

THE EFFECT OF HIV-1 TAT AND METHAMPHETAMINE ON EXPRESSION OF
CYTOKINES/CHEMOKINES AND SYNAPTIC GENES: IMPLICATIONS IN HIV NEUROAIDS

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by

ANANTHA RAM NOOKALA

B. Pharmacy, Jawaharlal Nehru Technological University, 2010

Kansas City, Missouri

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THE EFFECT OF HIV-1 TAT AND METHAMPHETAMINE ON EXPRESSION OF
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NEUROAIDS

Anantha Ram Nookala, Candidate for the Doctor of Philosophy Degree

University of Missouri-Kansas City, 2017

ABSTRACT

Human immunodeficiency virus-1 (HIV-1) enters the brain through blood brain barrier (BBB) early after the infection and leads to various neurological complications including HIV-associated dementia (HAD). Introduction of combined Anti Retro Viral therapy (cART) has significantly reduced the incidence of HAD and increased the life expectancy of people infected with HIV. Increase in the life span of people resulted in the development of less severe forms of cognitive dysfunctions, including asymptomatic neurocognitive impairment (ANI) and Mild neurocognitive disorder (MND). These neurological deficits affect approximately 50% of HIV-1 infected people. The neurotoxicity of HIV-1 has been attributed to the virus itself or the viral proteins like HIV-1 Tat shed after the infection via several mechanisms including production of cytokines/chemokines and decreased expression of neuroplasticity genes. *The present study was based on the central hypothesis that HIV-1 Tat and methamphetamine work synergistically in mediating pro-inflammatory cytokines and decreasing synaptic proteins.* The level of cytokine expression mediated by HIV-1 Tat is determined. Furthermore, we examined the cellular mechanisms responsible for HIV-1 Tat mediated cytokine production, and the role of synaptic proteins in HIV-1 Tat and methamphetamine induced neurocognitive impairment.

In the first chapter, we investigated the effect of HIV-1 Tat on pro-inflammatory cytokine expression in SVGA astrocytes. We measured levels of the IL-6, IL-8 and CCL5 in SVGA astrocytes after exposure with HIV-1 Tat. The results showed that HIV-1 Tat induced

expression of IL-6, IL-8 and CCL5 in a time-dependent manner. These results were also confirmed by detecting their intracellular expression in SVGA astrocytes using confocal microscopy. Next, we sought to explore the mechanism(s) by which HIV-1 Tat induces the cytokine response. The involvement of PI3K/Akt, p38 MAPK, JNK MAPK pathways and various transcription factors, including NF- κ B and AP-1 were determined by using selective inhibitors and siRNAs.

In the second chapter, we investigated combined the effect of HIV-1 Tat and methamphetamine on expression of IL-6 and its underlying signaling mechanism(s).

In the third chapter, the effect of HIV-1 Tat and methamphetamine on neurocognition was determined in HIV-1 Tat transgenic mice. Y maze and Morris water maze were employed to determine neurocognition. We also determined the effect of Tat and methamphetamine on expression of synaptic proteins in various brain regions. Furthermore, the role of HIV-1 Tat and methamphetamine on neurotrophic factors and CaMKII were determined by western blot.

In conclusion, we showed that HIV-1 viral protein Tat and methamphetamine were able to induce cytokine expression in astrocytes. The underlying mechanism(s) provides potential therapeutic target for neuroAIDS.

APPROVAL PAGE

The faculty listed below, appointed by the Dean of School of Graduate Studies have examined the dissertation titled “The effect of HIV-1 Tat and methamphetamine on expression of cytokines/chemokines and synaptic genes: Implications in HIV neuroAIDS” presented by Anantha Ram Nookala, candidate for the Doctor of Philosophy degree, and certify that in their opinion it is worthy of acceptance.

Supervisory Committee

Anil Kumar, Ph.D., Committee Chair
Division of Pharmacology and Toxicology

Kun Cheng, Ph.D.
Division of Pharmaceutical Sciences

Naveen Vaidya., Ph.D.
Department of Mathematics and Statistics

Santosh Kumar, Ph.D.
College of Pharmacy,
University of Tennessee Health Science Center

Dhirendra Pratap Singh, Ph.D.
College of Medicine,
University of Nebraska Medical Center

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ABBREVIATIONS

8-OHdG	8-hydroxy-2' –deoxyguanosine
ADC	AIDS dementia complex
AIDS	Acquired immunodeficiency syndrome
Akt	Protein kinase B
ANI	Asymptomatic neurocognitive impairment
ANOVA	Analysis of variance
AP-1	Activator protein-1
Arg3.1	Activity regulated cytoskeleton-associated protein
BBB	Blood brain barrier
BDNF	Brain-derived neurotrophic factor
C/CAT	CCAT enhancer binding proteins
C/EBP	CCAT/enhancer binding protein
CaMKII	Ca ²⁺ /calmodulin-dependent protein kinase II
cART	combined Anti Retro Viral therapy
CCL4	Macrophage inflammatory protein-1beta
CCL5	Regulated upon activation, normal T cell expressed and secreted
Cere	Cerebellum
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
CSF	Cerebro spinal fluid
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's modified eagle medium
EAA	Excitatory amino acids
ERK	Extracellular signal–regulated kinase
GABA	Gamma amino butyric acid

GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFAP	Glial fibrillary acidic protein
GFP	Green florescence protein
Gp120	glycoprotein120
HAART	Highly Active Antiretroviral Therapy
HAD	HIV associated dementia
HAND	HIV associated neurocognitive disorders
HDAC6	Histone deacetylase 6
Hippo	Hippocampus
HIV	Human Immunodeficiency Virus
HIV-1 Tat	HIV-1 Trans activator of transcription
HPRT	Hypoxanthine phosphoribosyltransferase
HTLV-III	Human T-cell lymphotropic retrovirus III
IKK	I κ B kinase
IKK2	I κ B kinase 2
IL-1 β	Interleukin-1 β
IL-6	Interleukin-6
IL-8	Interleukin-8
IP3	Inositol 1,4,5-trisphosphate
I κ B	I κ appaB
JNK	C-Jun N-terminal kinase
MAPKs	Mitogen activated protein kinases
MCMD	Minor cognitive motor disorders
MCP-1	Monocyte chemoattractant protein-1
METH	Methamphetamine
mGlu1	metabotropic glutamate receptor 1

MND	Minor neurocognitive disorder
Nef	Negative factor
NF- κ B	Nuclear factor kappa B
NMDA	N-methyl D-aspartate
Nrf2	Nuclear erythroid related factor 2
P38	p38 mitogen activated-protein kinase
PC	Parietal cortex
PD	Parkinson's disease
PFC	Prefrontal cortex
PI3K	phosphatidylinositol-4,5-bisphosphate 3-kinase
PSD-95	Post synaptic density protein 95
ROS	Reactive oxygen species
RT	Reverse transcriptase
RT-PCR	Real-time reverse transcription polymerase chain reaction
rTTA	reverse tetracycline transactivator
Scr	Scrambled
siRNA	small interfering RNA
SIV	Simian Immunodeficiency virus
TLR4	Toll like receptor 4
TNF- α	Tumor necrosis factor- α
VPR	Viral protein R

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DEDICATION

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CHAPTER 1

GENERAL INTRODUCTION

1.1 Human immunodeficiency virus

The first historical evidence of Human Immunodeficiency Virus (HIV) was obtained from Luc Montagnier and team when they isolated it from the lymph node of a patient. They initially named it as lymphadenopathy-associated virus (Barre-Sinoussi, Chermann et al. 1983). In another independent report, Robert Gallo had isolated the virus and named it as human T-cell lymphotropic retrovirus III (HTLV-III) (Broder and Gallo 1984). Furthermore, he reported for the first time that HTLV-III was responsible for pathogenesis of acquired immunodeficiency syndrome (AIDS). HIV belongs to the family of retroviridae. As the name implies, it contains reverse transcriptase (RT) enzyme that aids in the process of viral replication. Each virion contains a viral envelope that surrounds the capsid and matrix. The viral envelope is made from repeated trimers that are glycoproteins (Gelderblom, Ozel et al. 1989). The capsid encloses two copies of single stranded RNA that are associated with integrase and RT. The RT enzyme helps in the conversion of RNA into DNA and integrase helps in the insertion of viral DNA into host DNA. HIV genome contains several genes that encode for various structural and functional proteins. These have diverse functions in HIV life cycle.

Replication of HIV is associated with low fidelity, resulting in several mutations. HIV is found in two different strains, HIV-1 and HIV-2. HIV-1 bears close similarities to simian Immunodeficiency virus (SIV) isolated from chimpanzees whereas HIV-2 is related closely to SIV isolated from sooty mangabeys (Santiago, Range et al. 2005, Keele, Van Heuverswyn et al. 2006, Plantier, Leoz et al. 2009). Compared to HIV-2, HIV-1 is more pathogenic and predominant globally. Based on the similarities to SIV, HIV-1 is categorized into four different groups (M, N, O and P). Group M is the pathogenic form prevalent worldwide and is further divided into following sub-types/clades (A, B, C, D, F, G, H, J, K and circulating recombinant

forms). Clade B is common among people infected with HIV-1 in America, Europe and Australia. Clade C is dominant among people infected with HIV-1 in Sub-Saharan Africa, Brazil and India.

1.2 HIV epidemiology

According to recent UNAIDS statistics published in 2015, more than 36.9 million people worldwide are living with HIV-1. Majority of this infected population live in Sub-Saharan Africa (25.8 million). The number of HIV infected people living in North America and Western and Central Europe together constitute 2.4 million. The number of newly infected people for the year 2014 alone includes 2 million people. Deaths due to AIDS related illnesses for the year 2014 alone include 1.2 million people. There are more than 5,600 new infections per day that occur worldwide because of HIV. Clade B represents 12% of HIV infections that are present globally. From a devastating disease, HIV infection has become a more manageable chronic condition after the advent of Highly Active Antiretroviral Therapy (HAART). HIV

Adults and children estimated to be living with HIV | 2014

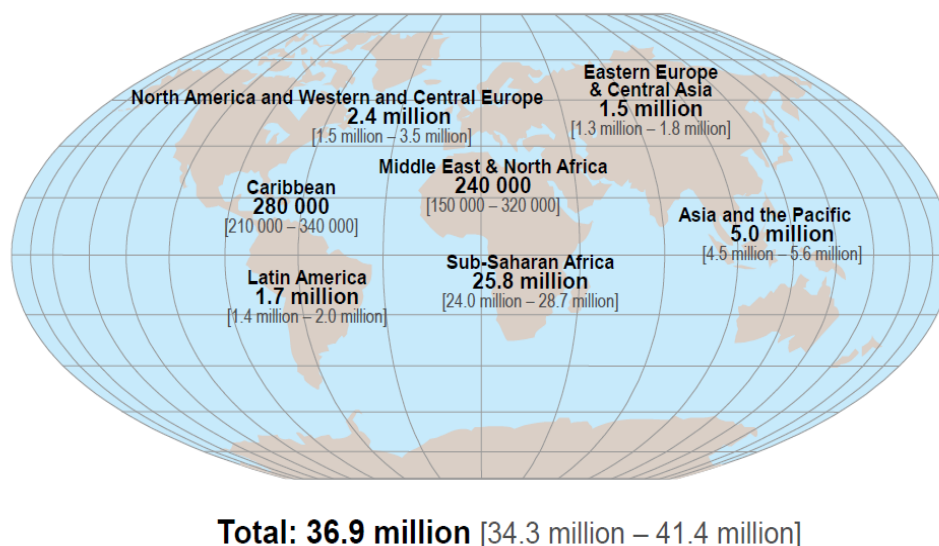


Figure 1: Adults and Children estimated to be living with HIV in 2014 (Source: UNAIDS)

presents as a huge economic burden on the society; estimated life time cost of treating a patient infected with HIV-1 is about \$379,000.

1.3 HIV infection and neuropathology

HIV crosses the blood brain barrier (BBB) soon after the infection (Davis, Hjelle et al. 1992, An, Groves et al. 1999). Similar to the viruses belonging to the family of lentivirus, the entry of the HIV into the central nervous system (CNS) is well explained by Trojan horse phenomenon (Peluso, Haase et al. 1985, Haase 1986). After HIV infects a person, it harbors itself in the monocytes. As a part of immunological surveillance, the infected monocytes pass through the BBB, allowing the virus to be released inside the CNS. The released virus infects the different brain cells, including microglia, resident perivascular macrophages, endothelial cells and astrocytes (Wiley, Schrier et al. 1986, Cosenza, Zhao et al. 2002, Fischer-Smith, Croul et al. 2004, Liu, Liu et al. 2004, Eugenin, Clements et al. 2011). Other minor mechanisms that HIV utilizes to enter the brain include crossing of epithelial cells by transcytosis (Bomsel 1997) and utilizing cholesterol rich lipid rafts and heparin sulfate proteoglycans to infect brain microvascular endothelial cells (Argyris, Acheampong et al. 2003). Infection of CNS by HIV results in the development of severe neurological complications, referred to as AIDS dementia complex (ADC). The numbers of HIV infected people with ADC were significantly higher (Portegies, Enting et al. 1993). However, after the onset of HAART, the incidence of people presenting with severe neurological complications have reduced significantly. Introduction of HAART has increased the life span of the people infected with HIV. This longevity has resulted in the development of milder forms of neurocognitive disorders. More than 50% of the people infected with HIV develop some form of neurocognitive impairment in their life time (Ozdener 2005). After the advent of HAART, neurological impairments associated with HIV were classified into three different groups by National institutes of health, including asymptomatic neurocognitive impairment (ANI), mild neurocognitive disorder (MND) and HIV associated dementia (HAD). The classification

referred to as Frascati criteria is widely accepted and brings all the impairments under one roof, referred to as HIV associated neurocognitive disorders (HAND). As the name implies, ANI does not affect the cognitive function whereas MND affects the cognitive function. Higher degree of neurocognitive impairment interferes with the individual's ability to perform day to day activities (Antinori, Arendt et al. 2007).

HIV infected people with neurological complications exhibit a distinct pattern of histopathological abnormalities. These anomalies include the presence of large multinucleated giant cells and other inflammatory cells in the CNS and damage to the brain white matter (Navia, Jordan et al. 1986, Budka, Wiley et al. 1991). Furthermore, the presence of activated astrocytes and microglia, elevated levels of $\beta 2$ -microglubulins, quinolinic acid and neopterin in cerebro spinal fluid (CSF) were used as diagnostic markers to characterize HIV-encephalitis (Brew, Bhalla et al. 1989, Brew, Bhalla et al. 1990, Heyes, Brew et al. 1991). Postmortem brains of HIV infected people with neurological impairments resulting in HAD demonstrated different pathological features including, loss of neurons, reactive astrocytosis, BBB impairment and decrease in synaptic density (Everall, Luthert et al. 1993, Thompson, Churchill et al. 2004).

Furthermore, apoptosis of neurons in the brain is a salient feature among the people infected with HIV (Ade-Biassette, Levy et al. 1995, Gelbard, James et al. 1995). However, neurons are not directly infected by the virus. The neuronal damage that is seen in HIV infected people with neurological complications can be mediated by direct effects and indirect effects of the virus. HIV, along with the released viral particles directly act on the neurons to induce pathological changes and eventually cause neurotoxicity. In addition to these direct effects, they can also act on the neighboring astrocytes and microglia to promote the release of various excitatory amino acids and other neurotoxic factors, including cytokines/chemokines, reactive oxygen species (ROS), nitric oxide synthase and quinolinic acid (Giulian, Vaca et al. 1990, Giulian, Wendt et al. 1993). The released excitatory amino

acids (EAA) act on the neurons to induce apoptosis by the process of exocytosis. This involves an increase in the intracellular levels of calcium, production of ROS and excessive stimulation of glutamate receptors (Bonfoco, Krainc et al. 1995). Even though there are insurmountable evidences indicating neurological impairments in HIV infected people, exact pathological mechanisms remain unknown.

Considerable efforts are being made to understand the role of neuroimmune function of the brain during the HIV pathology. Alterations in the levels of various cytokines/chemokines are known to be associated with immune dysfunction (Hopkins and Rothwell 1995). Elevated levels of cytokines/chemokines are consistently present in HIV infected individual in the pre HAART and post HAART era (Epstein and Gendelman 1993, Roberts, Passmore et al. 2010). They often serve as biological markers for identification of neurological impairments in these individuals (Yuan, Qiao et al. 2013).

1.4 Role of astrocytes in HAND

Astrocytes comprise a majority of cells in the brain and occupy more than 50% of the brain volume. They play important roles in many brain functions, including the maintenance of homeostasis of the neurons, modulation of neuronal activity and regulation of synaptic plasticity (Halassa and Haydon 2010, Navarrete, Perea et al. 2012). They also represent an important reservoir for the production of various mediators of inflammation, particularly in response to HIV-1 (Minagar, Shapshak et al. 2002, Thompson, Churchill et al. 2004, Williams, Dhillon et al. 2009). Astrocytes are activated to a pathological state termed as reactive astrocytes in response to HIV-1 infection. Unlike microglia, astrocytes are restrictively infected with HIV due to the lack of CD4 receptors on their surface (Gorry, Ong et al. 2003). Post mortem studies have shown that astrocytes infected with HIV demonstrate some early HIV genes such as Negative factor (Nef), gag and rev that help in viral replication (Ranki, Nyberg et al. 1995). However, some studies have identified CD4 independent mechanisms for HIV to infect astrocytes. HIV utilizes mannose receptors expressed on the surface of astrocytes to

infect them (Liu, Liu et al. 2004). Furthermore, it has been shown that astrocytes can uptake the virus into the vesicle compartments and also infect naive CD4+ T cells (Clarke, Lake et al. 2006). These two pathways indicate that astrocytes can be a source for persistence of virus inside the brain and also infecting naïve cells. Astrocytes harbor latently infected virus that becomes productively infectious after triggered by various activating factors. In the presence of pro-inflammatory cytokines like IL-1 β and TNF- α or co-culturing astrocytes with CD+ T cells, HIV recovers from dormant stage (Sabri, Tresoldi et al. 1999, Kore and Abraham 2014). These studies indicate a potential role for the favorable stimuli to generate new viral copies from astrocytes. Several HIV proteins including, glycoprotein120 (gp120), HIV-1 Trans activator of transcription (HIV-1 Tat), Nef and Viral protein R (vpr) can act on various CNS cells to stimulate the production of various inflammatory stimuli like cytokines. Advent of modern technology has enabled us with sensitive techniques to measure the percentage of astrocytes that are infected with HIV. By employing laser capture microdissection, Churchill et al have shown that more than 18% of the astrocytes are infected with HIV in individuals with dementia (Churchill, Wesselingh et al. 2009). The amount of infection coupled with the fact that astrocytes are the most abundant cell type in the brain suggests that these cells play a crucial role in harboring significant amount of virus inside the CNS.

1.5 HIV-1 Tat

Biochemical and mutation studies have helped to elucidate the structure of Tat. It is a basic protein of 86 to 101 amino acids in length depending upon the viral strain. Tat is divided into three different regions, including essential region (from 1-57 amino acids), auxiliary region (from 58-66 amino acids) and non-essential region (from 67-101 amino acids). Essential region is further divided into four functional domains (A, B, C and D). They include N-terminal domain (A), cysteine rich domain (B), Lys X Leu Gly Ile X Tyr domain (C) and basic domain (D). Mutations in cysteine rich region result in complete loss of transcriptional activity of Tat, indicating it is essential for Tat activity. Lysine amino acid of domain C is mainly responsible

for activity of Tat. Basic domain is required for localization of Tat into the cell and also essential for its transcriptional activity (Kuppuswamy, Subramanian et al. 1989). The basic domain along with the adjacent arginine domain specifically recognizes the hairpin loop structure rich in pyrimidines on transactivation RNA sequences (Weeks, Ampe et al. 1990). This results in the recruitment of positive transcription elongation factor b, resulting in the transcriptional elongation of viral promoter (Cann, Rosenblatt et al. 1985). As noted previously, Tat can be released from infected cells and can cross the BBB by various mechanisms. The free Tat protein inside the CNS causes neurotoxicity by various mechanisms.

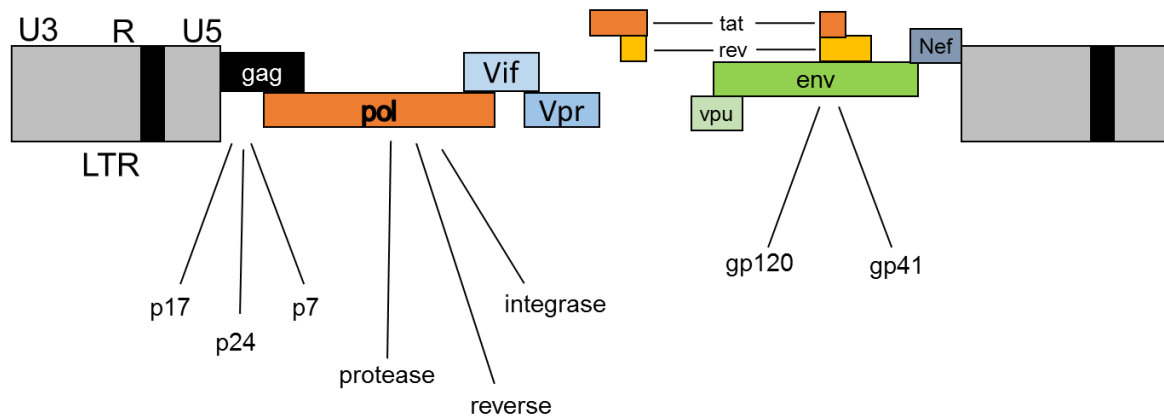


Figure 2: Structure of HIV-1 genome

1.5.1 Effect of HIV-1 Tat on neuroinflammation

Cytokines play a major role in inflammation and immune dysregulation and are implicated in several neurodegenerative disorders, including Alzheimer's disease, Parkinson's disease, multiple sclerosis and HIV-1 infection. Increased levels of various pro-inflammatory cytokines/chemokines are seen in individuals infected with HIV-1. Levels of cytokines/chemokines are elevated in plasma and CSF of HIV-1 infected individuals. Production of cytokine interleukin-6 (IL-6) has been shown to promote viral replication of latently infected cells through the activation of various transcription factors via toll like receptor 4 (Hoshino, Konishi et al. 2010) and also compromise proper function of BBB (Zidovetzki, Wang et al. 1998). HIV-1 Tat is one of the early viral proteins that is shown to promote the

release of cytokines/chemokines from various cells of CNS origin. Tat promotes the release of tumor necrosis factor- α (TNF α) and interleukin-10 (IL-10) from monocytes through the activation of toll like receptor 4 (TLR4) and various mitogen activated protein kinases (MAPKs) (Planes, Ben Haij et al. 2016). Tat also promotes the release of interleukin-6 (IL-6) and interleukin-8 (IL-8) from monocytes of HIV-1 infected and healthy individuals (Ben Haij, Planes et al. 2015). Tat induces the production of CCL2 to promote the activation of microglia and promote their migration (Eugenin, Dyer et al. 2005). Addition of HIV-1 Tat to primary microglia induces the down-regulation of fractalkine receptor and promotes up-regulation of various pro-inflammatory cytokines, including interleukin-1 β , (IL-1 β), IL-6, IL-10 and TNF- α (Duan, Yao et al. 2014). Also, Tat induces the phosphorylation of leucine rich repeat kinase 2 and cyclic AMP phosphodiesterase to induce the production of different cytokines (Kiebala and Maggirwar 2011, Marker, Puccini et al. 2012). Tat promotes the release of various beta chemokines, including regulated upon activation, normal T cell expressed and secreted (CCL5), macrophage inflammatory protein-1alpha (CCL3), and macrophage inflammatory protein-1beta (CCL4) (Hahn, Vo et al. 2010). HIV-1 Tat stimulated the expression of astrocyte elevated gene-1 to promote the production of IL-1 β and TNF- α cytokines from astrocytes (Vartak-Sharma, Gelman et al. 2014). Tat induced the diacetyl activity of Histone deacetylase 6 (HDAC6) to promote the production of various chemokines, including CXCL 10 and CXCL8 (Soo Youn, Ju et al. 2015). Tat interacts with various TLR's on astrocytes to affect their expression and promote the production of TNF- α and IL-6 (El-Hage, Podhaizer et al. 2011). Induction of monocyte chemoattractant protein-1 (MCP-1/CCL-2) by HIV-1 Tat is inhibited by the use of specific siRNA or inhibitors against cyclin dependent kinase 9 (Khiati, Chaloin et al. 2010). Tat also has been shown to promote the activation of vascular endothelial growth factor receptor and thereby induce up-regulation of various cytokines in brain endothelial cells to promote cytotoxicity (Khan, Di Cello et al. 2003). Tat has been shown to exhibit distinct effects

depending on the clade of virus, including, neuropathogenesis and neurotoxicity (Krishnan and Chatterjee 2015). Clade B has been shown to promote the production of cytokines/chemokines significantly compared to other clades, including clade A, clade C and clade D (Kennedy, Petrasca et al. 2014). Furthermore, Tat from clade B viral subtype is shown to be more toxic in brain microvascular endothelial cells in inducing inflammatory responses compared to Tat derived from HIV-1 circulating recombinant form, prominent viral subtype in Africa (Woollard, Bhargavan et al. 2014).

Increased cytokine expression is associated with neurotoxicity by various mechanisms, including disruption of BBB and abnormal activation of NMDA receptors (Jara, Singh et al. 2007, Wardill, Mander et al. 2016). Targeting various pathways (CD45 or protein tyrosine phosphate) that are associated with the production of cytokines/chemokines is associated with decrease in the activation of microglia (Song, Jung et al. 2016). Furthermore, targeting Ankyrin-rich membrane spanning protein on microglia is associated with decrease in the expression of several pro-inflammatory cytokines (Singh, Wooten et al. 2015). Both these studies indicate the prominent role of inflammatory mediators as a drug target to reduce the severity of HAND.

1.5.2 Effect of HIV-1 Tat on modulation of neurotransmitters

Various neurotransmitters play vital roles inside the CNS, important being the transmission of impulses throughout the body, memory and synaptic plasticity. The important neurotransmitters include glutamate and dopamine. Excessive release of glutamate has been shown to cause neurotoxicity and eventually result in neuronal death (Rahn, Slusher et al. 2012). Tat has been shown to affect the release of glutamate from astrocytes by down-regulating the expression of excitatory amino acid transporter-2 (Zhou, Liu et al. 2004, Rumbaugh, Li et al. 2007). Tat also promotes the release of glutamate from microglia (Gupta, Knight et al. 2010). Furthermore, Tat activates N-methyl D-aspartate (NMDA) receptors and potentiates glutamate mediated neurotoxicity through the low density lipoprotein receptor

related protein and Rho associated protein kinase (Krogh, Lyddon et al. 2015). Activation of NMDA receptors by Tat evokes an abnormal increase in intracellular calcium levels leading to excitotoxicity (Bonavia, Bajetto et al. 2001). Brailoiu et al have performed comprehensive studies demonstrating excitatory effects of Tat. They have shown that nanomolar to micromolar concentrations of Tat has induces long lasting polarization and decrease membrane resistance in cortical neurons. Pre-treatment with various glutamate receptor inhibitors, including 6-cyano-7-nitroquinoxaline-2,3-dione and d-2-amino-5-phosphonovaleric acid have significantly reduced Tat mediated excitatory effects (Brailoiu, Brailoiu et al. 2008). Furthermore, increase in the release of glutamate associated with Tat has been shown to be primarily mediated by metabotropic glutamate receptor 1 (mGlu1), but not metabotropic glutamate receptor 5 (Musante, Summa et al. 2010). Consistent with the glutamate release findings, Tat has been shown to enhance the production of inositol 1,4,5-trisphosphate (IP3). Apart from actions of Tat on glutamate, Tat has been shown to affect various other neurotransmitters. These include the release of acetylcholine from cholinergic nerve terminals and norepinephrine from adrenergic nerve terminals (Longordo, Feligioni et al. 2006). Tat also potentiates the inhibitory effect by decreasing the release of inhibitory neurotransmitter gamma amino butyric acid (Xu and Fitting 2016).

1.5.3 HIV-1 Tat and oxidative stress

Reactive oxygen species (ROS) are implicated in wide array of diseases, including neurodegenerative diseases and cancers. Oxidative stress arises as an imbalance of various processes inside the cell to neutralize ROS. These reactive species result in the formation of peroxides, superoxides and free radicals that interact with different structures in the cell, including DNA and lipids to induce toxicity. HIV-1 infection is associated with chronic oxidative stress, manifested by the reductions in the anti-oxidant defense systems and increased levels of various oxidative stress markers in the serum (Pace and Leaf 1995). These include decreased levels of superoxide dismutases, glutathione defense systems and elevated

levels of malonyldialdehyde and peroxides (Staal, Roederer et al. 1992, Ogunro, Ogunbamigbe et al. 2005). Induction of oxidative stress results in the damage to various cellular structures of the cell, including proteins, lipids and nucleic acids. 8-hydroxy-2' - deoxyguanosine (8-OHdG) is one of the major lesion reported as a result of oxidative nucleic acid damage (Valavanidis, Vlachogianni et al. 2009). Prefrontal cortex autopsy of HIV infected individuals showed decreased levels of mitochondrial DNA and increased levels of 8-OHdG. HIV-1 Tat has been shown to induce oxidative stress in cell culture and animal models. Tat promoted apoptosis of rat hippocampal neurons by promoting the production of ROS (Kruman, Nath et al. 1998). Synaptosomes isolated from wild type mice treated with Tat showed increased production of ROS, oxidation of lipid and alteration in the membrane potential of mitochondria (Pocernich, Sultana et al. 2004). Tat disrupts the normal mitochondrial function and increases the levels of calcium inside the cytoplasm. This in turn increases the production of ROS from mitochondria and results in apoptosis of cells (Norman, Perry et al. 2008). Tat promotes the induction of nitric oxide from glial cells which triggers the release of excessive glutamate from astrocytes. The released glutamate acts on neurons to cause neurotoxicity. Furthermore Tat potentiates the effect of glutamate by activating spermine oxidase that promotes the production of hydrogen peroxide (Capone, Cervelli et al. 2013). Tat induces ROS production to increase the expression of adhesion molecules (intercellular adhesion molecule-1 and vascular cell adhesion molecule-1) and thereby modulate the adhesion of monocytes (Song, Ryu et al. 2007). Furthermore, Tat can favor the entry of HIV into the CNS by mediating endothelial cytotoxicity through increased oxidative stress mediated inflammatory responses (Toborek, Lee et al. 2003). Tat also affects the enzymes such as glutathione, responsible for antioxidant defense systems to exacerbate oxidative stress mediated toxicity. Tat causes the up-regulation of anti-oxidant enzyme known as nuclear erythroid related factor 2 (Nrf2) that promotes the up-regulation of anti-oxidant defense systems. However, this increase in Nrf2 is not sufficient to counteract the oxidative

toxicity mediated by Tat and is required to promote HIV-1 long terminal repeat transactivation (Zhang, Li et al. 2009). Various other secondary markers of oxidative stress, including peroxidation of lipids have shown to be affected by Tat.

1.6 HIV and drugs of abuse

Use of recreational drugs for personal use is prominent worldwide, especially in the United States. According to the National Institute of Drug Abuse, more than 24 million people living in the United States have admitted to using at least one recreational drugs in one month for the year 2012 alone. This is 8.3% more than the number that is recorded in 2002. Drugs of abuse have been associated with HIV from its inception. Abuse of illicit drugs is higher among HIV seropositive individuals compared to HIV seronegative individuals (Garin, Velasco et al. 2015). Close to one-third of the population living with HIV abuse illicit drugs. Drug abuse can increase the risky sexual behavior and poor judgement, which may lead a person to acquire HIV. Furthermore, the sharing and use of needles and syringes between injection drug users increases the chances of acquiring HIV. Drug abuse exacerbates the disease progression towards AIDS and also presents itself as a risk factor for the neurocognitive dysfunctions seen in HIV infected individuals (Nath, Hauser et al. 2002).

1.7 Methamphetamine and HIV

Methamphetamine is the second most commonly abused illicit drug worldwide. In the USA alone, methamphetamine is abused in 10-15% of people infected with HIV (Cisneros and Ghorpade 2014). It poses a great risk for HIV-1 infection due to risky sexual behaviors, particularly among bisexual and gay men (Garofalo, Mustanski et al. 2007). It is mainly abused for its euphoric potential and stimulant properties on central and peripheral nervous systems. In small clinical doses, it is used to improve cognitive outcomes following brain injury. Also, it is used as a secondary drug of choice to treat patients with attention deficit hyperactivity disorder and refractory obesity (Kish 2008). In long term, it has severe effects on the central nervous system. It produces various behavioral problems, ranging from confusion, anxiety,

insomnia, delusions and hallucinations. Methamphetamine mainly affects the dopaminergic system and also GABAergic, serotonergic systems in different brain regions (Ricaurte, Schuster et al. 1980, Wilson, Kalasinsky et al. 1996). Methamphetamine abuse results in the neurotoxic effects on different regions of the brain, including frontal cortex, hippocampus, basal ganglia that are associated with memory, locomotion, cognition and executive functions (Belcher, O'Dell et al. 2005, Chang, Ernst et al. 2005, Berman, O'Neill et al. 2008). Consistent with these findings, methamphetamine use has been shown to impair learning and memory in human population and animal models (Scott, Woods et al. 2007, Siegel, Craytor et al. 2010, Kesby, Markou et al. 2015). The effects of methamphetamine on memory impairment are long lasting as measured by the decrease in prospective memory after 6 months of abstinence (Rendell, Mazur et al. 2009). Neurotoxicity mediated by methamphetamine is largely as a results of its action on the dopaminergic system (Riddle, Fleckenstein et al. 2006). Methamphetamine increases the concentration of dopamine in nerve terminals through various mechanisms, including decreased activity of dopamine transporter, decreased activity of tyrosine hydroxylase, reducing the activity of vesicular monoamine transporter and reduced degradation by monoamine oxidase (Silverstein, Shah et al. 2011, Coller and Hutchinson 2012).

Methamphetamine also binds to dopamine and trace amino acid 1 receptors to exhibit its neurotoxic potential (Ares-Santos, Granado et al. 2012). It also acts on pre-synaptic terminals to facilitate the release of glutamate and thereby further enhancing the neurotoxic potential (Zhang, Jin et al. 2014). Ubiquitin proteasome system and autophagy are majorly responsible for degradation process and methamphetamine affects both the systems to cause neurodegeneration. This is evident by the increased presence of ubiquitin reactive neurons in the substantia nigra and mid brain (Quan, Ishikawa et al. 2005). Moreover, astrocytosis and dendritic pathology that are seen in HIV-1 infection are also associated with methamphetamine abuse (Kuczenski, Everall et al. 2007, Raineri, Gonzalez et al. 2012).

1.7.1 Methamphetamine and oxidative stress

Methamphetamine has been shown for a long time to affect the redox balance in the body (Yamamoto and Bankson 2005). Oxidative stress induced by methamphetamine has been shown to play a major role in HIV pathogenesis. Release of nitric oxide by methamphetamine induces the release of dopamine that has been previously shown to be neurotoxic in in vitro and in vivo systems (Bowyer, Clausing et al. 1995). Use of specific nitric oxide inhibitors decrease the toxicity mediated by methamphetamine. Administration of methamphetamine to rats increased the expression of nitric oxide through the expression of dimethylarginine dimethylaminohydrolase 1, a major enzyme in nitric oxide synthesis pathway (Li, Wang et al. 2008). Furthermore, acute administration of methamphetamine resulted in the increased expression of various nitroproteins in the cell culture and rat striatum (Zhang, Chen et al. 2013). Increase in the expression levels of nitric oxide and glutamate further results in the activation of various molecules that result in the disruption of mitochondrial function (Sanchez-Alavez, Conti et al. 2013). These include disruption of complex II and complex II-III of mitochondrial electron transport chain. The effects are negated by the use of specific antagonists and scavengers against glutamate and peroxynitrite, respectively (Brown, Quinton et al. 2005). Furthermore, methamphetamine administration leads to increased expression of quinone, a secondary product of dopamine oxidation known to affect the function of mitochondrial enzymes (O'Shea, Urrutia et al. 2014).

