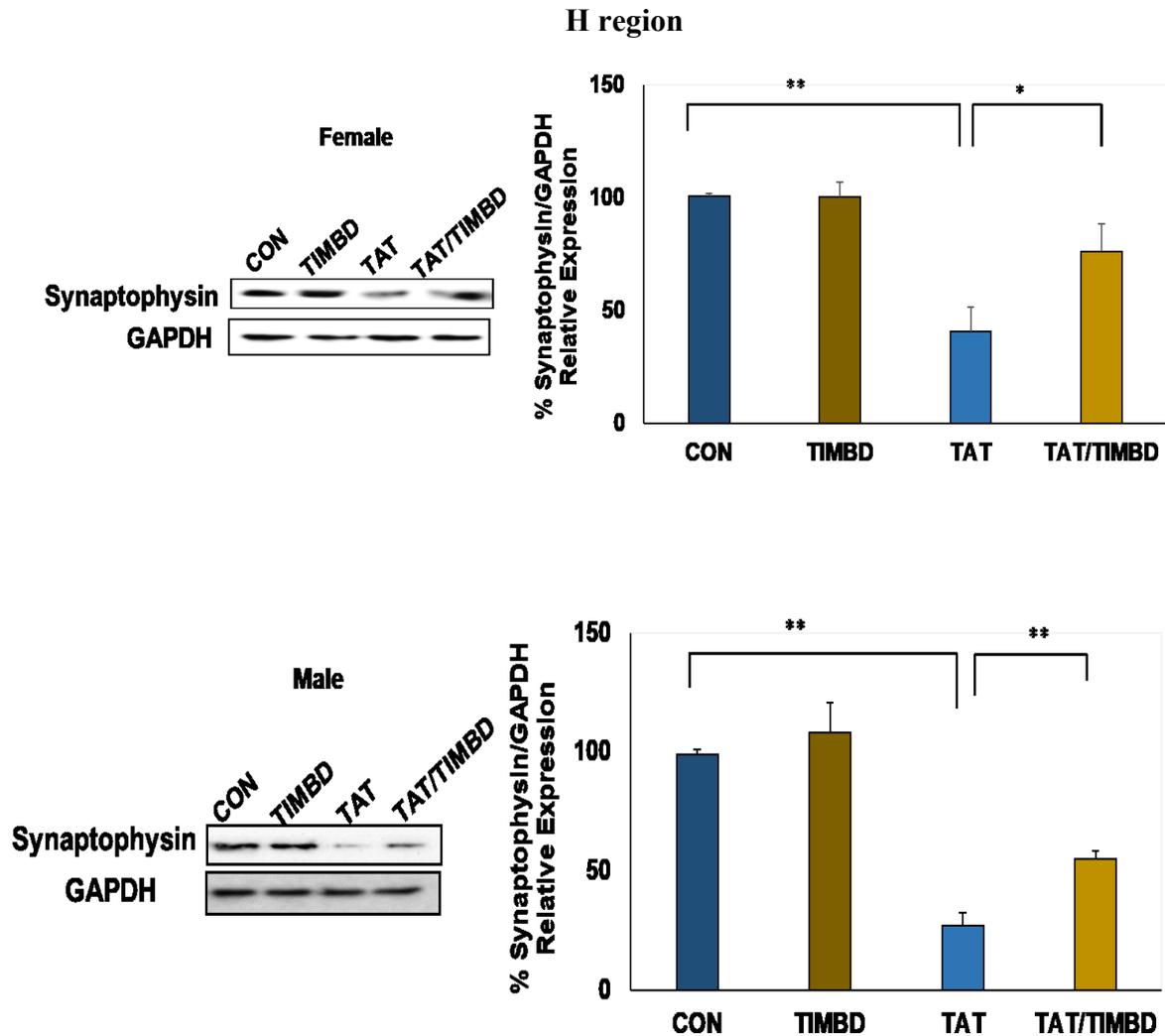


Figure 67. Brain regions were collected from HIV-1 Tat-tg mice and WT controls following 12 weeks of treatment with TIMBD or CON pellets. Expression levels of Synaptophysin were identified in H region as presented using western blotting for both female and male mice. Each bar represents mean \pm SEM values for at least 3 independent westerns. One-way Anova was used for statistical analysis and statistical significance is denoted as * (p-value \leq 0.05) and ** (p-value \leq 0.01).

TIMBD restores HIV-1 Tat-associated decrease in synaptophysin expression levels

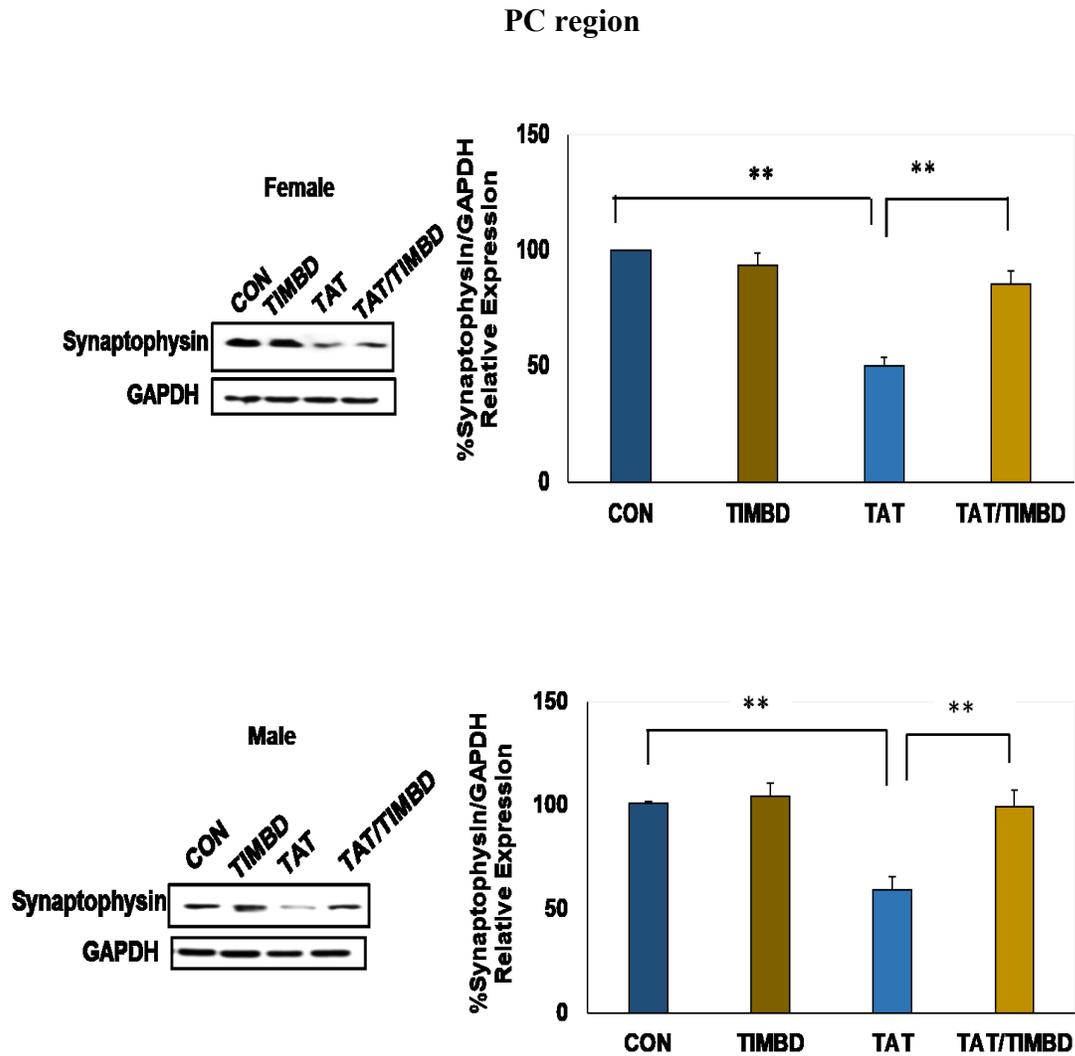


Figure 68. Brain regions were collected from HIV-1 Tat-tg mice and WT controls following 12 weeks of treatment with TIMBD or CON pellets. Expression levels of Synaptophysin were identified in PC region as presented using western blotting for both female and male mice. Each bar represents mean \pm SEM values for at least 3 independent westerns. One-way Anova was used for statistical analysis and statistical significance is denoted as * (p-value \leq 0.05) and ** (p-value \leq 0.01).

TIMBD restores HIV-1 Tat-associated decrease in synaptophysin expression levels

C region

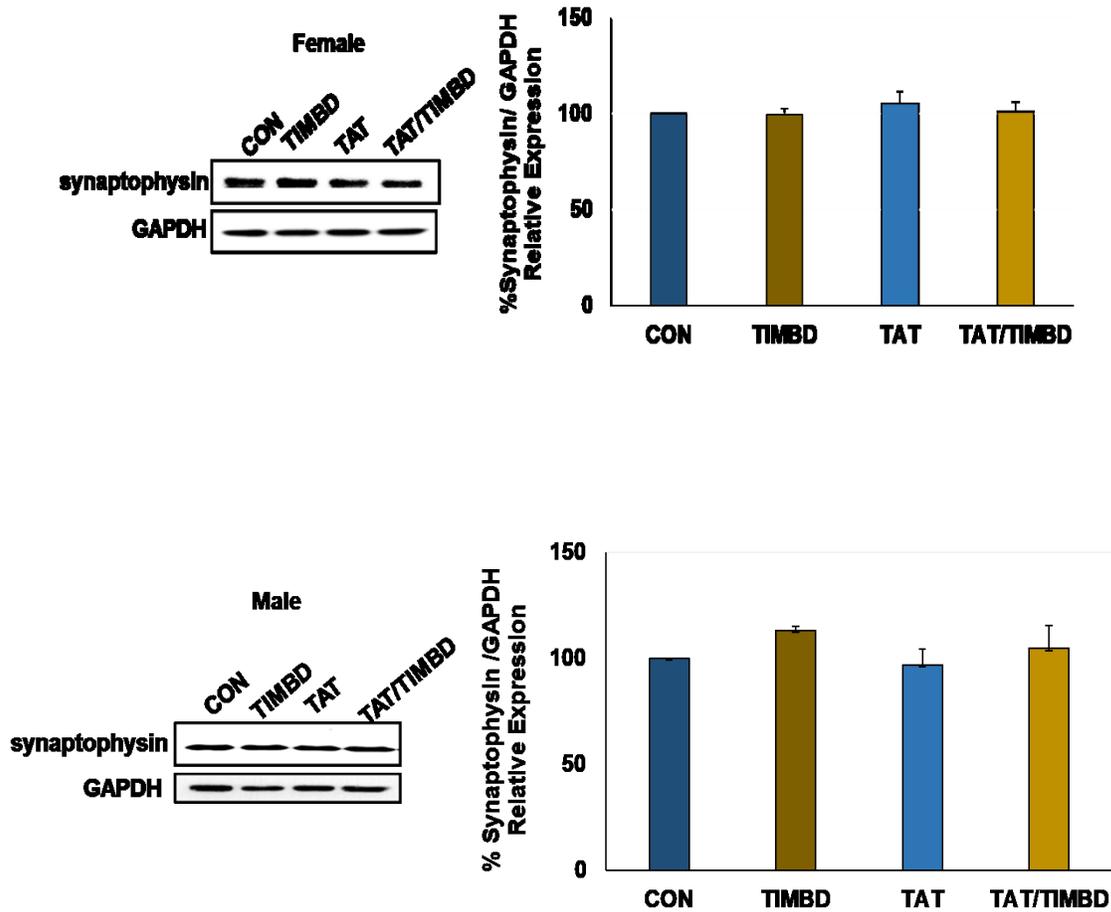


Figure 69. Brain regions were collected from HIV-1 Tat-tg mice and WT controls following 12 weeks of treatment with TIMBD or CON pellets. Expression levels of Synaptophysin were identified in C region as presented using western blotting for both female and male mice. Each bar represents mean \pm SEM values for at least 3 independent westerns. One-way Anova was used for statistical analysis and statistical significance is denoted as * (p-value \leq 0.05) and ** (p-value \leq 0.01).

TIMBD restores HIV-1 Tat associated decrease in Synapsin expression levels

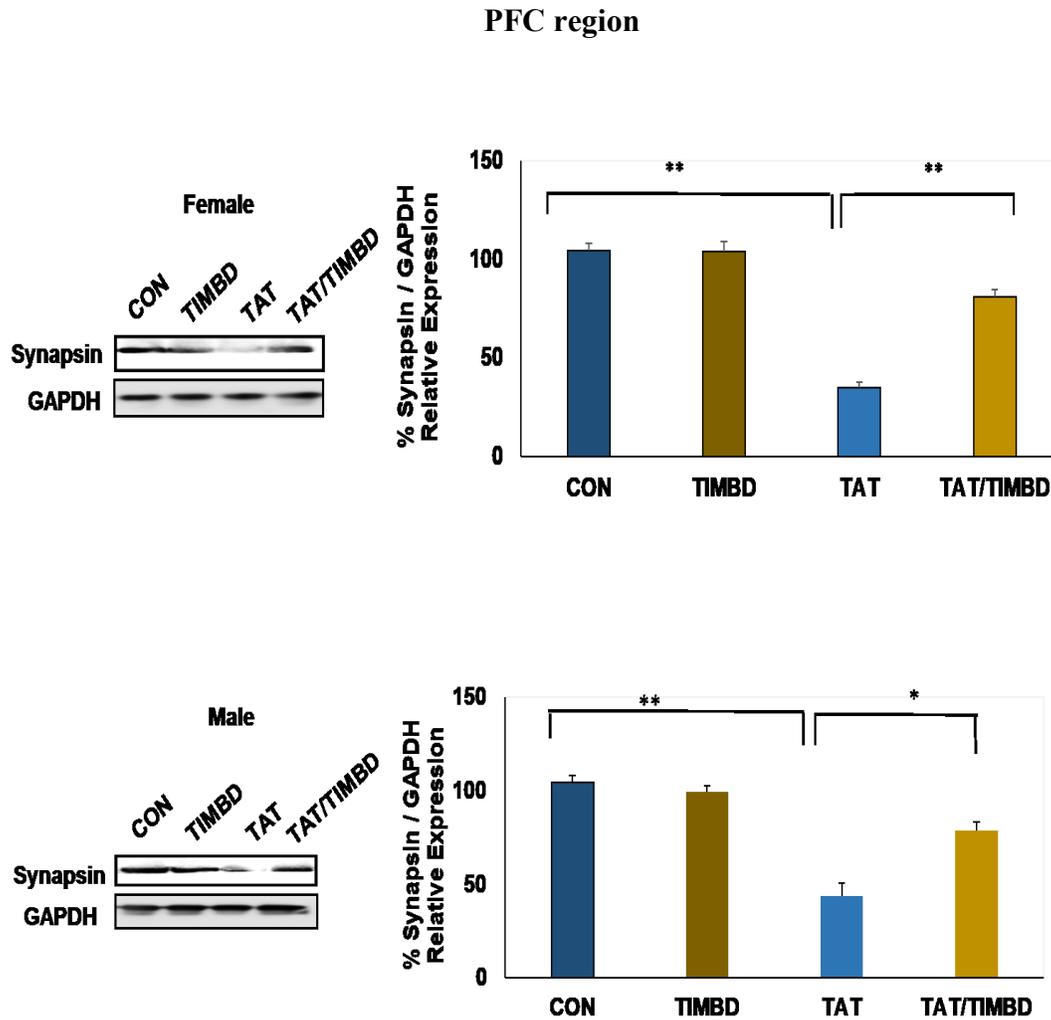


Figure 70. Brain regions were collected from HIV-1 Tat-tg mice and WT controls following 12 weeks of treatment with TIMBD or CON pellets. Expression levels of Synapsin were identified in PFC region as presented using western blotting for both female and male mice. Each bar represents mean \pm SEM values for at least 3 independent westerns. One-way Anova was used for statistical analysis and statistical significance is denoted as * (p-value \leq 0.05) and ** (p-value \leq 0.01).