1.7.2 Methamphetamine and neuroinflammation

Methamphetamine affects the normal functioning of the immune system. It affects the innate immune system function by stimulating the productions of various inflammatory stimuli. It shifts the balance to pro-inflammatory state by up-regulating the expression of pro-inflammatory cytokines like TNF- α and IL-8 and decreasing the expression of anti-inflammatory cytokines like CCL7 (Burns and Ciborowski 2016). Methamphetamine treatment increases the expression of trace amine-associated receptor 1 on T cells to induce the

activation of protein kinase C, a secondary messenger in immune activation and also stimulates the production of IL-2 (Sriram, Cenna et al. 2016). Methamphetamine induces the activation of various glial cells, including astrocytes and microglia. Activation of astrocytes and microglia is persistent for a long time even after methamphetamine withdrawal. Positron emission tomography of methamphetamine abusers showed elevated levels of (11)C]-(R)-[1-[2-chlorophenyl]-N-methyl-N-[1-methylpropyl]-3-isoquinoline carboxamide, radiotracer for the detection of reactive microglia. The levels of radiotracer were increased in different brain regions (Sekine, Ouchi et al. 2008). Furthermore, methamphetamine affects the cell morphology of microglia making it to increase in its size (LaVoie, Card et al. 2004). It binds to sigma 1 receptor expressed on the surface of astrocytes to induce the expression of glial fibrillary acidic protein (GFAP), marker of astrocyte activation (Zhang, Lv et al. 2015). Hyperthermia, a characteristic feature of methamphetamine administration and neurotoxicity are also attenuated by the use of sigma receptor antagonist (Kaushal, Robson et al. 2014). Methamphetamine also affects the function of voltage gated potassium channels on microglia. It specifically up-regulates the expression of potassium channel Kv1.3 and promotes the outward current that results in cell death of microglia (Wang, Qian et al. 2014). Furthermore, methamphetamine mediated increase in the expression of IL-6 and TNF- α were decreased by the use of specific Kv1.3 antagonist. Methamphetamine showed increased expression of IL-6 and IL-8 cytokines in astrocytes through the activation of NF- κ B pathway (Shah, Silverstein et al. 2012). Chronic methamphetamine treatment also reduces the expression levels of anti-inflammatory cytokines, including IL-2 and IFN- γ . Methamphetamine also increases the expression of cytotoxic inflammatory marker, cyclooxygenase-2 in striatum (Kita, Shimada et al. 2000). Methamphetamine decreases the expression of chemokines in dendritic cells, including MIP1 α and MIP1 β that can interact with HIV co-receptor (CCR5) to reduce the viral infection (Nair and Saiyed 2011).

1.8 Significance

Significant amount of work has been undertaken to determine HIV pathogenesis. The present study was undertaken to determine the role of HIV-1 Tat and/or methamphetamine to alter the production of various cytokines/chemokines and expression of various synaptic proteins and neurotrophic factors. Production of neurotoxicity is a multifaceted approach and our study attempted to investigate the molecular mechanisms underlying the production of these cytokines/chemokines. We have also performed behavioral paradigm to determine the effects of HIV-1 Tat and/or methamphetamine on locomotion, anxiety and memory. The present study was based on the hypothesis that HIV-1 Tat and methamphetamine will interact with each other to increase the neurotoxicity, mediated by the production of various pro-inflammatory cytokines/chemokines and also altering the expression of various synaptic proteins and neurotrophic factors. The long term goal of the current study is to develop a therapeutic strategy to block the production of various pro-inflammatory cytokines/chemokines or altered expression of various synaptic proteins and neurotrophic factors due to HIV-1 Tat and/or methamphetamine, which can decrease the progression towards HAND. The following specific aims were designed to elucidate our hypothesis:

- To measure the role of HIV-1 Tat and methamphetamine on the expression of various pro-inflammatory cytokines/chemokines in SVG astrocytes and human fetal astrocytes
- To investigate the molecular mechanisms underlying the expression of various pro-inflammatory cytokines/chemokines induced by HIV-1 Tat and methamphetamine
- To determine the effect of HIV-1 Tat and methamphetamine on locomotion, anxiety and memory by employing open field assay, light/dark box, Y maze and Morris water maze.
- To assess the involvement of various synaptic proteins and neurotrophic factors in HIV-1 and methamphetamine induced behavior changes

The findings of the present study are of clinical importance as understanding the molecular mechanisms of increased neuroinflammation via cytokine/chemokine secretion and altered expression of synaptic proteins and neurotrophic factors, can lead to effective development of therapeutic strategies aimed at intervening the progression of HAND.

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1 Cell culture

All the experiments were performed using SVGA cells (Astroglial cells modified from simian virus 40 (SV40)-transformed human glial cells (SVG), originally developed by Dr. Major (Major, Miller et al. 1985). The cells were a generous gift from Dr. Avindra Nath. For the experiments involving primary human astrocytes, cells were gifted by Dr. Anuja Ghorpade. These cells were isolated from aborted fetus brain tissues. Both the cells were maintained Dulbecco's modified eagle medium (DMEM) supplemented with 1% sodium bicarbonate, 1% Non-essential amino acids, 1% L-Glutamine, 10% heat inactivated fetal bovine serum and 50 µg/ml of Gentamycin. The cells were maintained in an incubator at 37°C and humidified air with 5% CO₂. The cells were cultured in 150 cm² flasks in 20 ml complete DMEM and passaged every alternate day. All the experiments were performed in either 6 well or 12 well plates with a seeding density of 0.7 million cells/well and 0.25 million cells/well, respectively. The cells were allowed to adhere overnight and the experiments were performed on the following day.

2.2 Reagents and chemicals

HIV-1 Tat expression plasmid was obtained from NIH aids research and reference reagent program (Catalog # 10453), initially developed by Dr. E Verdin, Gladstone institute, UCSF. Pharmacological inhibitors for NF-κB (BAY1170-82, SC-514), p38 (SB203580), JNK (SP600125), ERK1/2 (U0126), PI3K (LY294002) were purchased from Cayman chemicals (Ann Arbor, MI, USA). The recombinant HIV-1 Tat protein of 86 amino acids length (Catalog # 2222) was obtained from NIH aids research and reference reagent program. For all the experiments involving HIV-1 Tat protein, it was used at a concentration of 200 ng/ml. All the siRNA used in the experiments were pre-designed and validated. siRNA against p38 isoforms (α/β/γ/δ) (P/N AM51331; id 5213), p50 (P/N 4390420; id s9505), p65 (P/N AM4390824; id

s11914) and negative silencer control1 (scrambled) (# AM4611) were purchased from Ambion Inc (Carlsbad, CA, USA). siRNA against Akt1 (4390824; id s661), Akt2 (4390824; id s1215), akt3 (4392420; id s19429) isoforms, AP-1 (c-jun) (4392420; id s7660), C/EBP α (4392420; id s2890) and C/EBP γ (4392420; id s2902), JNK1 (4392420; id s28270) were purchased from Thermo Fisher Scientific (PA, USA). The primary antibodies for p65, p-c-jun, GAPDH, synapsin1, synaptophysin, Shank2, PSD95, p-CaMKII and all the secondary antibodies against rabbit and goat were purchased from Cell Signaling (Danvers, MA, USA), primary antibodies for ARG3.1, CNTF and BDNF were purchased from abcam (Cambridge, MA, USA) and primary antibodies for p-p38, p-akt, p-JNK and LaminB were purchased from Santa cruz biotechnology (Dallas, TX, USA). Mounting medium containing 4',6-diamidino-2-phenylindole (DAPI) was obtained from Vector Laboratories (Burlingame, CA).

2.3 Transfection

SVG astrocytes were transiently transfected with plasmid encoding HIV-1 Tat by using Lipofectamine 2000 (Life technologies, NY, USA) as previously described (Nookala, Shah et al. 2013). Briefly, astrocytes were plated in a 6 or 12 well plate and were allowed to adhere overnight. Next day, complete DMEM was removed and the cells were washed twice with PBS and serum free DMEM was added to the wells. A transfection mixture containing Lipofectamine and Optimem with or without 0.3 μ g of HIV-1 Tat plasmid was added to the wells containing serum free medium. After 5 h, transfection mixture was replaced with complete DMEM. The cells were harvested at 1, 3, 6, 12, 24 and 48 hours post-transfection to determine the expression levels of various cytokines/chemokines at mRNA. Cell culture supernatants were also collected at various time points, including 6, 12, 24, 48, 72 and 96 hours to determine the protein levels of various cytokines/chemokines. Experiments with pharmacological antagonists were performed by pretreating the cells 1 h prior to transfection with the plasmid coding for HIV-1 Tat. The doses of the antagonists were determined based on their IC₅₀ values and dosage used by others. For experiments with small interfering RNA

(siRNA), 0.55×10^6 cells/well were seeded in a 6-well plate and were allowed to adhere overnight before transfecting with 50 nM siRNA. Briefly, complete media was removed from the plates and cells were washed twice with PBS before addition of serum free medium. The transfection mixture containing siRNA, Opti-MEM and Lipofectamine were added into the wells. After 24 h, the transfection mixture was replaced with complete media and the cells were allowed to grow for 10 h. After the mentioned time, cells were collected by trypsinization, counted and re-seeded at a density of 0.275×10^6 cells per well in a 12 well plate. The transfection with siRNA was performed for 24 h as opposed to 5 h (for Tat plasmid) in order to ensure the maximum knockdown of the target as reported previously. The following day, these cells were then transiently transfected with the HIV-1 Tat plasmid for 5 h and the cells were harvested at 6 h for determining the expression of mRNA and at 48 h for determining the protein expression.

2.4 Quantitative real time RT-PCR

The cells were harvested at specific time points based on the experimental conditions and total RNA was extracted using RNeasy kit from Qiagen (Valencia, CA) as per the manufacturer's recommendations. Briefly, cells were lysed using 350 μ l of RLT buffer, followed by precipitation of RNA using 350 μ l of 70% ethanol. Then the cells were spun at 10,000 rpm for 20 sec and the eluate was discarded. The columns were washed once with 700 μ l of RW1 buffer and twice with 500 μ l of RPE buffer. 50 μ l of RNase free water was added into the column and the dissolved RNA was collected in the eluate in a pre-labeled tube. Concentration of RNA was measured and 150 ng was used to measure the mRNA expression levels of various cytokines/chemokines using real-time reverse transcription polymerase chain reaction (RT-PCR). Briefly, 150ng of RNA was reverse transcribed and amplified using the primers and PCR conditions as described in the following table (table 1). The expression levels of various genes were calculated by $2^{-\Delta\Delta C_t}$ method using hypoxanthine phosphoribosyltransferase (HPRT) as an internal housekeeping gene.

2.5 Multiplex cytokine assay

The protein levels of cytokines/chemokines in the cell culture supernatants were measured using multiplex cytokine assay kit (Bio-Rad, CA, USA) as per the manufacturer's protocol. Briefly, cell culture supernatants were collected from the plates at 6 h, 12 h, 24 h, 48 h, 72 h, 96 h post-transfection followed by centrifugation twice at 1000g for 5 min. 50 μ l of the samples and standards were mixed with magnetic beads and incubated on a shaker at room temperature for 30 min. The magnetic beads were washed and 25 μ l of detection antibody was added to each well followed by incubation for 30 min. The samples were washed and incubated with 50 μ l of streptavidin-PE conjugate for 10 min. Finally 125 μ l of the assay buffer was added and the samples were analyzed using Biorad Bioplex HTS (Bio-Rad, CA, USA). The protein concentrations of cytokines/chemokines was determined with the Bio-plex manager 5 using 5-PL statistics.

2.6 Western blotting

Cells were harvested at indicated time points to collect whole cell lysates and cytoplasmic and nuclear extracts. For whole cell extract preparation and animal tissues, RIPA buffer was used to lyse the cells and tissues, followed by homogenization and spinning at 10,000 rpm for 10 min to remove the cell debris. Nuclear and cytoplasm fractions were separated at indicated time points using the following protocol. Briefly, the cells were trypsinized and centrifuged at 10,000 rpm for 5min. The cell pellet was resuspended in 300 μ l of cytoplasmic buffer, followed by incubation on ice for 10 min and wash with 500 μ l of ice cold PBS. Then 200 μ l of nuclear buffer was added to the remaining pellet and was incubated on ice for 15 min. The suspension was vortexed intermittently and was centrifuged at 14,000 rpm for 10 min to obtain the nuclear portion. The concentrations of the proteins were determined from the standard curve using BCA kit (Pierce biotechnology, IL, USA). 20 μ g of protein was loaded into the wells of 12% polyacrylamide gel. Electrophoresis was performed at 80V for 3 h followed by transfer onto a PVDF membrane for 90 min at 350 mA. The membrane was

blocked in 5% Nonfat Dry Milk for 1 h. The membranes were incubated in primary antibody overnight at 4°C. The details and dilutions of antibodies are described in the table 3. The membranes were washed with PBST and incubated in the appropriate HRP conjugated secondary antibody for 90 min. The membranes were washed with PBST and the proteins were visualized by using BM Chemiluminescence Western Blotting Substrate (POD) (Roche Applied Sciences, Indianapolis, IN). The bands were analyzed and quantified by Flourchem HD2 software (Alpha Innotech, San Leandro, CA). GAPDH and LaminB were used as internal loading controls for cytoplasmic and nuclear protein, respectively, to normalize the expression of proteins of interest.

2.7 Immunocytochemistry

SVGA cells were seeded at a density of 0.6×10^6 cells/well in a 6-well plate on glass cover slips in each well. The cells were allowed to adhere overnight, followed by transfection with the HIV-1 Tat plasmid for 12 h on the following day. 6 h prior to the termination, 10 μ l of 1 mg/ml Golgi-stopTM (BD Biosciences, CA, USA) solution was added into each well in order to prevent the release of cytokines/chemokines into the supernatant. After specified time, complete DMEM was removed from the wells and the cells were fixed by the addition of 1:1 ice cold methanol and acetone solution and kept at -20°C for 20 min. The wells were air dried and the cover slips were incubated for 10 min in PBST (0.1% Triton-X in PBS). This was followed by 3 washes with PBS and blocking with 1% bovine serum albumin in PBST for 30 min. The cells were washed and further incubated for overnight with the mixture of antibodies for mouse anti-glial fibrillary acidic protein (anti-GFAP) (1:1000) and rabbit anti-IL-6 (1:500) or anti-IL-8 (1:500) or anti-CCL5 (1:500). On the following day, cells were washed thrice with PBST and the secondary antibodies (Alexafluor 555 labeled Anti-mouse IgG and Alexafluor 488 labeled anti-rabbit IgG) were added at a dilution of 1:1000 and the cover slips were incubated in dark for 1 h. The cover slips were gently taken out from the wells and washed with PBST before being transferred onto a glass slide containing the mounting medium with

4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA). The images were obtained using inverted confocal microscope, Leica TCS SP5 II (Leica Microsystems, Wetzlar, Germany). The intensity of cytokines/chemokines was calculated using imageJ software and GFAP was used as a house keeping gene to normalize the intensity values.

2.8 Animals

Doxycycline inducible HIV-1 Tat transgenic mice obtained from Dr. Kurt Hauser at Virginia Commonwealth University were used for the experiments. The expression of Tat in this transgenic line was under the control of glial fibrillary acidic promoter (GFAP) promoter and thereby, expression was restricted to astrocytes. Both Tat positive and Tat negative mice expressed reverse tetracycline transactivator gene whereas only Tat positive mice expressed Tat gene. All the mice were housed and bred at UMKC Laboratory Animal Research Core. Mice were genotyped to confirm the presence of Tat transgene. Eight week old HIV-1 Tat (+) and HIV-1 Tat (-) mice were administered doxycycline (6 mg/kg) for a duration of 16 weeks before they were subjected to behavior testing. All the behavioral tests were performed between 8:00 AM to 5:00 PM. All the procedures were approved and conducted in accordance with UMKC Institutional Animal Care and Use Committee.

2.9 Drug regimen

To simulate chronic methamphetamine use in human abusers, we employed escalating dosing regimen in mice. Methamphetamine (Sigma, St. Louis, USA) was dissolved in PBS and was administered intraperitoneally twice a day. The dose of methamphetamine was escalated stepwise from 0.1 mg/kg to 6 mg/kg over 7 day duration. After the escalation period, a maintenance dose of 6 mg/kg methamphetamine was given b.i.d for the remaining duration of experiment. Control mice received similar amount of PBS intraperitoneally.

2.10 Open field assay

Open field assay is used to determine the locomotor activity and level of anxiety. The chamber consists of a 40cm x 40 cm and covered on four sides with a black opaque plexi

glass. Infra-red photo beam arrays were placed 2 inches above the base of the testing floor on opposite sides. These beams are used to record the rearing activity of the mice. The procedure for performing the assay is as follows. The mice were brought in to the testing room and were allowed to acclimatize for 1 h. Mouse was placed into the chamber into one of the corners at random. Activity of the mice was recorded by means of an overhead camera over a duration of 6 min. The chamber was cleaned with 70% ethanol before testing the subsequent mouse to prevent odor trail. Total distance traveled by the mice over entire trial duration was calculated and serves as an indicator of locomotor activity. Furthermore, the amount of time spent and distance traveled by the mice at the center and the periphery were calculated. These two parameters served as an indicator of anxiety.

2.11 Light/dark box assay

Light/dark box is an important tool to measure the level of anxiety. The box consists of two chambers, a light chamber and a dark chamber. Two chambers are separated by a black opaque plexi glass with an opening in the center. Infra-red photo beam arrays were placed 2 inches above the base of the testing floor on opposite sides. These beams are used to record the rearing activity of the mice. The procedure for performing the assay is as follows. Animal was placed in the dark chamber and the test was started. The activity of the animal was monitored over a duration of 10 min by means of an overhead camera. The chamber was cleaned with 70% ethanol before testing the subsequent mouse to prevent odor trail. Time spent and distance traveled by the mice in the light compartment were calculated. These two parameters serve as measures of anxiety like phenomenon in mice.

2.12 Y Maze

Y maze measures spatial working memory in mice by using their innate tendency to explore new environments. The maze has three closed arms, or lanes of travel, that are each 12 inches in length and 120° from each other and are interconnected. The animal was placed into the end of one of the arms and was allowed to freely explore for 5 minutes. The starting

arm positions of the mice were chosen at random. The movement of the animal inside the Y maze was recorded by means of an overhead camera. The arms were thoroughly cleaned with disinfectant and 70% ethanol to eliminate any residual odors of previous mice. The number of arm entries were counted and acts as a marker of locomotor activity. The number of spontaneous alternations, as defined by entry into three different arms in sequence (triad), were

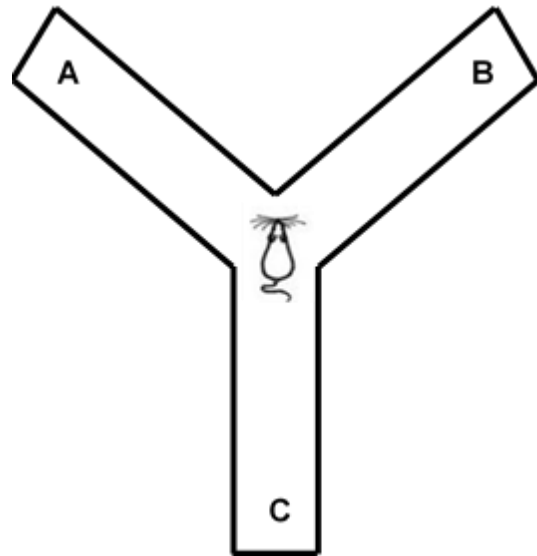


Figure 3: Diagram of Y maze

considered as a measure of working memory. Spontaneous alternations was calculated from the following equation:

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

2.13 Morris water maze

The Morris water maze is a standard test of measuring spatial learning and memory in rodents. Animals were brought to the testing facility in their home cages and allowed to acclimate for 1 hour and the water maze was prepared. Briefly, a 4 foot diameter galvanized tank was filled to a depth of 12 inches with 23-25°C water. A 10 cm escape platform was submerged 1 cm below the surface of the water in a fixed location. External navigation cues were placed outside the arena. The animal was placed in the water and was allowed 1 minute to freely explore the tank. If the animal does not find the escape platform within 1 minute, it was gently guided to the location of the platform. Once on the platform, the animal was given 60 seconds to rest within view of the external cues. The location trial was repeated 4 times a day for 5 days. The order of the location where the mice were placed in water during the acquisition trial was changed every day. The following order was employed to test the animals each day.

Latency to locate the escape platform was measured. A memory retention test was performed 24 hours following the final learning trial. Platform was removed and the animal was dropped from a novel location (SW quadrant) and was allowed to explore the tank for 60 seconds. Time spent in the target quadrant (where the platform was initially located) and distance traveled in the target quadrant were measured. The movement of the animal inside the maze was recorded by means of an overhead camera.

Table 1: Locations where the mice were dropped in the water maze at different trails during 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

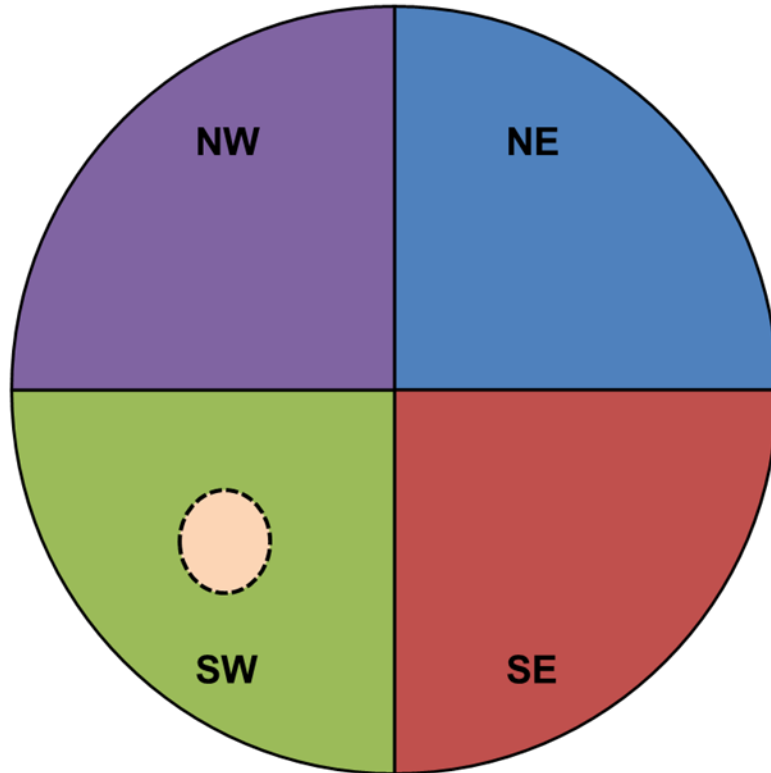


Figure 4: Diagram of Morris water maze. NW, NE, SW and SE represent northwest, northeast, southwest and southeast quadrants, respectively.

2.14 Genotyping

Tails were collected from the mice just before weaning. 400 μ l of lysis buffer and 2.5 μ l of proteinase K was added to the tail samples and were digested overnight in a water bath at 55°C. On the following day, 300 μ l of 6M sodium chloride was added and the tubes were spun at 10,000 rpm for 5 min. 300 μ l of supernatant was carefully transferred into a tube without collecting the debris. 350 μ l of isopropanol was added and the tubes were allowed to sit for 10 min, followed by centrifugation at 10,000 rpm for 5 min. The supernatants were discarded and the tubes were air dried. The pellet was washed with 70% ethanol and centrifuged at 10,000 rpm for 10 min. The supernatants discarded and air dried to completely remove traces of ethanol. The pellet was dissolved in 35 μ l of TE buffer and the concentration of DNA was recorded using a spectrophotometer. 100 ng of DNA was used for PCR to amplify Tat gene. The following forward and reverse primer sequences were used (Fwd: 5'-

GCGGATCCATGGAGCCAGTAGATCCTA-3' ; Rev: 5'-
GCGAATTCTCATTGCTTTGATAGAGAACTTG-3'). The PCR conditions that were used are as follows; denaturation at 95°C for 5 min; 40 cycles of denaturation at 95°C for 45sec, annealing at 54°C and extension at 72°C for 30 sec. 8 µl of amplified product was mixed with 2 µl of loading dye and was loaded into wells of 2% agarose gel. The gel was run at 80 V for 1 hour. Tat positive mice were identified by the presence of a band at 220 bp that corresponds to length of Tat.

2.15 Statistical analysis

Statistical analyses was performed using Graphpad Prism and Statistical package for social sciences (SPSS, version 23 for windows, IBM, Armonk, NY). For experiments involving cell culture, statistical analysis was performed to represent the data in mean \pm SE values. Results were based on at least three independent experiments with each experiment performed in triplicate. For experiments involving mice, values are represented as mean of all mice in a particular group. For the comparison between mock/control group and treatments, two-tailed student's t-test and one-way ANOVA were used to calculate p-value. Dunnet's post-hoc and Tukey's LSD post-hoc were performed appropriately as mentioned in the figure legends. For experiments involving morris water maze, two-way ANOVA with Tukey's post-hoc analysis was performed to calculate the significance for acquisition trial. $p < 0.05$ was considered as statistically significant.

Table 2: List of primers and PCR conditions

No	Gene	Primer Sequence	PCR Conditions			
			Reverse Transcription	Denaturation	Annealing	Extension
1	IL-6	Fwd: 5'-GGT ACA TCC TCG ACG GCA TC-3'	50°C for 10 min, 95°C for 15 min	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	IL-8	Fwd: 5'-CTC TTG GCA GCC TTC CTG ATT-3'	50°C for 10 min, 95°C for 15 min	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 10 min, 95°C for 15 min	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'				
		Probe: 5'-GCT GCT CAT AGC AGC CAC CTT C-3'				
4	MCP-1	Fwd: 5'-TAG CTC GCG AGC CTC TGC AC-3'	50°C for 10 min, 95°C for 15 min	95°C for 30 Sec	59°C for 30 sec	76°C for 30 sec
		Rev: 5'-FAM TCG CTC AGC CAG ATG CAA TCA ATG CC-3'-BHQ1				
		Probe: 5'-GCT TTC CTT GGT CAG GCA GTA-3'				
5	HPRT	Fwd: 5'-GCT TTC CTT GGT CAG GCA GTA-3'	50°C for 10 min, 95°C for 15 min	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'				
		Probe: 5'-GCT TTC CTT GGT CAG GCA GTA-3'				
6	MIP1α	Fwd: 5'-CCC GGT GTC ATC TTC CTA ACC-3'	50°C for 10 min, 95°C for 15 min	95°C for 15 Sec	54°C for 30 sec	76°C for 30 sec
		Rev: 5'-TTC TGG ACC CAC TCC TCA CTG-3'				
		Probe: 5'-AAG CTG AGA ACC AAG ACC CAG ACA-3'				
7	IP-10	Fwd: 5'-AAG CTG AGA ACC AAG ACC CAG ACA-3'	50°C for 10 min, 95°C for 15 min	95°C for 15 Sec	62°C for 30 sec	74°C for 30 sec
		Rev: 5'-AAA GGC ATT CTT CAC CTG CTC CAC-3'				
		Probe: 5'-CCA CCG ACA CAA GTG CGA TA-3'				
8	IL-4	Fwd: 5'-CCA CCG ACA CAA GTG CGA TA-3'	50°C for 10 min, 95°C for 15 min	95°C for 15 Sec	55°C for 30 sec	72°C for 30 sec
		Rev: 5'-CCC TGC AGA AGG TTT CCT TCT-3'				
		Probe: 5'-GCT TTC CTT GGT CAG GCA GTA-3'				

Table 3: List of antibodies and optimum dilutions

S. No	Name	Cat. No.	Company	Dilution
1	NK-κB p65 Rabbit mab	8242S	Cell signaling	(1:4000)
2	Phospho-IκBα (Ser32) (14D4) Rabbit mab	5209S	Cell signaling	(1:2000)
3	Phospho-Akt (Ser473) (D9E) XP® Rabbit mAb	4060S	Cell signaling	(1:2000)
4	Phospho-p38 MAPK (Thr180/Tyr182) (D3F9) XP® Rabbit mAb	4511S	Cell signaling	(1:2000)
5	Phospho-c-Jun (Ser73) (D47G9) XP® Rabbit mAb	3270S	Cell signaling	(1:4000)
6	p-JNK Antibody (G-7)	sc-6254	Santa cruz	(1:1500)
7	JNK Antibody (D-2)	sc-7345	Santa cruz	(1:1500)
8	Phospho-Synapsin (Ser9) Antibody	2311S	Cell signaling	(1:2000)
9	Synaptophysin (D35E4) XP® Rabbit mAb	5461S	Cell signaling	(1:2000)
10	PSD95 (D74D3) XP® Rabbit mAb	3409S	Cell signaling	(1:2000)
11	Anti-Arg 3.1 antibody	ab23382	Abcam	(1:2000)
12	SHANK2 Antibody	12218S	Cell signaling	(1:2000)
13	Anti-BDNF antibody	ab108319	Abcam	(1:2000)
14	Anti-CNTF antibody	ab46172	Abcam	(1:2000)
15	Phospho-CaMKII (Thr286) (D21E4) Rabbit mAb	12716S	Cell signaling	(1:2000)
16	GAPDH (D16H11) XP® Rabbit mAb	5174S	Cell signaling	(1:4000)
17	Lamin B Antibody (C-20)	sc-6216	Santa cruz	(1:1500)
18	Mouse IgG (conjugated with alexa fluor 555)	4409	Cell signaling	(1:2000)
19	Rabbit IgG (conjugated with alexa fluor 488)	4340	Cell signaling	(1:2000)
20	Rabbit anti-IL-6	ab6672	Abcam	(1:500)
21	Rabbit anti-IL-8	sc-7922	santacruz	(1:500)
22	Rabbit anti-CCL5	PA1-84182	Thermo Fisher	(1:500)
23	Anti-mouse IgG, HRP-linked Antibody	7076S	Cell signaling	(1:2000)
24	Anti-rabbit IgG, HRP-linked Antibody	7074S	Cell signaling	(1:2000)

CHAPTER 3

ROLE OF HIV-1 TAT IN THE EXPRESSION OF VARIOUS PRO-INFLAMMATORY CYTOKINES/CHEMOKINES AND THEIR UNDERLYING MECHANISM(S)

3.1 Introduction

One of the hallmarks of neurodegeneration is inflammation in the central nervous system (CNS). A major factor leading to neuroinflammation is the dysregulation of cytokines and chemokines in the CNS. Several pro-inflammatory cytokines, IL-1 β , IL-6, IL-8 and TNF- α have been implicated in neuroinflammation, leading to several neurodegenerative diseases including Alzheimer's disease (Forloni, Mangiarotti et al. 1997), Parkinson's disease (PD) (Nagatsu and Sawada 2005), multiple sclerosis (Maimone, Guazzi et al. 1997) and HIV associated neurocognitive disorders (HAND) (Kaul, Garden et al. 2001). In particular, in HIV infection, elevated levels of cytokines correlate with the degree of HAND (Yuan, Qiao et al. 2013). While IL-6, IL-8 and CCL5 have been extensively studied for their role in AD and PD, not much is known about the role of these cytokines/chemokines in HAND.

Human immunodeficiency virus-1 (HIV-1) enters the brain through blood brain barrier (BBB) early after the infection (Resnick, Berger et al. 1988). Prolonged infection of central nervous system further leads to various neurological complications including HIV-associated dementia (HAD). Introduction of combined Anti Retro Viral therapy (cART) has significantly reduced the incidence of HAD and increased the life expectancy of people infected with HIV. Increase in the life span of people resulted in the development of less severe forms of cognitive dysfunctions, including asymptomatic neurocognitive impairment (ANI) and Mild neurocognitive disorder (MND). The collection of these neurological deficits including HAD are referred HAND (Sacktor, Lyles et al. 2001). The neurotoxicity of HIV-1 has been attributed to the virus itself or the viral proteins shed after the infection via several mechanisms including production of cytokines/chemokines. The role of two HIV proteins, HIV-1 Tat and gp120 has been extensively studied for their role in neuroinflammation. We and others have recently

shown that gp120 induces pro-inflammatory cytokines and reactive oxygen species in different cells of the brain and thereby contributes to HAND (Yeung, Pulliam et al. 1995, Shah, Verma et al. 2011). In particular, presence of HIV-1 Tat has been reported in postmortem CNS tissue (hippocampus) of the HIV-1 infected patients, which underscores the significance of HIV-1 Tat in the HIV neuropathogenesis (Kruman, Nath et al. 1999). Furthermore, HIV-1 Tat has also been shown to promote the release of several pro-inflammatory cytokines and ROS from different brain cells (Nath, Conant et al. 1999, Toborek, Lee et al. 2003).

HIV-1 Trans activator of transcription (HIV-1 Tat or Tat) is an accessory viral protein produced at a very early stage of HIV-1 replication. It binds to the Tat associated region on the viral RNA and increases the replication of the virus (Dayton, Sodroski et al. 1986, Roy, Delling et al. 1990). Tat increases the transcription of HIV-1 genome by greater than 100 fold (Cann, Rosenblatt et al. 1985). Tat has been shown to be toxic to the mice when injected into the cerebroventricular region (Gourdou, Mabrouk et al. 1990, Jones, Olafson et al. 1998). The neurotoxicity of Tat is attributed to various mechanisms such as, over excitation of the neurons *via* N-methyl-D-aspartate receptor (Magnuson, Knudsen et al. 1995, Nath, Psooy et al. 1996, New, Maggirwar et al. 1998, Haughey, Nath et al. 2001) apoptosis of the neurons by increasing intracellular calcium levels (Mayne, Holden et al. 2000, Self, Mulholland et al. 2004) and disrupting the normal function of electron transport chain (Norman, Perry et al. 2007). It also affects the function of dopamine neurotransmission by deregulating the functions of dopamine transporter and vesicular monoamine transporter (Midde, Gomez et al. 2012). In addition to its direct effect on neurons, Tat has been shown to exhibit a bystander effect on neurons by promoting the release of several pro-inflammatory cytokines/chemokines, nitric oxide synthase and quinolinic acid from astrocytes and microglia (Li, Galey et al. 2005). Tat also affects the integrity of blood brain barrier by disrupting the tight junction proteins, by inducing oxidative stress (Price, Uras et al. 2006, Banerjee, Zhang et al. 2010) and apoptosis in brain microvascular endothelial cells (Andras, Pu et al. 2005).

Astrocytes comprise of majority of cells in the brain and occupy more than 50% of the brain volume. They also represent an important reservoir for the production of various mediators of inflammation, particularly in response to HIV-1 (Thompson, McArthur et al. 2001, Minagar, Shapshak et al. 2002). They play important roles in many brain functions, including the maintenance of homeostasis of the neurons, promoting the release of various neurotrophic factors, modulation of neuronal activity and regulation of synaptic plasticity (Halassa and Haydon 2010, Navarrete, Perea et al. 2012). Furthermore, they function as immune cells in the CNS by releasing myriad of cytokines/chemokines such as interleukins, (IL-1 β , IL-6, IL-8), Interferons (IFNs) and Chemokine ligands (CCLs) including CCL5 (Anderson and Swanson 2000). During viral infection, cytokines/chemokines directs the lymphocytes and monocytes to the site of inflammation (Appay and Rowland-Jones 2001). Post-mortem studies of brain samples of HIV-1 infected patients showed that a small portion of astrocytes are restrictively infected with the virus (Gorry, Ong et al. 2003). In a previous study by Churchill et al, they have demonstrated that in vivo astrocyte infection by HIV-1 occurs to a greater extent than previously known (Churchill, Wesselingh et al. 2009). A sub-population of astrocytes that are latently infected with HIV-1 undergoes apoptosis that correlates with the extent of HAND (Thompson, McArthur et al. 2001). The viral infection of astrocytes results in the production of viral proteins, which are neurotoxic. Tat has been shown to be produced by the astrocytes which were productively infected by the HIV-1 (Dou, Morehead et al. 2006). Tat has been shown to promote the up-regulation of many cytokines from astrocytes including MCP-1, IL-8, IL-6 and TNF- α (Chen, Mayne et al. 1997, Nath, Conant et al. 1999, Kutsch, Oh et al. 2000).

Although HIV-1 Tat has been shown to induce cytokines/chemokines in astrocytes, the mechanism(s) remains largely unknown. The present study was undertaken to conduct a systematic study of cytokine/chemokine expressions, time kinetics and identification of various transcription factors and upstream signaling molecules.

3.2 Results

3.2.1 HIV-1 Tat induces the expression of various cytokines/chemokines in SVG astrocytes

Previous studies have demonstrated the expression of various cytokines/chemokines by HIV-1 Tat from astrocytes. We wanted to determine the expression of a panel of cytokines/chemokines that are upregulated/downregulated by HIV-1 Tat. We tested the mRNA expressions of 9 different cytokines/chemokines at 6 hours post transfection. We found that the expressions of IL-6, IL-8 and CCL5 were significantly upregulated (more than 10 fold) and the expression of MCP-1 was slightly increased whereas the expressions of IL-1 β , TNF- α , MIP-1 and IL-10 remained unaffected by HIV-1 Tat compared to others (Fig 5). We then tested for the protein levels of these different cytokines at 48 h post transfection. We observed significant increase in the expression levels of IL-6, IL-8 and CCL5. Therefore, all the further

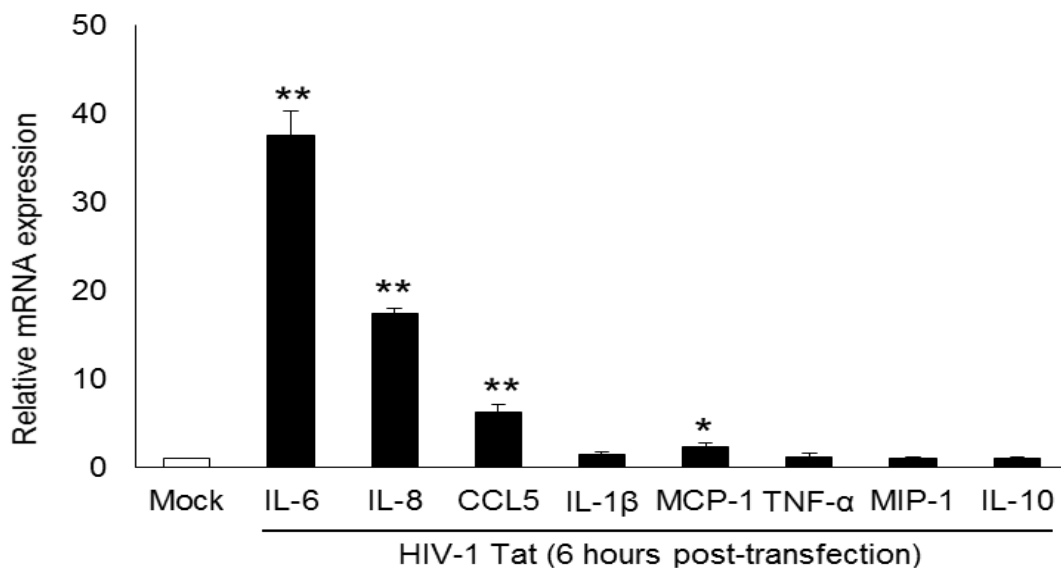


Figure 5: HIV-1 Tat-mediated cytokine/chemokine expression in SVG astrocytes: 0.7×10^6 million cells were transfected with $0.3 \mu\text{g}$ of plasmid encoding HIV-1 Tat using lipofectamine 2000. The cells were harvested 6 hours post-transfection. The mRNA expressions were measured using real time RT-PCR and levels are presented as fold difference between HIV-1 Tat transfected cells and mock-transfected control. Each bar represents mean \pm SE. The statistical significance was calculated using student's t-test and ** denotes p-value ≤ 0.01 and * denotes p-value ≤ 0.05 .

studies were focused on the IL-6, IL-8 and CCL5 expression and delineation of the mechanistic pathway(s) involved in their expression.