TIMBD restores HIV-1 Tat associated decrease in Synapsin expression levels

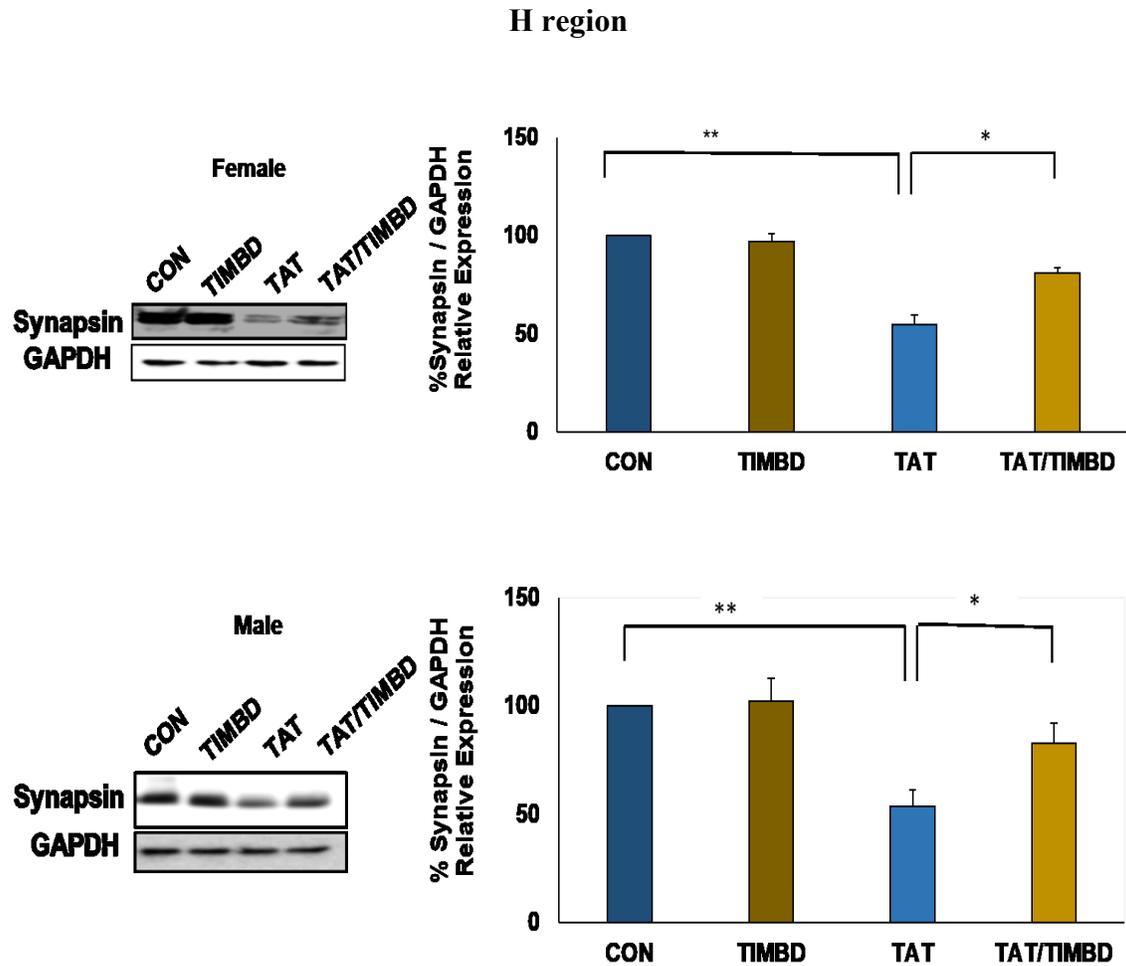


Figure 71. Brain regions were collected from HIV-1 Tat-tg mice and WT controls following 12 weeks of treatment with TIMBD or CON pellets. Expression levels of Synapsin were identified in H region as presented using western blotting for both female and male mice. Each bar represents mean \pm SEM values for at least 3 independent westerns. One-way Anova was used for statistical analysis and statistical significance is denoted as * (p-value \leq 0.05) and ** (p-value \leq 0.01).

TIMBD restores HIV-1 Tat associated decrease in Synapsin expression levels

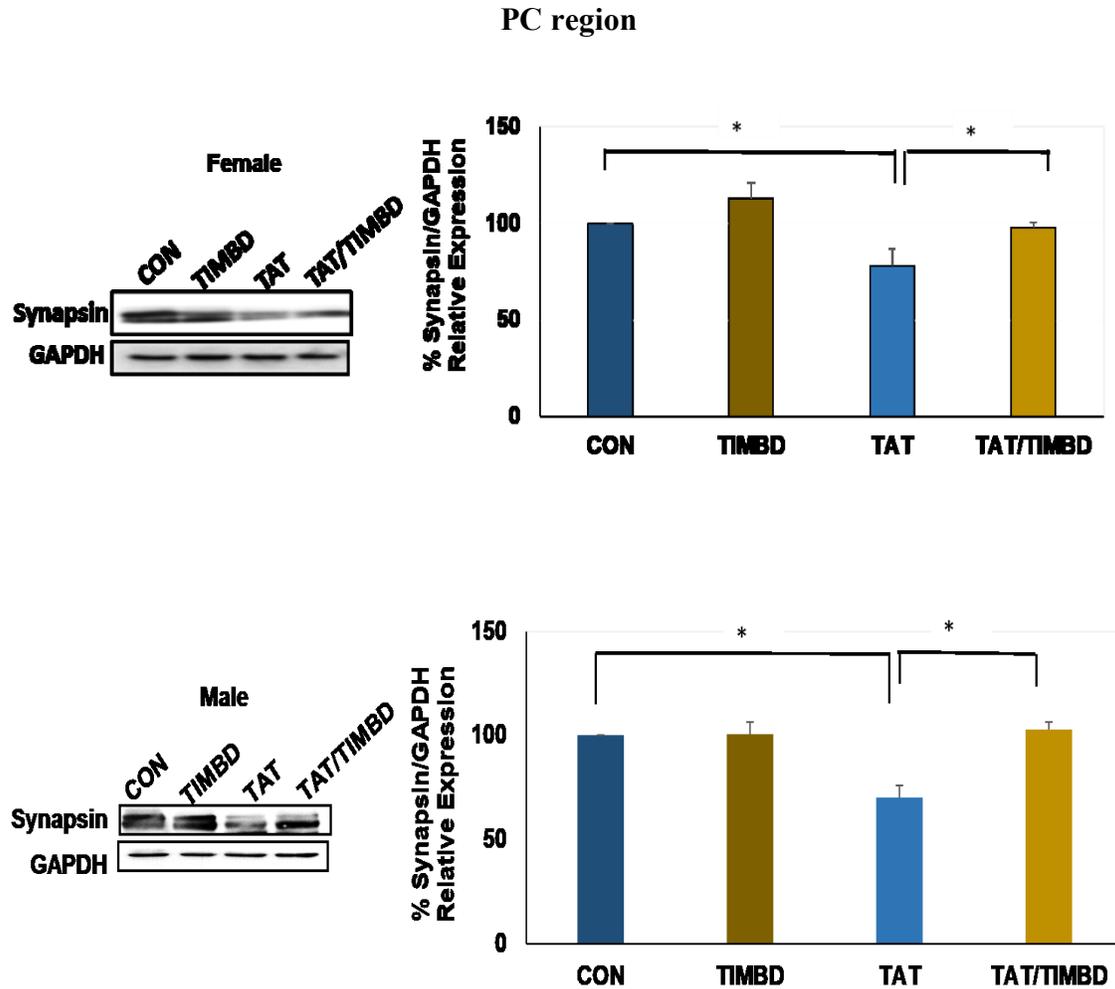


Figure 72. Brain regions were collected from HIV-1 Tat-tg mice and WT controls following 12 weeks of treatment with TIMBD or CON pellets. Expression levels of Synapsin were identified in PC region as presented using western blotting for both female and male mice. Each bar represents mean \pm SEM values for at least 3 independent westerns. One-way Anova was used for statistical analysis and statistical significance is denoted as * (p-value \leq 0.05) and ** (p-value \leq 0.01).

TIMBD restores HIV-1 Tat associated decrease in Synapsin expression levels

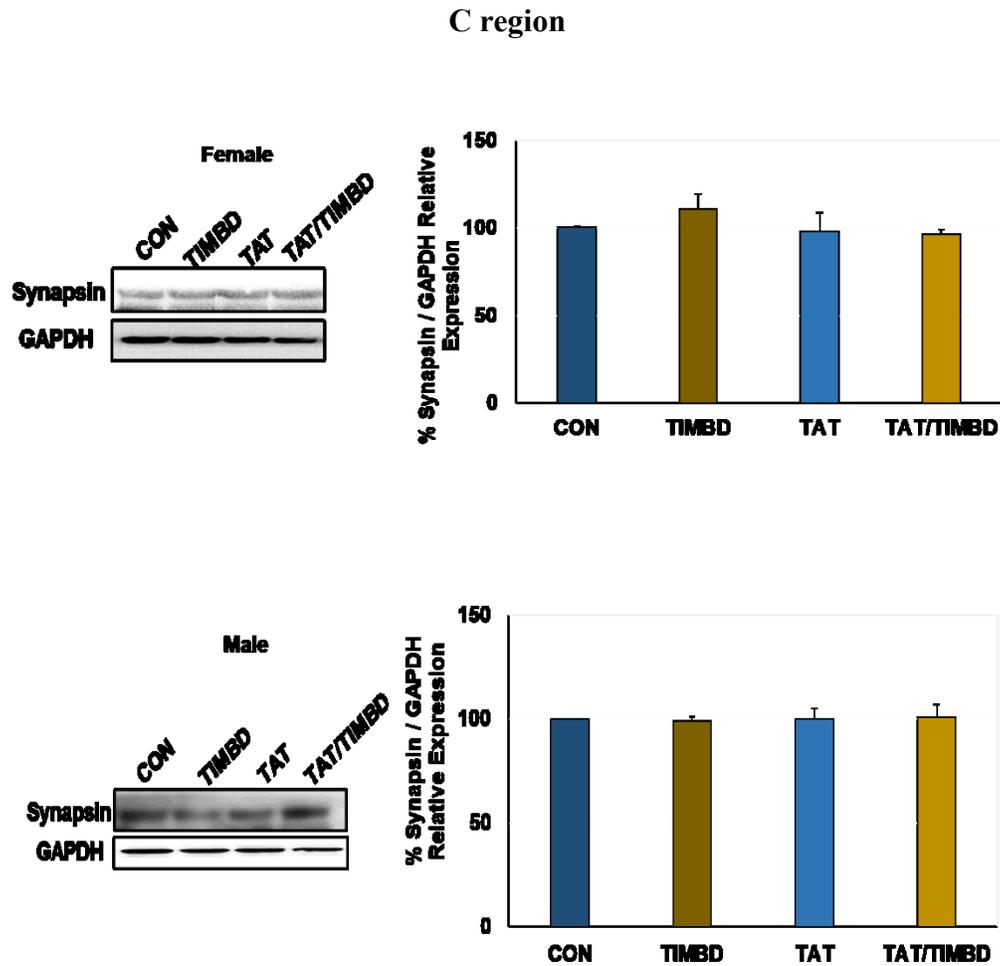


Figure 73. Brain regions were collected from HIV-1 Tat-tg mice and WT controls following 12 weeks of treatment with TIMBD or CON pellets. Expression levels of Synapsin were identified in C region as presented using western blotting for both female and male mice. Each bar represents mean \pm SEM values for at least 3 independent westerns. One-way Anova was used for statistical analysis and statistical significance is denoted as * (p-value \leq 0.05) and ** (p-value \leq 0.01).

6.2.7. Effect of HIV-1 Tat and TIMBD on postsynaptic proteins

Dysregulation of postsynaptic proteins including postsynaptic density protein-95 (PSD-95) and activity regulated cytoskeleton-associated protein (Arg3.1) play important roles in development of cognition and memory illnesses (Fitzgerald et al., 2015; Plath et al., 2006; Tzingounis & Nicoll, 2006). PSD-95 is a scaffolding protein and a central organizer of many postsynaptic proteins (Fernandez et al., 2009; Husi, Ward, Choudhary, Blackstock, & Grant, 2000). Arg3.1 which is considered an effector early gene, is implicated in the consolidation of memories (Plath et al., 2006; Tzingounis & Nicoll, 2006). Our results suggest that HIV-1 Tat decreased protein expression of PSD-95 in PFC, H and PC regions in both female and male mice significantly compared to WT control mice (**Fig. 74, 75 and 76**). TIMBD treatment was able to restore the expression levels of PSD-95 in those regions, compared to Tat-tg mice, in both female and male mice (**Fig. 74, 75 and 76**). However, there was no change in PSD-95 levels observed for C in either Tat mice or TIMBD-treated Tat mice (**Fig. 77**). TIMBD treatment of WT controls did not affect the expression levels of PSD-95 in all brain regions tested for both female and male mice (**Fig. 74 - 77**). Moreover, Arg3.1 expression levels were decreased by HIV-1 Tat in PFC, H and PC regions in both female and male mice significantly, compared to WT control mice (**Fig. 78, 79 and 80**). TIMBD treatment restored expression levels of Arg3.1 in those regions, compared to Tat-tg mice, in both female and male mice (**Fig. 78, 79 and 80**). However, no change in Arg3.1 levels was reported for the C region in either Tat mice or TIMBD-treated Tat mice (**Fig. 81 D**). Additionally, TIMBD treatment of WT controls did not change the expression levels of Arg3.1 (**Fig. 78 – 81**).

These results indicate that HIV-1 Tat can decrease the expression levels of presynaptic and postsynaptic proteins in the brain and that TIMBD treatment can restore

this expression significantly. These findings suggest that TIMBD might have potential as a therapeutic candidate that can modify the neurotransmission lost by HIV-1 Tat in the brain.

TIMBD restores HIV-1 Tat associated decrease in PSD95 expression levels

PFC region

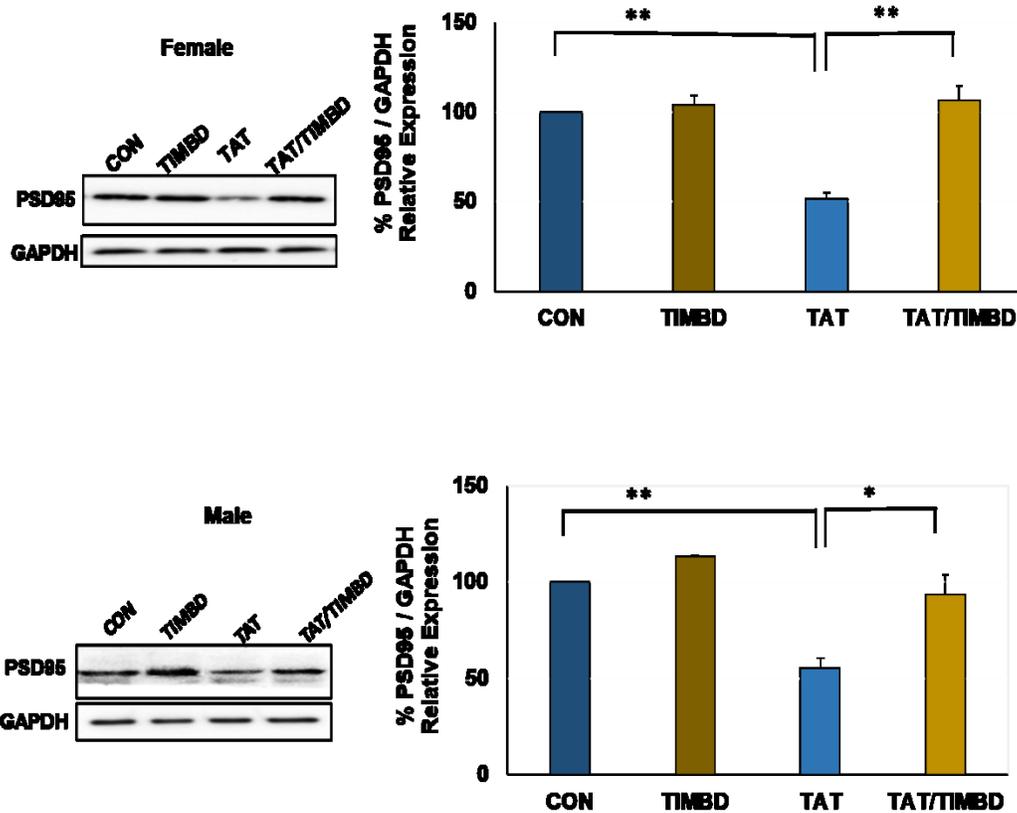


Figure 74. Brain regions were collected from HIV-1 Tat-tg mice and WT controls following 12 weeks of treatment with TIMBD or CON pellets. Expression levels of PSD95 were identified in PFC region as presented using western blotting for both female and male mice. Each bar represents mean \pm SEM values for at least 3 independent westerns. One-way Anova was used for statistical analysis and statistical significance is denoted as * (p-value \leq 0.05) and ** (p-value \leq 0.01).

TIMBD restores HIV-1 Tat associated decrease in PSD95 expression levels

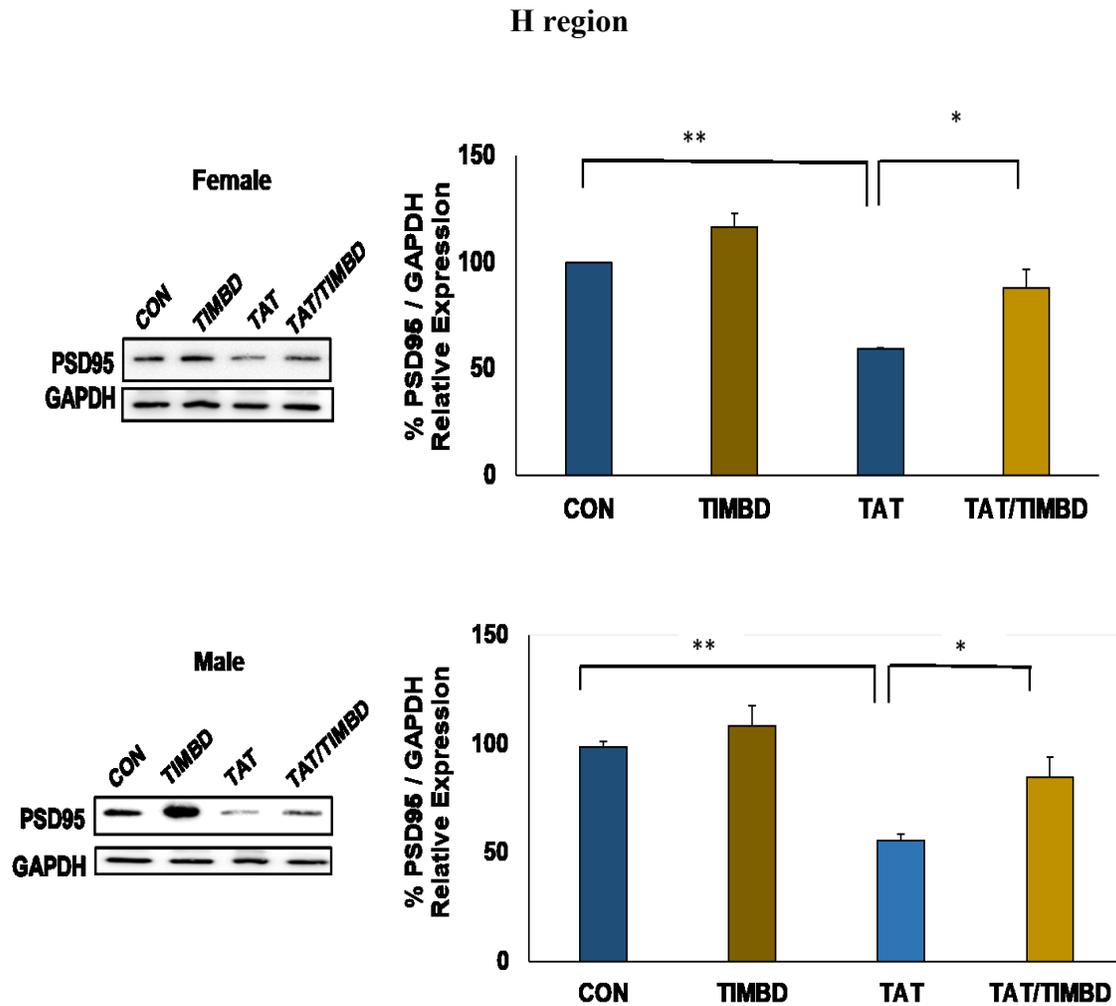


Figure 75. Brain regions were collected from HIV-1 Tat-tg mice and WT controls following 12 weeks of treatment with TIMBD or CON pellets. Expression levels of PSD95 were identified in H region as presented using western blotting for both female and male mice. Each bar represents mean \pm SEM values for at least 3 independent westerns. One-way Anova was used for statistical analysis and statistical significance is denoted as * (p-value \leq 0.05) and ** (p-value \leq 0.01).

TIMBD restores HIV-1 Tat associated decrease in PSD95 expression levels

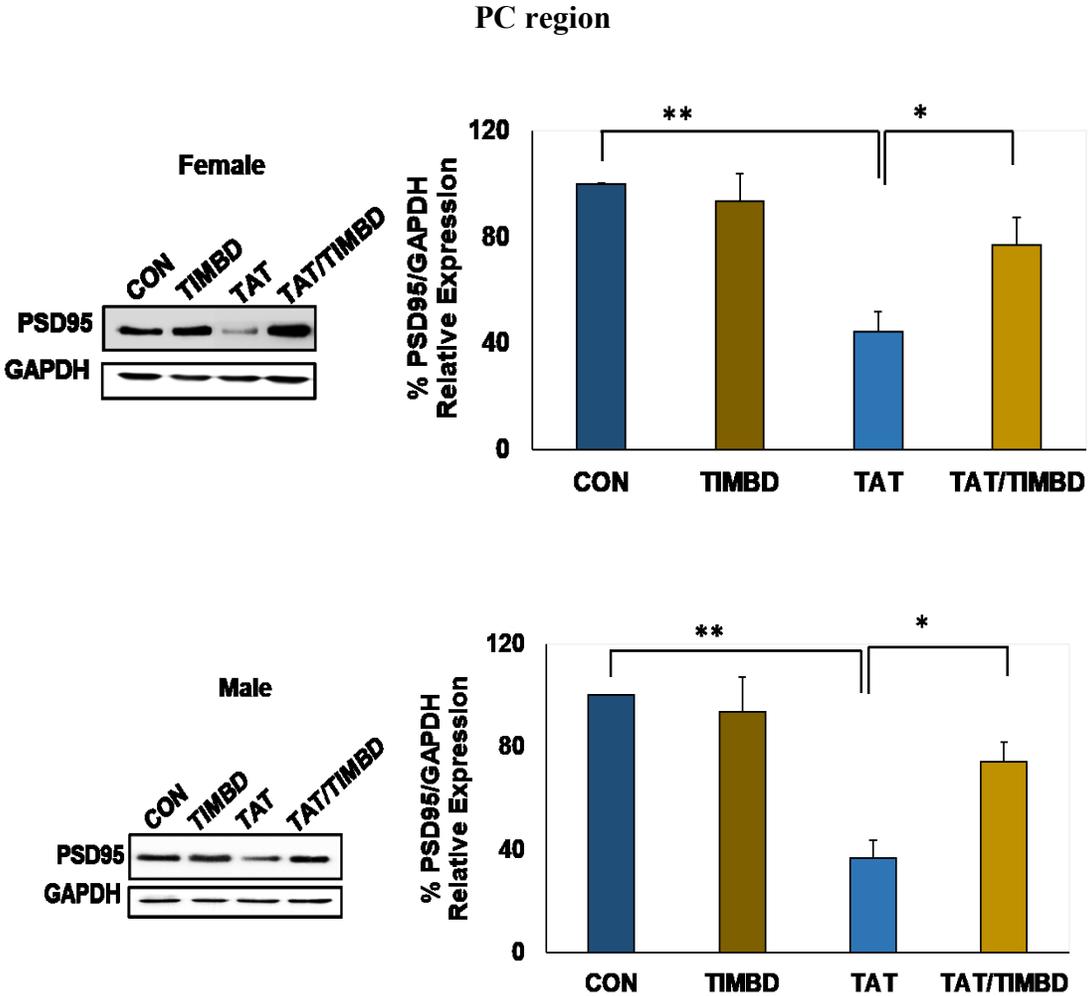


Figure 76. Brain regions were collected from HIV-1 Tat-tg mice and WT controls following 12 weeks of treatment with TIMBD or CON pellets. Expression levels of PSD95 were identified in PC region as presented using western blotting for both female and male mice. Each bar represents mean \pm SEM values for at least 3 independent westerns. One-way Anova was used for statistical analysis and statistical significance is denoted as * (p-value \leq 0.05) and ** (p-value \leq 0.01).

TIMBD restores HIV-1 Tat associated decrease in PSD95 expression levels

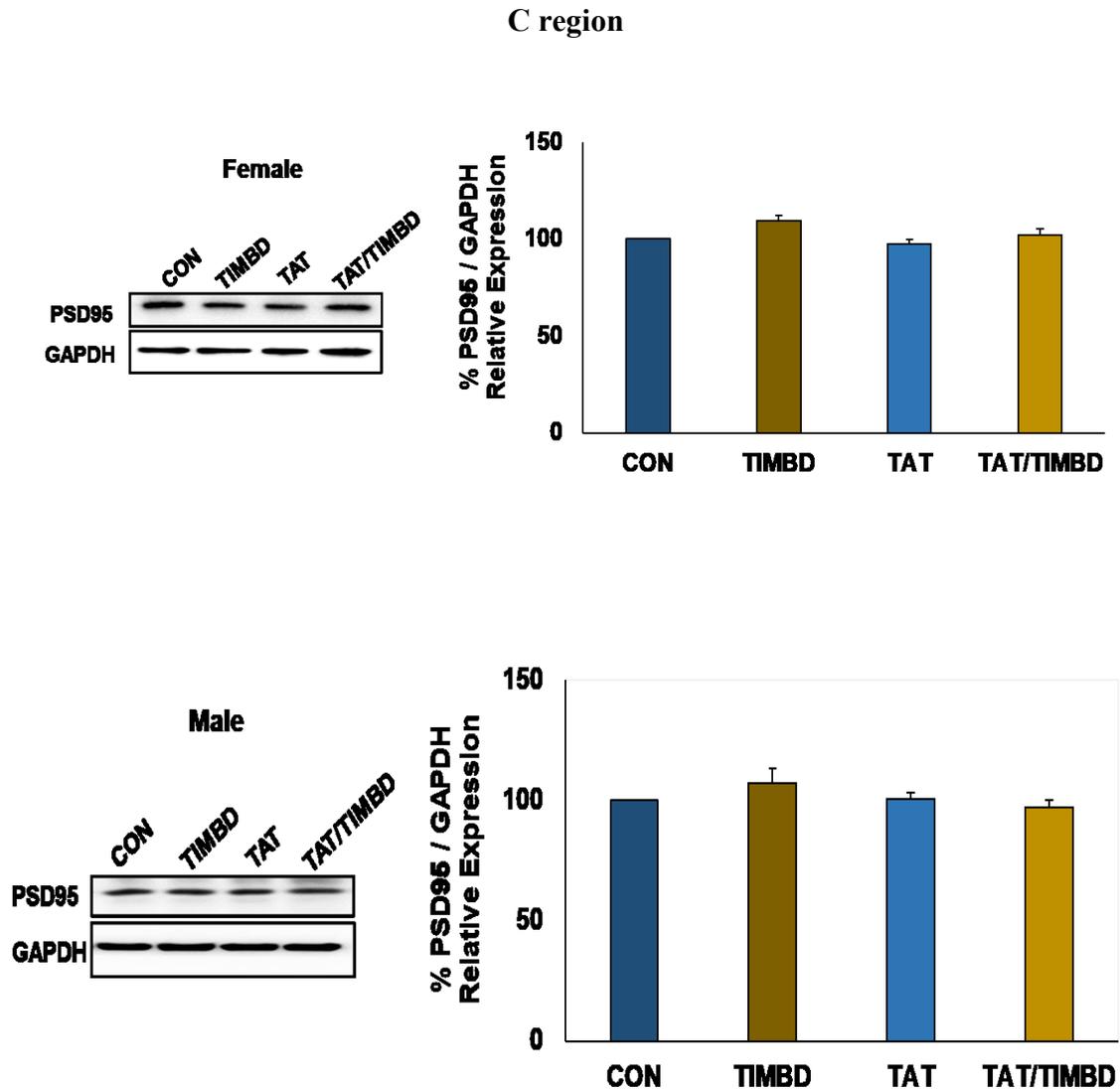


Figure 77. Brain regions were collected from HIV-1 Tat-tg mice and WT controls following 12 weeks of treatment with TIMBD or CON pellets. Expression levels of PSD95 were identified in C region as presented using western blotting for both female and male mice. Each bar represents mean \pm SEM values for at least 3 independent westerns. One-way Anova was used for statistical analysis and statistical significance is denoted as * (p-value \leq 0.05) and ** (p-value \leq 0.01).

TIMBD restores HIV-1 Tat associated decrease in Arg3.1 expression levels

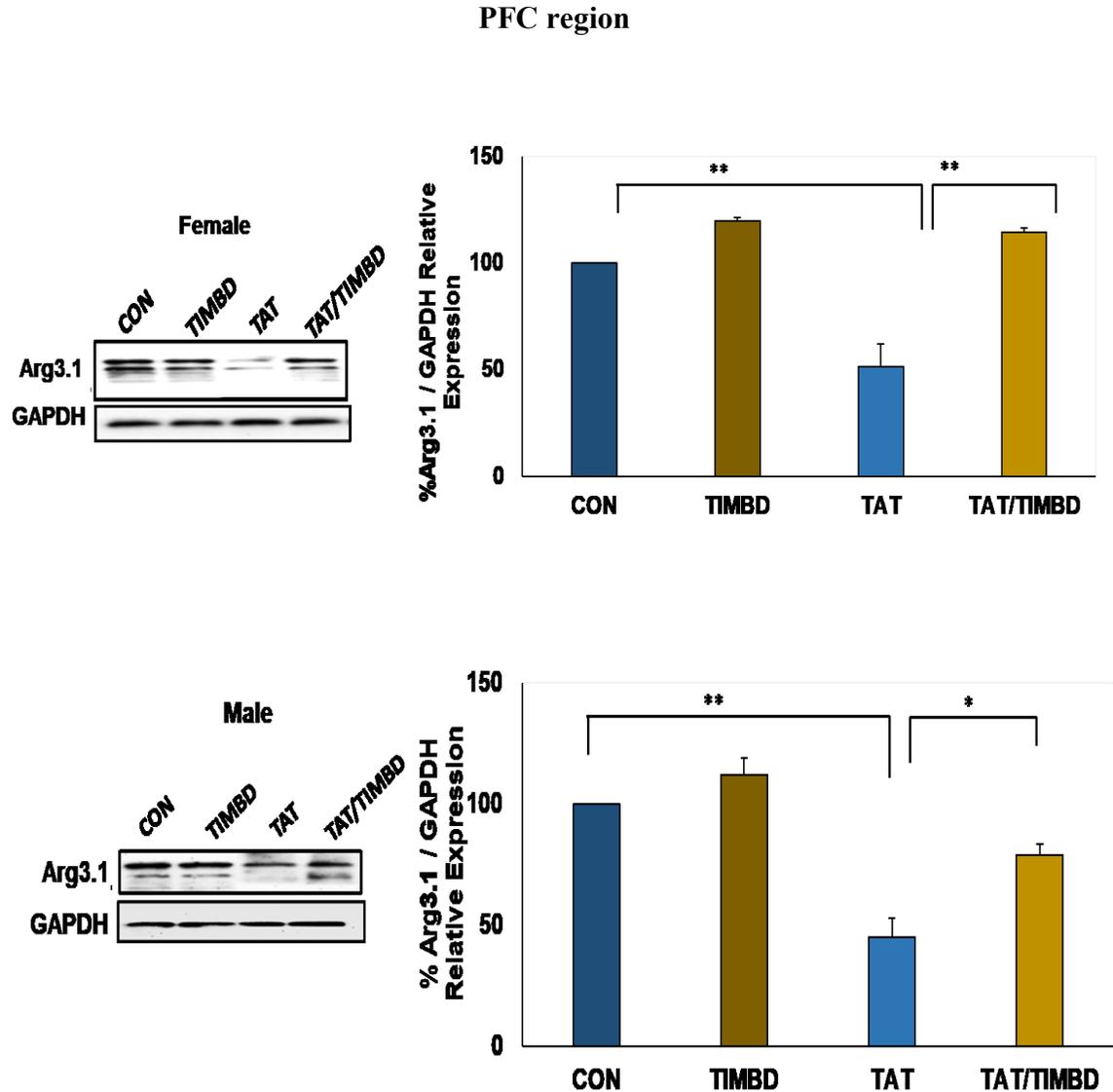


Figure 78. Brain regions were collected from HIV-1 Tat-tg mice and WT controls following 12 weeks of treatment with TIMBD or CON pellets. Protein expression levels of arg3.1 were identified in PFC region as presented using western blotting for both female and male mice. Each bar represents mean \pm SEM values for at least 3 independent westerns. One-way Anova was used for statistical analysis and statistical significance is denoted as * (p-value \leq 0.05) and ** (p-value \leq 0.01).