3.2.2 HIV-1 Tat induces the expression of IL-6 in a time dependent manner at mRNA and protein level

A myriad of pro-inflammatory cytokines including IL-6 has been shown to be elevated in the CSF of individuals infected with HIV-1. The role of HIV-1 Tat in the up-regulation of this cytokine from astrocytes has been shown in previous studies. However, the molecular mechanisms behind the up regulation of this cytokine remains largely unknown. In the present study, we sought to determine the molecular mechanisms behind the up regulation of this cytokine by HIV-1 Tat in astrocytes. To this end, we employed the transfection approach, whereby the astrocytes were transfected with plasmid encoding HIV-1 Tat. Transfection efficiency was monitored by setting a parallel transfection with plasmid encoding green fluorescence protein (GFP). The efficiency as measured and analyzed by BD FACScanto flow cytometer ranged in between 50-65% (data not shown). We first performed a time kinetics experiment to determine the peak expression levels of IL-6 at mRNA and protein levels. Compared to mock transfected cells, the levels of IL-6 mRNA increased as early as 1 h, peaked at 6 h and declined steadily until 72 h (Fig 6A). The peak expression levels of IL-6 after 6 h of transfection was found to be 29.2 ± 2.5 and 26.2 ± 1.7 fold, respectively.

To determine the protein levels of IL-6, a multiplex cytokine assay was performed at indicated time points (6 h, 12 h, 24 h, 48 h, 72 h and 96 h). The levels of IL-6 protein started to increase significantly from 6 h (6.10 ± 0.36 ng/ml vs 2.10 ± 0.19 ng/ml) after transfection and gradually increased until 96 h (14.08 ± 1.59 ng/ml vs 1.79 ± 0.22 ng/ml) where it showed the maximum increase (until observation period) compared to the mock transfected cells (Fig 6B). There was not a significant change in IL-6 protein levels in mock transfected cells. These results clearly demonstrate that HIV-1 Tat induces the expression of IL-6 from astrocytes in a time dependent manner at the level of mRNA as well as protein level. These results were also

confirmed in primary astrocytes isolated from two fetal brains collected after abortion where treatment with HIV-1 Tat protein (200 ng/ml) for 2 hours showed significant IL-6 up-regulation (10.2 ± 3.4 fold) (Fig 6C).

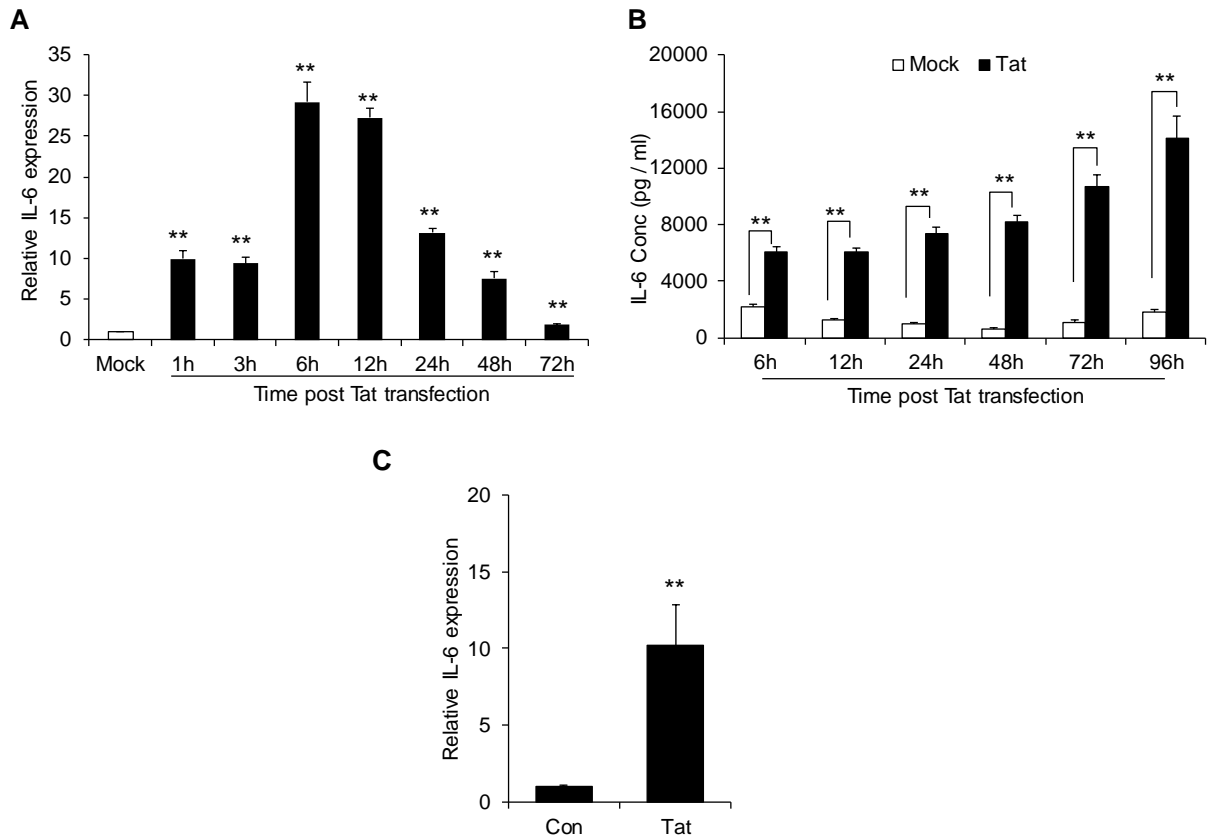


Figure 6: HIV-1 Tat induces time dependent expression of IL-6 in SVG astrocytes. Seven hundred thousand SVG astrocytes were transfected with 0.3 μ g of HIV-1 Tat plasmid using Lipofectamine 2000. **(A)** Cells were harvested at 1, 3, 6, 12, 24, 48 and 72 hours and total RNA was isolated. The expression levels of IL-6 were determined by real time RT-PCR. The values represented are normalized to their mock-transfected controls. **(B)** The concentrations of IL-6 in cell culture supernatants were measured at 6, 12, 24, 48, 72 and 96 hours after transfection by multiplex cytokine assay. Open bars and closed bars represent protein concentrations of mock- and HIV-1 Tat- transfected cells, respectively. Each experiment was done at least in triplicate and each bar represents the \pm SE of three individual experiments. **(C)** Primary astrocytes from 2 independent donors were treated with 200 ng/ml of Tat protein for 2 hours and total RNA was isolated. The expression level of IL-6 was determined by real time RT-PCR. Statistical analyses was performed by Student's *t*-test and ** denotes p-value of ≤ 0.01

3.2.3 HIV-1 Tat induces the expression of IL-8 in a time dependent manner at mRNA and protein level

Similar to IL-6, we measured the expression levels of IL-8 at different time points at both mRNA and protein. The time kinetics experiment showed an increase in the mRNA expression levels of IL-8 as early as 1 hour post transfection. The mRNA expression continued to increase until 6 hours, where it peaked and then started to gradually decrease until last time point (72h) of the observation period. The peak expression of IL-8 was found to be 26.2 ± 1.7 fold compared to mock transfected cells (Fig 7A). We then performed bioplex to quantify the expression levels of IL-8 at protein level at various time points (6 h, 12 h, 24 h, 48 h, 72 h and 96 h). The expression of IL-8 started to increase from 6 h post transfection (0.172 ± 0.01 compared to 0.112 ± 0.006 ng/ml in controls), continued gradually until 96 h (0.813 ± 0.09 ng/ml compared to 0.185 ± 0.005 ng/ml in controls), last time point of the observation period (Fig 7B). There was not a significant change in IL-8 protein level in mock transfected cells. These results clearly demonstrate that HIV-1 Tat induces the expression of IL-8 from astrocytes in a time dependent manner at the level of mRNA as well as protein level. These results were also confirmed in primary astrocytes isolated from two fetal brains collected after abortion where treatment with HIV-1 Tat protein (200 ng/ml) for 2 hours showed significant IL-8 up-regulation (17.3 ± 5.2 fold) (Fig 7C).

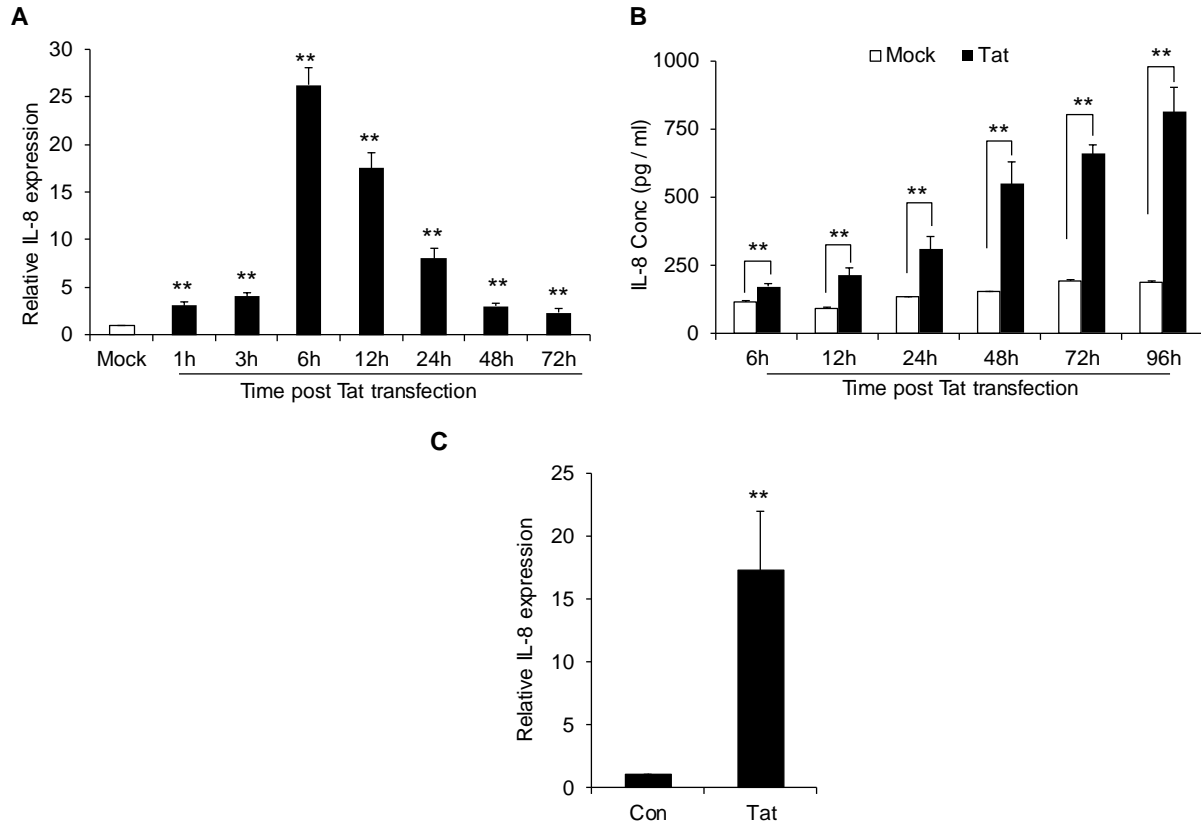


Figure 7: HIV-1 Tat induces time dependent expression of IL-8 in SVG astrocytes. Seven hundred thousand SVG astrocytes were transfected with 0.3 μ g of HIV-1 Tat plasmid using Lipofectamine 2000. **(A)** Cells were harvested at 1, 3, 6, 12, 24, 48 and 72 hours and total RNA was isolated. The expression levels of IL-8 were determined by real time RT-PCR. The values represented are normalized to their mock-transfected controls. **(B)** The concentrations of IL-8 in cell culture supernatants were measured at 6, 12, 24, 48, 72 and 96 hours after transfection by multiplex cytokine assay. Open bars and closed bars represent protein concentrations of mock- and HIV-1 Tat- transfected cells, respectively. Each experiment was done at least in triplicate and each bar represents the \pm SE of three individual experiments. **(C)** Primary astrocytes from 2 independent donors were treated with 200 ng/ml of Tat protein for 2 hours and total RNA was isolated. The expression level of IL-8 was determined by real time RT-PCR. Statistical analyses was performed by Student's *t*-test and ** denotes p-value of ≤ 0.01

3.2.4 HIV-1 Tat induces the expression of CCL5 in a time dependent manner at mRNA and protein level

In view of the findings that elevated CCL5 has been detected in the CSF of HIV-1 infected individuals suffering from HAD and that HIV-1 Tat induces CCL5 production in astrocytes (Kelder, McArthur et al. 1998, El-Hage, Podhaizer et al. 2011), we sought to investigate the underlying mechanism(s) responsible for HIV-1 Tat-mediated CCL5 expression in astrocytes. We observed elevated CCL5 mRNA level within 1 h of transfection (17.09 ± 0.59 fold), which gradually declined in a time-dependent manner over 72 h observation period (Fig. 8A). Similarly, the expressions at protein levels were measured in cell culture supernatants at various time intervals (6 h, 12 h, 24 h, 48 h, 72 h and 96 h) (Fig. 8B). The protein levels of CCL5 showed significant increase as early as 6 h (0.48 ± 0.04 ng/ml vs 0.04 ± 0.001 ng/ml in control). The peak CCL5 expression was observed at 48h post-transfection (2.04 ± 0.17 ng/ml compared to 0.27 ± 0.01 ng/ml in controls) followed by time-dependent decrease over 96 h observation period (Fig. 8B). These results indicate that HIV-1 Tat-mediated induction of CCL5 expression follows a time-dependent kinetics at both mRNA as well as protein levels.

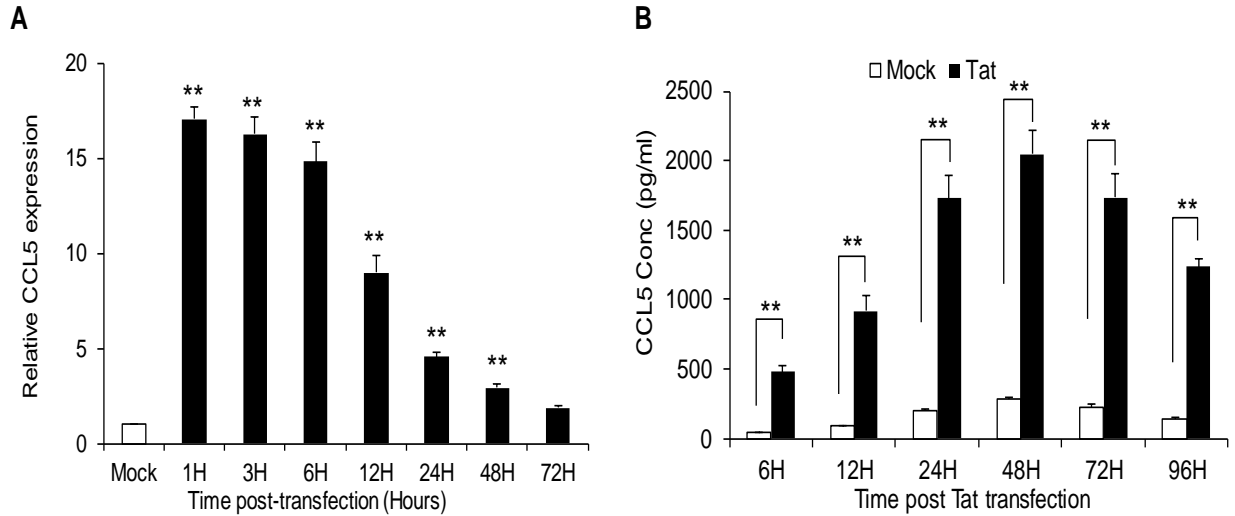


Figure 8: HIV-1 Tat induces time dependent expression of CCL5 in SVG astrocytes. Seven hundred thousand SVG astrocytes were transfected with 0.3 μg of HIV-1 Tat plasmid using Lipofectamine 2000. **(A)** Cells were harvested at 1, 3, 6, 12, 24, 48 and 72 hours and total RNA was isolated. The expression levels of CCL5 were determined by real time RT-PCR. The values represented are normalized to their mock-transfected controls. **(B)** The concentrations of CCL5 in cell culture supernatants were measured at 6, 12, 24, 48, 72 and 96 hours after transfection by multiplex cytokine assay. Open bars and closed bars represent protein concentrations of mock- and HIV-1 Tat-transfected cells, respectively. Each experiment was done at least in triplicate and each bar represents the \pm SE of three individual experiments. Statistical analyses was performed by Student's *t*-test and ** denotes *p*-value of ≤ 0.01

3.2.5 HIV-1 Tat-mediated induction of intracellular protein expression of various cytokines/chemokines

In order to further confirm the findings observed at mRNA and protein levels, we employed immunocytochemistry on the HIV-1 Tat-transfected astrocytes to visualize the production of IL-6, IL-8 and CCL5. The cells were incubated with a cocktail of GFAP and IL-6 or IL-8 or CCL5 specific antibodies. These proteins were visualized by staining with a secondary antibody labeled with Alexafluor 488 and Alexafluor 555 for GFAP and the cytokine, respectively. DAPI staining was performed to visualize the nucleus of the cells. The results clearly show that IL-6, IL-8 and CCL5 are increased in HIV-1 Tat-transfected cells when compared with mock-transfected and untransfected cells (Fig. 9-11). Our results also indicate that astrocyte marker GFAP did not significantly change with either control or mock or HIV-1 Tat transfection (Fig. 9-11). The intensity of IL-6 over GFAP was 3 ± 0.21 fold higher in HIV-1 Tat-transfected cells when compared to the control cells (Fig. 9j). Similarly, the intensity of IL-8 was 2.46 ± 0.28 fold (Fig. 10t) and intensity of CCL5 was 2.6 fold more when compared to the untransfected cells (Fig. 11j). The change in intensities of either IL-6 or IL-8 or CCL5 was not significant in mock-transfected cells when compared to the untransfected cells.

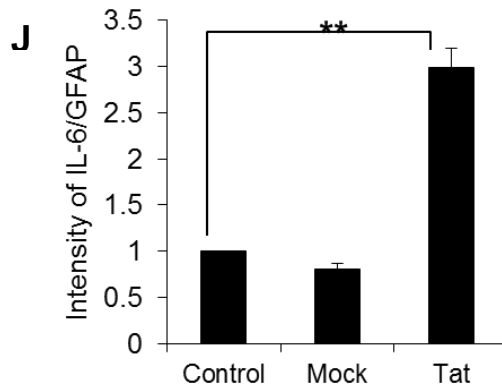
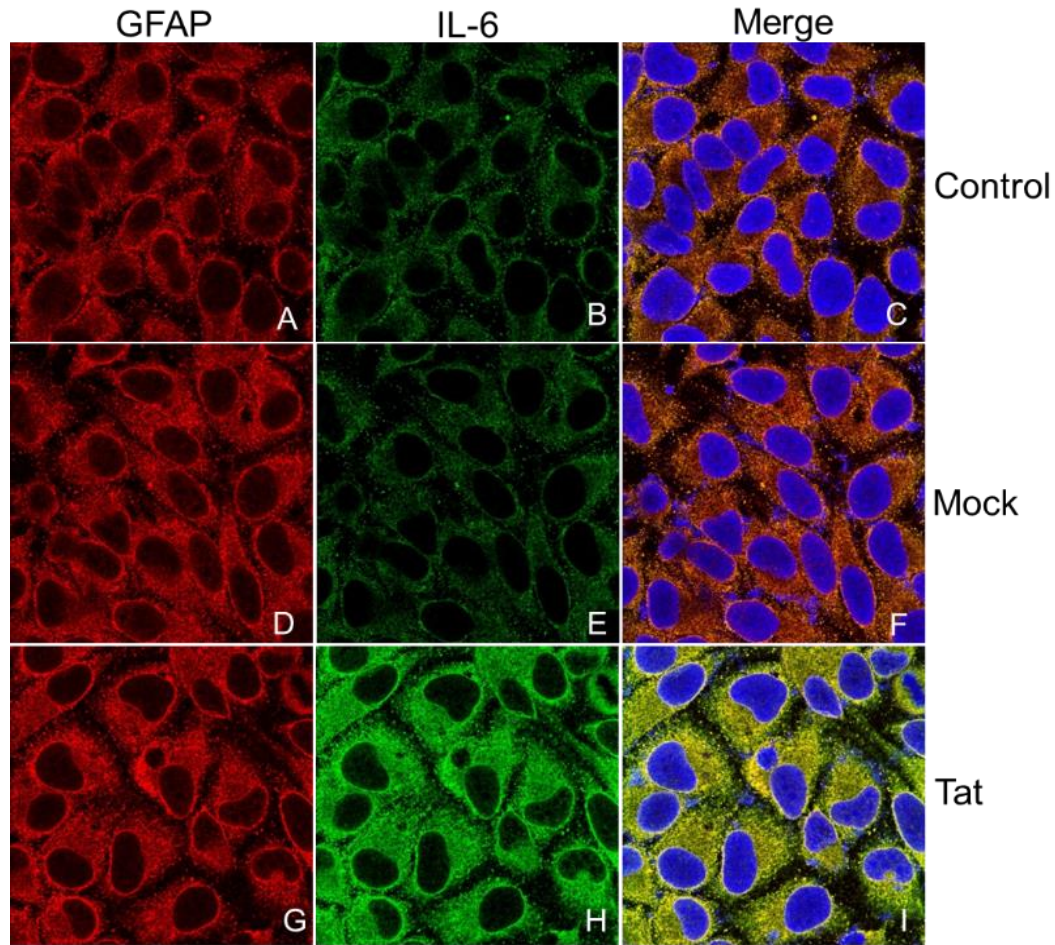


Figure 9: Immunocytochemistry of IL-6 mediated by HIV-1 Tat in astrocytes: (a-i) Five hundred thousand SVG astrocytes were grown on a cover slip and transfected with plasmid encoding HIV-1 Tat. The over expression of IL-6 by HIV-1 Tat (g-i) was compared with control cells (a-c) and mock-transfected cells (d-f). The cells were costained with a mixture of antibodies against GFAP (red) and IL-6 (green). The nucleus was stained blue using 4',6-diamidino-2-phenylindole (DAPI). The images for different fluorophores were obtained using an inverted confocal microscope. The quantification of IL-6 was done using imageJ software (j). Each experiment was done at least in triplicate and each bar represents the \pm SE of three individual experiments. Statistical analyses was performed by Student's t-test and ** denotes P-value of ≤ 0.01 .

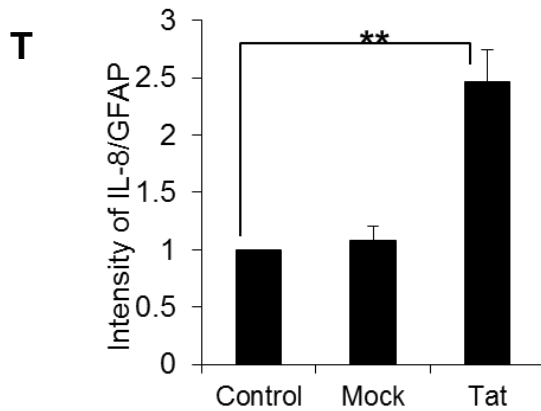
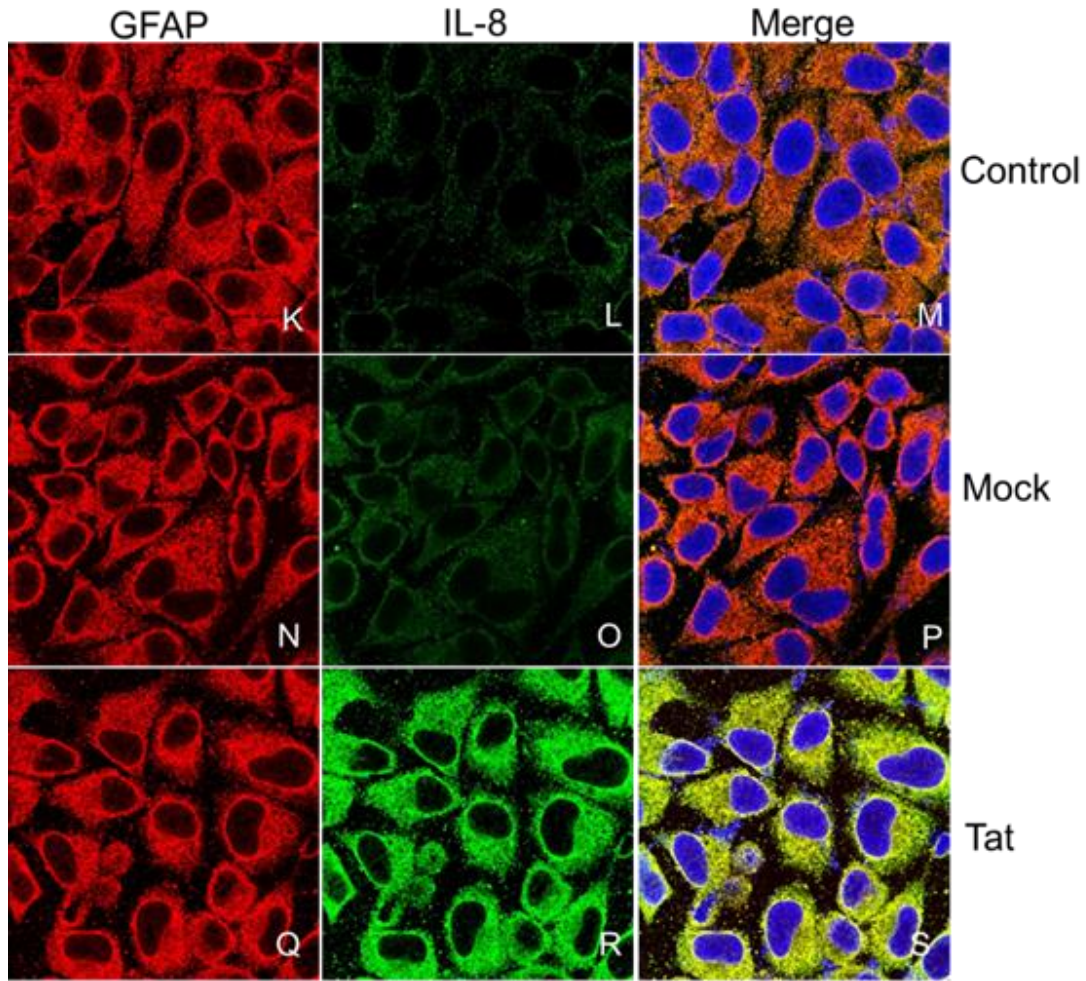


Figure 10: Immunocytochemistry of IL-8 mediated by HIV-1 Tat in astrocytes: (k-s) Five hundred thousand SVG astrocytes were grown on a cover slip and transfected with plasmid encoding HIV-1 Tat. The over expression of IL-6 and IL-8 by HIV-1 Tat (q-s) was compared with control cells (k-m) and mock-transfected cells (n-p). The cells were costained with a mixture of antibodies against GFAP (red) and IL-8 (green). The nucleus was stained blue using 4',6-diamidino-2-phenylindole (DAPI). The images for different fluorophores were obtained using an inverted confocal microscope. The quantification of IL-8 was done using imageJ software (t). Each experiment was done at least in triplicate and each bar represents the \pm SE of three individual experiments. Statistical analyses was performed by Student's t-test and ** denotes P-value of ≤ 0.01 .

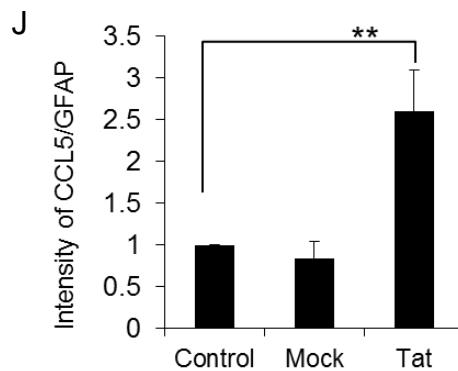
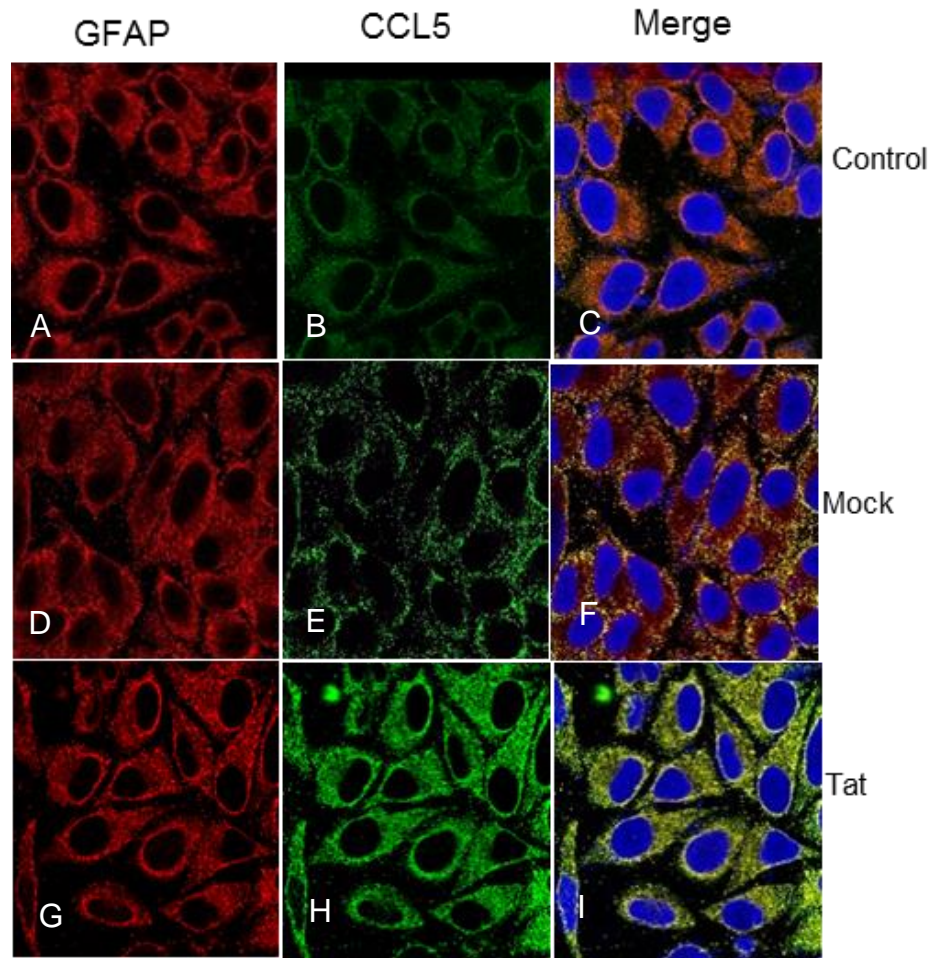


Figure 11: Immunocytochemistry of CCL5 induced by HIV-1 Tat in astrocytes: (A–I) SVGA astrocytes were grown on a cover slip before transfecting with HIV-1 Tat plasmid. Untransfected control (A–C) and mock-transfected cells (D–F) were used to compare the up-regulation of CCL5 in cells transfected with HIV-1 Tat (G–I). The cells were stained with the primary antibodies against CCL5 and GFAP and secondary antibody labeled with Alexafluor 555 (GFAP) and Alexafluor 488 (CCL5). Finally the cover slips were mounted on medium containing DAPI to stain the nucleus. The merge panels represent the co-localization of CCL5 with GFAP. The images were captured using inverted confocal microscope, Leica TCS SP5 II. (J) The intensity of CCL5 over GFAP was calculated using imageJ software. Student's t-test was employed to calculate the significance and ** denotes the p-value ≤ 0.01 .

3.2.7 Activation of NF- κ B by HIV-1 Tat in SVG astrocytes

After determining the up-regulation of IL-6, IL-8 and CCL5 in astrocytes by HIV-1 Tat, we wanted to determine the underlying signal transduction pathway(s). We initially looked at the role of nuclear factor kappa B (NF- κ B) in the up-regulation of HIV-1 Tat mediated IL-6, IL-8 and CCL5 in the astrocytes as these cytokines/chemokines contain binding sites for NF- κ B (Libermann and Baltimore 1990, Kunsch and Rosen 1993). NF- κ B is a master transcription factor involved in many inflammatory processes, including the up-regulation of several pro-inflammatory cytokines/chemokines in several different systems (Shea, Beehler et al. 1996). Canonical activation of NF- κ B occurs through the phosphorylation of I κ B kinase (IKK), generally achieved through the activation of upstream signaling molecules or signals from outside the cell. Activated IKK phosphorylates serine residues located on the regulatory domain of I κ B (I κ B). Phosphorylation of I κ B results in its detachment from NF- κ B complex and is degraded by proteasomes through the process of ubiquitination. The active NF- κ B complex is released and goes into the nucleus to bind to specific binding sites on DNA to turn on the transcription of various genes, including the pro-inflammatory cytokines/chemokines (Lawrence 2009).

Previous reports have indicated the role of NF- κ B in the production of cytokines by HIV-1 Tat in astrocytes and other cells (Nicolini, Ajmone-Cat et al. 2001, Nookala, Shah et al. 2013). In order to confirm the activation of NF- κ B by HIV-1 Tat, we transfected the cells with plasmid encoding HIV-1 Tat and measured the translocation of p65 (indicator of NF- κ B activation) into the nucleus. After confirming the activation of NF- κ B by HIV-1 Tat, we performed time kinetics (3h, 6h, 9h and 12h) to determine the peak increase in p65 translocation. Compared to mock-transfected cells, p65 translocation in HIV-1 Tat-transfected cells started to increase as early as 3 hours, peaked at 9 hours (2.04 ± 0.04 fold) and remained constant until the indicated time point (12 hours) (Fig. 12A). We used GAPDH and LaminB as loading controls for cytoplasmic and nuclear fractions, respectively and the data was

normalized to them. We also confirmed the activation of NF- κ B in primary astrocytes obtained from two different donors by measuring p-I κ B α expression after treatment with HIV-1 Tat protein. The level of p-I κ B α increased as early as 5 minutes and peak level was observed at 20 minutes post-treatment (Fig. 12B). These results demonstrate that HIV-1 Tat induces the activation of NF- κ B transcription in astrocytes.