TIMBD restores HIV-1 Tat associated decrease in Arg3.1 expression levels

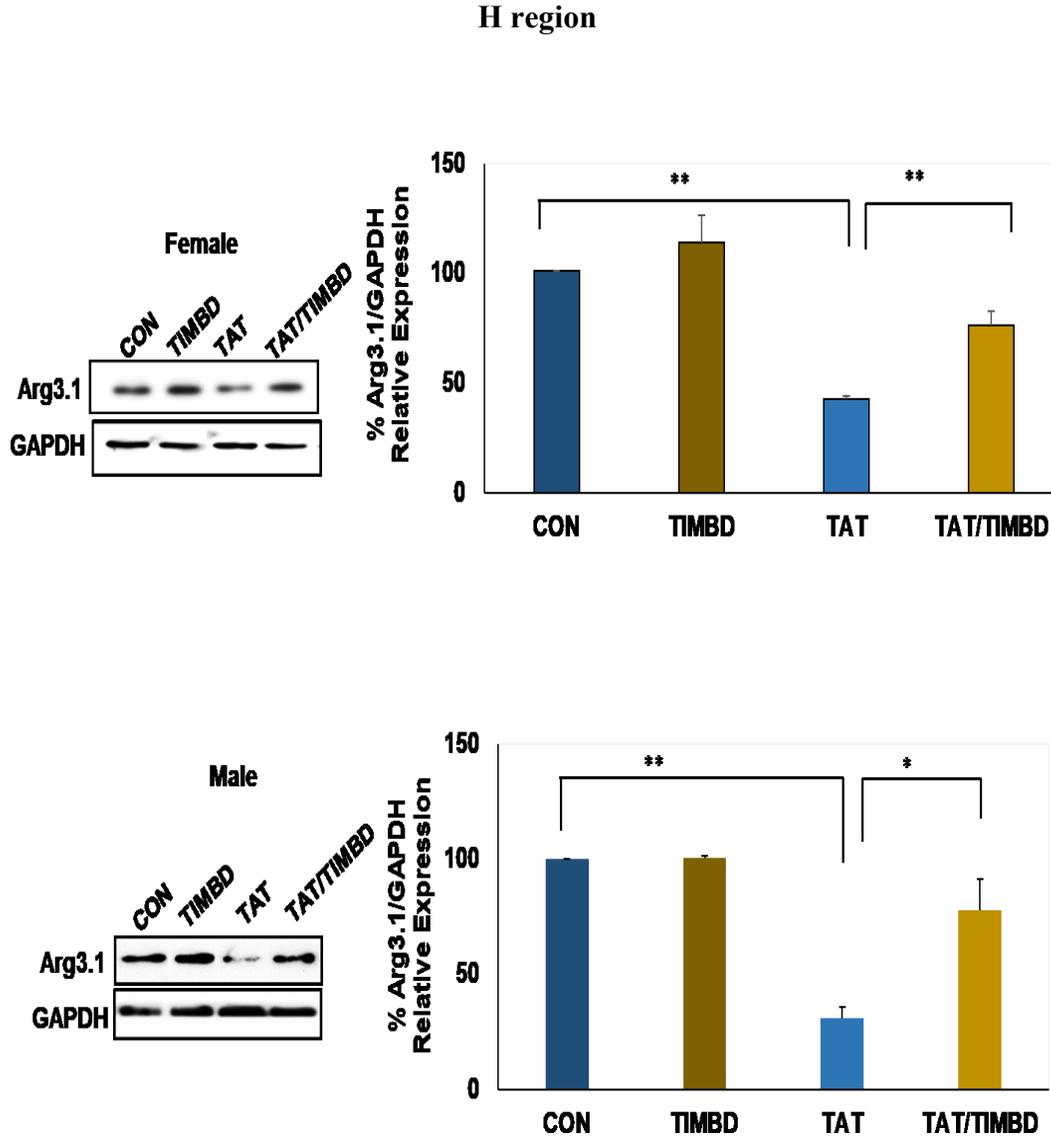


Figure 79. Brain regions were collected from HIV-1 Tat-tg mice and WT controls following 12 weeks of treatment with TIMBD or CON pellets. Protein expression levels of arg3.1 were identified in H region as presented using western blotting for both female and male mice. Each bar represents mean \pm SEM values for at least 3 independent westerns. One-way Anova was used for statistical analysis and statistical significance is denoted as * (p-value \leq 0.05) and ** (p-value \leq 0.01).

TIMBD restores HIV-1 Tat associated decrease in Arg3.1 expression levels

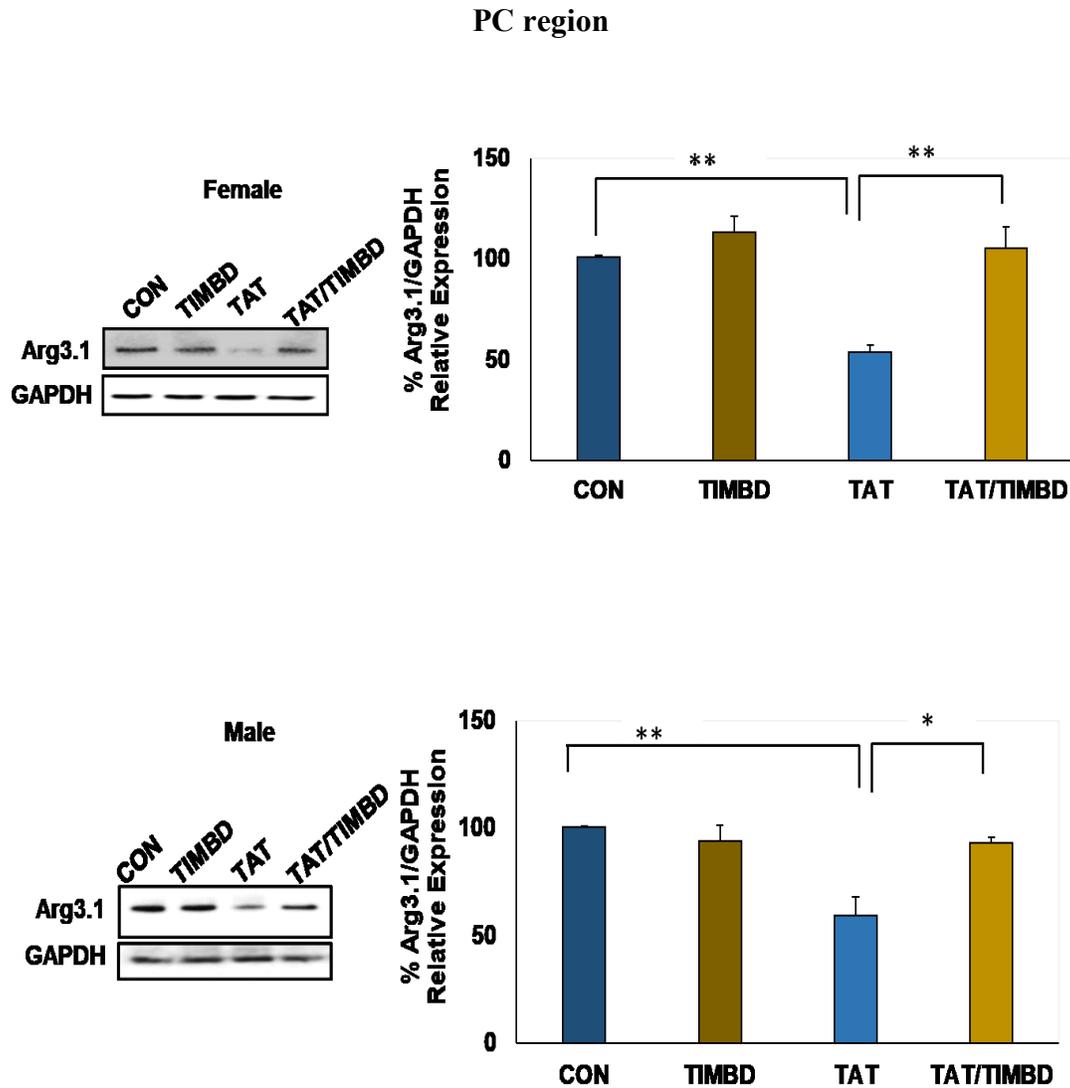


Figure 80. Brain regions were collected from HIV-1 Tat-tg mice and WT controls following 12 weeks of treatment with TIMBD or CON pellets. Protein expression levels of arg3.1 were identified in PC region as presented using western blotting for both female and male mice. Each bar represents mean \pm SEM values for at least 3 independent westerns. One-way Anova was used for statistical analysis and statistical significance is denoted as * (p-value \leq 0.05) and ** (p-value \leq 0.01).

TIMBD restores HIV-1 Tat associated decrease in Arg3.1 expression levels

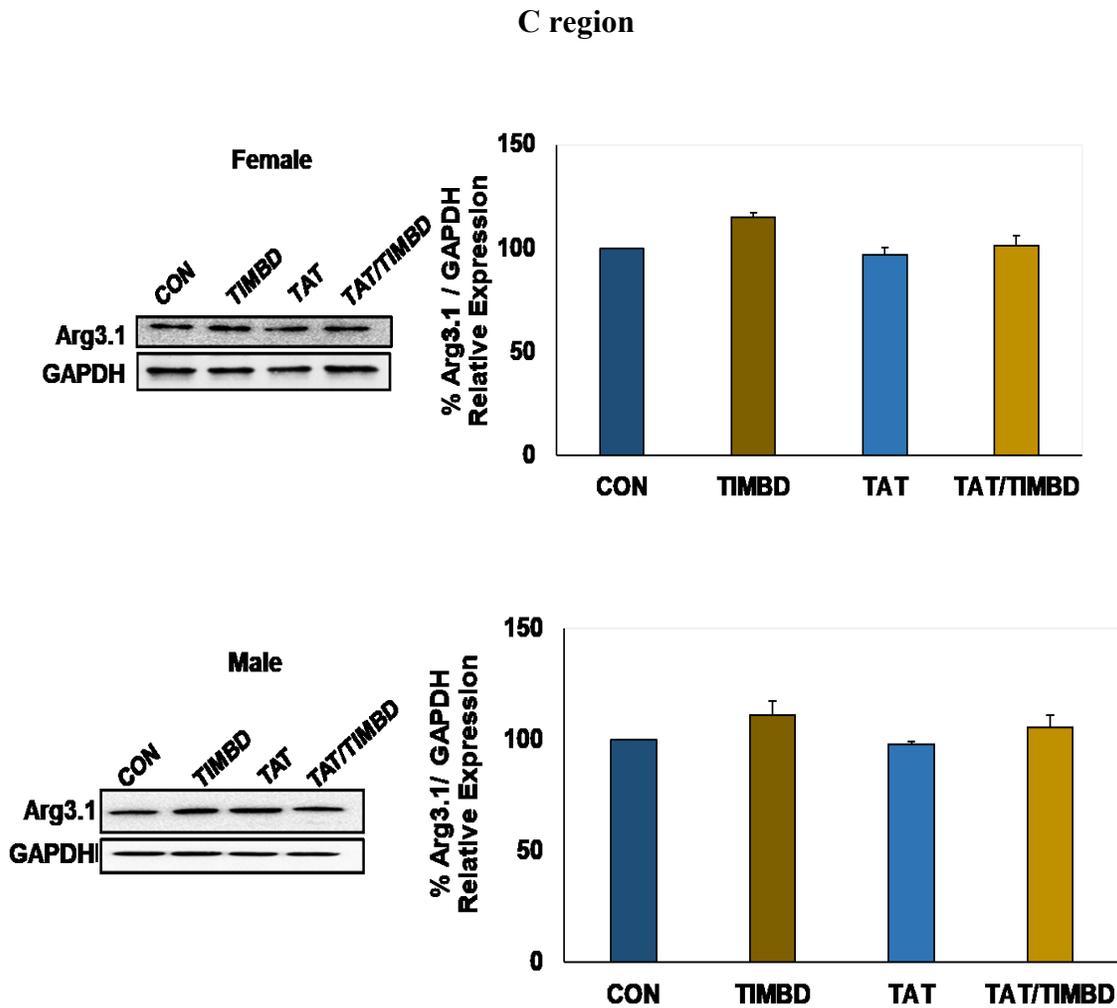


Figure 81. Brain regions were collected from HIV-1 Tat-tg mice and WT controls following 12 weeks of treatment with TIMBD or CON pellets. Protein expression levels of arg3.1 were identified in C region as presented using western blotting for both female and male mice. Each bar represents mean \pm SEM values for at least 3 independent westerns. One-way Anova was used for statistical analysis and statistical significance is denoted as * (p-value \leq 0.05) and ** (p-value \leq 0.01).

6.2.8. Effect of HIV-1 Tat and TIMBD on CAMK-II expression

Calcium/calmodulin-dependent kinase II (CAMK-II) is a multifunctional protein kinase in neurons (Ashpole et al., 2012; Hudmon & Schulman, 2002; Lisman, Schulman, & Cline, 2002; Soderling, Chang, & Brickey, 2001). CAMK-II is considered detrimental to neuronal survival and known for its critical role in learning and memory (Ashpole et al., 2012; Hudmon & Schulman, 2002; Lisman et al., 2002; Soderling et al., 2001). CAMK-II protein can regulate neurotransmission and synaptic plasticity in response to calcium signaling produced by neuronal activity (Ashpole et al., 2012; Hudmon & Schulman, 2002). The expression levels of phosphorylated CAMK-II (pCAMK-II) decreased by HIV-1 Tat in both female and male mice in PFC, H and PC regions (**Fig. 82, 83 and 84**). This decrease was significant, compared to WT control mice, in both female and male mice. TIMBD treatment of Tat-tg mice restored the expression levels of pCAMK-II compared to Tat in both female and male mice in PFC, H and PC regions (**Fig. 82, 83 and 84**). However, the expression levels of pCAMK-II were not altered in C region with TIMBD treatment of WT control mice alone (**Fig. 85**). These findings indicate that TIMBD might be able to modulate pCAMK-II expression and thus might play a significant role in memory modulation.

TIMBD restores HIV-1 Tat associated decrease in pCAMK-II expression

levels

PFC region

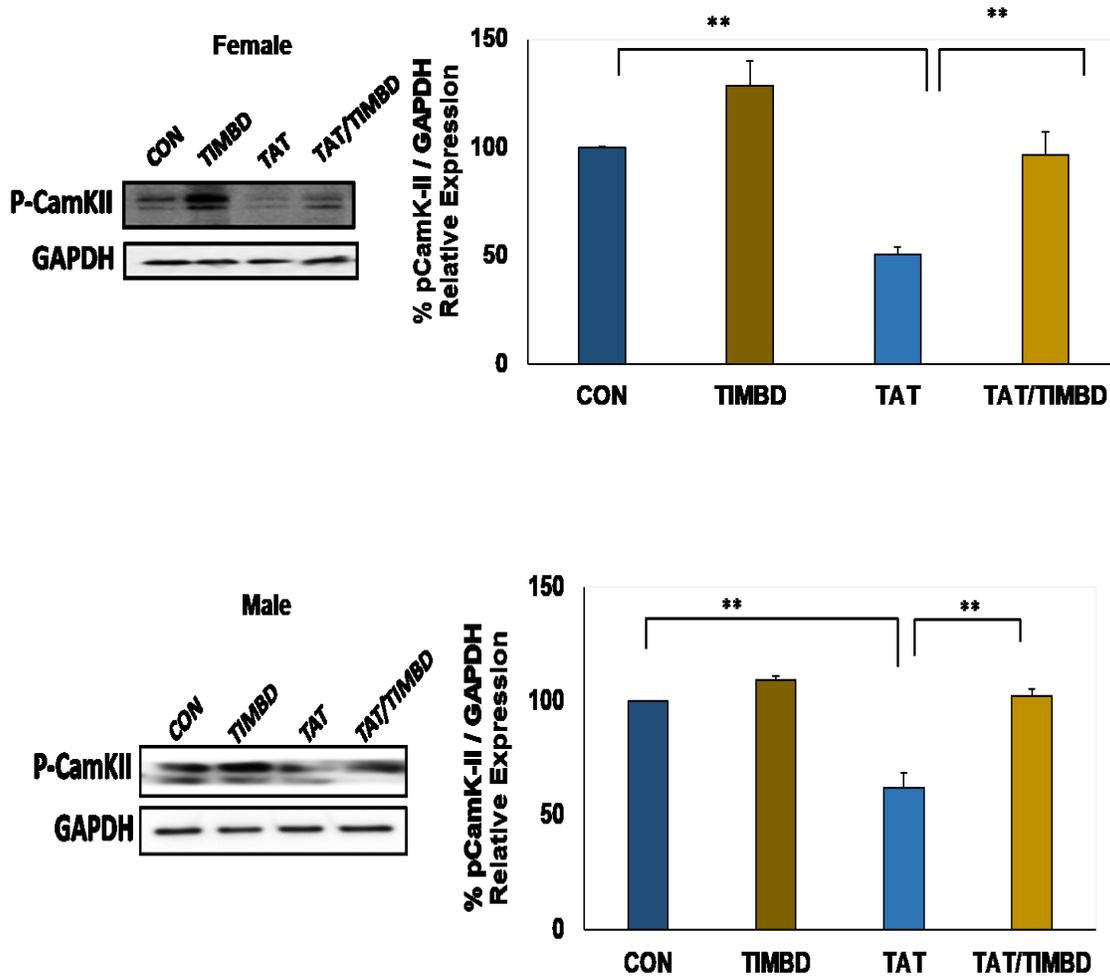


Figure 82. Brain regions were collected from HIV-1 Tat-tg mice and WT controls following 12 weeks of treatment with TIMBD or CON pellets. Expression levels of pCAMK-II were determined in PFC region as presented using western blotting for both female and male mice. Each bar represents mean \pm SEM values for at least 3 independent westerns. One-way Anova was used for statistical analysis and statistical significance is denoted as * (p-value \leq 0.05) and ** (p-value \leq 0.01).

TIMBD restores HIV-1 Tat associated decrease in pCAMK-II expression

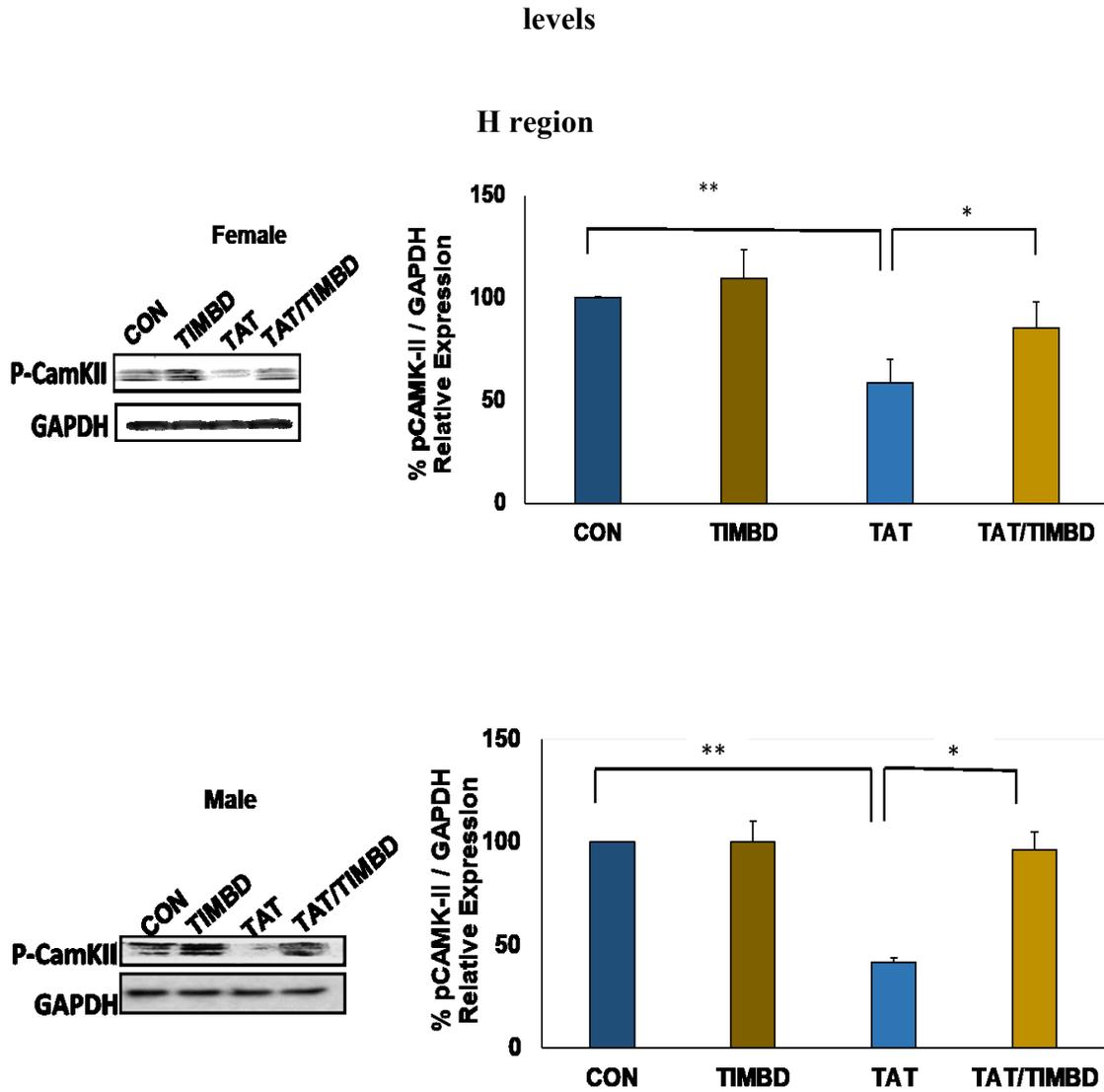


Figure 83. Brain regions were collected from HIV-1 Tat-tg mice and WT controls following 12 weeks of treatment with TIMBD or CON pellets. Expression levels of pCAMK-II were determined in H region as presented using western blotting for both female and male mice. Each bar represents mean \pm SEM values for at least 3 independent westerns. One-way Anova was used for statistical analysis and statistical significance is denoted as * (p-value \leq 0.05) and ** (p-value \leq 0.01).

TIMBD restores HIV-1 Tat associated decrease in pCAMK-II expression levels

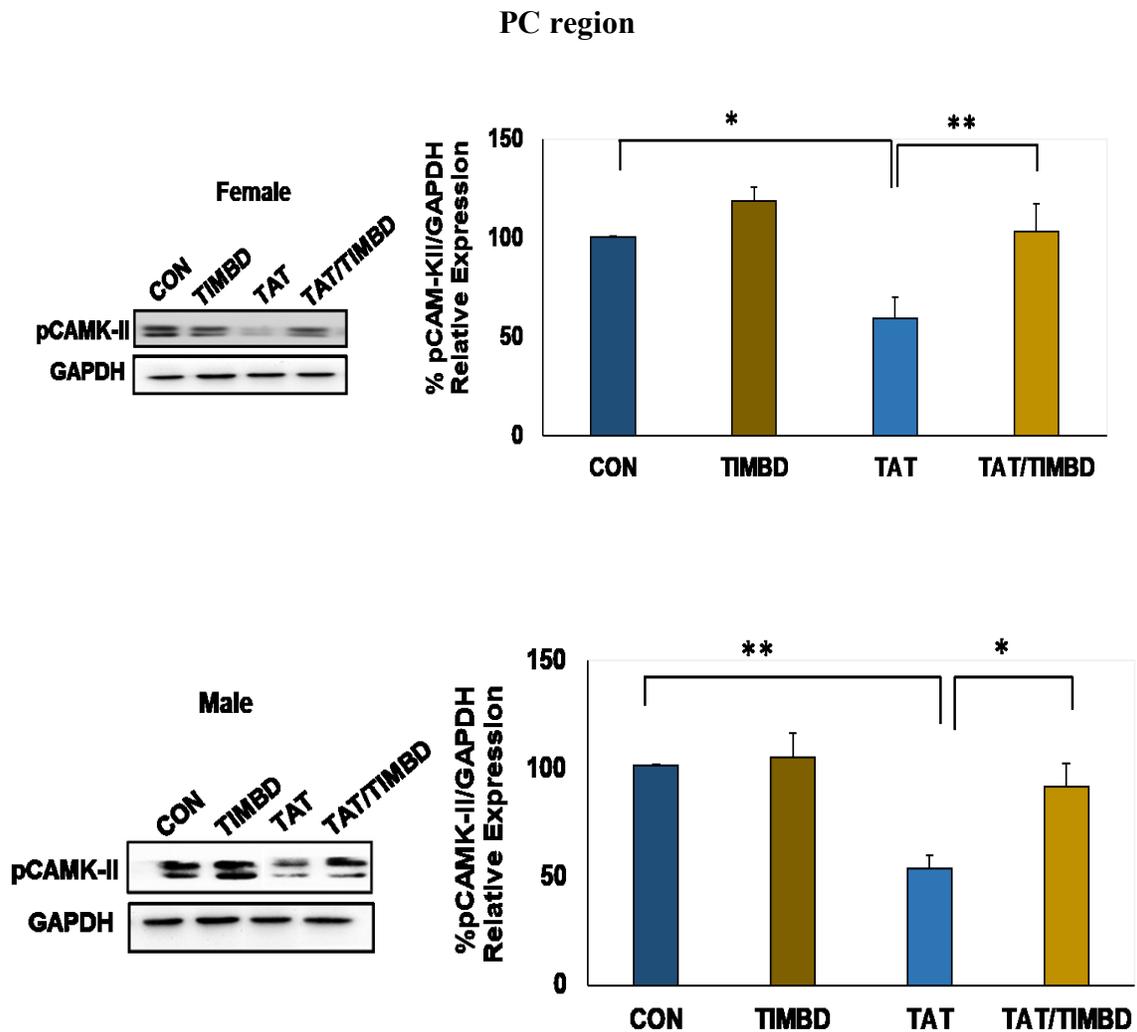


Figure 84. Brain regions were collected from HIV-1 Tat-tg mice and WT controls following 12 weeks of treatment with TIMBD or CON pellets. Expression levels of pCAMK-II were determined in PC region as presented using western blotting for both female and male mice. Each bar represents mean \pm SEM values for at least 3 independent westerns. One-way Anova was used for statistical analysis and statistical significance is denoted as * (p-value \leq 0.05) and ** (p-value \leq 0.01).

TIMBD restores HIV-1 Tat associated decrease in pCAMK-II expression

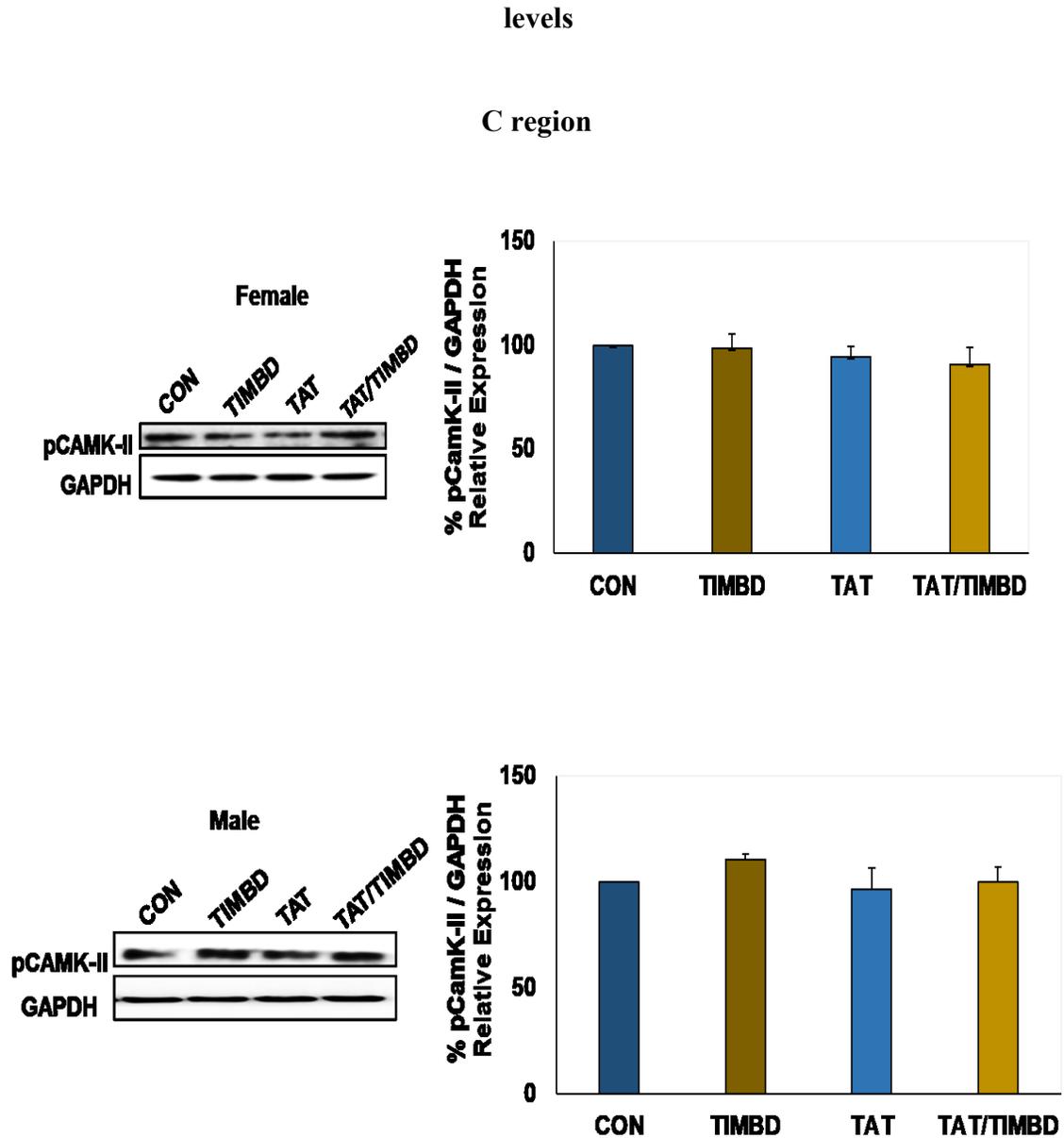


Figure 85. Brain regions were collected from HIV-1 Tat-tg mice and WT controls following 12 weeks of treatment with TIMBD or CON pellets. Expression levels of pCAMK-II were determined in C region as presented using western blotting for both female and male mice. Each bar represents mean \pm SEM values for at least 3 independent westerns. One-way Anova was used for statistical analysis and statistical significance is denoted as * (p-value \leq 0.05) and ** (p-value \leq 0.01).