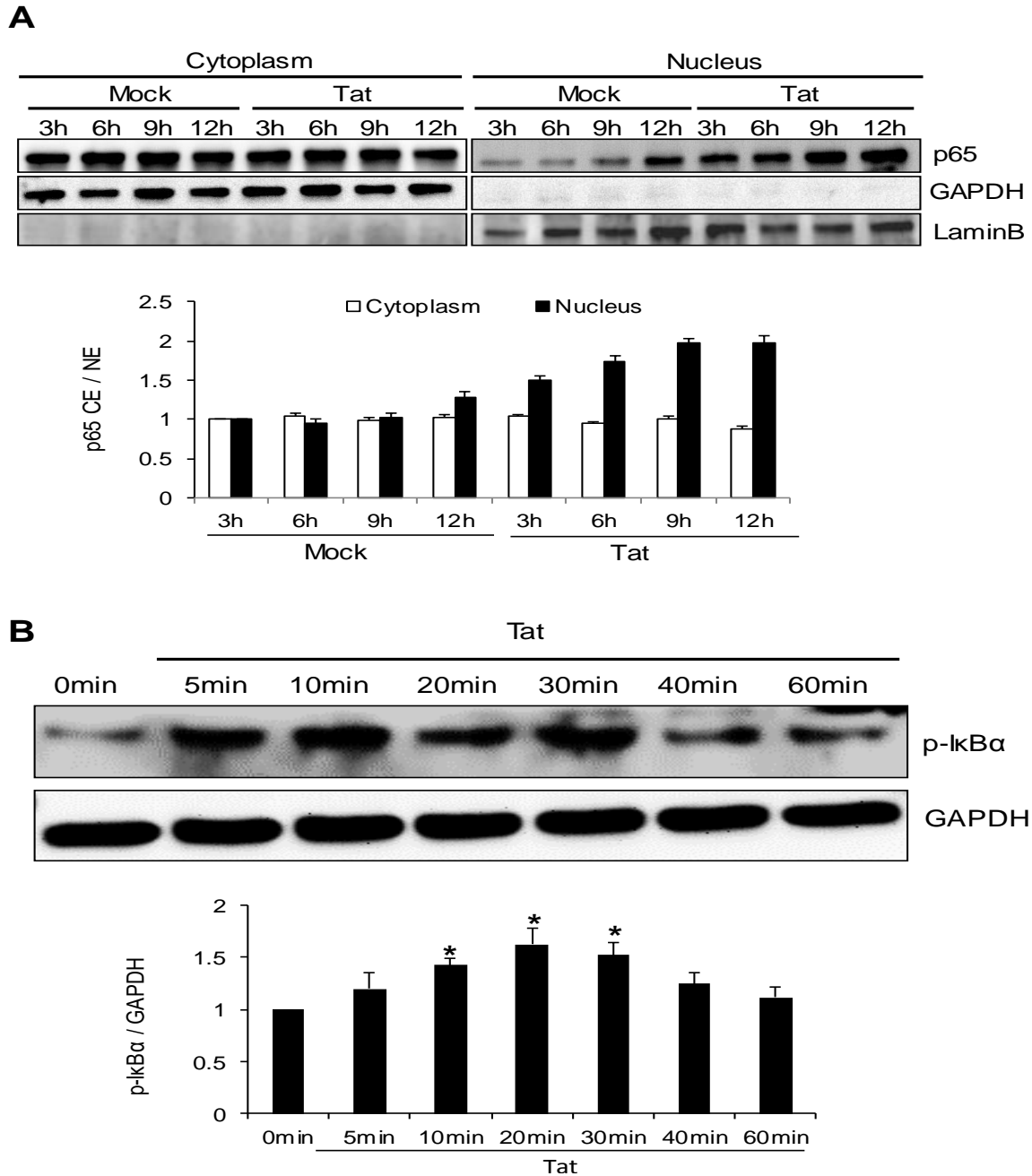


Figure 12: HIV-1 Tat mediated activation of NF- κ B in astrocytes: (A) SVG astrocytes were either mock-transfected or transfected with HIV-1 Tat plasmid and translocation of p65 was measured at 3, 6, 9 and 12 hours. Open bars and closed bars represent cytoplasm and nuclear fractions, respectively. (B) Primary astrocytes were treated with 200 ng/ml Tat protein and p-I κ B α protein levels were measured from 0 minutes to 60 minutes. The bar graph represents the mean values obtained from two independent donors. Each experiment was done at least in triplicate and each bar represents the \pm SE of three individual experiments. Statistical analyses was performed by one-way ANOVA and ** denotes P-value of ≤ 0.01

3.2.8 Involvement of NF- κ B in HIV-Tat mediated up-regulation of IL-6, IL-8 and CCL5 in astrocytes

After establishing that HIV-1 Tat-induced p65 translocation in SVG astrocytes, we wanted to determine if NF- κ B was involved in up-regulation of cytokines/chemokines. To determine this, we employed pharmacological inhibitor approach. We used BAY11-7082 or SC-514, those specifically inhibit I κ B kinase, enzyme responsible for the phosphorylation of I κ B α . The starting dose of the inhibitors was based on IC₅₀ values from previous literature and was further optimized based on their effect on cell viability (data not shown) (Kishore, Sommers et al. 2003, Strickson, Campbell et al. 2013). Cells were pretreated with 10 μ M concentration of specific inhibitory kinase kinase (IKK) inhibitor, BAY11-7082 (IC₅₀=11.2 μ M) or SC-514 (IC₅₀=14.5 μ M), 1 hour prior to transfection with HIV-1 Tat. The expression levels of IL-6, IL-8 and CCL5 were determined at 6 hours and 48 hours post transfection for mRNA and protein respectively. BAY11-7082 decreased the expression of IL-6 by 47.1 \pm 6.1% and 63.3 \pm 4.7% at mRNA and protein levels respectively (Fig. 13A, B). Similarly, the expression levels of IL-8 were decreased by 41.1 \pm 7.5% and 47.4 \pm 4.7% at the levels of mRNA and protein respectively (Fig. 13C, D). Furthermore, use of 10 μ M concentration of SC-514 decreased the expression of CCL5 by 46.6 \pm 14.2% at the level of mRNA and by 47.7 \pm 11.9% at the level of protein, respectively (Fig. 14E, F).

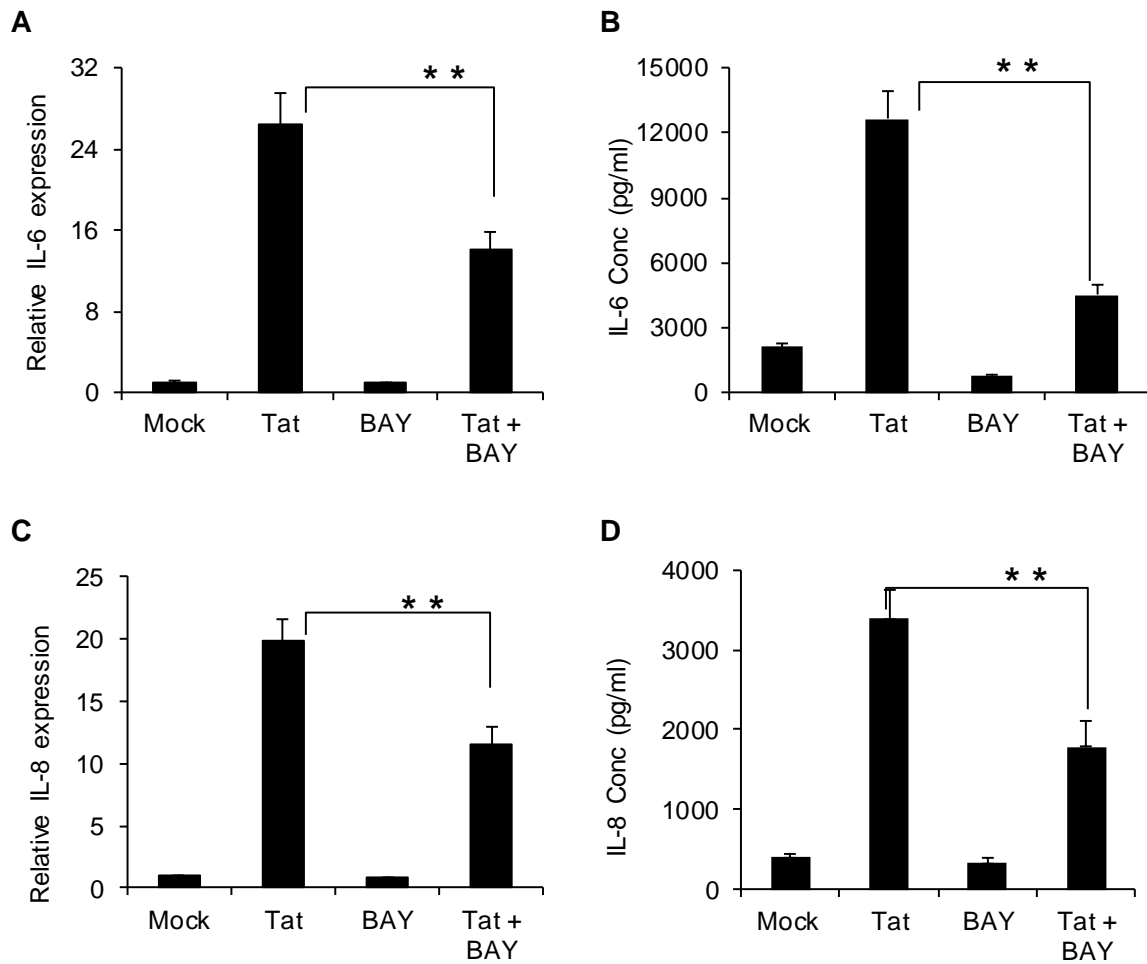


Figure 13: Involvement of NF- κ B in HIV-Tat mediated up-regulation of IL-6 and IL-8: SVG astrocytes were pretreated with 10 μ M concentration of NF- κ B inhibitor (BAY11-7082) 1 hour prior to the transfection. The expressions of IL-6 and IL-8 were determined at 6 hours and 48 hours post transfection for mRNA (**A, C**) and protein (**B, D**), respectively. The values represented are normalized their mock-transfected controls. Each experiment was done at least in triplicate and each bar represents the \pm SE of three individual experiments. Statistical analyses was performed by one-way ANOVA and ** denotes P -value of ≤ 0.01 .

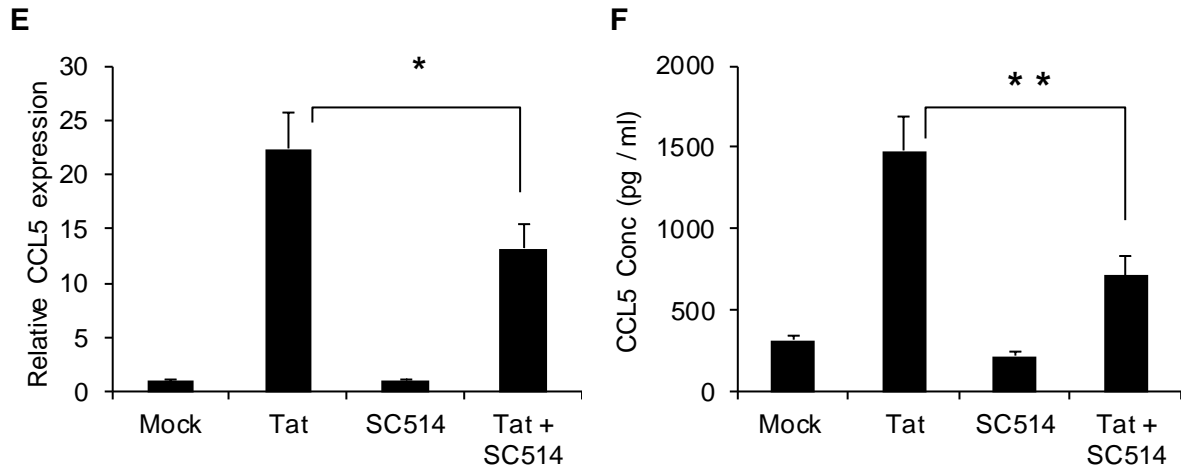


Figure 14: Involvement of NF-κB in HIV-1 Tat mediated production of CCL5 from astrocytes: (E, F) SVG astrocytes were treated with 10 μM of NF-κB inhibitor (SC514) prior to transfection with plasmid encoding HIV-1 Tat. The expression of CCL5 at the mRNA and protein levels were measured at 6 h and 48 h post-transfection by using RT-PCR (E) and multiplex cytokine assay (F), respectively. The values represented for mRNA are expressed relative to the mock-transfected controls. Each experiment was performed in triplicate and each bar in the figure represents the mean ± SE of at least three individual experiments. One-way ANOVA was used to perform the statistical analysis and ** denotes p-value ≤0.01 and * denotes p-value of ≤0.05.

To confirm the results of the pharmacological inhibitor, we employed siRNA approach to individually knock down major subunits of NF-κB which include p50 (NF-κB1) and p65 (RelA) subunits. 50 nmoles of siRNA was used to knock down the individual subunits and the efficiency of knock down was previously verified (Gangwani, Noel et al. 2013). Our results show that p65, but not p50 knock down decreased the expression of IL-6 by $40.8 \pm 3.8\%$ and $48.2 \pm 5.8\%$ at mRNA and protein levels respectively (Fig. 15A, B). Similar to the inhibitor results, knock down of p65 decreased the expression levels of IL-8 by $60.1 \pm 4.7\%$ at the level of mRNA and by $69.3 \pm 2.6\%$ at the level of protein (Fig. 15C, D). Knock down of the p50 subunit of NF-κB did not affect the expression levels of IL-6 and IL-8 at either level of mRNA or protein. Knock down of p50 subunit of NF-κB did not have any effect on the expression

levels of IL-6, but has increased the expression levels of IL-8 at mRNA and protein. However, knock down of p65 and p50 subunits have partially decreased the expression levels of CCL5 at both mRNA and protein levels. Knock down of p65 has decreased the expression of CCL5 by $42.8 \pm 8.3\%$ at mRNA and $48.9 \pm 6.07\%$ at protein level respectively. Similarly, knock down of p50 subunit has decreased the expression of CCL5 by $69.8 \pm 10.5\%$ at mRNA and $68.9 \pm 4.86\%$ at protein level, respectively (Fig. 15E, F). The results of siRNA were in accordance with the inhibitor results and show that NF- κ B played an important role in the up-regulation of cytokines/chemokines mediated by HIV-1 Tat in astrocytes.

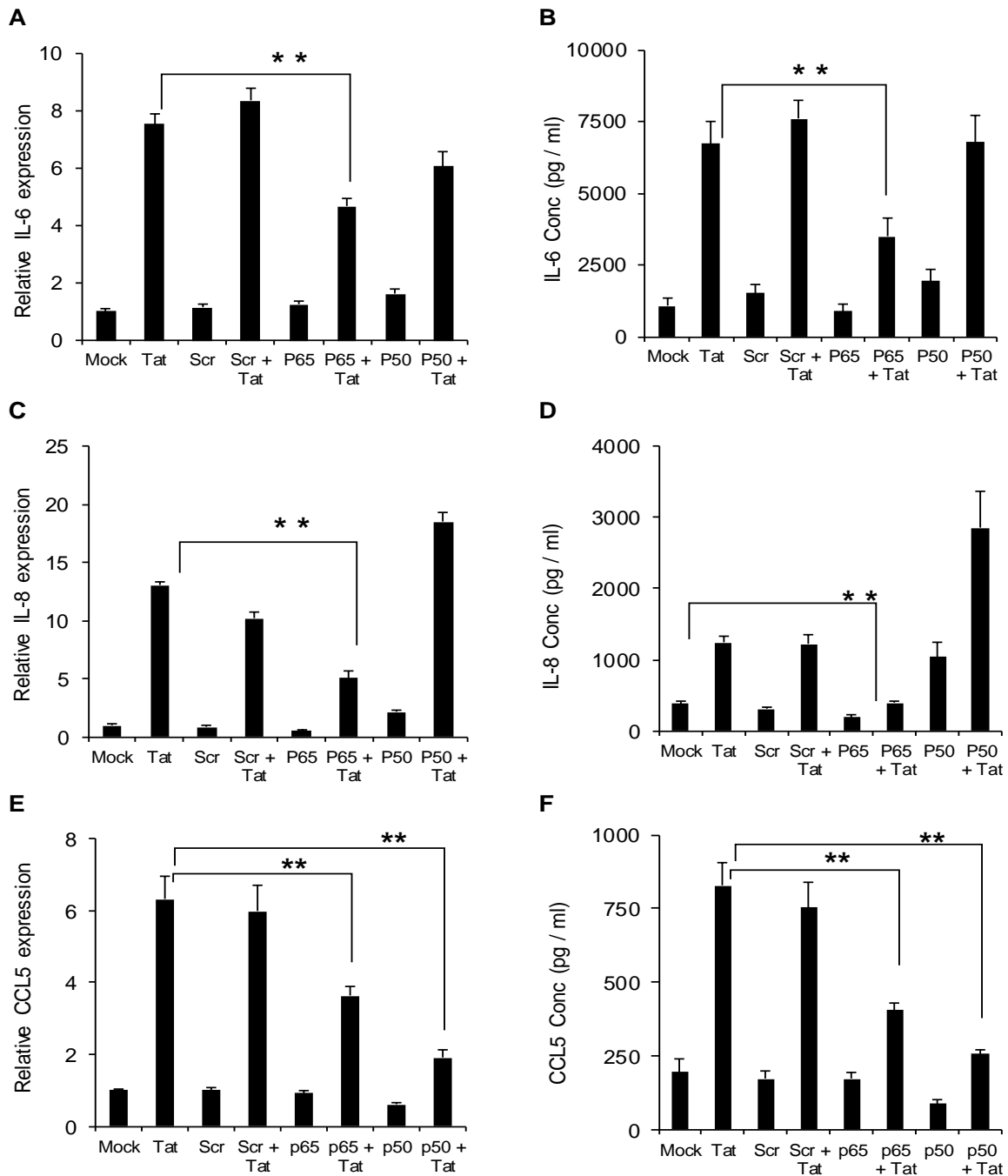


Figure 15: Involvement of NF- κ B in HIV-Tat mediated up-regulation of cytokines/chemokines: cells were transfected with the siRNA followed by Tat transfection as mentioned in the Materials and Methods. The expression of IL-6, IL-8 and CCL5 was determined at 6 hours and 48 hours post transfection for mRNA (**A**, **C**, **E**) and protein (**B**, **D**, **E**), respectively. The values represented for mRNA are expressed relative to the mock-transfected controls. Each experiment was done at least in triplicate and each bar represents the \pm SE of three individual experiments. Statistical analyses was performed by one-way ANOVA and ** denotes P -value of ≤ 0.01 .

We also measured the translocation of p65 into the nucleus upon pretreatment with BAY11-7082. The translocation of p65 decreased by $32 \pm 3.3\%$ in BAY11-7082 pretreated cells compared to the HIV-1 Tat-transfected cells (Fig. 16A).

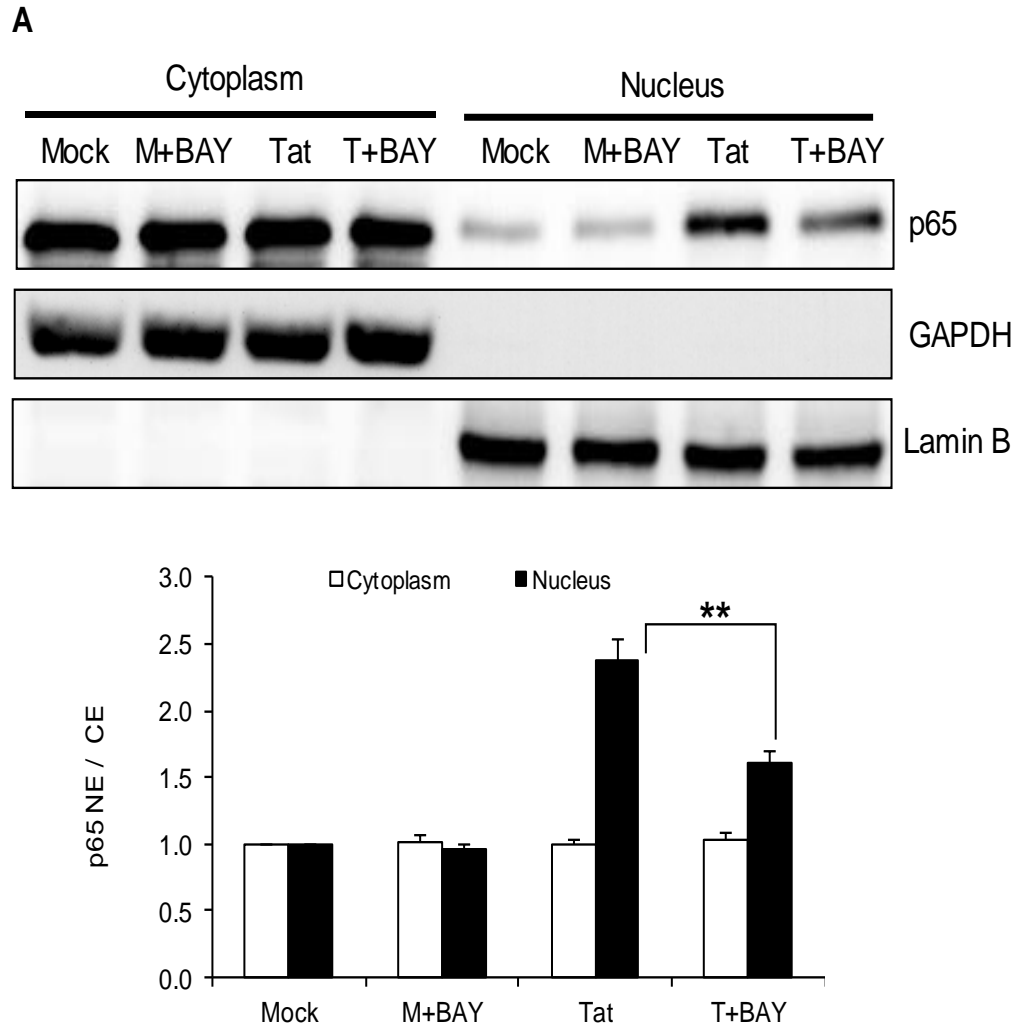


Figure 16: Involvement of NF- κ B in HIV-1 Tat mediated induction of cytokines/chemokines: Astrocytes were either mock-transfected or transfected with HIV-1 Tat plasmid for a duration of 6 hours and translocation of p65 was measured. Each experiment was done at least in triplicate and each bar represents the \pm SE of three individual experiments. Statistical analyses was performed by one-way ANOVA and ** denotes P -value of ≤ 0.01 .

3.2.9 Involvement of MAPK Pathway in Induction of cytokines/chemokines by HIV-1 Tat

After determining the role of NF- κ B, we wanted to explore the upstream signaling pathways involved in the activation of NF- κ B. Various Mitogen-activated protein kinases (MAPKs) such as p38 MAPK, ERK MAPK and JNK MAPK are known to activate NF- κ B as reported previously (Craig, Larkin et al. 2000). We, therefore, used specific pharmacological antagonists against p38 MAPK (SB203580), JNK MAPK (SP600125) and ERK MAPK (U0126) to verify their involvement.

3.2.10 Role of p38 MAPK molecules in the Induction of cytokines/chemokines by HIV-1 Tat.

p38 mitogen activated-protein kinase (p38 MAPK) is a family of important upstream MAPKs that can activate NF- κ B and regulate the expression of many cytokine/chemokines. To determine the role of p38 MAPK in the up-regulation of IL-6, IL-8 and CCL5 by HIV-1 Tat, we used SB203580, a specific inhibitor of p38 MAPK. The starting dose of the inhibitor was based on IC₅₀ values from previous literature and was further optimized based on its effect on cell viability (data not shown) (Warny, Keates et al. 2000). Pretreatment of astrocytes with 10 μ M concentration of SB203580 decreased the expression levels of IL-6 by $47.8 \pm 4.1\%$ and $82.4 \pm 0.5\%$ at levels of mRNA and protein respectively (Fig. 17A, B). Similarly, pretreatment with SB203580 decreased the expression levels of IL-8 by $43.2 \pm 5.6\%$ and $66.1 \pm 5.8\%$ at mRNA and protein levels (Fig. 17C, D). However, pretreatment of astrocytes with SB203580 did not affect the expression levels of CCL5 at both mRNA and protein levels (Fig. 17E, F).

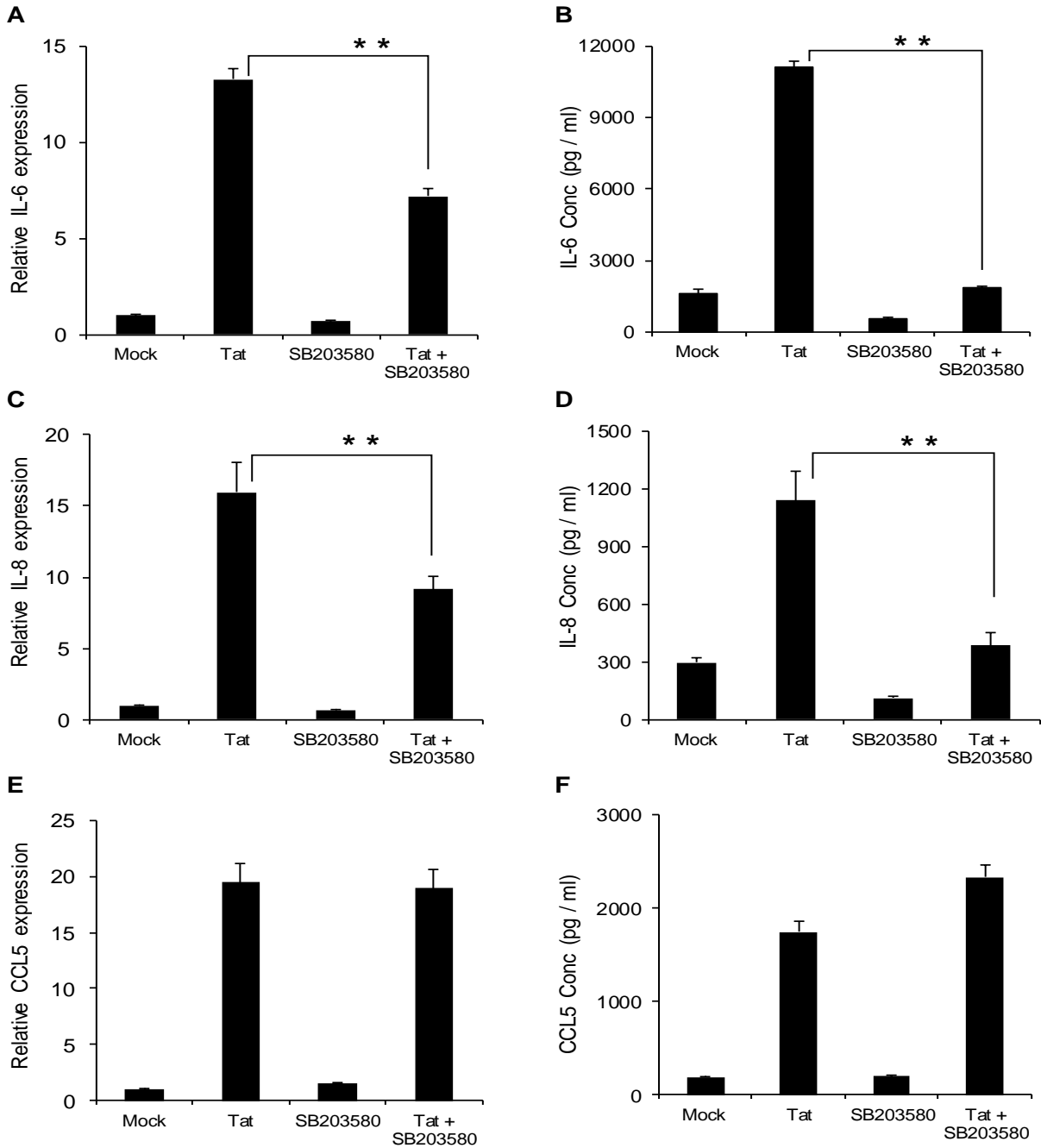


Figure 17: Inhibition of HIV-1 Tat-induced expression of IL-6 and IL-8 but not CCL5 by inhibitor of p38 mitogen activated-protein kinase (MAPK): (A-F) Astrocytes were pretreated with 10 μ M concentration of p38 MAPK inhibitor (SB203580) 1 hour prior to the transfection. (A, C, E) The expression levels of IL-6, IL-8 and CCL5 at mRNA were determined by real time RT-PCR at 6 hours post transfection. The values represented are normalized to their mock-transfected controls. (B, D, F) The protein concentrations of IL-6, IL-8 and CCL5 were determined in the cell culture supernatants at 48 hours post transfection by multiplex cytokine assay. Each experiment was done at least in triplicate and each bar represents the \pm SE of three individual experiments. Statistical analyses was performed by one-way ANOVA and ** denotes P -value of ≤ 0.01 .

The involvement of p38 MAPK was further confirmed by the fact that HIV-1 Tat-mediated increase in phosphorylated p38 levels were decreased by pretreatment with SB203580 (Fig. 18A).

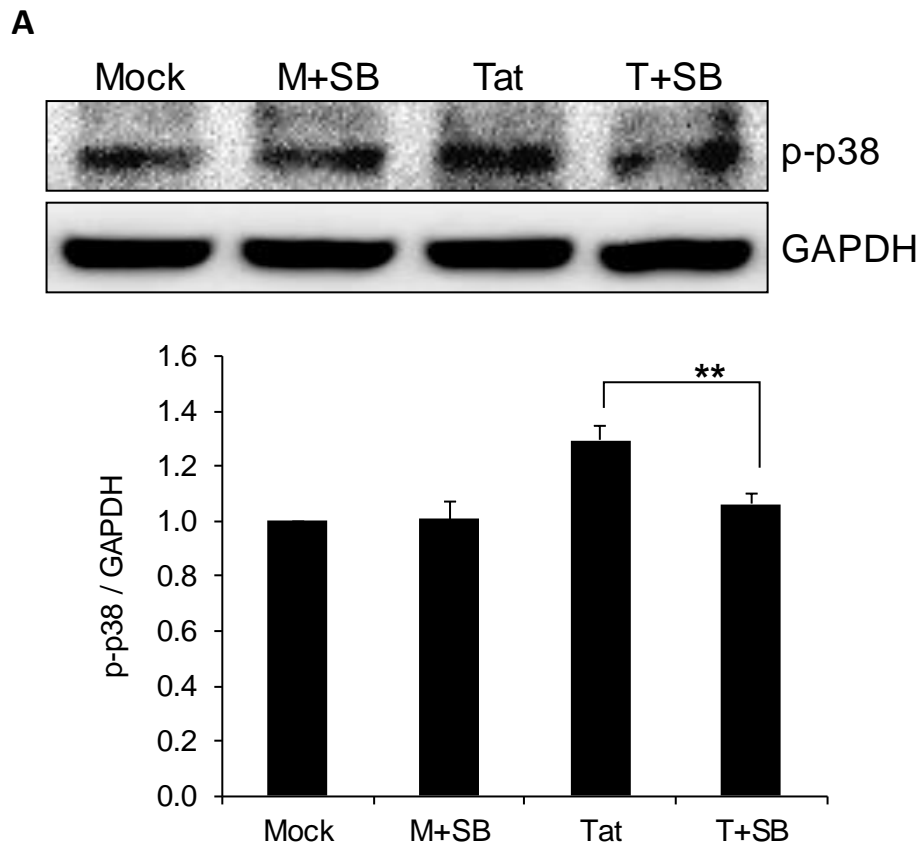


Figure 18: HIV-1 Tat mediated phosphorylation of p38 is inhibited by SB203580: Astrocytes were either mock-transfected or transfected with HIV-1 Tat plasmid for a duration of 6 hours and p-p38 levels were measured in whole cell extracts. A representative Western blot is shown in the figure. Each experiment was done at least in triplicate and each bar represents the \pm SE of three individual experiments. Statistical analyses was performed by one-way ANOVA and ** denotes p -value of ≤ 0.01 .

p38 MAPK exists in four different isoforms ($\alpha/\beta/\gamma/\delta$) of which SB203580 affects only p38 α and p38 β isoforms of p38 but not the other two isoforms (p38 γ and p38 δ) (Lee, Kassis et al. 1999). In order to validate the roles of p38 α and p38 β and also to determine the roles of

the other two isoforms in the up-regulation of IL-6, IL-8 and CCL5, we individually knocked down all the isoforms of p38 ($\alpha/\beta/\gamma/\delta$) using siRNA. The gene knock down was generally partial (>60%) except in the case of p38 β where silencing was complete (Gangwani, Noel et al. 2013, Liu, Shah et al. 2014). Knocking down p38 β and p38 δ isoforms decreased the expression of IL-6. Specifically, knock down of p38 β decreased the expression of IL-6 by $42.6 \pm 5.6\%$ and $41.4 \pm 4.9\%$ at the levels of mRNA and protein, respectively (Fig. 19A, B). Further, p38 δ knock down decreased the expression of IL-6 by $49.7 \pm 3.5\%$ and $43.8 \pm 4.3\%$ at mRNA and protein levels respectively (Fig. 19C, D). Individual knock down of only p38 β decreased the expression level of IL-8 by $47.1 \pm 6.4\%$ and $36.2 \pm 6.4\%$ at the level of mRNA and protein respectively (Fig. 19E, F). As expected with the inhibitor results, knock down of p38 α and p38 β and p38 γ did not affect the expression levels of CCL5 at the level of both mRNA and protein. Surprisingly, knocking down p38 δ decreased the expression of CCL5 by $56.1 \pm 5.5\%$ at the level of mRNA and by $43.26 \pm 2.21\%$ at the level of protein.

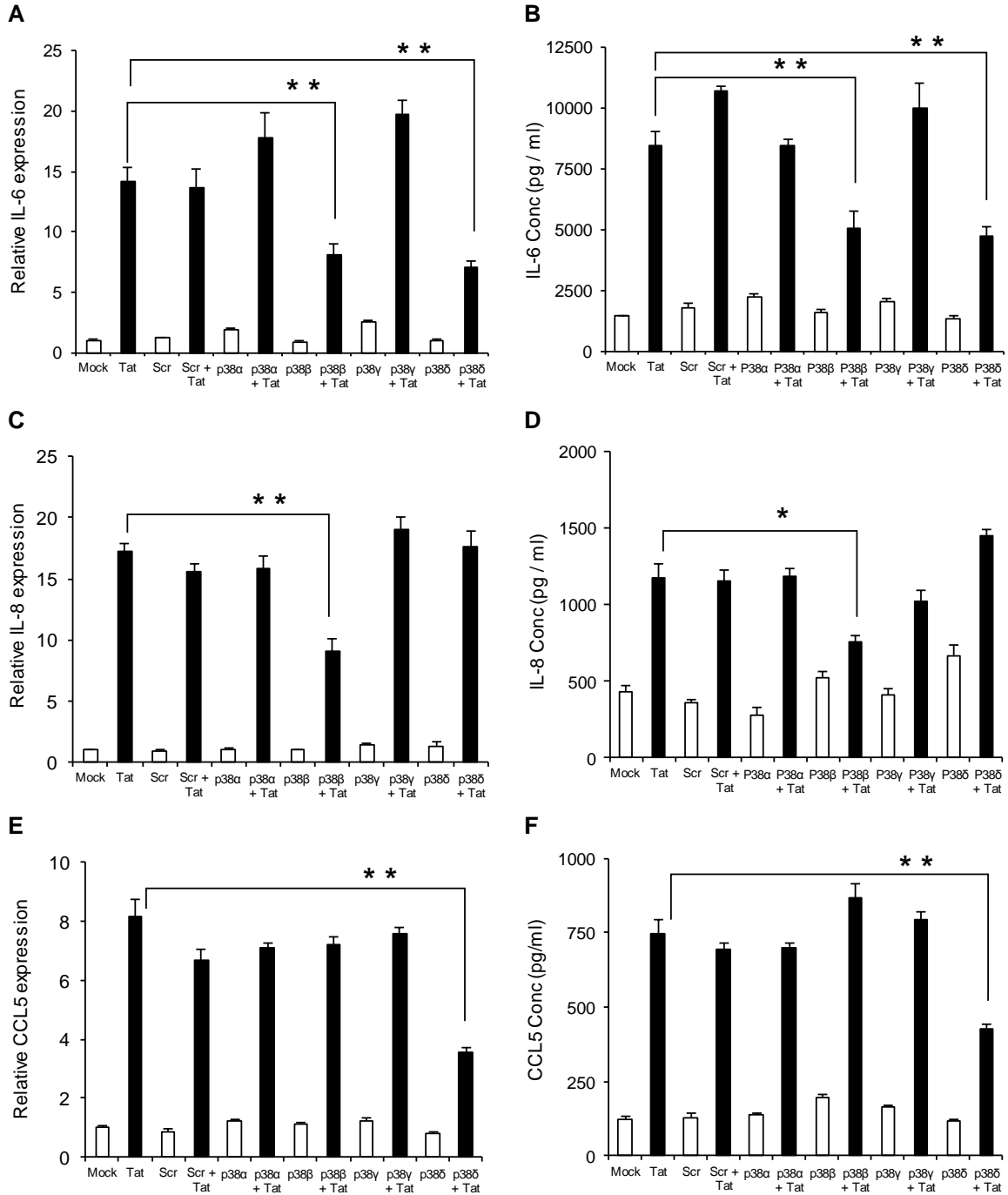


Figure 19: Involvement of p38 mitogen activated-protein kinase (MAPK) in the induction of IL-6, IL-8 and CCL5 by HIV-1 Tat. (A-F) SVG astrocytes were transfected with siRNA (scrambled or p38α or p38β or p38γ or p38δ) for 48 hours. Then they were mock-transfected or transfected with plasmid encoding HIV-1 Tat. (A, C, E) The expression levels of IL-6, IL-8 and CCL5 at mRNA were determined by real time RT-PCR at 6 hours post transfection. The values represented are normalized to their mock-transfected controls. (B, D, F) The protein concentrations of IL-6, IL-8 and CCL5 were determined in the cell culture supernatants at 48 hours post transfection by multiplex cytokine assay. Each experiment was done at least in triplicate and each bar represents the ± SE of three individual experiments. Statistical analyses was performed by one-way ANOVA and ** denotes p-value of ≤ 0.01.

Of all the p38 isoforms, p38 α and p38 β are known to activate NF- κ B. To see if p38 MAPK was leading to the activation of NF- κ B, we pretreated the astrocytes with SB203580 and measured p65 translocation into the nucleus. Pretreatment with SB203580 decreased the translocation of p65 by 33.9 \pm 4.6% compared to HIV-1 Tat-transfected astrocytes without the inhibitor (Fig. 20A). To specifically determine if p38 β isoform led to the activation of NF- κ B in our model system, we individually knocked down p38 β isoform with siRNA and measured the translocation of p65 into the nucleus. Knocking down p38 β isoform decreased p65 translocation by 28.5% \pm 3.6% compared to HIV-1 Tat- transfected cells without inhibitor (Fig. 21A).