6.3. Discussion

HIV-associated neurocognitive disorder is still one of the detrimental disorders that affect majority of HIV-infected patients (Clifford & Ances, 2013; Saylor et al., 2016). HIV-1 Tat is one of the HIV viral proteins that is considered an important regulator for HAND (Decrion et al., 2005; S. H. Kim et al., 2015; Louboutin & Strayer, 2014; Pu et al., 2003; Toborek et al., 2003). HIV-1 Tat is known to affect both glutamatergic and dopaminergic neurotransmission systems. It has been shown in the literature that HIV-1 Tat associated behavioral deficits might be correlated with glial activation and synaptic damage which play critical roles in developing HAND symptoms (El-Hage et al., 2006; Hahn et al., 2015; Lu et al., 2011). In this study, the effect of TIMBD was studied on behavioral deficits associated with HIV-1 Tat using Tat-tg mice. We also determined whether TIMBD can improve HIV-1 Tat-associated decrease in expression of neuronal transmission genes. Previous studies suggested that natural compounds like RES might have neuroprotective effects in neurodegenerative diseases (Poulose, Thangthaeng, Miller, & Shukitt-Hale, 2015; Wang et al., 2002). However, human studies have revealed that orally administered RES has a low bioavailability due to its extensive first pass metabolism and short half-life (Cottart, Nivet-Antoine, Laguillier-Morizot, & Beaudeau, 2010). Animal studies have also revealed that due to the rapid metabolism of RES, only a limited amount of almost 1% of the parent compound is actually in circulation (Rotches-Ribalta, Andres-Lacueva, Estruch, Escribano, & Urpi-Sarda, 2012). RES has been reported to have around an 8-minute half-life following intravenous administration in rats (Marier et al., 2002). Thus, the limited potency and poor metabolic profile has limited its clinical use and led us to develop an analog that may potentially work more effectively. The synthesis of TIMBD is previously

reported (Siddiqui et al., 2013), which was shown to be effective against breast cancer (Chatterjee et al., 2017; Ronghe, Chatterjee, Singh, et al., 2016). In this study, we studied its effects on behavioral deficits associated with HIV.

We have shown for the first time that, TIMBD treatment of Tat-tg mice can improve their impaired memory, as demonstrated using the MWM test (**Fig. 56 and Fig. 57**). The MWM test is considered a very sensitive test for detecting any impairment in learning and memory associated with brain damage (Brandeis et al., 1989; D'Hooge & De Deyn, 2001; Morris, 1984; Vorhees & Williams, 2006). The use of TIMBD as a neurotherapeutic agent that can modulate memory may be a novel advancement in memory management. Our results showed that Tat can cause anxiety-like behavior using open field test and light/dark box test which have been reported previously in the literature as sensitive methods for measuring anxiety-like behavior in mice (Hahn et al., 2015). Tat specifically decreased the distance, time and number of entries to the center area in the open field test in female but not in male mice. TIMBD treatment was able to increase the distance, time and number of entries to the center area in female mice, compared to Tat-tg mice (**Fig. 53 and Fig. 54 panel A**). However, the total distance was decreased in Tat-tg mice in both female and male mice significantly, compared to their WT control mice (**Fig. 54 panel B**). TIMBD treatment of Tat-tg mice was able to increase the total distance traveled compared to Tat-tg mice in both female and mice (**Fig. 54 panel B**). We suggest that the difference in data collected using different parameters might be attributed to the sensitivity of the parameter to gender differences. That might explain the significant difference when total distance was used to compare between control and treatment groups. Using total distance travelled as a measure has been widely used in many studies (Hahn et al., 2015). Additionally, we also

confirmed our findings using light/dark box test which demonstrated that Tat-tg mice have anxiety-like behavior and that TIMBD treatment can improve this behavior significantly in both female and male mice (**Fig. 55**).

Neurotrophic factors are required to maintain normal neuronal development (Chao, 2003; Chao et al., 2006). Changes in levels of neurotrophins have been reported in HAND patients (Bachis et al., 2003). The neurotrophic factor BDNF is required for hippocampal-associated learning (Bekinschtein, Cammarota, & Medina, 2014; Tyler, Alonso, Bramham, & Pozzo-Miller, 2002). Studies have demonstrated that HIV infection causes BDNF imbalance that might lead consequently to neuronal death (Bachis, Avdoshina, Zecca, Parsadanian, & Mocchetti, 2012; J. Fields, Dumaop, Langford, Rockenstein, & Masliah, 2014). This neuronal death can lead to impairment in learning, memory, and plasticity in the hippocampus (J. L. Lee, Everitt, & Thomas, 2004). In our study, we found that Tat could reduce the protein expression levels of BDNF in PFC and H (**Fig. 58 and 59**), which might be related to the memory impairment that is reported in the behavior results section (**Fig. 56 and 57**). This study has demonstrated for the first time that TIMBD is able to restore the expression levels of BDNF in PFC and H, indicating that it might improve memory and plasticity through modulation of neurotrophic factors.

Degradation of synaptic proteins has been linked to many neurodegenerative diseases, including HAND (Fiala, Spacek, & Harris, 2002; Masliah & Terry, 1993; Swann, Al-Noori, Jiang, & Lee, 2000). HIV-1 Tat can cause loss of dendritic structure which is responsible for the synaptic plasticity impairment within neural networks (H. J. Kim et al., 2008; S. T. Li et al., 2004). Arc/Arg3.1 is selectively expressed in CAMK-II glutamatergic neurons in the brain which then get phosphorylated by CAMK-II (Donai et al., 2003;

Vazdarjanova et al., 2006). Arc/Arg3.1 is also found in PSD proteins (Husi et al., 2000). That indicates that any impairment in those proteins involved in behavior development will lead consequently to deficits in behavior. In this study, we reported for the first time that TIMBD can recover the protein expression levels of presynaptic and postsynaptic proteins significantly compared to that of Tat. These findings suggest that neurotherapeutic agents like TIMBD could be valuable intervention treatment strategies to improve the neurodeficits affecting HAND patients.

In conclusion, TIMBD treatment of Tat-tg mice showed improvement in anxiety-like behavior which was illustrated by the increase in the total distance traveled in the open field test and the increase in the time spent in the light zone in the light/dark box test. Moreover, TIMBD treatment of Tat-tg mice could significantly ameliorate the memory impairment as demonstrated by the MWM test results. Additionally, TIMBD restored expression levels of neurotrophic factors which are impaired by Tat. TIMBD treatment also increased the expression levels of presynaptic and postsynaptic proteins, as well as pCAMK-II in Tat-tg mice indicating that TIMBD can improve the synaptic loss associated with HIV-1. Future studies should focus on identifying the upstream mechanism of TIMBD and optimizing the dose to be used therapeutically to improve the behavioral deficits associated with HIV.

CHAPTER 7

TIMBD SUPPRESSES INFLAMMATION AND OXIDATIVE STRESS ASSOCIATED WITH HIV-1 TAT IN ASTROCYTES

7.1. Introduction

Human immunodeficiency virus (HIV)-associated diseases including neurodegeneration have been potential health concerns globally (Anzala et al., 1995; Morgan et al., 2002). HIV is known to infiltrate the brain and potentially infect different cells of the brain. In the years following HIV infection, patients show signs of neurocognitive problems termed HIV-associated neurocognitive disorders (HAND) (Alfahad & Nath, 2013; Atashili et al., 2013; Barber et al., 2014). Following infection of the central nervous system (CNS), viral proteins like gp120, Tat and vpr are released from the infected brain cells, causing injury to the brain, and potentially leading to cognitive and motor dysfunction in the infected individuals (Clifford & Ances, 2013; Toborek et al., 2005). Patients suffer from memory loss, personality changes, diminished mental capacity and the inability to use acquired knowledge (Fellows et al., 2013; Letendre et al., 2010; Letendre et al., 2009). Complete understanding of HIV-associated neurotoxicity has been difficult. HIV viral proteins released from infected microglia and monocytes have been generally considered as direct and indirect contributors to HAND (Al-Harathi et al., 2014; Markowitz et al., 2007). Additionally, astrocytes can also be infected with HIV, leading to activation of astrocytes and subsequent release of inflammatory mediators which in turn lead to neuronal death, directly or indirectly (Gorry et al., 2003; Gray et al., 2014).

HIV-1 Tat is one of the HIV viral proteins that plays a key role in the expression and replication of the viral genome (Harrich et al., 1997; Kameoka et al., 2002; Morrow et

al., 1994). Studies have demonstrated the presence of HIV-1 Tat mRNA and protein in the brain of HIV patients (Del Valle et al., 2000; Hudson et al., 2000; Wiley et al., 1996). The Tat protein, secreted from infected cells, can exert its effect on uninfected cells through Tat-responsive genes (Ensoli et al., 1993; Watson & Edwards, 1999). Based on these findings, HIV-1 Tat is considered an important regulator of HIV neuroAIDs. Studies have shown that HIV-1 Tat acts through modulating the expression of adhesion molecules like VCAM-1 and ICAM-1 in human endothelial cells (Dhawan et al., 1997). Additionally, HIV-1 Tat is suggested to impair the BBB by decreasing occludin production levels (R. Xu et al., 2012). HIV-1 Tat can increase VCAM-1 in both human vein endothelial cells and in astrocytes (Song et al., 2007; Woodman et al., 1999). In addition, studies have shown that HIV-1 Tat might play an important role in trafficking of monocytes into the CNS which is critical in the development of HIV-neurocognitive disorders (Rappaport et al., 1999).

Previous studies have shown that HIV-1 Tat can freely penetrate neuronal cell membranes and lead to an increase in oxidative stress markers like lipid peroxidation (Ivanov et al., 2016; Jeong et al., 2012). The HIV-1 Tat-associated oxidative stress occurs through the generation of reactive oxygen species (ROS). Oxidative stress is caused by the imbalance between free radicals and the antioxidants defense enzymes which is shifted towards ROS. Presence of HIV-1 Tat protein in rat brains caused an increase in protein oxidation which may lead to oxidative damage of the neurons (Aksenov et al., 2001). HIV-1 Tat-induced oxidative stress was found to be mediated by NADPH-oxidase (NOX) in astrocytes that can increase adhesion of monocytes to astrocytes (Song et al., 2011). NADPH-oxidases are a family of enzymes that are involved in the generation of ROS in

many neurological disorders (Hernandes & Britto, 2012; G. H. Kim et al., 2015; Song et al., 2011). Studies have shown that HIV-1 Tat increases oxidative stress in brain endothelial cells (Price et al., 2005). Increase in ROS levels in the brain causes an increase in MMPs levels which is considered a major contributor to BBB dysfunction (Lakhan et al., 2013; Pun et al., 2009). Additionally, studies have shown that HIV-1 Tat increased ROS levels in astrocytes which in turn lead to alteration in the levels of ICAM-1 and VCAM-1 adhesion molecules (Song et al., 2007). The disruption of the BBB by Tat may lead to transmigration of infected cells to the brain. This will lead eventually to an accumulation of inflammatory cells that can cause neuropathological changes in the CNS (King et al., 2006).

HIV-1 Tat triggers neuroinflammation through NF κ B and AP1, SP1 signaling and subsequent upregulation of cytokines (Gonek et al., 2017; Lim & Garzino-Demo, 2000; Nath et al., 1999). HIV-1 Tat can induce the production of several cytokines, including IL6, IL8, IL2, TNF- α , MCP-1 (Buonaguro et al., 1992; Conant et al., 1998; Y. W. Lee et al., 2005; Sastry et al., 1996; Westendorp et al., 1994). *In vitro* studies have demonstrated that Tat treatment of mouse neurons led to activation of p38 and JNK MAPK pathways. However, only the p38MAPK pathway was required for Tat-mediated neuronal death (J. Cao et al., 2005; I. N. Singh et al., 2005). Tat-induced neurotoxicity was also shown to be not only dependent on caspase activity but many additional pathways are involved in Tat-associated neuronal cell death (I. N. Singh et al., 2004).

Natural compounds are suggested to have wide therapeutic effects in different pathological conditions (Koehn & Carter, 2005; Nobili et al., 2009). Although natural products have shown therapeutic effects in many studies, poor specificity and

bioavailability have presented a challenge for their clinical use (Ginsburg & Deharo, 2011; Walle, 2011). Our group has developed novel RES analog; 4-(E)-{(p-tolylimino)-methylbenzene-1,2-diol} (TIMBD). Previous studies have shown that TIMBD has potent antioxidant effects in breast cancer (Chatterjee et al., 2017; Ronghe, Chatterjee, Singh, et al., 2016; Siddiqui et al., 2013). Therefore, confirming that TIMBD would exert the same effects against HIV-1 Tat in astrocytes is significant and can strengthen its potential for using clinically to inhibit the development of HIV-proteins induced-oxidative stress and neuroinflammation.

7.2. Results

The synthesis of 4-(E)-{(p-tolylimino)-methylbenzene-1,2-diol} (TIMBD) has been reported previously (Siddiqui et al., 2013). The effect of TIMBD against HIV-1 Tat-induced inflammation and oxidative stress was investigated using astrocytes.

7.2.1. TIMBD inhibits HIV-1 Tat-induced IL6 and IL8 mRNA expression levels

It has been previously reported that Tat induces mRNA and protein expression levels of proinflammatory cytokines IL6, IL8 and CCL5 in SVG astrocytes (Nookala & Kumar, 2014; Nookala, Shah, Noel, & Kumar, 2013). Tat induces maximal mRNA expression at 6 h post transfection (Nookala & Kumar, 2014). To determine the effects of TIMBD on the expression levels of IL6 and IL8 mRNA, SVG astrocytic cells were treated with doses ranging from 6 to 50 μ M of TIMBD or RES 1 h prior to transfection with Tat plasmid using serum-free medium. The cells were transiently transfected with 0.3 μ g plasmid encoding HIV1-Tat for 5 h, followed by medium change and incubation for 6 h. RNA was isolated using established protocols (Shah & Kumar, 2010; Shah, Singh, et al., 2011; Shah, Verma, et al., 2011). HIV1 Tat induced IL6 mRNA expression levels of 24.1 ± 2.2 while treated with TIMBD decreased expression levels of IL6 mRNA significantly in a dose dependent manner (**Fig. 86 panel A**) while RES decreased Tat-induced IL6 mRNA expression levels less (**Fig. 86 panel B**). Additionally, RES at 50 μ M dose was somewhat cytotoxic to SVG astrocytes (**Fig. 11**).

The effect of TIMBD on IL8 mRNA expression levels was studied. It has previously been shown that Tat induces maximal mRNA expression at 6 h post transfection (Nookala & Kumar, 2014). To determine the effects of TIMBD on the expression levels of IL8 mRNA, SVG astrocytic cells were treated with doses ranging from 6 to 50 μ M of

TIMBD or RES 1 h prior to transfection with Tat plasmid using serum-free medium. The cells were transiently transfected with 0.3 μ g plasmid encoding HIV1-Tat for 5 h and then the medium was changed, and the cells were incubated for 6 h. RNA was isolated using established protocols as described in the Methods section. HIV-1 Tat induced IL8 mRNA expression levels of 21.8 ± 1.3 while treatment with TIMBD decreased expression levels of IL8 mRNA significantly in a dose dependent manner (**Fig. 87 panel A**), while RES decreased IL8 mRNA expression levels less significantly than TIMBD (**Fig. 87 panel B**). Thus, our results indicate that TIMBD, and to a lesser degree RES, decrease proinflammatory cytokines associated with HIV-1 Tat, IL6 and IL8, at the mRNA level in SVG astrocytes.