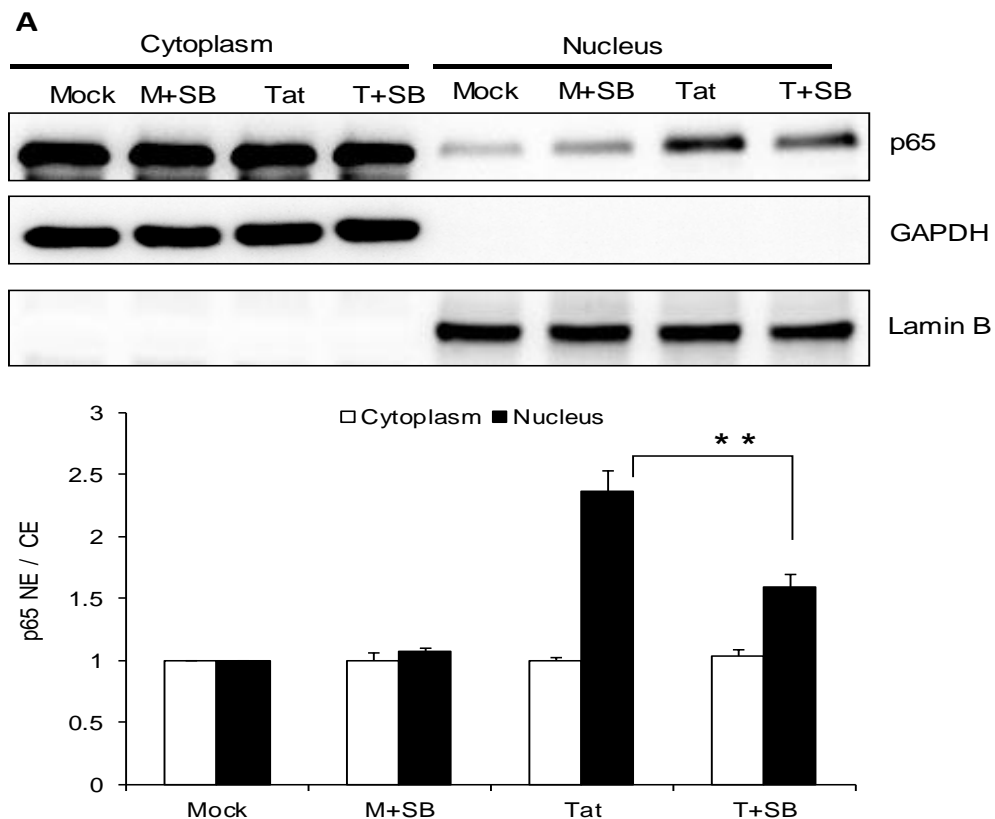


Figure 20: HIV-1 Tat mediated activation of NF- κ B involves p38 MAPK: (A) Astrocytes were either mock-transfected or transfected with HIV-1 Tat plasmid for duration of 6 hours and translocation of p65 was measured. A representative Western blot is shown in the figure. Each experiment was done at least in triplicate and each bar represents the \pm SE of three individual experiments. Statistical analyses was performed by one-way ANOVA and ** denotes P -value of ≤ 0.01 .

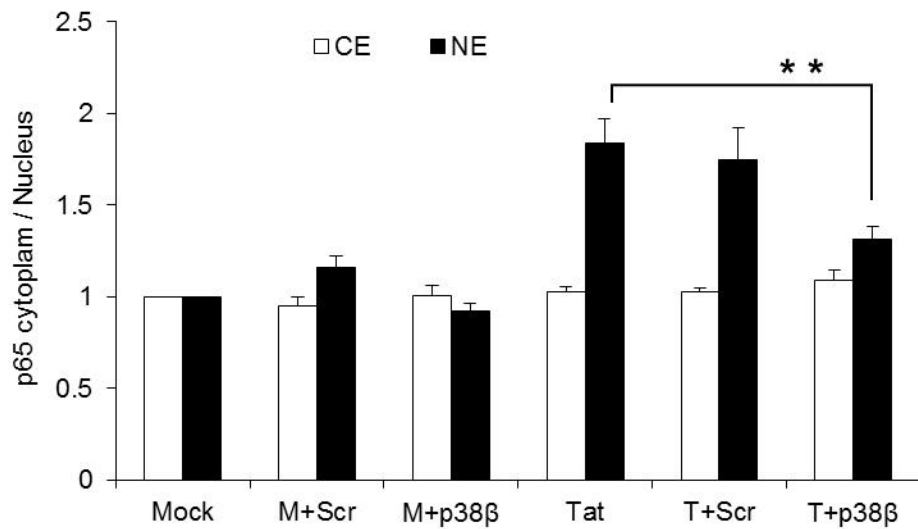
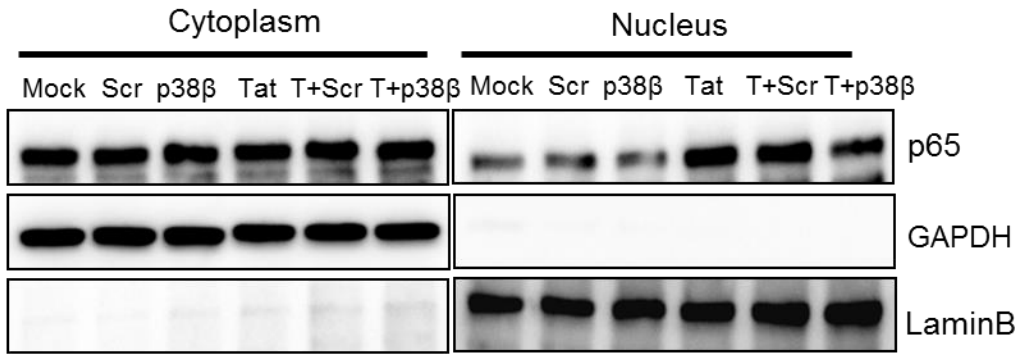


Figure 21: HIV-1 Tat mediated activation of NF- κ B involves p38 MAPK: (A) Astrocytes were transfected with p38 β siRNA for 48 hours prior to transfection with HIV-1 Tat plasmid. The translocation of p65 is measured 6 hours after transfection. Open bars and closed bars represent cytoplasm and nuclear fractions, respectively. A representative Western blot is shown in the figure. Each experiment was done at least in triplicate and each bar represents the \pm SE of three individual experiments. Statistical analyses was performed by one-way ANOVA and ** denotes p -value of ≤ 0.01 .

3.2.11 HIV-1 Tat-mediated induction of cytokines/chemokines involves multiple transcription factors activated by different upstream signaling molecules

In view of our results that p38 δ knock down decreased IL-6 and CCL5 expression; we wanted to determine the possible transcription factors that could be activated by p38 δ . Activator protein-1 (AP-1) and CCAT enhancer binding proteins (C/CAT), are known to be activated by p38 δ (Efimova, Broome et al. 2003). There are 6 isoforms of CCAT/enhancer binding protein (C/EBP) known in the literature of which p38 δ can specifically lead to the activation of α and γ isoforms of C/EBP (Lekstrom-Himes and Xanthopoulos 1998). To ascertain the involvement of these transcription factors in the up-regulation of cytokines/chemokines by HIV-1 Tat, we individually knocked them down using specific siRNA. The expressions of cytokines/chemokines at the mRNA and protein levels were measured 6 h and 48 h post-transfection, respectively. We used scrambled (Scr) siRNA as negative control. AP-1 (c-jun component) knock down by siRNA decreased the IL-6 expression by $43.2 \pm 3.4\%$ and $51.2 \pm 6.3\%$ at mRNA and protein level, respectively (Fig. 22A, B). The knock down of C/EBP α or C/EBP γ did not affect the expression levels of IL-6 at either level of mRNA or protein. Similar to IL-6, the expression of IL-8 was not affected by the knock down of C/EBP α or C/EBP γ . Even though knock down of p38 δ did not affect the expression level of IL-8, surprisingly, knocking down AP-1 transcription factor decreased the levels of IL-8 by $42.1 \pm 8.4\%$ and $44.3 \pm 3.2\%$ at the levels of mRNA and protein, respectively (Fig. 22C, D). Knock down of all the transcription factors have affected the expression levels of CCL5. The C/EBP α knock down declined CCL5 production at mRNA and protein levels by $44.8 \pm 4.1\%$ and $30.1 \pm 5.9\%$, respectively. Knockdown of C/EBP γ and AP-1 also decreased CCL5 production at comparable level. Knockdown of C/EBP γ has decreased the expression of CCL5 by 48% and 32% at the level of mRNA and protein, respectively. Similarly, knockdown of AP-1 has decreased the expression of CCL5 by 45.3% at the level of mRNA and by 39.6% at the level of protein (Fig. 22E, F).

In all these experiments, the Scr siRNA showed slight decrease in CCL5 expression at RNA level but the change was statistically insignificant. Furthermore, the Scr siRNA did not show any effect on CCL5 expression at protein level suggesting specificity of the effects obtained by using C/EBP α , C/EBP γ and AP-1 siRNA.

We have also confirmed the involvement of p38 δ isoform in the activation of AP-1 by performing a western blot. We transfected SVG astrocytes with p38 δ siRNA for 48 hours. These cells were either mock transfected or transfected with plasmid coding HIV-1 Tat. Whole cell lysates were prepared and p-c-jun levels were measured 6 hours post-transfection. HIV-1 Tat mediated phosphorylation of c-jun has decreased by 30% upon knock down of p38 δ (Fig. 23A).

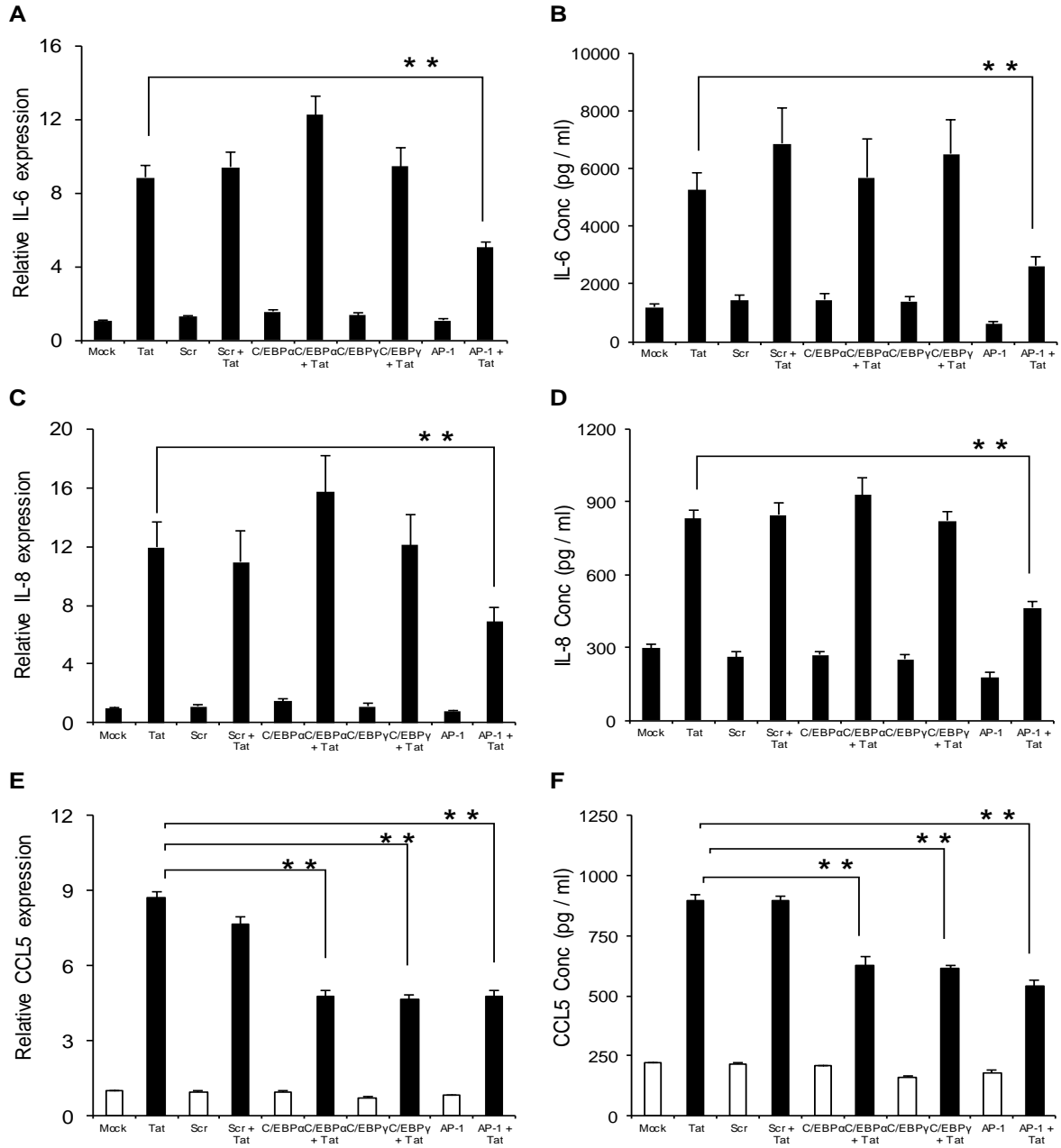


Figure 22: HIV-1 Tat-mediated expression of cytokines/chemokines involves C/EBP α , C/EBP γ and activator protein-1 (AP-1) transcription factors: (A-F) Astrocytes were transfected with either scrambled or C/EBP α or C/EBP γ or AP-1 siRNA for a duration of 48 hours, followed by either mock transfection or transfection with HIV-1 Tat plasmid. (A, C, E) The expression levels of IL-6, IL-8 and CCL5 at mRNA were determined by real time RT-PCR at 6 hours post transfection. The values represented are normalized to their mock-transfected controls. (B, D, F) Multiplex cytokine assay was employed to measure the protein concentrations of IL-6, IL-8 and CCL5 in the cell culture supernatants at 48 hours post transfection. Each experiment was done at least in triplicate and each bar represents the \pm SE of three individual experiments. Statistical analyses was performed by one-way ANOVA and ** denotes P -value of ≤ 0.01

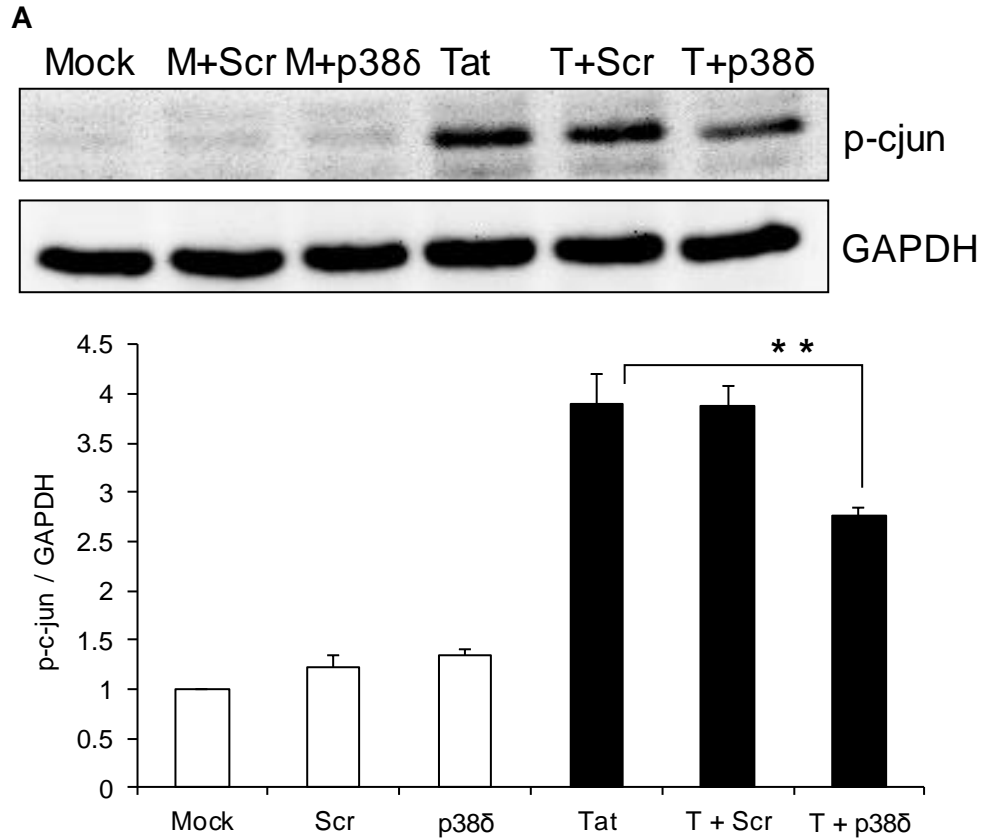


Figure 23: Involvement of p38 mitogen activated-protein kinase (MAPK) delta isoform in the induction of cytokines/chemokines by HIV-1 Tat: (A) Astrocytes were transfected with p38δ siRNA for 48 hours, followed by transfection with HIV-1 Tat plasmid. The levels of p-c-jun were measured after 6 hours of transfection. A representative Western blot is shown in the panel. Each experiment was done at least in triplicate and each bar represents the \pm SE of three individual experiments. Statistical analyses was performed by one-way ANOVA and ** denotes p-value of ≤ 0.01 .

3.2.12 Role of JNK MAPK signaling molecule in the Induction of cytokines/chemokines by HIV-1 Tat

In view of the IL-8 expression results obtained from knock down of AP-1, we wanted to explore the role of other upstream MAPKs that can lead to its activation. C-Jun N-terminal kinase (JNK) is another upstream signaling molecule that can activate AP-1. To determine the role of JNK in the up-regulation of IL-8 and other cytokines in astrocytes by HIV-1 Tat, we pretreated cells with a specific JNK inhibitor (SP600125) and measured the levels of cytokines/chemokines at 6 hours and 48 hours post transfection for mRNA and protein, respectively. The starting dose of the inhibitor was based on IC₅₀ values from previous literature and was further optimized based on its effect on cell viability (data not shown) (Hsieh, Wang et al. 2010). Pretreatment of astrocytes with 10 μ M concentration of SP600125 did not affect the expression levels of either IL-6 or CCL5 (Fig. 24A, C, E, F). However, pretreatment of astrocytes with SP600125 significantly affected the expression of IL-8. The expression of IL-8 was decreased by $42.4 \pm 9.0\%$ at the level of mRNA and by $58.3 \pm 7.0\%$ at the level of protein (Fig. 24C, D).

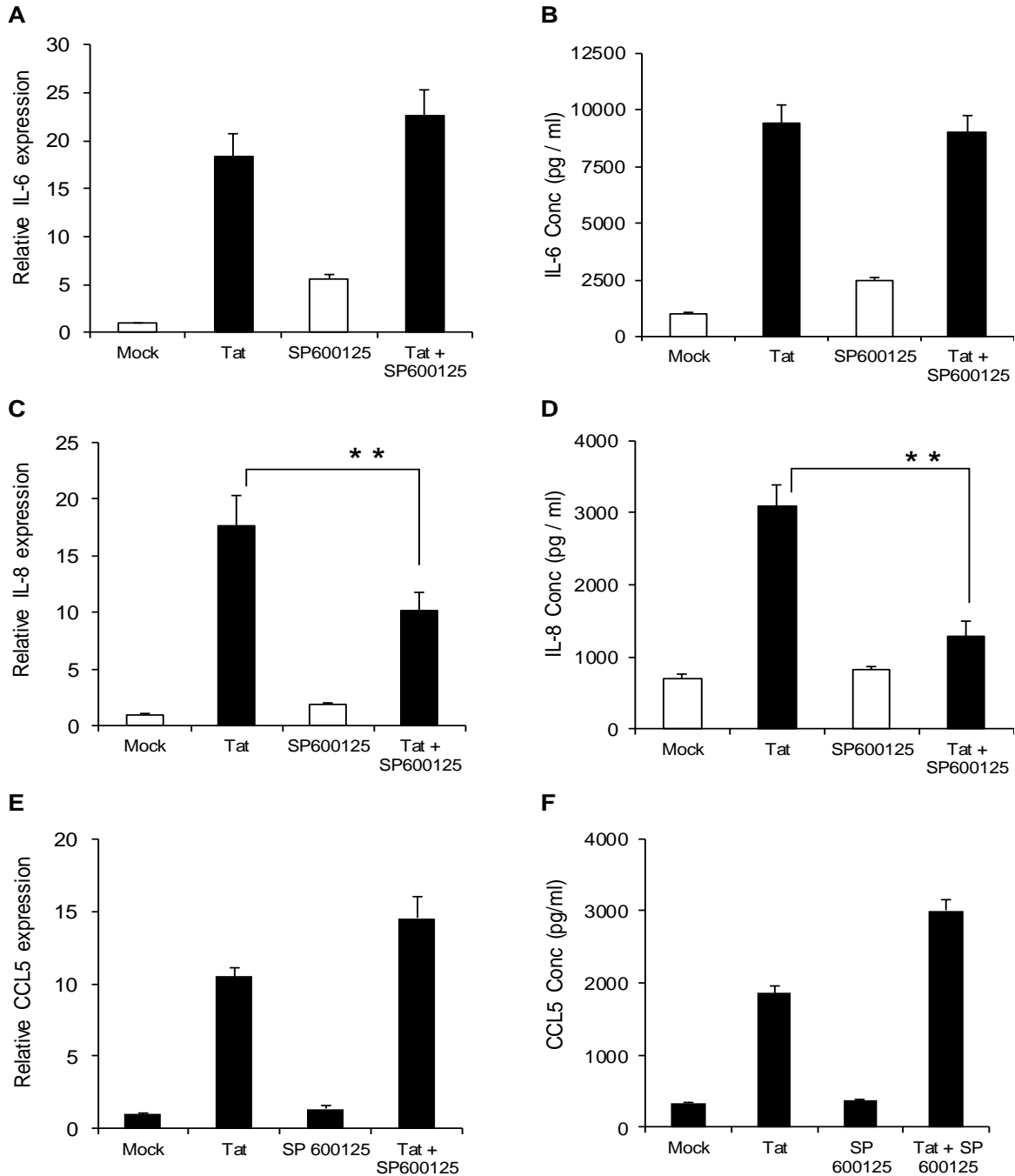


Figure 24: Involvement of C-Jun N-terminal kinase/mitogen-activated protein kinase (JNK MAPK) in HIV-1 Tat-mediated expression of IL-8. (A-F) SVG astrocytes were pretreated with JNK MAPK inhibitor (SP600125) for 1 hour prior to transfection. **(A, C, E)** The expression levels of IL-6, IL-8 and CCL5 at mRNA level were determined at 6 hours post transfection by real time RT-PCR. The values represented are normalized to their mock- transfected controls. **(B, D, E)** IL-6, IL-8 and CCL5 protein concentrations in the cell culture supernatants at 48 hours post transfection were determined by multiplex cytokine assay. Each experiment was done at least in triplicate and each bar represents the \pm SE of three individual experiments. Statistical analyses was performed by one-way ANOVA and ** denotes P -value of ≤ 0.01 .

To verify the activation of JNK, we measured the phosphorylation of JNK by western blot. Pretreatment with SP600125 decreased the HIV-1 Tat-mediated increase in phosphorylated JNK from 1.25 fold to 1 fold (Fig. 25A).

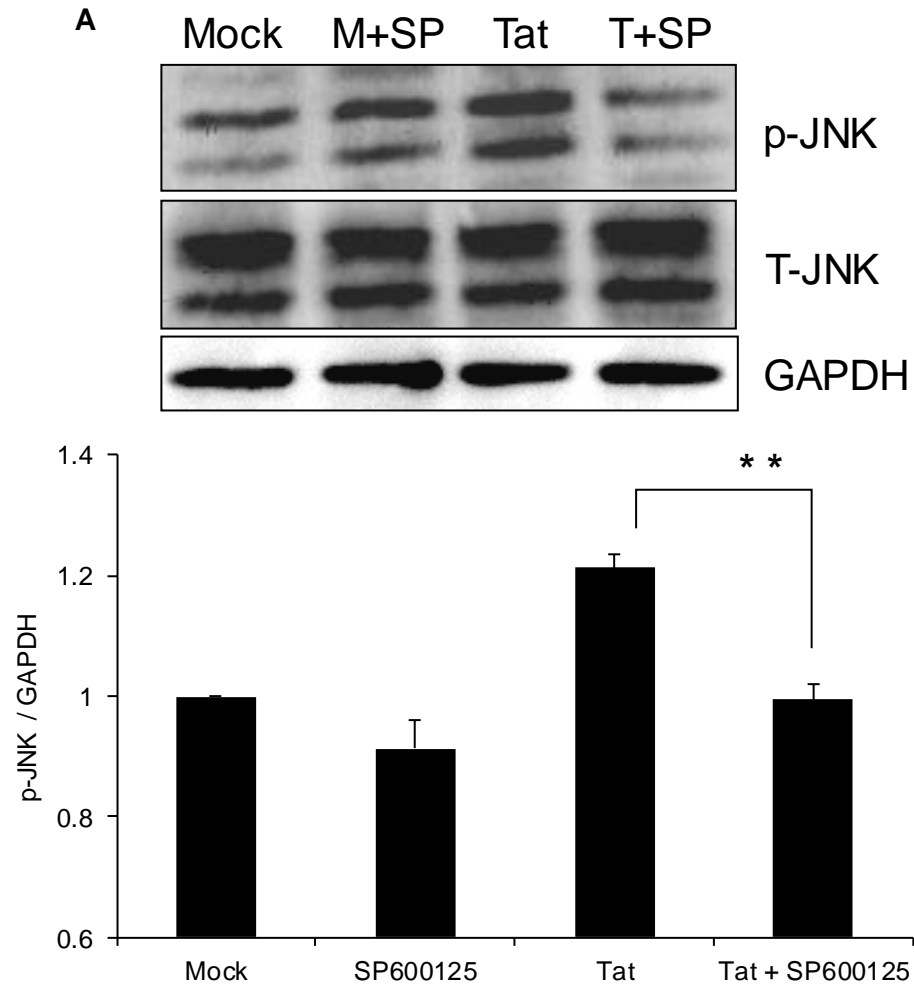


Figure 25: Involvement of JNK MAPK in HIV-1 Tat mediated induction of IL-8: Astrocytes were either mock-transfected or transfected with HIV-1 Tat plasmid for duration of 6 hours and p-JNK and JNK were measured in whole cell extracts. A representative Western blot is shown in the panel. Each experiment was done at least in triplicate and each bar represents the \pm SE of three individual experiments. Statistical analyses was performed by one-way ANOVA and ** denotes *P*-value of ≤ 0.01 .

Next, to determine whether JNK MAPK can lead to the activation of AP-1 transcription factor, we pretreated the astrocytes with SP600125 and measured the phosphorylation of c-jun in whole cell lysates. As shown in figure 26 A, densitometric analysis of western blot shows that pretreatment with SP600125 decreased the levels of p-c-jun by $28.3 \pm 3.3\%$ compared to the HIV-1 Tat-transfected cells. To confirm the role of JNK MAPK in the activation of AP-1, we specifically knocked down JNK1 isoform using siRNA and measured the phosphorylated c-jun levels in whole cell lysates. Knock down of JNK1 decreased p-c-jun levels by $26.1 \pm 5.2\%$ compared to HIV-1 Tat-transfected cells (Fig. 26B). These results demonstrate that HIV-1 Tat mediated induction of IL-8 involves activation of JNK MAPK which in turn activates AP-1 transcription factor.

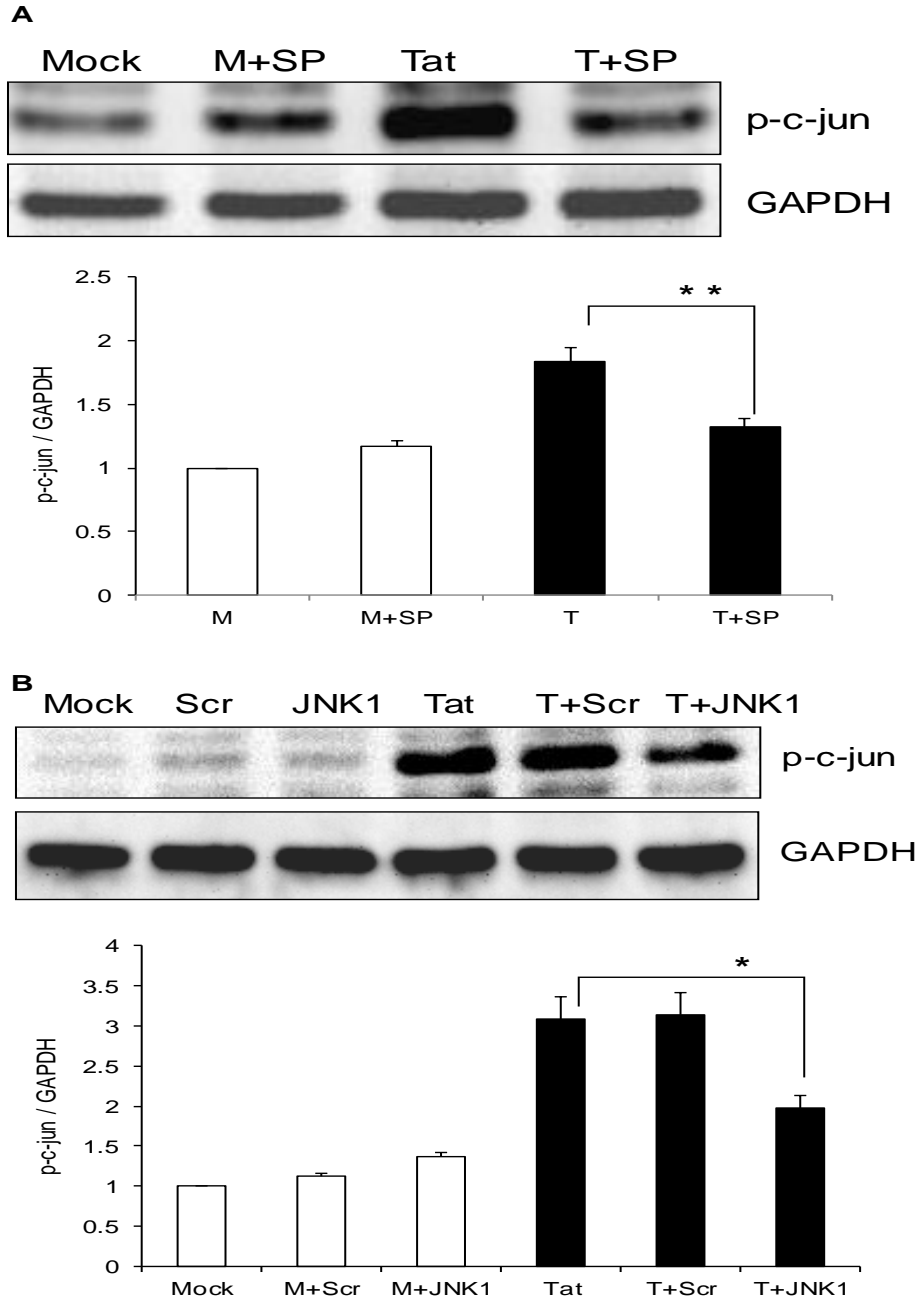


Figure 26: HIV-1 Tat mediated activation of JNK MAPK activates AP-1 transcription factor: (A) SVGA cells were pretreated with 10 μ M concentration of SP 600125. The levels of p-c-jun were measured in whole cell extracts 6 hours after the transfection. (B) Astrocytes were transfected with JNK1 siRNA for 48 hours, followed by transfection with HIV-1 Tat plasmid. The levels of p-c-jun were measured after 6 hours of transfection. A representative Western blot is shown in the panels A and B. Each experiment was done at least in triplicate and each bar represents the \pm SE of three individual experiments. Statistical analyses was performed by one-way ANOVA and ** denotes p -value of ≤ 0.01 .

3.2.13 Role of ERK MAPK signaling molecule in the Induction of cytokines/chemokines by HIV-1 Tat

Extracellular signal-regulated kinase (ERK) is another upstream signaling molecule that can activate NF- κ B and AP-1 transcription factors to induce the production of various pro-inflammatory cytokines. To determine the role of ERK MAPK in the up-regulation of cytokines/chemokines in astrocytes induced by HIV-1 Tat, we pretreated cells with a specific ERK inhibitor (U0126) and measured the levels of cytokines/chemokines at the level of mRNA at 6 hours post transfection. The starting dose of the inhibitor was based on IC50 values from previous published literature and was further optimized based on its effect on cell viability (data not shown) (Song, Tanaka et al. 2004). Pretreatment of astrocytes with 10 μ M concentration of U0126 did not affect the expression levels of either IL-6 or IL-8 or CCL5 (Fig. 27A-C). Furthermore, U0126 has significantly increased the expression levels of IL-6 and IL-8. Since, there was no decrease at the level of mRNA, we did not quantify the expression of these cytokines in the cell culture supernatants. These results indicate that HIV-1 Tat mediated induction of cytokines/chemokines does not involve ERK MAPK pathway.

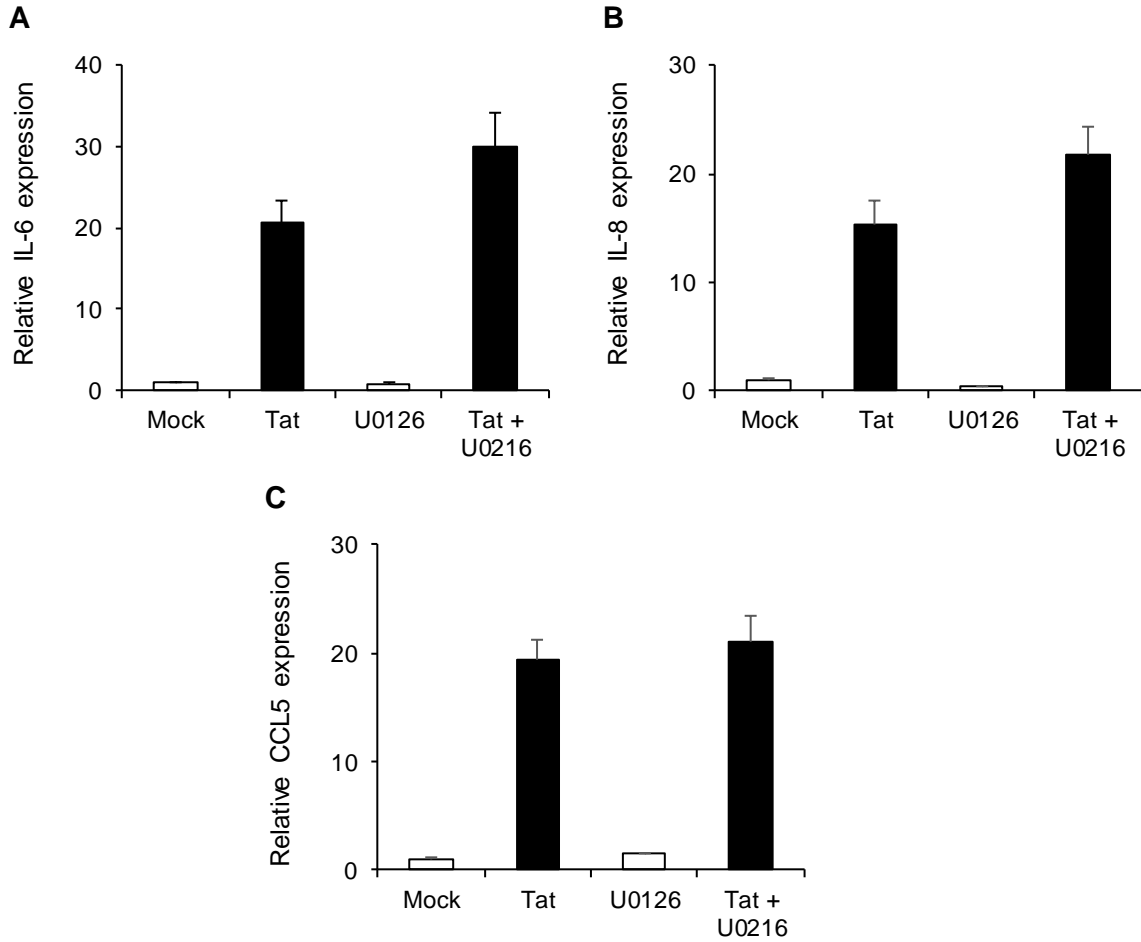


Figure 27: HIV-1 Tat mediated induction of cytokines/chemokines does not involve ERK MAPK pathway: SVG astrocytes were pre-treated with ERK MAPK inhibitor, U0126 1 hour prior to the transfection. (A-C) The expression levels of IL-6, IL-8 and CCL5 were measured 6 h after post-transfection with plasmid encoding HIV-1 Tat. The values are represented to their mock transfected controls. Each experiment was performed in triplicate and each bar represents the \pm SE of three individual experiments.

3.2.14 Involvement of PI3K/Akt in HIV-Tat mediated up-regulation of cytokines/chemokines in astrocytes

After determining the involvement of NF- κ B and MAPK regulators in the up-regulation of IL-6, IL-8 and CCL5, we wanted to explore the role of further upstream signaling molecules that can activate NF- κ B. The phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)/Akt pathway is a major upstream signaling mechanism that is involved in the regulation of cytokines by activation of NF- κ B through I κ B kinase (IKK)-mediated phosphorylation of I κ B α (Kane, Shapiro et al. 1999). To ascertain the role of PI3K/Akt in the up-regulation of cytokines/chemokines by HIV-1 Tat, we initially employed an inhibitor approach wherein the cells were pretreated with LY294002, a reversible PI3K inhibitor. The starting dose of the inhibitor was based on IC₅₀ values from previous literature and was further optimized based on its effect on cell viability (data not shown) (Fotheringham, Coalson et al. 2012). Pretreatment with 10 μ M concentration of LY294002 decreased HIV-1 Tat mediated expression of IL-6 by 73.5 \pm 2.6% and 81.3 \pm 0.7% at the levels of mRNA and protein, respectively (Fig. 28A, B). The expression of IL-8 was also decreased by 43.3 \pm 3.5% and 55.1 \pm 1.9% at mRNA and protein levels, respectively (Fig. 22C, D). Furthermore, pretreatment with LY294002 decreased CCL5 expression by 46.2 \pm 4.3% at mRNA and 53.2 \pm 7.44 at protein level (Fig. 28E, F). These results suggest that HIV-1 Tat mediated induction of IL-6, IL-8 and CCL5 involve PI3K/Akt pathway.

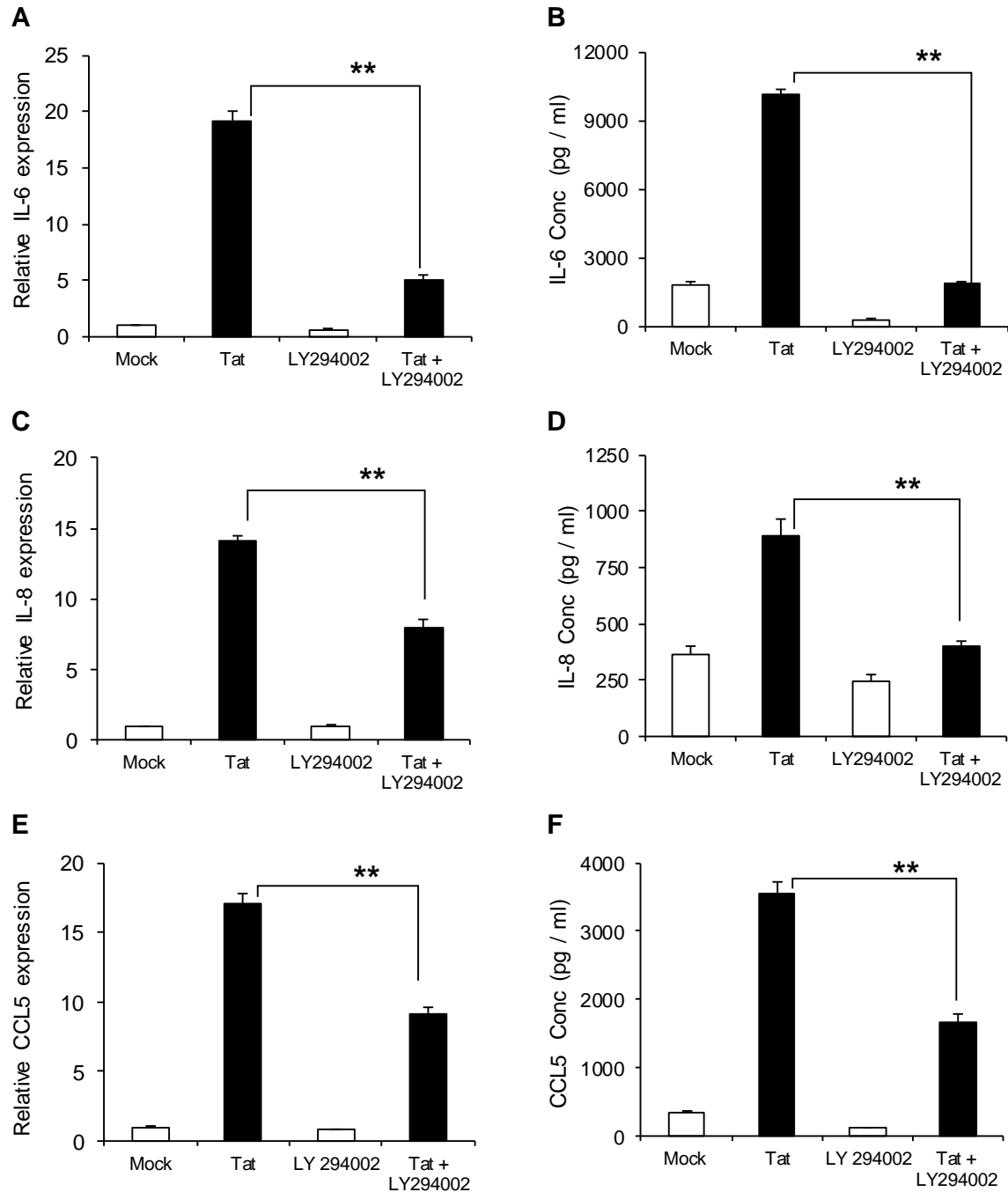


Figure 28: HIV-1 Tat-mediated expression of IL-6 and IL-8 involves PI3K/Akt pathway. (A-F) Astrocytes were pretreated with a specific PI3K inhibitor (LY294002) for 1 hour prior to transfection. (A, C, E) The expression levels of IL-6, IL-8 and CCL5 at the level of mRNA were determined at 6 hours post transfection by real time RT-PCR. The values represented are normalized to their mock-transfected controls. (B, D, F) IL-6, IL-8 and CCL5 protein concentrations in the cell culture supernatants at 48 hours post transfection were determined by multiplex cytokine assay. Each experiment was done at least in triplicate and each bar represents the \pm SE of three individual experiments. Statistical analyses was performed by one-way ANOVA and ** denotes P -value of ≤ 0.01 and * denotes P -value of ≤ 0.05 .

Activation of PI3k leads to the activation of downstream signaling molecule, Protein kinase B (Akt). Results with LY294002 indicate that PI3K/Akt pathway might be involved in HIV-1 Tat mediated induction of cytokines/chemokines. The involvement of PI3K/Akt was further confirmed by measuring the phosphorylation of Akt. The cells were mock transfected or HIV-1 Tat transfected and whole cell lysates were prepared 6 h post-transfection. The results show that HIV-1 Tat-mediated increase in phosphorylated Akt levels were decreased by pretreatment with LY294002 (Fig. 29A). HIV-1 Tat mediated 1.3 fold increase in Akt activation was completely abrogated by pre-treatment with LY294002. Activation of NF- κ B by PI3K pathway was determined by measuring the translocation of p65 upon pretreatment with LY294002. Compared to HIV-1 Tat transfected cells, pretreatment with LY294002 decreased the translocation of p65 by $29.9 \pm 5.9\%$ (Fig. 30A).

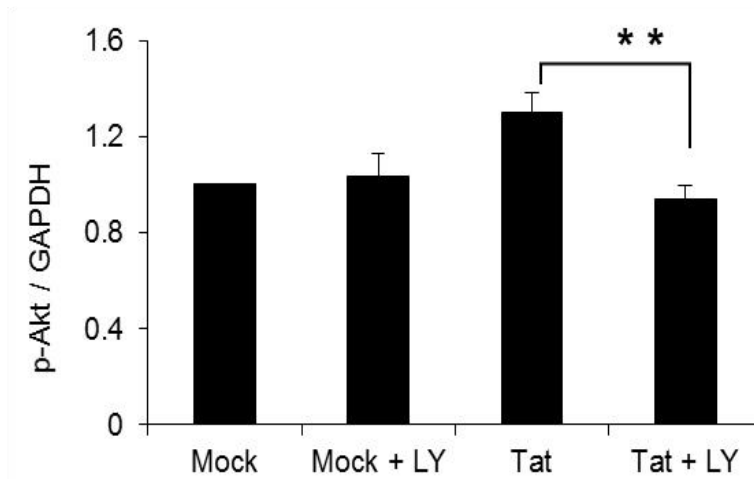
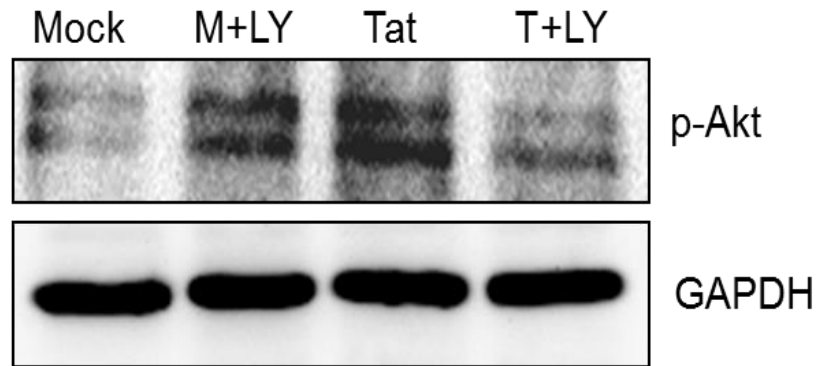


Figure 29: HIV-1 Tat mediates activation of PI3K/Akt pathway: (A) Astrocytes were either mock-transfected or transfected with HIV-1 Tat plasmid for duration of 6 hours and p-Akt levels were measured in whole cell extracts. Experiment was done at least in triplicate and each bar represents the \pm SE of three individual experiments. Statistical analyses was performed by one-way ANOVA and ** denotes p-value of ≤ 0.01 .

A

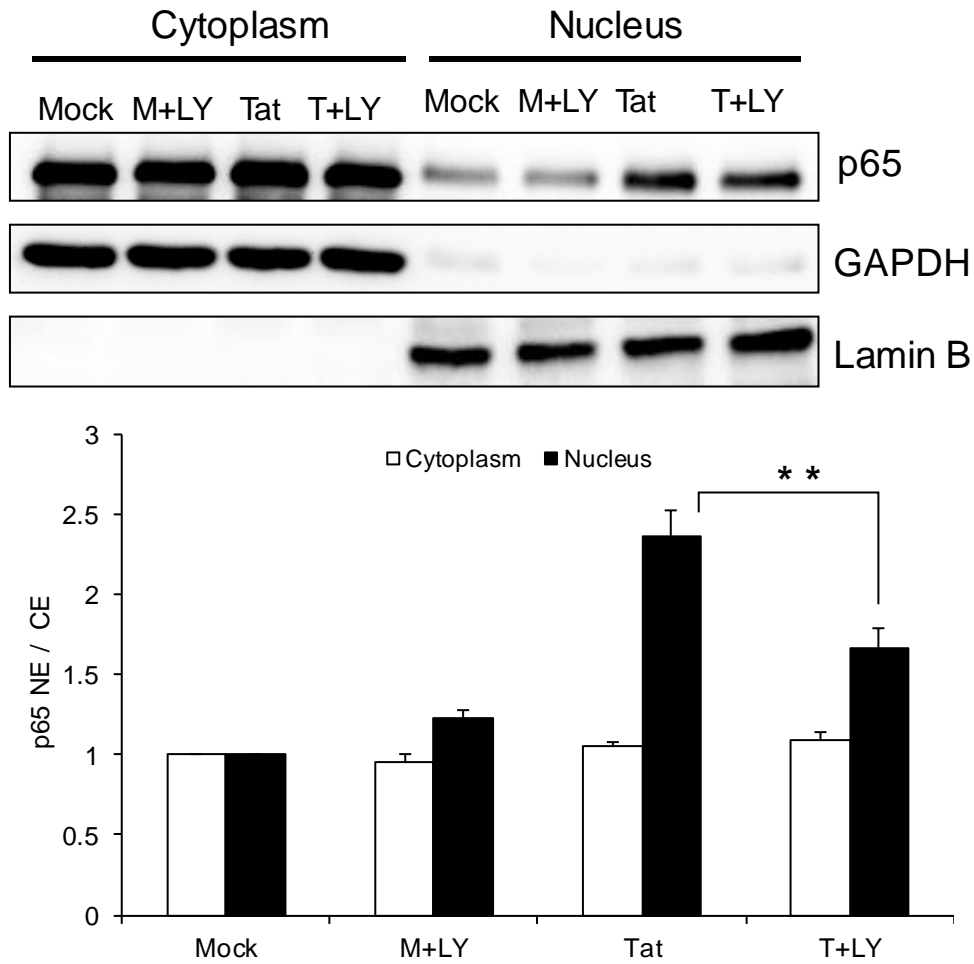


Figure 30: HIV-1 Tat-mediated expression of cytokines involves PI3K/Akt and NF- κ B pathway: (A) Astrocytes were transfected for a duration of 6 hours and translocation of p65 was measured. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and LaminB were used as internal loading controls for cytoplasmic and nuclear protein fractions, respectively. A representative Western blot is shown in figure. Experiment was done at least in triplicate and each bar represents the \pm SE of three individual experiments. Statistical analyses was performed by one-way ANOVA and ** denotes p-value of ≤ 0.01 .

Akt is the effector downstream signaling molecule that is activated by upstream PI3K. To verify the involvement of PI3K/Akt pathway in the up-regulation of HIV-1 Tat mediated cytokines/chemokines, we have used siRNA against Akt. Akt exists in three different isoforms, (Akt1/Akt2/Akt3). The efficiency of gene knock down was verified previously. Individual knock down of all the isoforms decreased the expression levels of IL-6 and IL-8 by 35% and 30%, respectively at the levels of mRNA (Fig. 31A, C). Similar results were obtained at the protein level, where the decrease for IL-6 and IL-8 was found to be 35% and 28%, respectively (Fig. 31B, D). The knockdown of Akt2 and Akt3 but not Akt1 reduced the expression of CCL5 by $34.05 \pm 7.7\%$ and $42.8 \% \pm 6.3\%$ at the level of mRNA and by $29.25 \pm 2.86\%$ and $46.4 \pm 3.03\%$ at protein levels (Fig. 31E, F). The results with inhibitor and siRNA demonstrate that HIV-1 Tat mediated induction of cytokines/chemokines involve activation of NF- κ B through PI3K/Akt pathway.

In conclusion, results indicate that HIV-1 Tat mediates the up-regulation of IL-6, IL-8 and CCL5 in astrocytes involving different upstream signaling molecules and transcription factors (Fig. 32-34).

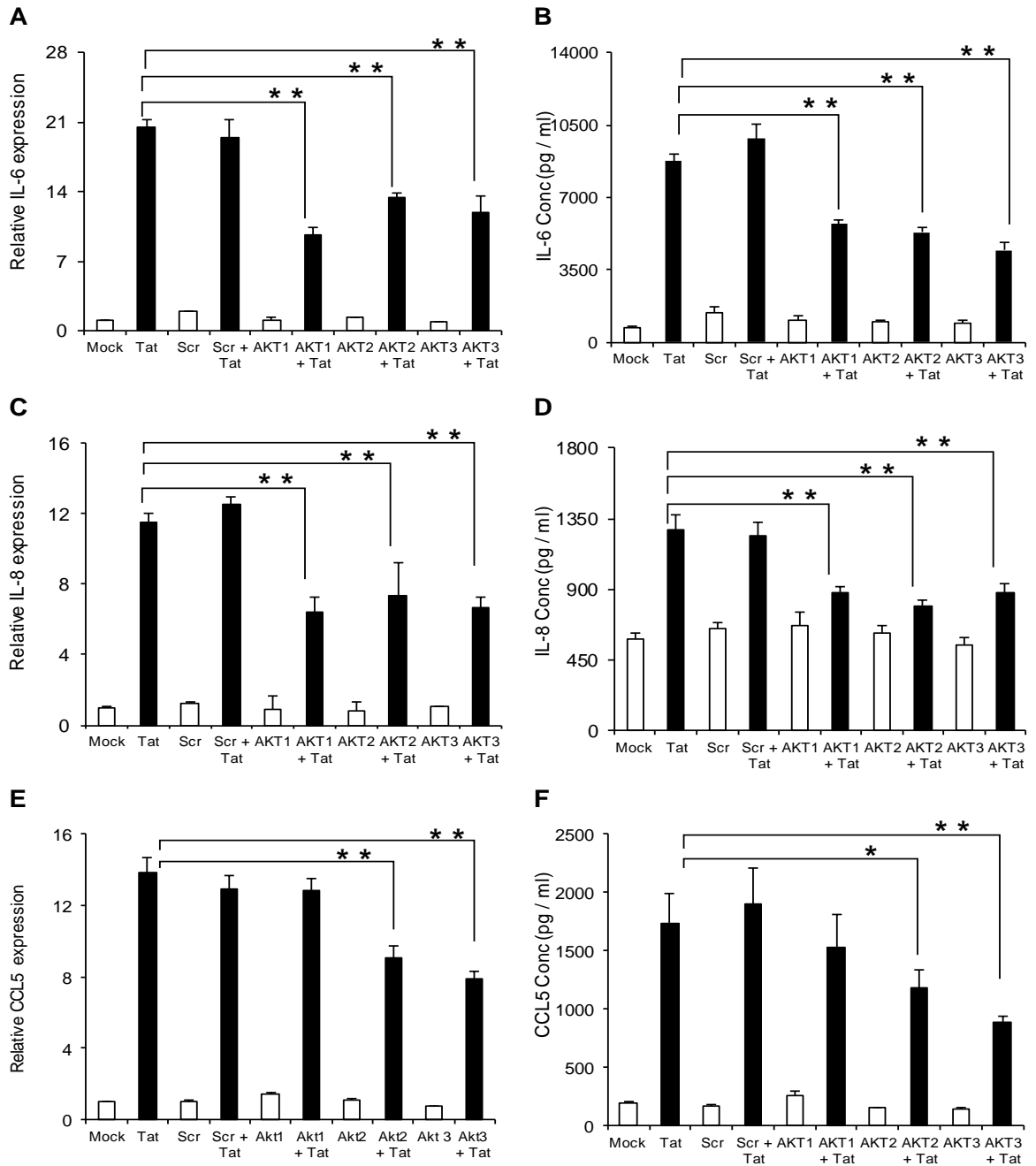


Figure 31: HIV-1 Tat-mediated expression of IL-6, IL-8 and CCL5 involves PI3K/Akt pathway: (A-F) Astrocytes were transfected with either scrambled or Akt1 or Akt2 or Akt3 siRNA for a duration of 48 hours, followed by either mock transfection or transfection with HIV-1 Tat plasmid. (A, C, E) The levels of IL-6 and IL-8 at mRNA level were determined by real time RT-PCR at 6 hours post transfection. (B, D, F) The protein concentrations of IL-6 and IL-8 in cell culture supernatants at 48 hours post transfection were determined by multiplex cytokine assay. Each experiment was done at least in triplicate and each bar represents the \pm SE of three individual experiments. Statistical analyses was performed by one-way ANOVA and ** denotes P -value of ≤ 0.01 and * denotes p -value of ≤ 0.05 .

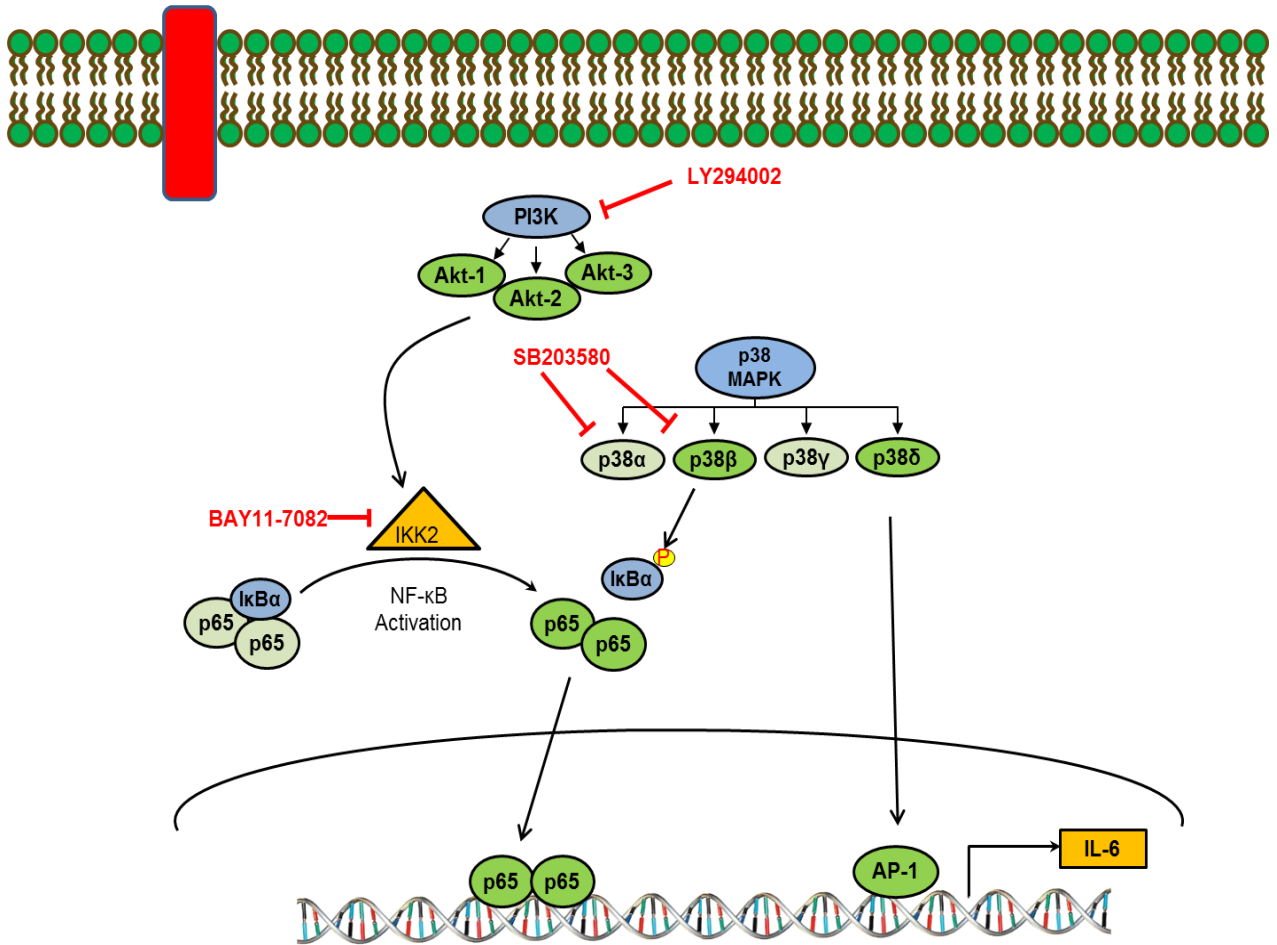


Figure 32: Schematic of signaling pathways involved in HIV-1 Tat-mediated up-regulation of IL-6 in astrocytes. The major signaling pathways involved in HIV-1 Tat-mediated up-regulation of IL-6 are PI3K/Akt and p38 MAPK which lead to the activation of NF- κ B and AP-1 (solid line). The siRNAs used to target various isoforms are indicated by a green color whereas drug targets are indicated by a blue color. The involvement and absence of a particular isoform or a signaling molecule is indicated by a dark color and a pale color, respectively. Specific inhibitors used for targeting signaling molecules are indicated by a red color.

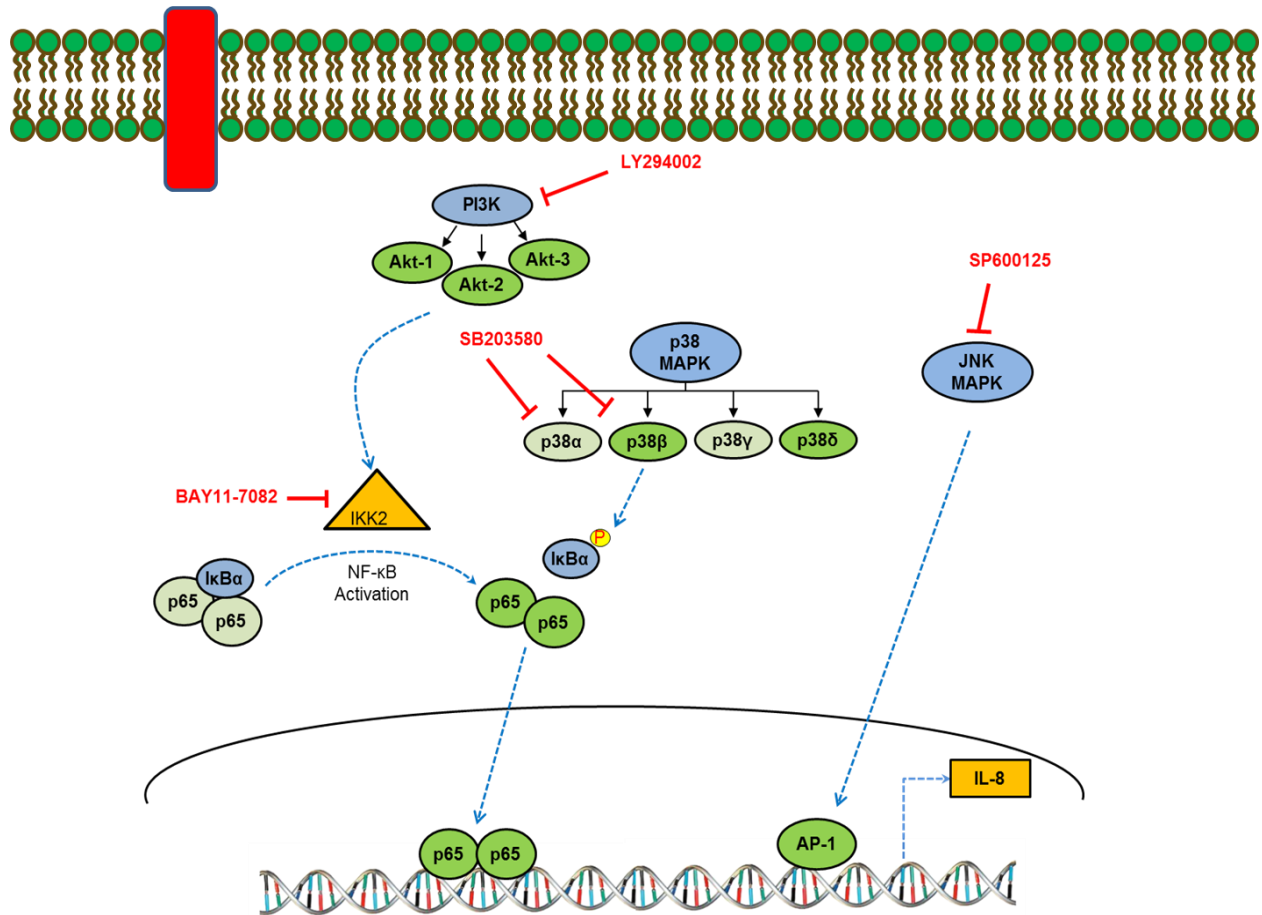


Figure 33: Schematic of signaling pathways involved in HIV-1 Tat-mediated up-regulation of IL-8 in astrocytes: Induction of IL-8 by HIV-1 Tat involves PI3K/Akt, p38 and C-Jun N-terminal kinase/mitogen activated-protein kinase (JNK MAPK) signaling pathways, leading to the activation of NF-κB and AP-1 transcription factors (broken line). The siRNAs used to target various isoforms are indicated by a green color whereas drug targets are indicated by a blue color. The involvement and absence of a particular isoform or a signaling molecule is indicated by a dark color and a pale color, respectively. Specific inhibitors used for targeting signaling molecules are indicated by a red color.

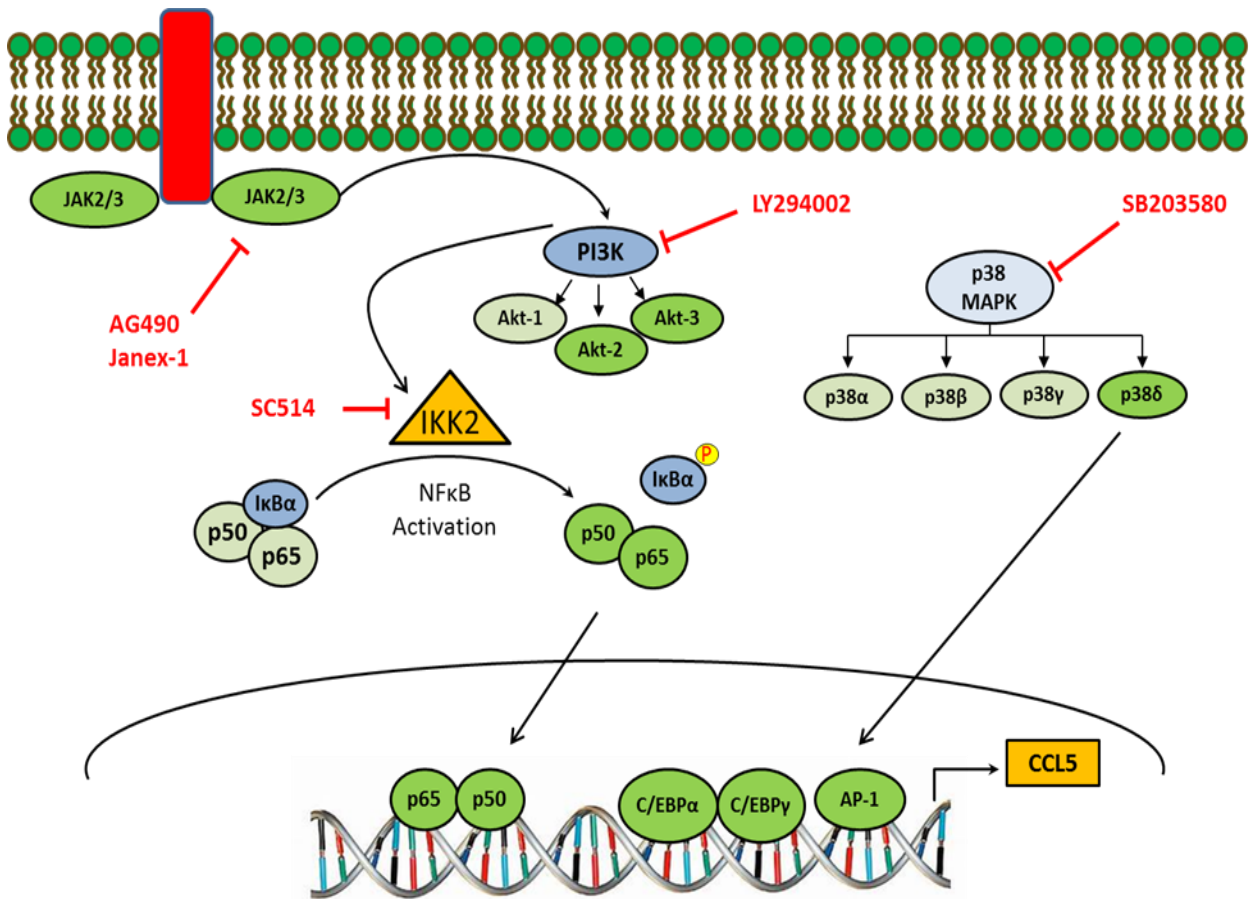


Figure 34: Schematic representation of the signaling pathways involved in HIV-1 Tat mediated up-regulation of CCL5 in astrocytes: The induction of CCL5 by HIV-1 Tat involved JAK/PI3K/Akt and p38 MAP kinase pathways. These signaling pathways differentially regulated the induction of CCL5 by activating various transcription factors, including NF- κ B, C/EBP α , C/EBP γ and AP-1. The target molecules of siRNA are indicated in green color and the involvement of a specific isoform is shown in brighter color and the absence is shown in pale color. The specific inhibitors for their respective targets are shown in red.

3.3 Discussion

HIV-1 associated neurological deficits range from minor cognitive motor disorders (MCMD) to a more severe form of dementia referred to as HAD. Several mechanisms, including the dysregulation of cytokine profile and infiltration of various inflammatory cells and oxidative stress have been proposed for the development of HAND in patients infected with HIV-1 (Rappaport, Joseph et al. 1999, Li, Galey et al. 2005). Elevated levels of pro-inflammatory cytokines including IL-6, IL-8, and IFN- γ have been found in the various regions of the brain in HIV infected people (Griffin, McArthur et al. 1991, Mastroianni, Paoletti et al. 1992, Mamik and Ghorpade 2012). Further, increased CCL5 has not only been detected in the CSF, but it has been directly correlated with viral load in CSF and MCMD (Kelder, McArthur et al. 1998, Letendre, Lanier et al. 1999). Moreover, elevated levels of IL-6 and IL-8 have been shown to play important roles in many inflammatory responses such as recruitment of leukocytes, accumulation of neutrophils and production of acute phase proteins (Baggiolini and Clark-Lewis 1992, Gabay 2006). Viral proteins, especially HIV-1 Tat and gp120, have been implicated in this phenomenon. Additionally, HIV-1 proteins (gp120 and Tat) have been shown to increase the cytokine/chemokines expression in the cells of CNS origin (El-Hage, Gurwell et al. 2005, Shah, Verma et al. 2011). However, very little is known about the molecular mechanisms behind the up regulation of these cytokines/chemokines. In the present study, we sought to dissect the molecular mechanisms behind the up regulation of cytokines/chemokines by HIV-1 Tat in SVG astrocytes. The astrocytes were transfected with an expression plasmid encoding HIV-1 Tat and the expression levels of cytokines/chemokines were determined at various time points. The results showed a time dependent increase in the expression of IL-6, IL-8 and CCL5 at the level of mRNA and protein. These results are in agreement with the previous literature showing the over expression of IL-6, IL-8 and CCL5 induced by HIV-1 Tat (Kutsch, Oh et al. 2000, Ju, Song et al. 2009). However, the secretion of IL-6 was different compared to that shown in the previous studies

(Ju, Song et al. 2009). This can be attributed to the difference in the type of cell line used (CRT-MG vs SVG astrocytes) and also different methods of HIV-1 Tat exposure (protein treatment vs transfection). However, the expression of IL-8 at protein level was consistent to that obtained by Kutch et al, where they demonstrated increased IL-8 protein expression with HIV-1 Tat protein treatment in primary astrocytes (Kutsch, Oh et al. 2000). The role of CCL5 in the context of HIV is uncertain. CCL5 interacts with CCR5 receptor to inhibit the replication of various macrophage-tropic strains of HIV-1 at a concentration of 50-250 ng/ml (Arenzana-Seisdedos, Virelizier et al. 1996, Trkola, Paxton et al. 1998). On the other hand, CCL5 at 1-10 µg/ml concentration increased the HIV-1 infectivity by activating p44/p42 MAPK (Gordon, Muesing et al. 1999, Chang, Gordon et al. 2002). CCL5 has also been shown to exhibit neuroprotective properties by decreasing the neuronal apoptosis induced by gp120 (Kaul and Lipton 1999, Catani, Corasaniti et al. 2000). However these concentrations are very high when compared to the concentrations that have been reported in the CSF of HIV infected patients suffering from dementia and opportunistic infections where CCL5 concentration have been reported to be in the range of 50-200 pg/ml (Kelder, McArthur et al. 1998, Christo, Vilela Mde et al. 2009). In another case of *Neisseria meningitides*/meningioma cell culture system, 5-10 ng/ml concentrations of CCL5 were shown to play a role in inflammatory responses (Fowler, Yin et al. 2006). These studies suggest that, a lower concentration of CCL5 perhaps causes inflammation whereas higher concentration might be involved in protective effect. In our study, we achieved the peak concentration of CCL5 in the range of 2-4 ng/ml, which would be expected to lead into pro-inflammatory response. Our results with immunocytochemistry also confirm the elevated presence of IL-6, IL-8 and CCL5 in HIV-1 Tat transfected astrocytes.

NF-κB is a major transcription factor involved in regulating the expression of many cytokines and chemokines. It binds to the promoter region of many genes, including IL-6 and IL-8 to regulate their expression (Matsusaka, Fujikawa et al. 1993). Previous studies have shown increased binding and activation of NF-κB by HIV-1 Tat in various cells, including

astrocytes and microglia (Conant, Ma et al. 1996, Nicolini, Ajmone-Cat et al. 2001). Recent study by Fiume and coworkers has shown that HIV-1 Tat increased the binding of p65 DNA and also its transcriptional activity (Fiume, Vecchio et al. 2012). In agreement with these reports, we have also observed a time dependent increase in the translocation of p65 into the nucleus by HIV-1 Tat in SVG astrocytes. Next, we demonstrated the involvement of NF- κ B in the up regulation of IL-6 and IL-8 by pretreating the astrocytes with BAY 11-7082, a specific inhibitor of I κ B kinase 2 (IKK2). In order to determine involvement of NF- κ B, we used SC514, a specific inhibitor of I κ B kinase 2 (IKK2). SC514 prevents the degradation of I κ B α and thereby prevents the translocation of NF- κ B (Kishore, Sommers et al. 2003). In accordance with the previous reports, we also observed SC514 mediated reduction of CCL5 expression suggesting a role for NF- κ B.