TIMBD decreases IL6 RNA expression levels in a dose dependent manner

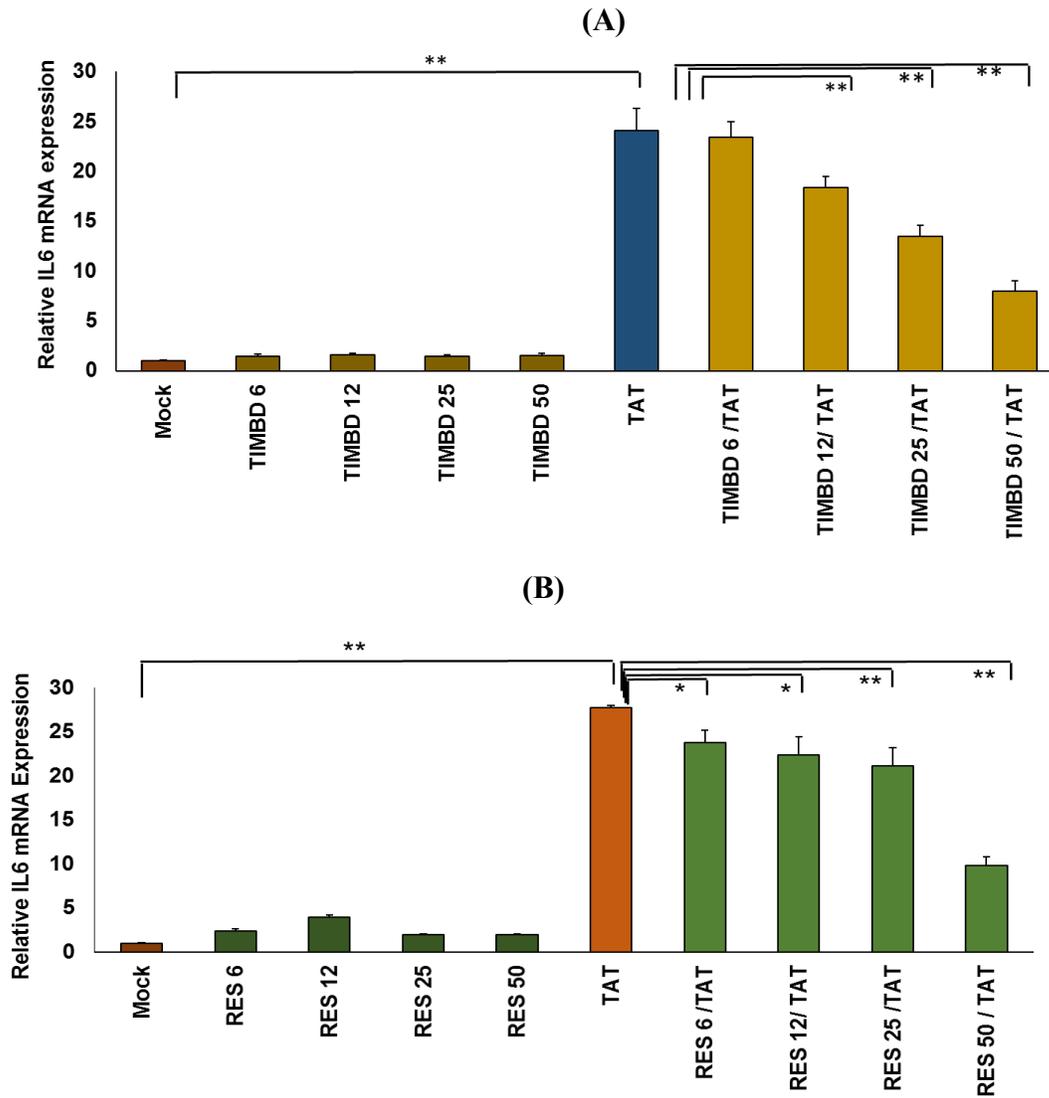
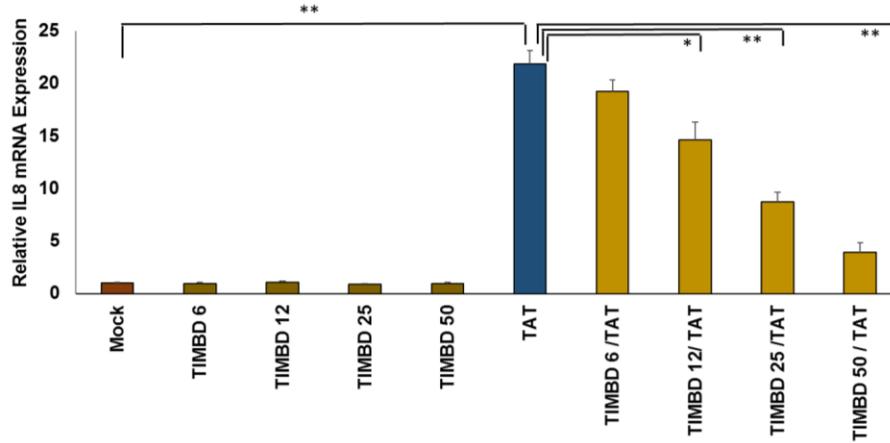


Figure 86. SVG astrocytes were treated with 6 to 50 μ M TIMBD (A) or RES (B) 1 h prior to transfection with plasmid encoding HIV-1 Tat for 5 h. The cells were harvested at 6 h and the expression levels of IL6 mRNA were determined by RT-PCR. Each bar represents mean \pm SEM for at least 3 independent experiments, with each experiment done in triplicate. One-way Anova was used for statistical analysis and statistical significance is denoted as * (p-value \leq 0.05) and ** (p-value \leq 0.01).

TIMBD decreases IL8 RNA expression levels in a dose dependent manner

(A)



(B)

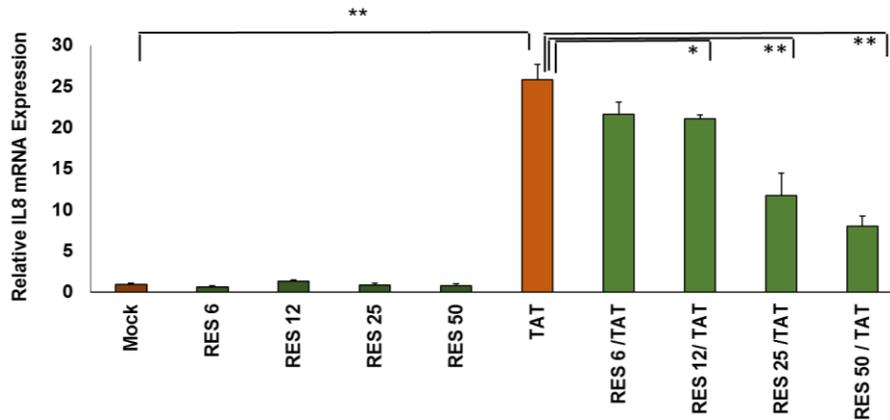


Figure 87. SVG astrocytes were treated with 6 to 50 μ M TIMBD (A) or RES (B) 1 h prior to transfection with plasmid encoding HIV-1 Tat for 5 h. The cells were harvested at 6 h and the expression levels of IL8 mRNA were determined by RT-PCR. Each bar represents mean \pm SEM for at least 3 independent experiments, with each experiment done in triplicate. One-way Anova was used for statistical analysis and statistical significance is denoted as * (p-value \leq 0.05) and ** (p-value \leq 0.01).

7.2.2. TIMBD decreases oxidative stress associated with HIV-1 Tat in astrocytes

Previous studies have shown that Tat induces ROS in astrocytes (Song et al., 2007). This is suggested to be attributed to an imbalance between the oxidative stress and the antioxidant defense system. In our current studies, SVG astrocytic cells were treated with 25 μ M of either RES or TIMBD 1 hour prior to transfection with Tat plasmid using serum-free medium. The cells were then transiently transfected with 0.3 μ g plasmid encoding HIV-1 Tat for 5 h, the medium was refreshed with serum, followed by incubation for 48 h. Mean fluorescence intensity (MFI) was measured to indicate the levels of ROS in SVG astrocytes in response to Tat and TIMBD or RES treatments. HIV-1 Tat increased ROS levels, expressed as MFI fold change, among Tat-treated cells, TIMBD showed a more powerful effect of limiting the increase of ROS levels due to Tat compared to RES (**Fig. 88**).

These findings were confirmed using primary astrocytes. Primary astrocytes were treated with 25 μ M of TIMBD 1 hour prior to treatment with 200 nM of HIV-1 Tat protein for 3 h and 24 h. Mean fluorescence intensity was measured to indicate levels of ROS in primary astrocytes in response to Tat protein and TIMBD treatment. HIV-1 Tat increased ROS levels at both 3 and 24 h while TIMBD decreased ROS levels significantly compared to Tat (**Fig. 89**).

TIMBD effect on ROS producing enzyme NADPH-Oxidase (NOX2) was also measured. It has been reported that Tat increases the expression levels of NOX2 in astrocytes (Song et al., 2011). Our results demonstrated that Tat increases protein expression levels of NOX2 while TIMBD reduces Tat-mediated increase of NOX2 levels

(Fig. 90). These results indicate that TIMBD decreases Tat-associated ROS and the expression levels of enzymes involved in ROS production.

TIMBD decreases HIV-1 Tat induced oxidative stress compared to RES in SVG

astrocytes

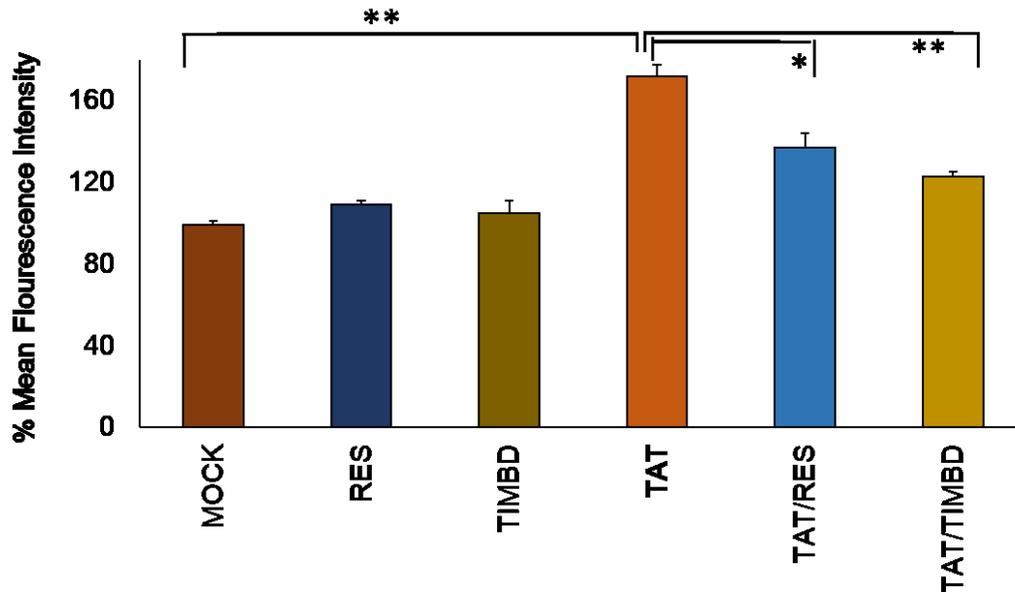


Figure 88. SVG astrocytes were treated with 50 μ M TIMBD or RES for 1 h prior to transfection with 0.3 μ g plasmid encoding HIV-1 Tat for 48 h. The cells were incubated with CM-H26 and fluorescence intensity was measured using flow cytometry. Each bar represents mean \pm SE from at least three independent experiments with three replicates in each experiment. One-way Anova was used for statistical analysis and statistical significance is denoted as * (p-value \leq 0.05) and ** (p-value \leq 0.01).

TIMBD decreases HIV-1 Tat induced oxidative stress in primary astrocytes

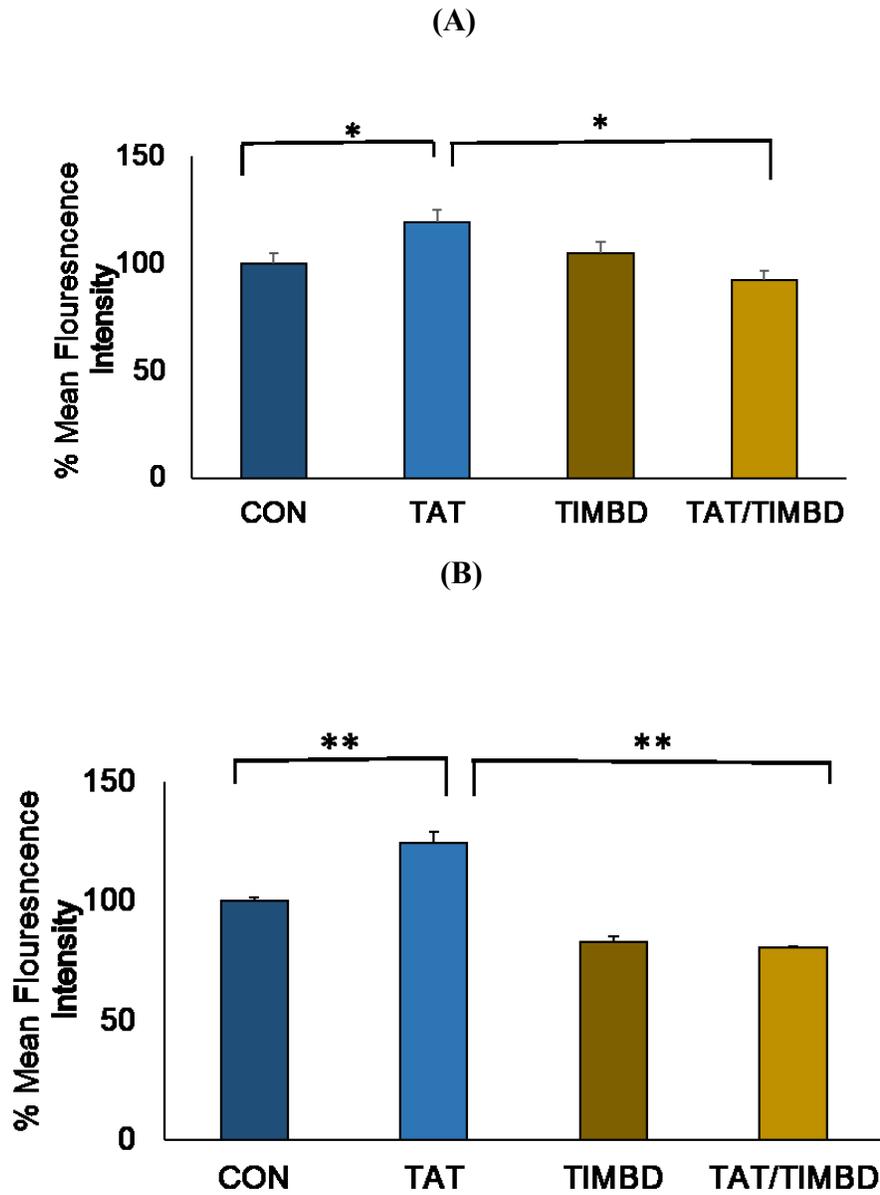


Figure 89. Primary astrocytes were treated with 50 μ M TIMBD 1 h prior to treatment with 200 nm HIV-1 Tat protein for 3 h (A) or 24 h (B). The cells were incubated with CM-H2DCFDA and fluorescence intensity was measured using flow cytometry. Each bar represents mean \pm SE from at least three independent experiments with three replicates in each experiment. One-way Anova was used for statistical analysis and statistical significance is denoted as * (p-value \leq 0.05) and ** (p-value \leq 0.01).