To confirm the data with the pharmacological inhibitor, we have employed the siRNA approach. NF- κ B is comprised of different subunits of which p65 and p50 are important in regulating the expression of several cytokines/chemokines. The transcriptional activity is mainly attributed to p65 subunit of NF- κ B (Vallabhapurapu and Karin 2009). The two subunits were knocked down individually by using siRNA and expression of IL-6, IL-8 and CCL5 were measured. Knock down of p65 but not p50 has decreased the expression levels of both IL-6 and IL-8 in astrocytes by HIV-1 Tat. This suggests the possibility that p65 homodimers are more important in regulating the expression of IL-6 and IL-8 from astrocytes by HIV-1 Tat. This is in agreement with the findings by Georganas et al showing p65 homodimers but not p50 homodimers are important in regulating the expression of both IL-6 and IL-8 in rheumatoid arthritis fibroblast like synoviocytes (Georganas, Liu et al. 2000). Surprisingly, knock down of p50 has increased the expression levels of IL-8 at the level of mRNA and protein. This can be attributed to the role of p50 homodimer as transcriptional repressor (Plaksin, Baeuerle et al. 1993). (Plaksin, Baeuerle et al. 1993)

Therefore, it seems that HIV-1 Tat does not use p50 homodimer for up regulating the expression of IL-8. However, further investigation needs to be done to determine the role of p50 homodimer on the expression of IL-8. However, knock down of p50 and p65 has significantly decreased the expression levels of CCL5 at mRNA and protein, confirming our results with the chemical antagonist. These results indicate that p50 and p65 heterodimer is involved in HIV-1 Tat mediated expression of CCL5 in astrocytes. Overall, our results indicate that HIV-1 Tat differentially affects various NF- κ B transcription factors to regulate the expression of IL-6, IL-8 and CCL5.

After determining the role of NF- κ B, we sought to look at the role of upstream signaling molecules that can lead to up regulation of IL-6, IL-8 and CCL5 by HIV-1 Tat. MAPKs are important upstream signaling molecules that can result in the activation of many cytokines mediated through NF- κ B. Particularly, p38 MAPK, belonging to the family of serine/threonine protein kinases has been shown to be involved in the up regulation of many cytokines/chemokines (Lee, Laydon et al. 1994). The involvement of p38 MAPK in the up regulation of IL-6, IL-8 and CCL5 by HIV-1 Tat was determined by pretreating the astrocytes with SB203580, a specific inhibitor of p38 MAPK. SB203580 significantly decreased the expression levels of IL-6 and IL-8, but did not affect the expression of CCL5. p38 MAPK exists in four different isoforms ($\alpha/\beta/\gamma/\delta$), of which SB203580 inhibits p38 α and p38 β isoforms (Lee, Kassis et al. 1999). Both these isoforms of p38

lead to the activation of NF- κ B (Kumar, Behera et al. 2010). To verify the involvement of NF- κ B activation (p38 α and p38 β isoforms) and role of other two isoforms (p38 γ and p38 δ) in the up regulation of IL-6, IL-8 and CCL5 by HIV-1 Tat, we have individually knocked down all the isoforms using siRNA. Consistent with the results of SB203580, knock down of p38 β but not p38 α has partially decreased the expression levels of IL-6 and IL-8. The decrease in the translocation of p65 into the nucleus with SB203580 pretreatment demonstrated the connection between p38 MAPK and NF- κ B. Specifically decrease in the translocation of p65

upon knock down p38 β shows that this isoform is more important for the activation of NF- κ B by HIV-Tat. As knock down of p38 δ partially reduced the expression levels of IL-6 and CCL5 and it does not lead to the activation of NF- κ B, we wanted to determine the role of various other transcription factors that can be activated by it. p38 δ activation is associated with increased activation of various transcription factors, including AP-1, C/EBP α and C/EBP γ (Efimova, Broome et al. 2003). Our results demonstrated that knock down of c-jun (important component of AP-1) but not C/EBP α and C/EBP γ has partially decreased the expression levels of IL-6. Our results showed reduction of CCL5 levels after knocking down AP-1, C/EBP α and C/EBP γ which clearly suggests the involvement of these transcription factors in the regulation of HIV-1 Tat-mediated CCL5 expression. The western blot showing a decrease in the phosphorylation of c-jun by HIV-1 Tat upon knock down of p38 δ isoform demonstrates that this isoform is important in HIV-1 Tat mediated expression of IL-6 and CCL5.

As p38 δ knock down did not alter the expression levels of IL-8 mediated by HIV-1 Tat, we were surprised to see the decreased expression levels of IL-8 by HIV-1 Tat upon knock down of AP-1. Then we tested the possibility of other upstream signaling molecules that can activate c-jun to induce the expression levels of IL-8 by HIV-1 Tat. Several previous studies have shown the role of JNK MAPK in the activation of c-jun (Papachristou, Batistatou et al. 2003). JNK belongs to the family of MAPK and is involved in the activation of transcription factors in response to various stimuli. We demonstrated the role of JNK MAPK in the expression of IL-8 by HIV-1 Tat by pretreatment with SP600125, a specific inhibitor of JNK. It acts by competing with ATP to inhibit the phosphorylation of c-jun. Pretreatment of astrocytes with SP600125 and also siRNA against JNK has decreased the phosphorylation levels of c-jun by HIV-1 Tat. These results unequivocally demonstrate that JNK MAPK is important in the expression of IL-8 mediated by HIV-1 Tat. JNK did not affect the expression levels of IL-6 and CCL5.

PI3K/Akt is a major signaling molecule that can modulate the activation of NF- κ B by promoting the phosphorylation of I κ B α (Heck, Lezoualc'h et al. 1999). Several previous studies have indicated the role for HIV-1 and HIV-1 Tat in the activation of PI3K/Akt pathway in macrophages (Chugh, Bradel-Tretheway et al. 2008, Lucas, Kim et al. 2010). In our study, pretreatment of SVG astrocytes with reversible PI3K inhibitor, LY294002, decreased HIV-1 Tat mediated increase in the phosphorylation of PI3K. It also partially decreased HIV-1 Tat mediated increase in IL-6, IL-8 and CCL5. Akt or Protein Kinase B is a downstream signaling molecule of PI3K. It belongs to the family of serine/threonine protein kinases and is known to exist in three different isoforms (Akt1/PKB α , Akt2/PKB β , Akt3/PKB γ). All three isoforms of Akt are present in the brain and differ in their phosphorylation sites (Easton, Cho et al. 2005). Akt3 is predominantly important in the brain since it contributes for more than 50% of all the Akt isoforms found in the brain. In our study, individual knock down of all the isoforms by siRNA has decreased the expression levels of IL-6 and IL-8 mediated by HIV-1 Tat. These findings indicate the importance of all the isoforms in the expression of IL-6 and IL-8 mediated by HIV-1 Tat. In our study, siRNA against Akt2 and Akt3, but not Akt1 showed substantial reduction in the expression of CCL5. These findings suggest that perhaps the brain specific isoforms of Akt; i.e. Akt2 and Akt3, play an important role in the expression of CCL5, *via* activation of NF- κ B, which serves as one of the several transcription factor in this process.

CHAPTER 4

HIV-1 TAT AND METHAMPHETAMINE MEDIATED EXPRESSION OF IL-6 INVOLVES NF-Kb AND PI3K/Akt PATHWAYS

4.1 Introduction

More than 30% of the people infected with HIV-1 suffer from a range of neurological disorders, collectively referred to as HIV associated neurocognitive disorders (HAND) (Sacktor, Skolasky et al. 2016). HIV-1 crosses the blood brain barrier soon after infection through the infected monocytes and replicates inside microglia and astrocytes (Gonzalez-Scarano and Martin-Garcia 2005). Several mechanisms have been implicated in the development of HAND, including ongoing viral replication and neurotoxicity mediated by virotoxins like gp120 and HIV-1 Tat. Soluble Tat has been shown to be present in HIV-1 infected individuals even after the initiation of combined antiretroviral therapy (cART) (Mediouni, Darque et al. 2012).

HIV-1 Transactivator of transcription (HIV-1 Tat) is early protein expressed by the proviral DNA and greatly enhances the viral replication (Dayton, Sodroski et al. 1986). Tat is actively secreted by the infected cells and affects various signaling molecules of bystander uninfected cells through the interaction of their surface receptors or by internalizing into the cells (Tyagi, Rusnati et al. 2001). Intraventricular injection of HIV-1 Tat has been shown to be neurotoxic to mice (Jones, Olafson et al. 1998). Neurotoxicity mediated by HIV-1 Tat can occur through direct interaction with neurons and indirect actions on surrounding cells to promote the release of soluble mediators. These mediators include production of various pro-inflammatory cytokines, reactive oxygen species, quinolinic acid and glutamate (Gupta, Knight et al. 2010, Li, Yim et al. 2010, Kim, Smith et al. 2015). Furthermore, HIV-1 Tat interacts with neurons to promote neuronal apoptosis through various mechanisms, including activation of NMDA receptors, glutamate excitotoxicity, increase in the levels of intracellular calcium concentration, alteration of mitochondria potential and activation of cytochrome c and

endonuclease G pathways (Singh, Goody et al. 2004, Zhu, Yao et al. 2009, Lecoecur, Borgne-Sanchez et al. 2012, Krogh, Wydeven et al. 2014).

Drugs of abuse are an important comorbid factor in individuals infected with HIV-1. More than 50% of the people with HIV-1 use and abuse one of the illicit drugs. Methamphetamine is a psychostimulant and is one of the most commonly abused illicit drugs among HIV infected individuals in the United States. It promotes neurotoxicity by primarily affecting dopaminergic neurons and also by affecting the levels of various neurotransmitters at the synapse (Larsen, Fon et al. 2002, Yamamoto, Moszczynska et al. 2010, Yu, Zhu et al. 2015). Moreover, methamphetamine has been shown to affect the integrity of blood brain barrier by up-regulating the expression of various pro-inflammatory cytokines including IL-6, IL-8, TNF- α and inducing oxidative stress (Ramirez, Potula et al. 2009, Shah, Silverstein et al. 2012, Wang, Qian et al. 2014, Coelho-Santos, Leitao et al. 2015, Wongprayoon and Govitrapong 2015). Furthermore, methamphetamine exacerbates neurocognitive impairments seen in HIV infected individuals (Rippeth, Heaton et al. 2004, Carey, Woods et al. 2006). Several recent studies have indicated the role of HIV-1 Tat in augmentation of neurotoxicity mediated by methamphetamine. However, there are no studies indicating the combined role of methamphetamine and HIV-1 Tat in the up-regulation of cytokines from astrocytes, an important contributor of neurotoxicity.

In previous studies, we have shown the up-regulation of cytokines by HIV-1 and methamphetamine from astrocytes involving PI3K/Akt and NF- κ B pathways. The present study was undertaken to determine the combined effect of HIV-1 Tat and methamphetamine in the up-regulation of cytokines from astrocytes. We also sought to determine the role of PI3K/Akt and NF- κ B signaling pathway in HIV-1 Tat and methamphetamine mediated up-regulation of cytokines. We chose SVGA astrocytic cell line and primary astrocytes to investigate our hypothesis.

4.2 Results

4.2.1 Methamphetamine exacerbates the production of IL-6 mediated by HIV-1 Tat in astrocytes

In our earlier studies, we have demonstrated that HIV-1 Tat or methamphetamine treatment alone induces the production of IL-6 and IL-8 from astrocytes (Shah, Silverstein et al. 2012). Furthermore, several studies have shown interaction of methamphetamine with HIV-1 Tat to potentiate its effect by several mechanisms, including increased oxidative stress (Flora, Lee et al. 2003), activation of matrix metalloproteinases (Conant, St Hillaire et al. 2004), alteration of dopaminergic neurons function (Maragos, Young et al. 2002), and promoting neuronal apoptosis (Qi, Gang et al. 2011). Along similar lines, we hypothesized that methamphetamine augments HIV-1 Tat mediated production of cytokines from astrocytes. To test our hypothesis, we treated SVGA cells with 500 μ M methamphetamine for three days and transfected them with or without plasmid coding HIV-1 Tat. We tested the mRNA expression levels of IL-6 and IL-8 at 6 hour post-transfection as we previously have demonstrated their induction with methamphetamine and HIV-1 Tat alone. We found that HIV-1 Tat and methamphetamine combination treatment has significantly increased the expression levels of IL-6 compared to treatment with either Tat or methamphetamine alone (Fig 35A). Even though, there was induction of IL-8 by HIV-1 Tat and methamphetamine alone, combination treatment has significantly decreased its expression levels at both mRNA and protein levels (Fig 35B).

Therefore, we have performed a time kinetics to determine the expression of IL-6 at mRNA and protein levels. We found that the expression levels of IL-6 for mRNA levels have started to increase from 3 h, peaked at 6 h (24.6 ± 1.1 fold for Tat, 6.9 ± 0.8 fold for Methamphetamine and 38.8 ± 1.4 fold for combination treatment) and started to decrease until the observation period (until 24 h) (Fig 36A). We also determined the protein level of IL-6 at 6 h, 12 h, 24 h and 48 h post-transfection by bioplex assay. The expression of IL-6 started

to increase from 6 h until indicated time point (48 h). The peak increase in the expression of IL-6 at 48 h was found to be 9.36 ± 0.36 ng/ml for Tat treatment, 3.27 ± 0.11 ng/ml for methamphetamine treatment and 20.94 ± 1.63 ng/ml for combination treatment (Fig 36B). We have also confirmed our results using primary astrocytes isolated from aborted human fetus. We quantified IL-6 mRNA expression 2 h after treating primary astrocytes with 200 ng/ml of recombinant Tat protein and/or 500 μ M methamphetamine. Combination treatment has increased the expression of IL-6 by 28.0 ± 5.1 fold compared to either HIV-1 Tat (8.9 ± 2.7 fold) or methamphetamine (2.5 ± 0.1 fold) treatments alone (Fig 36C).

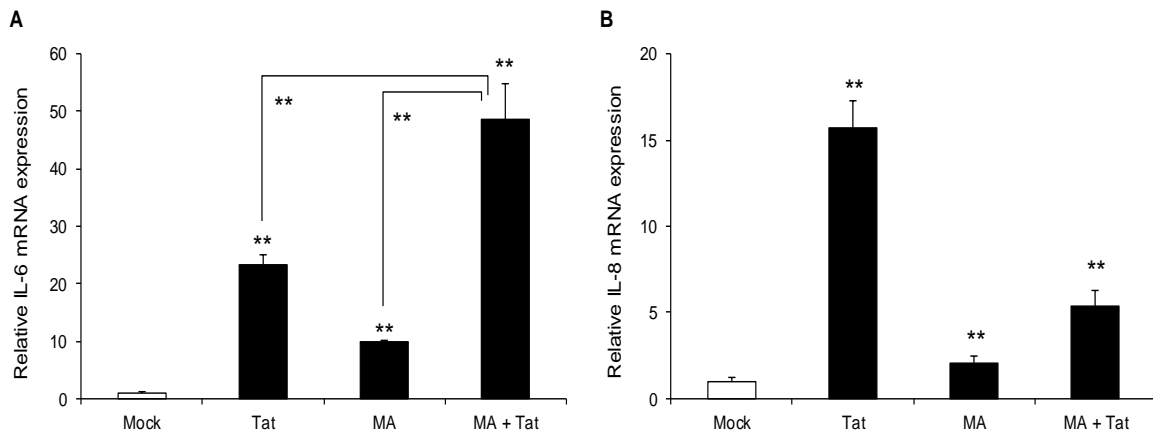


Figure 35: Methamphetamine exacerbates HIV-1 Tat mediated induction of IL-6 but not IL-8 in astrocytes: SVG astrocytes were treated with 500 μ M methamphetamine for 3 days before transfecting them with HIV-1 Tat. Expression levels of IL-6 and IL-8 were determined 6 hours post transfection by using real time RT-PCR. Each experiment was performed in triplicate and SE represents mean of three independent experiments. Statistical significance was calculated using one-way ANOVA with Tukey's post-hoc analysis and p-value ≤ 0.05 is considered as statistically significant.

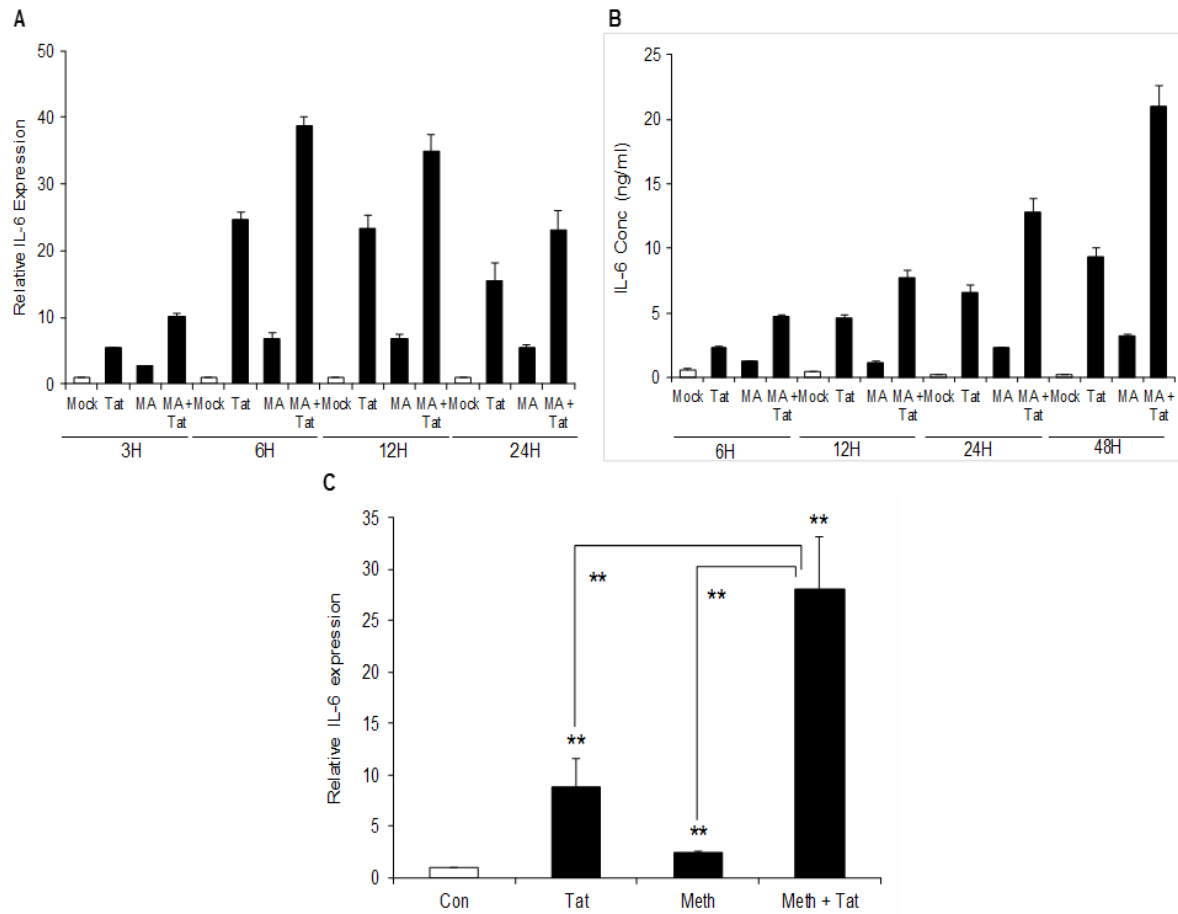


Figure 36: Methamphetamine exacerbates HIV-1 Tat mediated induction of IL-6 at RNA and protein levels in astrocytes: SVG astrocytes were treated with 500 μ M methamphetamine for 3 days before transfecting them with HIV-1 Tat. (A) Cells were harvested at 3, 6, 12 and 24 hours and expression levels of IL-6 mRNA were determined using real time RT-PCR. (B) Cell culture supernatants were collected at 6, 12, 24 and 48 hours post-transfection and IL-6 protein levels were determined using bioplex assay. (C) Primary human astrocytes from two independent donors were treated with 200 ng/ml of HIV-1 Tat protein and 500 μ M of methamphetamine and harvested after 2 h to determine IL-6 mRNA using real time RT-PCR. Each experiment was performed in triplicate and SE represents mean of three independent experiments. Statistical significance was calculated using one-way ANOVA with Tukey's post-hoc analysis and p-value ≤ 0.05 is considered as statistically significant.

4.2.2 Methamphetamine and HIV-1 Tat mediated induction of IL-6 involves NF- κ B pathway

We and several other groups have previously shown the activation of NF- κ B by HIV-1 Tat and methamphetamine in astrocytes. Furthermore, we have previously shown that NF- κ B is involved in HIV-1 Tat or methamphetamine mediated production of IL-6. In order to determine the involvement of NF- κ B in the interaction of methamphetamine and HIV-1 Tat mediated production of IL-6, we measured the expression p-I κ B α in whole cell lysates and p65 translocation in nuclear and cytoplasmic extracts. Phosphorylation of I κ B α was increased by HIV-1 Tat and methamphetamine treatments either in combination or alone. Combination treatment increased the expression of p-I κ B α by $47.6 \pm 12\%$ compared to mock transfected cells (Fig 37A). As expected, expression of p-I κ B α also was also increased to a similar extent by individual treatment of HIV-1 Tat or methamphetamine. We have previously shown the translocation of NF- κ B subunits into nucleus upon treatment with HIV-1 Tat and methamphetamine. In order to determine the involvement of NF- κ B, we measured the translocation of p65 subunit upon HIV-1 Tat transfection and/or methamphetamine treatment for 6 hours and there was significant increase in all the treatment groups. Even though, there was slight increase in the translocation of p65 with the combination treatment compared to HIV-1 Tat transfection ($82.9 \pm 17.8\%$ vs $57.7 \pm 2.1\%$), statistical significance was not achieved (Fig 37B). Results from p-I κ B α and p65 translocation experiments indicate that NF- κ B is involved in HIV-1 Tat and methamphetamine mediated production of IL-6. To further confirm our findings, we have pretreated SVGA cells with 10 μ M BAY 11-7082 1 hour prior to treatment with methamphetamine and/or transfection with HIV-1 Tat and harvested at 6 h and 48 h post-transfection for the quantification of IL-6 at mRNA and protein levels. Results show that BAY 11-7082 significantly decreased HIV-1 Tat and/or methamphetamine mediated production of IL-6 at mRNA and protein levels (Fig 38A, B).

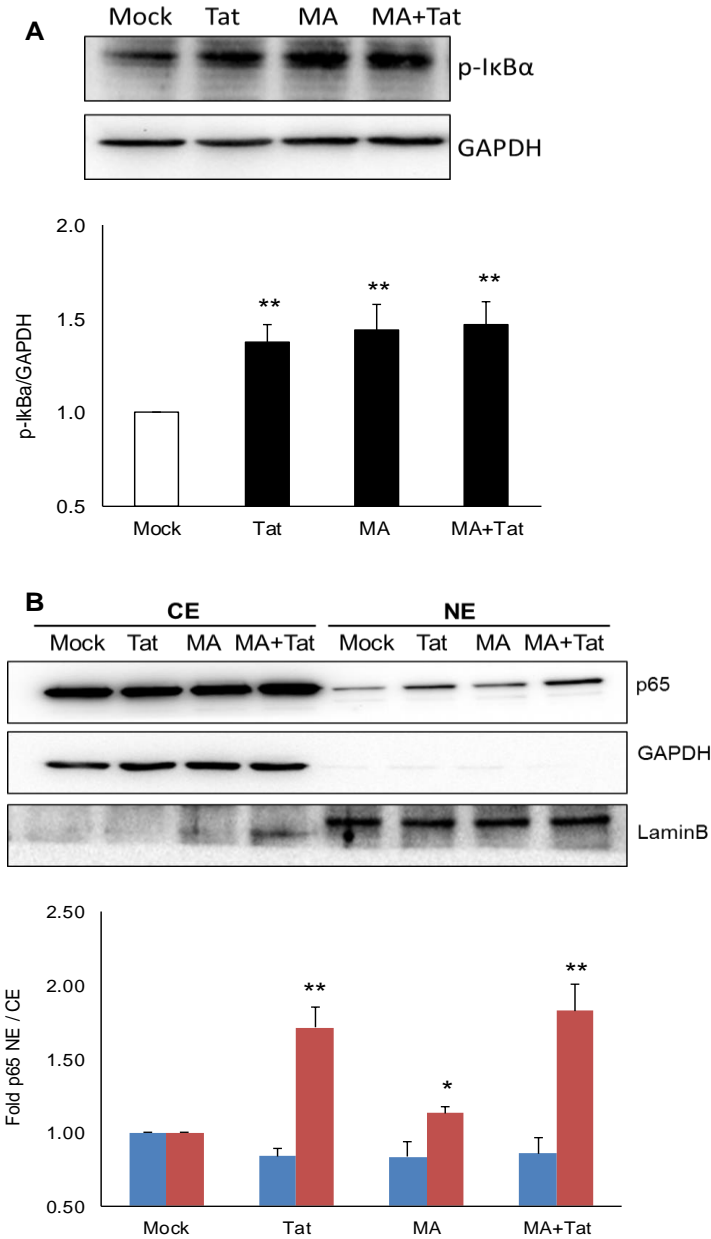


Figure 37: Methamphetamine and HIV-1 Tat induces NF-κB activation in astrocytes: SVG astrocytes were treated with 500 μM methamphetamine for 3 days before transfecting them with HIV-1 Tat. (A) Cells were harvested after 3 hours post-transfection and expression of p-IκBα was determined by western blot. (B) Cells were harvested after 6 hours post-transfection to separate nuclear and cytoplasmic fractions and measure p65 translocation into nucleus. GAPDH was used as a loading control for whole cell lysates and cytoplasmic fraction and LaminB was used as loading control for nuclear fraction. Each experiment was performed in triplicate and SE represents mean of three independent experiments. Statistical significance was calculated using one-way ANOVA with Tukey's post-hoc analysis and p-value ≤ 0.05 is considered as statistically significant.

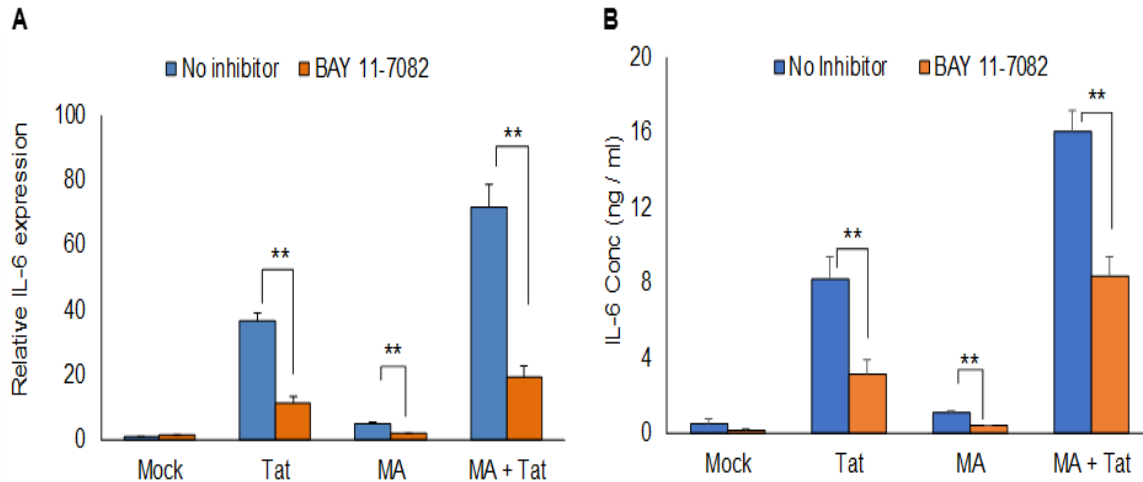


Figure 38: NF- κ B is involved in the induction of IL-6 by methamphetamine and HIV-1 Tat: SVG astrocytes were treated with 500 μ M of MA for 72 hours (3 days). The cells were seeded at 2.75×10^5 cells/well in 12-well plates. The cells were transfected with 0.3 μ g of plasmid expressing HIV-1 Tat. Cells were pre-treated with specific inhibitors for NF- κ B pathway (BAY 11-7082) 1 h prior to the addition of MA and/or HIV-1 Tat. The cells were harvested 6 hours and 48 hours after post-transfection for the determination of mRNA (A) and protein (B) levels, respectively. Each bar shows the mean \pm SE of three independent experiments performed in triplicate. The p-values ≤ 0.01 were considered statistically significant when calculated using one-way ANOVA.

4.2.3 Involvement of PI3K/Akt pathway in HIV-1 Tat and methamphetamine mediated potentiation of IL-6

We have investigated the role of PI3K/Akt pathway in HIV-1 Tat and methamphetamine mediated production of IL-6 in astrocytes. PI3K/Akt is an important upstream signaling pathway that is involved in the activation of NF- κ B and also has been shown to involve in HIV-1 Tat or methamphetamine mediated production of cytokines from astrocytes. In order to demonstrate the involvement of PI3K/Akt pathway, SVGA cells were transfected with HIV-1 Tat and/or methamphetamine for 3 hours and p-Akt levels were measured in whole cell lysates. There was significant increase in the expression of phosphorylated Akt by all the treatment groups (approximately by 50-100%) (Fig 39A). Similar to p-I κ B α expression levels, there was no significant difference in the expression levels of p-Akt by different treatment groups. To further confirm the involvement of PI3K/Akt pathway, SVGA cells were pretreated with 10 μ M LY294002, a specific PI3K inhibitor and the

expression levels of IL-6 was measured at 6 h post-transfection for mRNA and 48 h post-transfection for protein (Fig 40A, B). Together, these results indicate that HIV-1 Tat mediated induction of IL-6 was potentiated by methamphetamine and involves P3K/Akt pathway.

Overall, our results indicate that methamphetamine potentiates the induction of IL-6 at mRNA and protein levels by HIV-1 Tat from astrocytes. Furthermore, potentiation of IL-6 occurs through the involvement of PI3K/Akt and NF- κ B pathways.

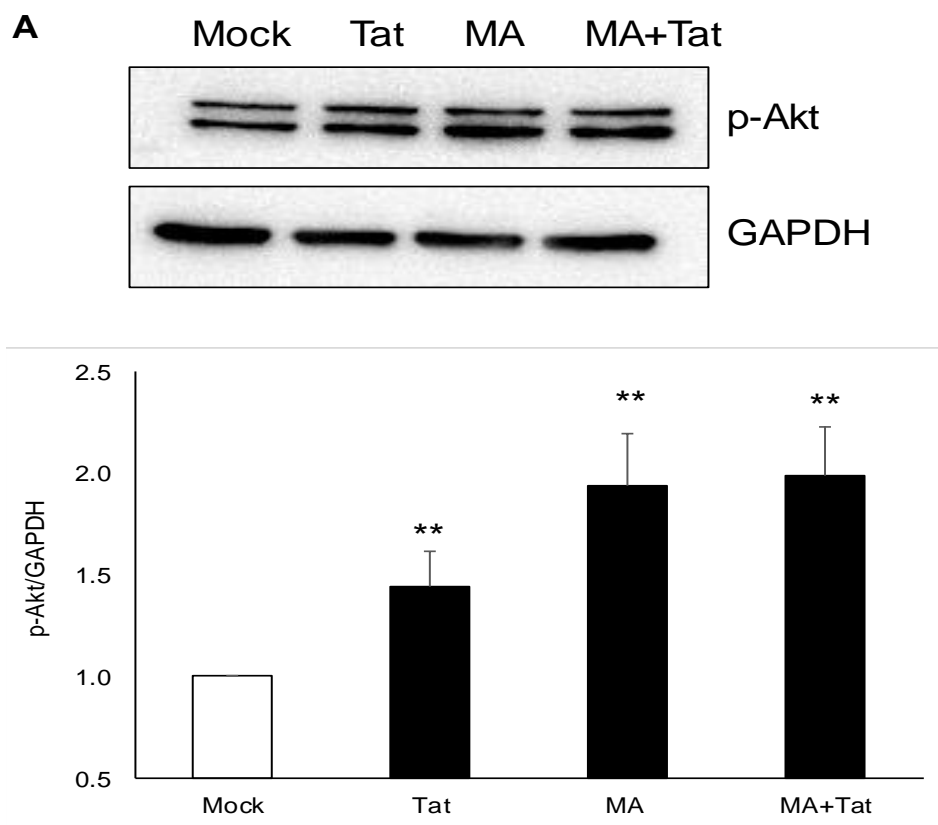


Figure 39: Methamphetamine and HIV-1 Tat induces PI3K/Akt activation in astrocytes: SVG astrocytes were treated with 500 μ M methamphetamine for 3 days before transfecting them with HIV-1 Tat. (A) Cells were harvested after 3 hours post-transfection and expression of p-Akt was determined by western blot. GAPDH was used as a loading control to normalize the expression. Each experiment was performed in triplicate and SE represents mean of three independent experiments. Statistical significance was calculated using one-way ANOVA with Tukey's post-hoc analysis and p-value ≤ 0.05 is considered as statistically significant.

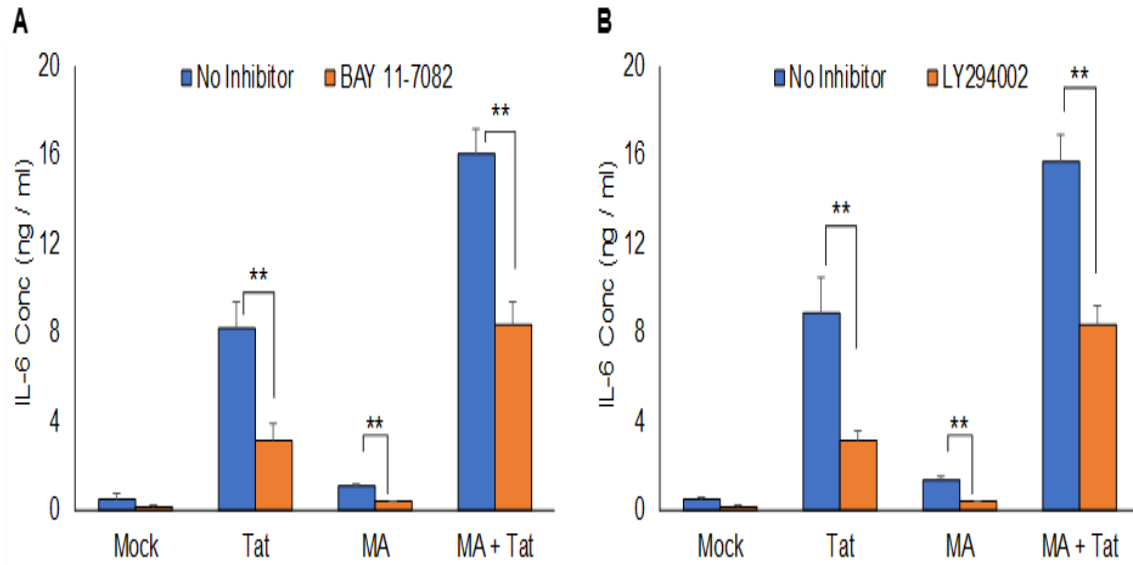


Figure 40: PI3K/Akt pathway is involved in the induction of IL-6 by methamphetamine and HIV-1 Tat: SVG astrocytes were treated with 500 μ M of MA for 72 hours (3 days). The cells were seeded at 2.75×10^5 cells/well in 12-well plates. The cells were transfected with 0.3 μ g of plasmid expressing HIV-1 Tat. Cells were pre-treated with specific inhibitors for PI3K pathway (LY 294002) 1 h prior to the addition of MA and/or HIV-1 Tat. The cells were harvested 6 hours and 48 hours after post-transfection for the determination of mRNA (A) and protein (B) levels, respectively. Each bar shows the mean \pm SE of three independent experiments performed in triplicate. The p-values \leq 0.01 were considered statistically significant when calculated using one-way ANOVA.

4.3 Discussion

Cytokines and chemokines are crucial for the regulation of immune responses in the CNS. However, increased production of pro-inflammatory cytokines is associated with pathology in several disease states, including Alzheimer's disease, Parkinson's disease, meningitis and HAND (Nagatsu, Mogi et al. 2000, Hanisch, Prinz et al. 2001, Brosseron, Krauthausen et al. 2014, de Almeida, Rotta et al. 2016). Elevated levels of various pro-inflammatory cytokines and chemokines often serve as biomarkers to correlate with the severity of HAND in individuals infected with HIV-1 (Yuan, Qiao et al. 2013, de Almeida, Rotta et al. 2016). One-third of the population infected with HIV-1 abuse one of the recreational drugs, including methamphetamine, cocaine and opioids and are associated with faster disease progression. Methamphetamine use in particular, promotes risky sexual behavior and increases the risk of acquiring HIV (Plankey, Ostrow et al. 2007). An earlier study, Rippeth et al have shown that HIV infected individuals who abuse methamphetamine exhibit a greater degree of neuropsychological impairment compared to individuals who abuse methamphetamine or infected with HIV (Rippeth, Heaton et al. 2004). Furthermore, several studies have shown that methamphetamine increases HIV replication and also increases brain viral load using in vitro and in vivo models (Liang, Wang et al. 2008, Nair, Saiyed et al. 2009, Marcondes, Flynn et al. 2010). Methamphetamine has also been shown to promote the expression of various pro-inflammatory cytokines (Shah, Silverstein et al. 2012, Robson, Turner et al. 2013). Interaction of methamphetamine and Tat have shown to promote higher expression of cytokines and induce neurotoxicity (Theodore, Cass et al. 2006, Theodore, Cass et al. 2006).

Therefore, we wanted to determine the underlying signaling mechanisms that are involved in methamphetamine and HIV-1 Tat mediated production of cytokines in astrocytes. We have shown that HIV-1 Tat and methamphetamine interact to induce higher expression of IL-6 from astrocytes at mRNA and protein levels. For the first time, we have shown that

HIV-1 Tat mediated induction of IL-6 in astrocytes is exacerbated by treatment with methamphetamine. We also confirmed our findings using primary human fetal astrocytes.