TIMBD decreases HIV-1 Tat-mediated increase in pro-oxidant enzyme NOX2

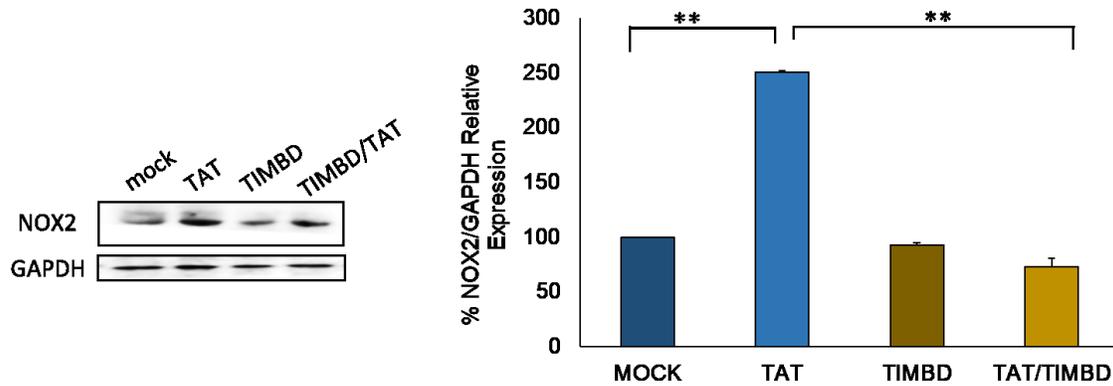


Figure 90. SVG astrocytes were treated with 50 μ M TIMBD 1 h prior to transfection with plasmid encoding HIV-1 Tat for 48 h. Expression levels of pro-oxidant enzyme NOX2 were identified using western blotting following 24 h HIV-1 Tat transfection and TIMBD treatment. Each bar represents mean \pm SEM for at least 3 independent experiments, with each experiment done in triplicate. One-way Anova was used for statistical analysis and statistical significance is denoted as * (p-value \leq 0.05).

7.2.3. TIMBD increases the protein expression levels of antioxidant enzymes in SVG astrocytes

It is known that the antioxidant defense system in the cell neutralizes ROS production in response to any damage (Szymonik-Lesiuk et al., 2003; Weydert & Cullen, 2010). However, any impairment to the defense system can lead to oxidant stress damage. The antioxidant system known to be involved includes many enzymes like catalase which is one of the major enzymes that can convert ROS to neutral molecules (Mates, 2000; Price et al., 2005). Other antioxidant enzymes have also been reported to be involved in balancing ROS levels including Flavine Monooxygenase (FMO1) (B. Singh et al., 2014). To identify the mechanism by which TIMBD decreases ROS levels induced by Tat, SVG astrocytic cells were treated with 50 μ M of TIMBD 1 hour prior to transfection with Tat plasmid using serum-free medium. The cells were transiently transfected with 0.3 μ g plasmid encoding HIV-1 Tat for 5 h and after a medium change and then were incubated for 48 h. Protein was isolated using established protocols as described for western blotting analysis in the Methods section. HIV-1 Tat decreased the protein expression levels of antioxidant enzymes catalase and FMO1 while TIMBD treatment recovered their expression levels significantly, compared to Tat (**Fig. 91 panels A and B**). These results suggest that TIMBD can recover the antioxidant enzyme expression levels which might lead to a decrease in ROS production and the consequent oxidative-stress damage.

TIMBD increases protein expression levels of anti-oxidant enzymes in SVG

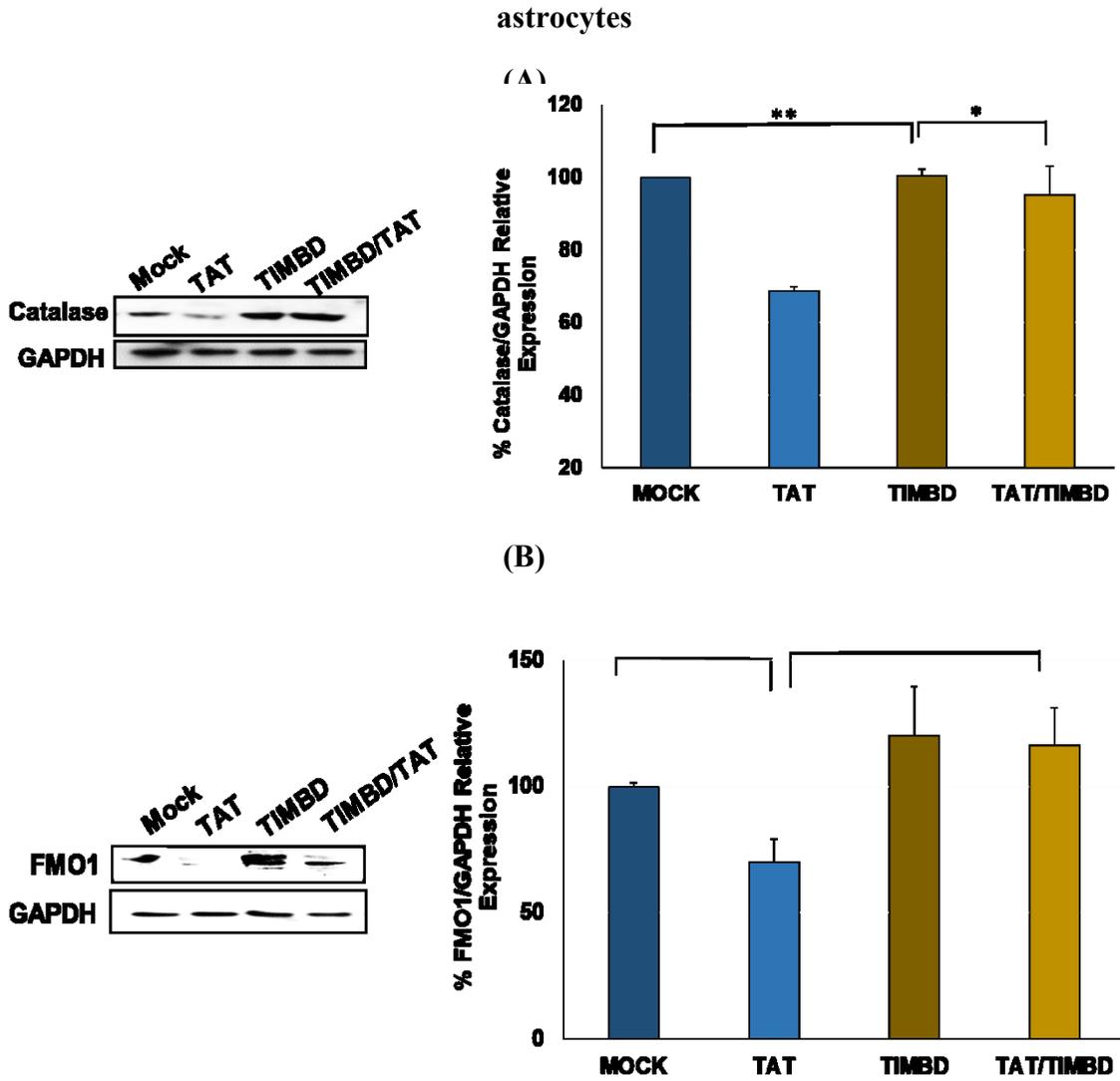


Figure 91. SVG astrocytes were treated with 50 μ M TIMBD 1h prior transfection with plasmid encoding HIV-1 Tat for 48 h. Protein expression levels of Catalase (A) and FMO1 (B) were measured per well with GAPDH used as loading control. Each bar represents mean \pm SEM for 3 independent experiments, with each experiment done in triplicate and the western blots are representative images. One-way Anova was used for statistical analysis and statistical significance is denoted as * (p-value \leq 0.05) and ** (p-value \leq 0.01).

7.2.4. TIMBD decreases the expression levels of MMP9 in SVG astrocytes

Neurodegeneration has been correlated with an increase in protein expression of matrix-metalloproteinase-9 (MMP9) (Louboutin, Agrawal, et al., 2010; Louboutin et al., 2011). MMP9 plays an important role in breaking the integrity of the BBB, leading to leakage through the CNS (Shigemori, Katayama, Mori, Maeda, & Kawamata, 2006). Tat has been shown to increase MMP9 expression in astrocytes which is correlated with the neuronal damage (Ju et al., 2009). To determine the effects of Tat and TIMBD treatment on MMP9 expression levels, SVG astrocytic cells were treated with 50 μ M of TIMBD 1 hour prior to transfection with Tat plasmid using serum-free medium. Cells were transiently transfected with 0.3 μ g plasmid encoding HIV-1 Tat for 5 h and after medium change incubated for 48 h. Protein was isolated and western blotting analysis was performed. HIV-1 Tat increased protein expression levels of MMP9 while TIMBD treatment decreased the expression levels of MMP9 significantly, compared to Tat (**Fig. 92**). These results suggest that TIMBD might be able to preserve the BBB integrity by decreasing MMP9 expression levels.

TIMBD decreases protein expression levels of MMP9 in SVG astrocytes

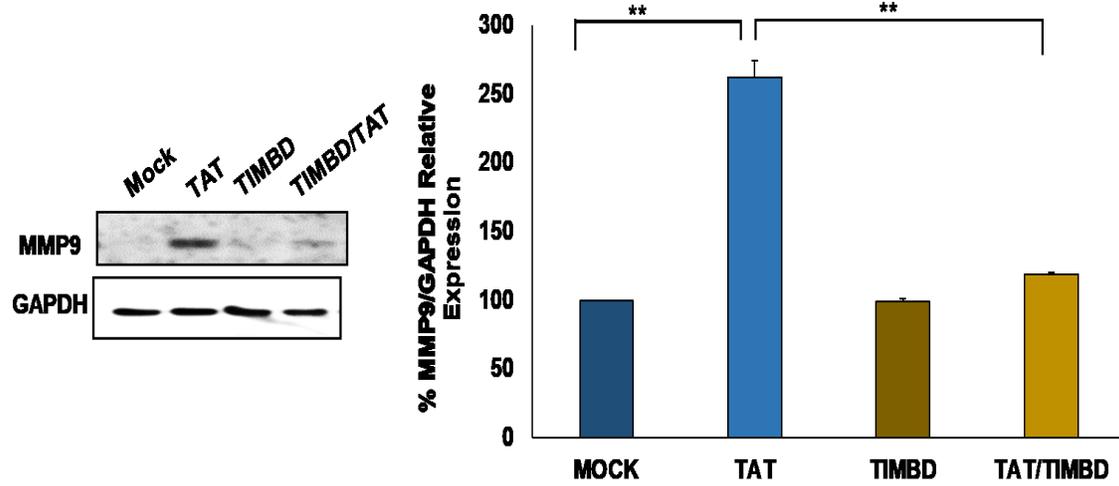


Figure 92. SVG astrocytes were treated with 50 μ M TIMBD 1 h prior to transfection with plasmid encoding HIV-1 Tat for 48 h. Protein expression levels of MMP9 were measured in wells using GAPDH as loading control. Each bar represents mean \pm SE for 3 independent replicates, and the western blots are representative images. One-way Anova was used for statistical analysis and statistical significance is denoted as ** (p-value \leq 0.01).

7.3. Discussion

Previous studies have indicated that HIV-1 Tat protein-associated inflammation and oxidative stress play a major role in neurodegeneration in HIV patients. Thus, efforts are being made to develop intervention molecules to inhibit HIV Tat-associated inflammation and oxidative stress. Although neuronal death is considered the major cause for the neurodegeneration occurring in HIV patients, astrocytes are considered vital because they are involved directly and indirectly in the neuronal death (J. Cao et al., 2005; I. N. Singh et al., 2005). HIV Tat has been shown to induce oxidative stress and lead to the induction of proinflammatory cytokines in astrocytes (Aksenov et al., 2001; Nookala & Kumar, 2014; Nookala et al., 2013).

In the present study, we have shown that TIMBD has a higher potency than RES towards inhibiting production of Tat-induced inflammatory cytokines. Since inflammation plays a vital role in neuropathogenesis, availability of TIMBD to prevent neurodegenerative disease affecting individuals infected with HIV is useful clinically. Our results indicate that TIMBD can decrease Tat-mediated increase in oxidative stress by modulating the expression of anti-oxidant enzymes.

In summary, our results show that TIMBD decreases HIV1-Tat-associated production of IL6 and IL8 cytokines in astrocytes. Our studies further demonstrate that TIMBD decreases Tat-mediated increase in oxidative stress as well as pro-oxidant enzyme NOX2. TIMBD is suggested to be working through increasing the expression levels of anti-oxidant enzymes and thus subsequently decreasing oxidative stress. Further studies are needed to confirm the mechanism by which TIMBD decreases the production of

inflammatory cytokines and increases the antioxidant enzymes-decreased with Tat in astrocytes.

CHAPTER 8

CONCLUSION AND FUTURE DIRECTIONS

HIV is still infecting a considerable number of patients in the US. Although the use of antiretroviral therapy has controlled the progression of HIV infection, the neurocognitive damage associated with it is still affecting many HIV patients. The prevalence of the less severe form of HIV-associated neurocognitive disorders (HAND) has increased compared to the more severe forms. HAND patients suffer from cognitive, motor and behavioral impairment that hinders their daily life performance. While antiretroviral therapy has been successful, it does not completely eliminate the disease reservoir cells including resting memory CD4⁺ T cells, macrophages, and astrocytes. Following HIV invasion to brain, the virus persists permanently in brain cells, mainly in microglia, macrophages, and astrocytes. Neurotoxicity in patients with HIV-1 infection is mediated by HIV-1 proteins such as gp120 and Tat, as well as other products released from infected cells. The mechanisms of neurotoxicity are thought to be both direct and indirect and include oxidative stress, increase in apoptosis, stimulation of inflammation, altered calcium homeostasis etc., which can eventually lead to the neurocognitive behavioral deficits.

The current study was conducted to determine the effect of novel compound TIMBD in alleviating the neurotoxic processes/effects associated with HIV-1 proteins. The study determined the effect of TIMBD in decreasing oxidative stress associated with HIV-gp120 in astrocytes. Gp120 induces ROS production in astrocytes, which can consequent lead to cell death. Treating the astrocytic cells with TIMBD caused a significant decrease in ROS production and oxidative stress marker. The decreases of oxidative stress by

TIMBD was determined to be through increasing the nuclear translocation of NRF2 transcription factor. Once NRF2 is translocated to the nucleus, it can then lead to an increase in the expression of antioxidant enzymes which subsequently lead to decrease in oxidative stress. Additionally, the effect of TIMBD on antioxidant enzymes has been confirmed using an *in vivo* model. Furthermore, TIMBD was able to decrease cell death associated with gp120 in astrocytes indicating a neuroprotection effect of TIMBD.

We also determined the effect of TIMBD on the inflammatory markers associated with gp120. While gp120 increased the expression levels of IL6 and IL8, TIMBD treatment showed a significant effect in decreasing the expression levels of these inflammatory cytokines in astrocytes in a dose dependent manner. The increase of IL6 and IL8 is known to be through many signaling pathways. The mechanism of TIMBD action is suggested to be through modulating the activity of transcription factors AP1, STAT3 and NFκB as well as that of the upstream regulators including AKT, IKKs, and p38MAPK. Additionally, we showed that TIMBD can also decrease the oxidative stress and inflammation associated with HIV-1 Tat in astrocytes.

We further conducted an *in vivo* study to determine the effect of TIMBD on HIV-1 Tat associated behavioral deficits and the corresponding neurotransmission genes. Since HIV-1 Tat-transgenic (tg) mice have shown a pronounced anxiety-like behavior and memory impairment. TIMBD treatment of Tat-tg mice decreased the anxiety-like behavior and improved the memory impairment compared to non-treated Tat-tg mice. Furthermore, the effect of TIMBD was correlated with many neurotransmission genes that are involved in controlling behavior. TIMBD was able to recover the expression levels of neurotrophic factors, synaptic proteins, and calcium signaling molecule.

Following future directions are required to explain the role of TIMBD as a neuroprotective agent:

1. The effect of TIMBD on oxidative stress and inflammatory cytokines *in vivo* and their correlation with the behavioral impairment.
2. The effect of TIMBD on BBB integrity.
3. Identifying the molecular targets of TIMBD.
4. Studying the bioavailability, stability and toxicity of TIMBD.

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VITA

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