NF- κ B is a major transcriptional factor that is involved in the regulation of various cytokines (Lawrence 2009, Hoesel and Schmid 2013). We and several other groups have previously shown the involvement of NF- κ B in the induction of cytokines by HIV-1 Tat or methamphetamine (Shah, Silverstein et al. 2012, Nookala and Kumar 2014). We therefore investigated the role of NF- κ B as an overlapping mechanism in HIV-1 Tat and methamphetamine mediated production of IL-6. We have shown increased translocation of p65 and phosphorylation of I κ B α upon treatment with HIV-1 Tat and methamphetamine. Furthermore, increased expression of IL-6 by methamphetamine and/or HIV-1 Tat treatment was decreased upon pre-treatment with BAY 11-7082, a specific NF- κ B inhibitor. These results confirm the involvement of NF- κ B in HIV-1 Tat mediated induction of IL-6 that is exacerbated by methamphetamine in astrocytes.

PI3K is an upstream signaling pathway that is involved in the activation of NF- κ B by inducing the phosphorylation of I κ B α (Heck, Lezoualc'h et al. 1999). We and several other groups have previously shown the involvement of PI3K in the induction of cytokines by HIV-1 Tat or methamphetamine (Shah, Silverstein et al. 2012, Nookala and Kumar 2014). Our results with the use of LY294002, a specific PI3K inhibitor decreased HIV-1 Tat and methamphetamine mediated production of IL-6 in astrocytes.

In conclusion, our results for the first time demonstrated exacerbated production of IL-6 at mRNA and protein levels by combination treatment with methamphetamine and HIV-1 Tat in astrocytes. Exacerbation of IL-6 is induced by overlapping mechanisms involving PI3K/Akt and NF- κ B pathways.

CHAPTER 5

EFFECT OF HIV-1 Tat AND METH ON COGNITION AND EXPRESSION OF VARIOUS SYNAPTIC GENES

5.1 Introduction

HIV-1 infection results in the development of wide range of neurocognitive disorders, together referred to as HIV-Associated Neurocognitive Disorders (HAND). Introduction of combination Anti-Retroviral Therapy (cART) has increased the life expectancy of people infected with HIV-1 and thereby, prevalence of HAND has increased over the past decade (Dore, McDonald et al. 2003). More than 50% of the people infected with HIV-1 develop some form of neurocognitive impairment (Heaton, Clifford et al. 2010). Higher degree of neurocognitive impairment interferes with the individual's ability to perform day to day activities (Antinori, Arendt et al. 2007). The incidence and degree of neurocognitive impairments are higher among individuals abusing illicit drugs, especially methamphetamine (Boddiger 2005, Potula and Persidsky 2008). HIV-1 does not infect the neurons, but neuronal damage and toxicity is the major feature of HAND. This neuronal toxicity is explained by direct and indirect actions of released viral proteins (gp120, HIV-1 Tat, Nef, Vpr). Viral proteins can directly act on the neurons or indirectly promote the release of various soluble mediators from different brain cells or change the environment of brain to cause neurotoxicity. The neurocognitive impairments associated with HIV-1 infection produces correlates with changes in synaptic proteins and dendrites (Masliah, Heaton et al. 1997, Everall, Heaton et al. 1999).

HIV-1 transactivator of transcription (HIV-1 Tat or Tat) is a viral protein that is released very early during the viral replication. It binds to the transactivation response element of the viral mRNA to promote transcriptional elongation (Cann, Rosenblatt et al. 1985). HIV-1 infected individuals with dementia and encephalitis show elevated levels of Tat mRNA in their brain tissues (Del Valle, Croul et al. 2000, Hudson, Liu et al. 2000). Tat is released in active form from infected cells and uptaken by different brain cells and neurons by passive diffusion

or by interacting with various receptors (Liu, Jones et al. 2000, Tyagi, Rusnati et al. 2001, King, Eugenin et al. 2006). Tat is known to promote neurotoxicity by various mechanisms; including glutamate excitotoxicity (Haughey, Nath et al. 2001), NMDA mediated increase in intracellular calcium levels (Krogh, Lyddon et al. 2015), increase in reactive oxygen species and nitric oxide levels (Kim, Smith et al. 2015), increase in cytokines (Nookala and Kumar 2014, Mediouni, Jablonski et al. 2015) and disruption of blood brain barrier (Huang, Chen et al. 2014). Tat alters the expression of various genes associated with synaptic plasticity, thereby preventing the consolidation of short term events into long term memory. Neuropathology that is seen in doxycycline inducible Tat transgenic mice is similar to that observed in HIV-1 infected individuals. For instance, reactive astrocytosis that is considered to be the prominent feature of HIV-1 induced pathology is seen in the brain of Tat transgenic mice (Vitkovic and da Cunha 1995, Kim, Liu et al. 2003). Also, dendritic pathology, a prominent feature associated with neurocognitive impairment in HIV-1 infected individuals is seen in Tat expressing transgenic mice (Hagen, Haram et al. 1991, Masliah, Heaton et al. 1997). Consistent with these findings, Tat alone has been shown to impair learning and memory (Carey, Sypek et al. 2012, Fitting, Ignatowska-Jankowska et al. 2013).

Methamphetamine is the second most commonly abused illicit drug world wide. It poses a great risk for HIV-1 infection due to risky sexual behaviors, particularly among bisexual and gay men (Garofalo, Mustanski et al. 2007). It is mainly abused for its euphoric potential and stimulant properties on central and peripheral nervous systems. Methamphetamine mainly affects the dopaminergic system and also GABAergic, serotonergic systems in different brain regions (Ricaurte, Schuster et al. 1980, Wilson, Kalasinsky et al. 1996). Methamphetamine abuse results in the neurotoxic effects on different regions of the brain, including frontal cortex, hippocampus, basal ganglia that are associated with memory, locomotion, cognition and executive functions (Belcher, O'Dell et al. 2005, Chang, Ernst et al. 2005, Berman, O'Neill et al. 2008). Consistent with these findings, methamphetamine use has

been shown to impair learning and memory in human population and animal models (Scott, Woods et al. 2007, Siegel, Craytor et al. 2010, Kesby, Markou et al. 2015). The effects of methamphetamine on memory impairment are long lasting as measured by the decrease in prospective memory after 6 months of abstinence (Rendell, Mazur et al. 2009). Moreover, astrocytosis and dendritic pathology that are seen in HIV-1 infection are also associated with methamphetamine abuse (Kuczenski, Everall et al. 2007, Raineri, Gonzalez et al. 2012).

The study was undertaken to determine the combined effects of HIV-1 Tat and methamphetamine on locomotion, anxiety, learning and spatial memory. Open field was used to measure general locomotor activity and parameters of anxiety and light/dark box was employed to measure anxiety. Y maze and Morris water maze were employed to evaluate the spatial learning and working memory in the mice. Furthermore, we also aimed to determine the effect of methamphetamine and HIV-1 Tat on expression of various genes associated with synaptic plasticity.

5.2 Results

5.2.1 Effect of HIV-1 Tat and METH on ambulation

HIV-1 infected individuals increased the prevalence of people suffering with minor forms of neurocognitive disorders. These HIV-1 infected individuals are associated with several psychiatric disorders, including distress and anxiety (Myers, Satz et al. 1997, Bing, Burnam et al. 2001). These influence the daily living of the people and also present a huge economic burden on the society. Apart from socioeconomic factors, molecular and epigenetic factors induced by viral proteins, especially HIV-1 Tat might play a role in mediating these effects. To test this hypothesis, we employed open field and light/dark box to determine general locomotor activity and anxiety.

Experiments were performed on both male and female mice to determine the gender differences. Doxycycline inducible HIV-1 Tat transgenic mice were used in all the experiments. Mice that do not express HIV-1 Tat and reverse tetracycline transactivator (rtTA) genes served as controls. Dose of METH was escalated from 0.1mg/kg to 6mg/kg over 1 week and maintained at 6mg/kg for 2 weeks before the commencement of behavioral testing. Mice were given equal volumes of either saline or METH twice a day through intraperitoneal injection. Weight of the animals were recorded every week. METH treatment regimen did not cause any change in the weight of the animals over the duration of the experiment (Fig. 41A, B). HIV-1 Tat expression was determined by RT-PCR different regions of the brain (Fig. 42). Mice which expressed Tat and rTTA genes showed significantly high expression of HIV-1 Tat RNA compared to control mice. Expression was higher in cerebellum compared to prefrontal cortex or parietal cortex or hippocampus.

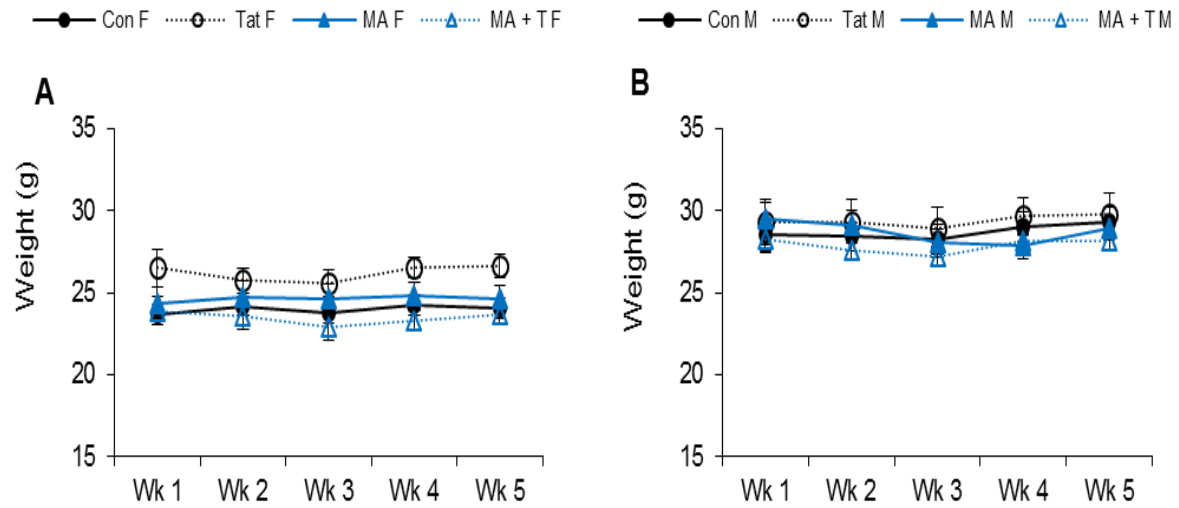


Figure 41: Effect of METH administration on body weight: (A, B) 6 month old wild type mice and HIV-1 Tat mice were administered either PBS or METH for 4 weeks. Body weight of the mice were taken once a week until euthanasia. METH treatment did not change body weight between different treatment groups in females (A) and males (B).

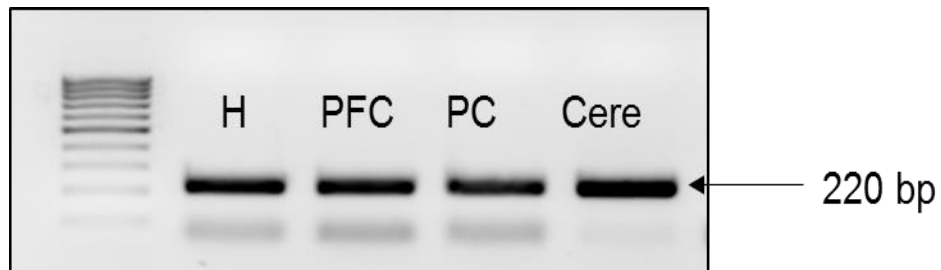


Figure 42: Doxycycline formulated chow induced HIV-1 Tat expression in different brain regions: Different brain regions were isolated from mice after euthanasia. Total RNA was isolated and 150 ng RNA was used to run RT-PCR. Amplified product was run on 2% agarose gel and HIV-1 Tat was identified by presence of band at 220 bp in hippocampus, prefrontal cortex, parietal cortex and cerebellum. A representative image of Tat expression in one mice is shown in the figure.

5.2.2 Effect of HIV-1 Tat and METH in open field assay

Generalized locomotor activity and anxiety were evaluated in control mice and HIV-1 Tat mice that were treated with either saline or METH. Mice were placed at one of the corner in the apparatus and allowed to explore for 5 min. The starting position of mice was chosen at random. Ambulatory activity was determined by measuring the total distance traveled by the mice over 5 min duration. Total distance traveled by female mice was significantly higher compared to male mice (14.71 ± 1.3 m vs 10.75 ± 0.87 m). Chronic administration of METH increased the ambulatory activity in mice. The percentage increase in the total distance traveled compared to vehicle treated controls is 45.0 ± 18.6 % in females and 24.3 ± 20.1 % in males (Fig. 43A, B). Also, the total distance traveled by METH treated female mice was significantly higher compared to METH treated male mice (21.3 ± 2.7 m vs 13.4 ± 2.16 m) ($p < 0.01$). We also calculated the distance traveled and number of entries made by the mice in the center zone. These two measures serve as indicators of anxiety. The distance traveled in center zone was significantly lower in Tat +ve mice in female (1.18 ± 0.39 vs 2.78 ± 0.57 in controls) and male (0.73 ± 0.22 vs 2.03 ± 0.22 in controls). Distance traveled by mice in the center increased in METH treated animals. The increase in the distance traveled in the center in METH treated HIV-1 Tat –ve mice was higher than saline administered HIV-1 Tat –ve controls in males and females. Similarly, distance traveled in the center was higher in METH treated HIV-1 Tat +ve mice compared to saline administered HIV-1 Tat +ve mice in males and females (Fig. 43C, D). Number of entries made into the center zone was significantly lower in Tat +ve mice in female (7.5 ± 2.37 vs 18.17 ± 3.61 in controls) and male (6.25 ± 1.87 vs 14.64 ± 1.70 in controls). Number of entries made by mice into the center increased in METH treated animals. The increase in the number of entries to the center in METH treated HIV-1 Tat –ve mice was higher than saline administered HIV-1 Tat –ve controls in males and females. Similarly, number of entries made into the center was higher in METH treated HIV-1 Tat +ve mice compared to saline administered HIV-1 Tat +ve mice in males and females (Fig. 43E, F).

These results indicate that HIV-1 Tat induction induced anxiety in both male and female mice. However, METH treatment did not exacerbate the anxiety induced by HIV-1 Tat. It further decreased the anxiety in HIV-1 Tat –ve mice and HIV-1 Tat +ve mice.

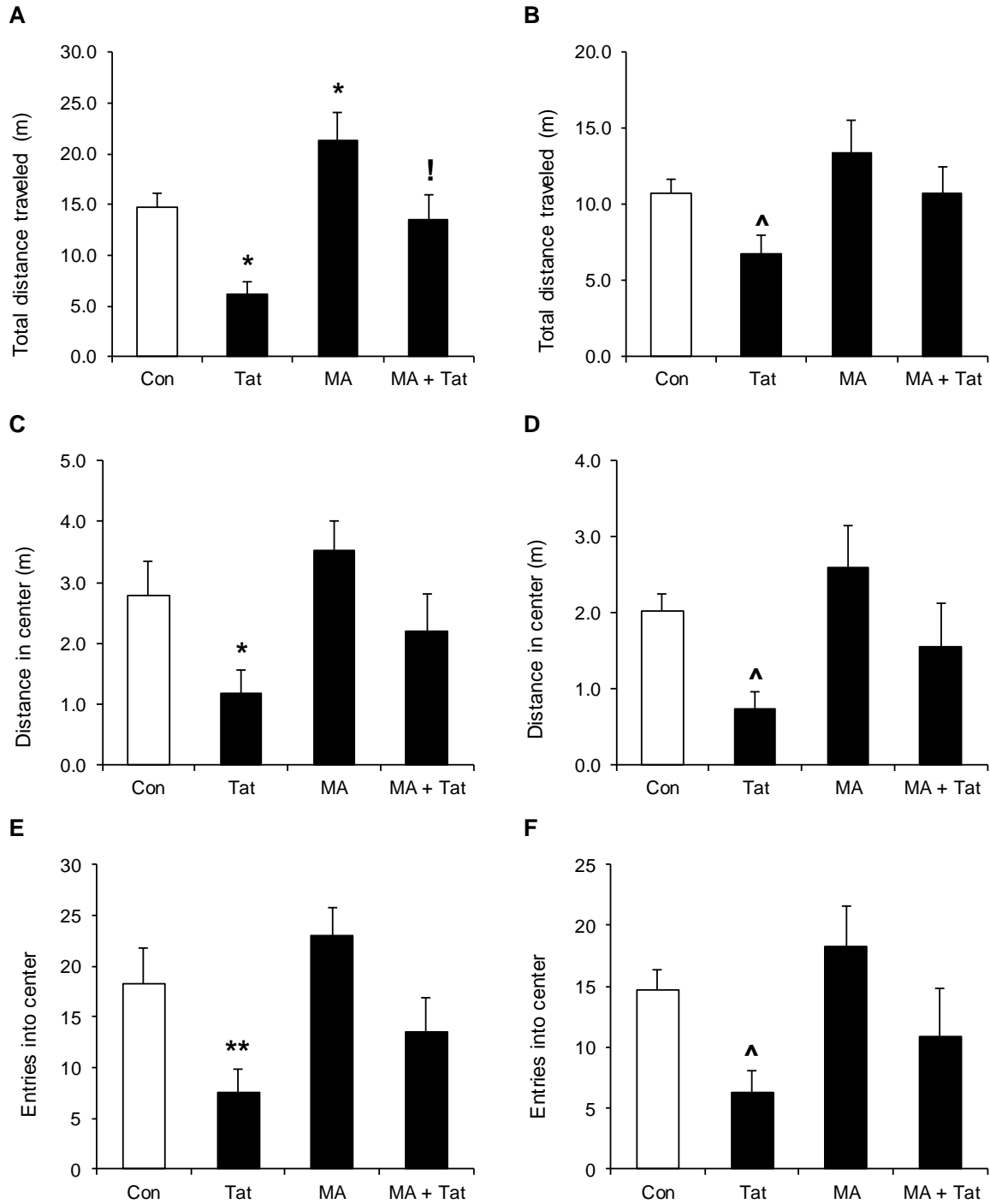


Figure 43: Effect of HIV-1 Tat and METH on ambulatory activity in open field assay: (A-F) 6 month old wild type mice and HIV-1 Tat mice were administered either PBS or METH for 3 weeks and tested on open field assay. (A,B) Total distance traveled by mice over 5 min test duration was measured in females (A) and males (B). (C, D) Total distance traveled by mice in the center zone over 5 min test duration was measured in females (C) and males (D). (E, F) Number of entries into center zone over 5 min test duration was measured in females (E) and males (F). * indicates p-value < 0.05 compared to WT controls; ^ indicates p-value < 0.1 compared to WT controls.

5.2.3 Effect of HIV-1 Tat and METH in Light/dark box assay

We have employed light dark box to evaluate anxiety like phenomenon in mice after chronic HIV-1 Tat induction and/or METH treatment. Mice were placed into the apparatus and activity was monitored over a duration of 10 min. Amount of time spent and number of entries made into light zone were measured and served as indicators of anxiety. Chronic HIV-1 Tat induction decreased the time spent in light zone compared to control mice of both sexes (31.8 ± 5.35 sec vs 39.14 ± 4.85 sec in females and 20.45 ± 4.84 sec vs 52.72 ± 11.09 in males). (Fig. 44A, B). However, statistical significance was achieved only in male mice, but not in female mice. Chronic administration of METH has increased the time spent in the light zone. It increased from 39.14 ± 4.85 sec to 49.10 ± 6.67 sec in females and from 52.72 ± 11.09 sec to 67.46 ± 12.75 sec in males. Administration of METH to HIV-1 Tat +ve mice also showed similar phenomenon compared to saline administered HIV-1 Tat +ve mice in both the sexes. Results from entries made into light zone also showed similar phenomenon (Fig. 44C, D). Thus, the results from open field and light dark box assays indicate that chronic HIV-1 Tat induction results in anxiety like phenomenon and this is over come in mice that were administered METH.

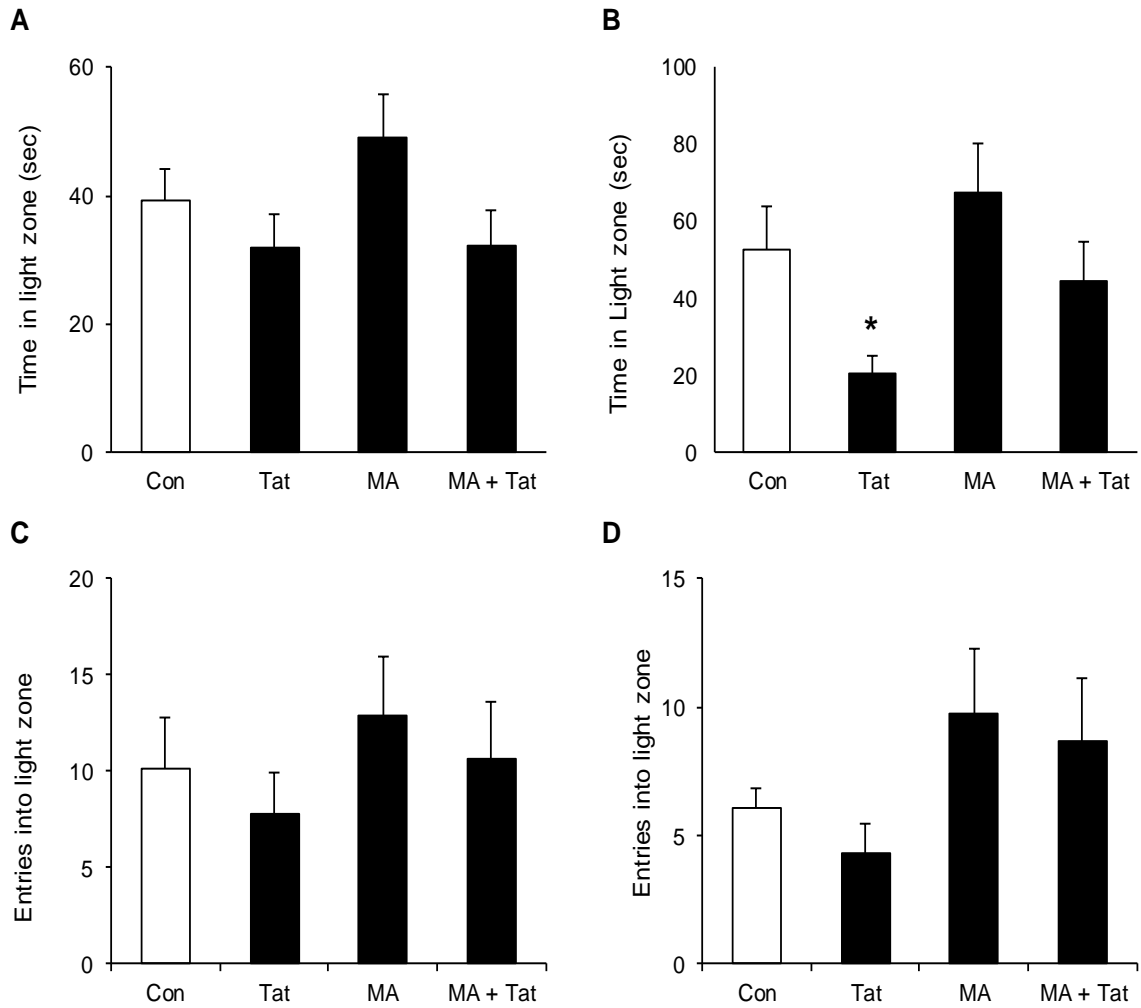


Figure 44: Effect of HIV-1 Tat and METH on anxiety in Light/dark box assay: (A-D) 6 month old wild type mice and HIV-1 Tat mice were administered either PBS or METH for 3 weeks and tested on Light/dark box assay. (A, B) Time spent by mice in light zone over 10 min test duration was measured in females (A) and males (B). (C, D) Entries made into light zone over 10 min test duration was measured in females (C) and males (D). * indicates p-value < 0.05 compared to WT controls.

5.2.4 Effect of HIV-1 Tat and METH on working memory

HIV-1 seropositive individuals who abuse drugs are associated with increased working memory deficits compared to HIV-1 seronegative drug users (Bartok, Martin et al. 1997). Also, HIV-1 infection alone is associated with decline in working memory (Stout, Salmon et al. 1995, Hinkin, Hardy et al. 2002). Furthermore, long term METH administration lead to deficits in memory (Recinto, Samant et al. 2012). However, the combined effect of HIV-1 Tat and METH is unknown. In order to determine the combined effect of HIV-1 Tat and METH on neurocognition, we have employed Y-maze and Morris water maze. Experiments were performed on both male and female mice to determine the sex differences. Doxycycline inducible HIV-1 Tat transgenic mice were used in all the experiments. Mice that do not express HIV-1 Tat and rTTA genes served as controls. Dose of METH was escalated from 0.1mg/kg to 6mg/kg over 1 week and maintained at 6mg/kg for 2 weeks before the commencement of behavioral testing. Mice were given equal volumes of either saline or METH twice a day through intraperitoneal injection.

Spontaneous alternation was evaluated using Y-maze and serves as an indicator of working memory. A significant decrease in the spontaneous alternation was observed in HIV-1 Tat mice administered METH in both male and female mice compared to vehicle treated control group (n=8-13 per group; $p < 0.05$; Fig. 45A, B).). Despite the trend, HIV-1 Tat induction alone did not affect spontaneous alteration compared to the control group in both the sexes ($p > 0.05$). However, there was a significant decrease in the spontaneous alteration of methamphetamine treated male mice compared to the control group ($p = 0.09$), but not the female mice. Compared to HIV-1 Tat male group, there was significant decrease in spontaneous alternation in HIV-1 Tat mice treated with METH ($p < 0.05$). There was no difference in any of the groups based on the gender. Furthermore, there were no significant differences in the number of total arm entries made by mice in each group in both the sexes (Fig. 45C, D). Also, the number of arm entries between the sexes remained similar across

different groups. This indicates that general locomotor function was not altered and did not influence the effects of HIV-1 Tat and METH alone or in combination on spontaneous alternation.

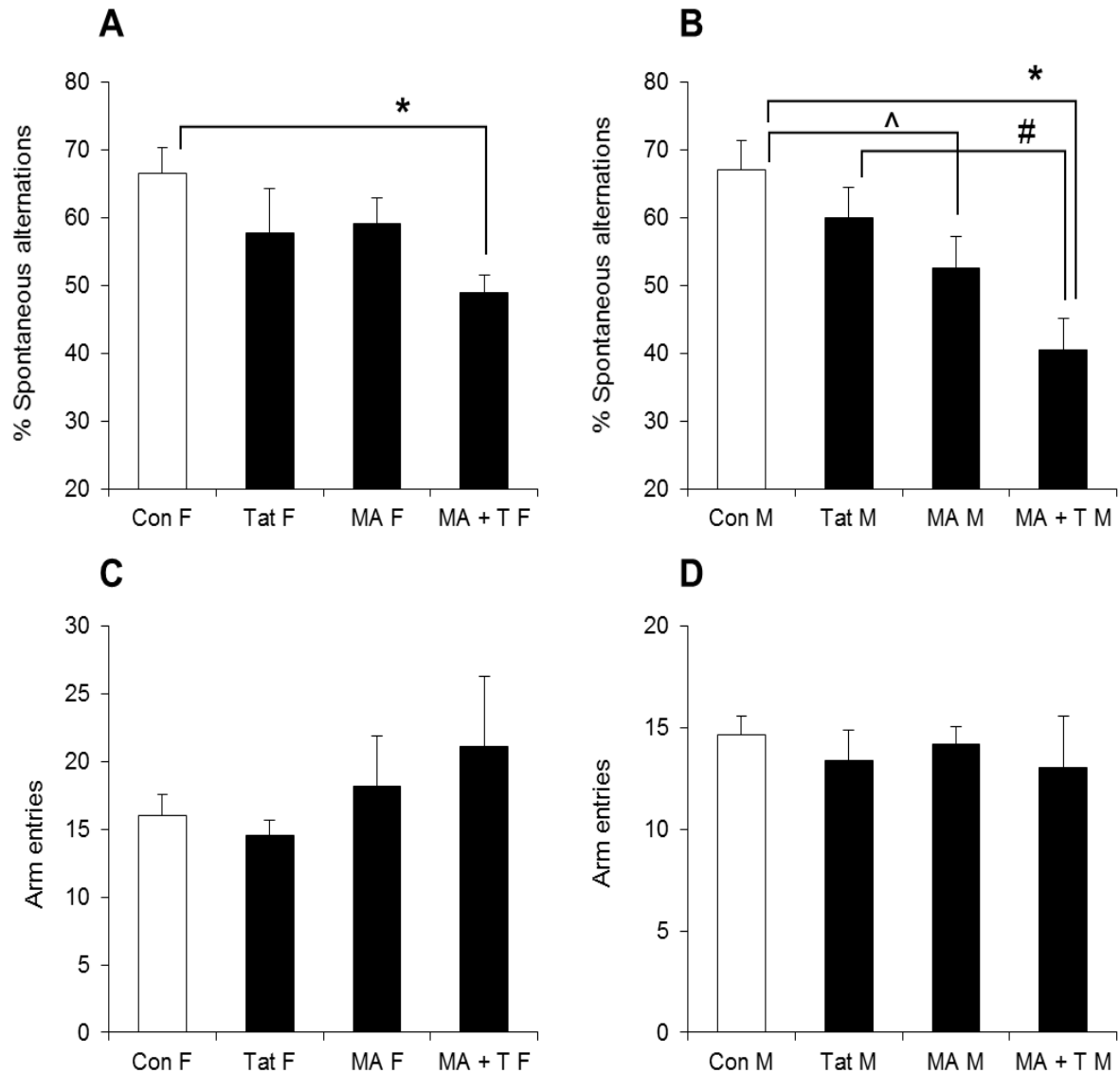


Figure 45: Effect of HIV-1 Tat and METH on spontaneous alternations in Y-maze: (A, B) 6 month old wild type mice and HIV-1 Tat mice were administered either PBS or METH for 3 weeks and tested on Y maze for 5 min. HIV-1 Tat positive mice that were administered METH showed significantly lower number of spontaneous alternations compared to WT mice in females (A) and males (B) ($p < 0.05$, one-way ANOVA with Tukey's post hoc analysis). (C, D) The total number of arm entries made by mice were not significantly different between different treatment groups in females (C) and males (D). * indicates p -value < 0.05 compared to WT controls; ^ indicates p -value < 0.1 compared to WT controls; # denotes p -value < 0.05 compared to HIV-1 Tat mice.

5.2.5 Effect of HIV-1 Tat and methamphetamine on spatial learning and memory

Morris water maze is commonly employed to demonstrate deficits in spatial learning and memory in rodents. Previous studies have indicated decline in spatial learning and memory with HIV-1 Tat or METH alone (Camarasa, Rodrigo et al. 2010, Carey, Sypek et al. 2012, Chen, Liu et al. 2012, Fitting, Ignatowska-Jankowska et al. 2013). Therefore, we determined the combined effect of HIV-1 Tat and METH on spatial learning and memory using Morris water maze. Spatial learning was evaluated by determining the time taken to reach the escape platform over 5 days of acquisition trial, and reference memory was evaluated by determining time spent in target quadrant and number of annulus entries (platform crossings) in probe trial. Data was normally distributed in all the groups between sexes (Shapiro Wilk test; $p > 0.05$ for all groups). Mice in control group from both sexes learnt to find the escape platform quickly compared to the other groups. Escape latency was 38.2 ± 3.6 sec on D1 compared to 14.6 ± 1.9 sec on D5 for male mice and 39.2 ± 3.1 sec on D1 compared to 13.1 ± 1.4 sec on D5 for female mice (Fig. 46A, B). Induction of HIV-1 Tat resulted in longer times to find the escape platform in male and female mice ($p = 0.059$ for males and $p < 0.01$ for females). METH treatment resulted in longer times to find the escape platform, but reached statistical significance only in females ($p < 0.01$). However, HIV-1 Tat mice that are treated with METH took significantly longer time to reach the hidden platform compared to other treatment groups in both the sexes ($p < 0.01$). We also determined the swim speed of the animals over the course of acquisition trial (Fig. 46C, D). Average swim speed of the animals between different groups remained consistent over the duration of acquisition trial, indicating that treatment did not affect the swim speed of the animals. No significant differences were observed in the swim speeds of the animals between the sexes.

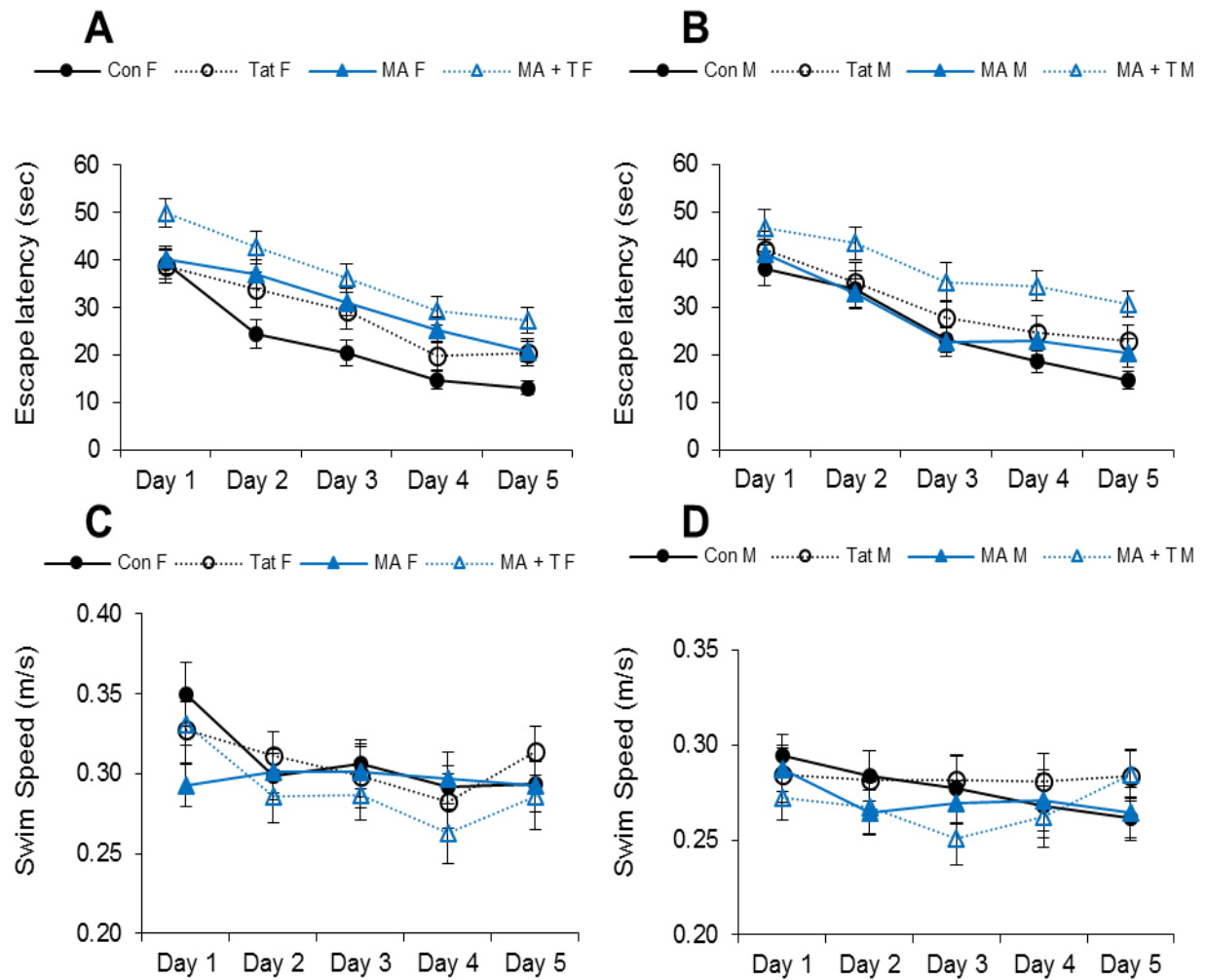


Figure 46: METH treated HIV-1 Tat mice showed impaired spatial learning in Morris water maze: (A, B) 6 month old wild type mice and HIV-1 Tat mice were administered either PBS or METH for 3 weeks and tested on Morris water maze. Escape latency (sec) was measured over of five day acquisition trial. Values represented are mean \pm SE of all trials in all the mice of a particular group. There was significant increase in the escape latency of HIV-Tat female mice ($p < 0.01$) and male mice ($p = 0.059$), METH treated female mice ($p < 0.01$) and METH treated HIV-1 Tat female mice ($p < 0.01$) and male mice ($P < 0.01$). (C, D) Swimming speed was calculated over five day acquisition trial and no significant difference was observed between different groups in females (C) and males (D).

A probe trial was conducted 24 h after the acquisition trial to evaluate reference memory. Animals from control group from both the sexes memorized the platform location better than animals from other groups, indicated by the time spent in the target quadrant (22.18 ± 1.58 sec for females vs 20.09 ± 1.24 sec for males) (Fig. 47A, B). There was a significant decrease in the time spent by the animals in the target quadrant from HIV-1 Tat group and combination group (HIV-1 Tat + METH) in both males and females ($p < 0.05$). Time spent by the animals in the target quadrant was significantly less in the combination group compared to only METH treated group in males, but not in females (Fig. 41A, B). Furthermore, data from the number of platform crossings showed similar results that align with the time spent in target quadrant (Fig. 47C, D). There was no difference in the number of platform crossings between similar groups in males and females. These results indicate that reference memory was affected by HIV-1 Tat and METH did not worsen it.

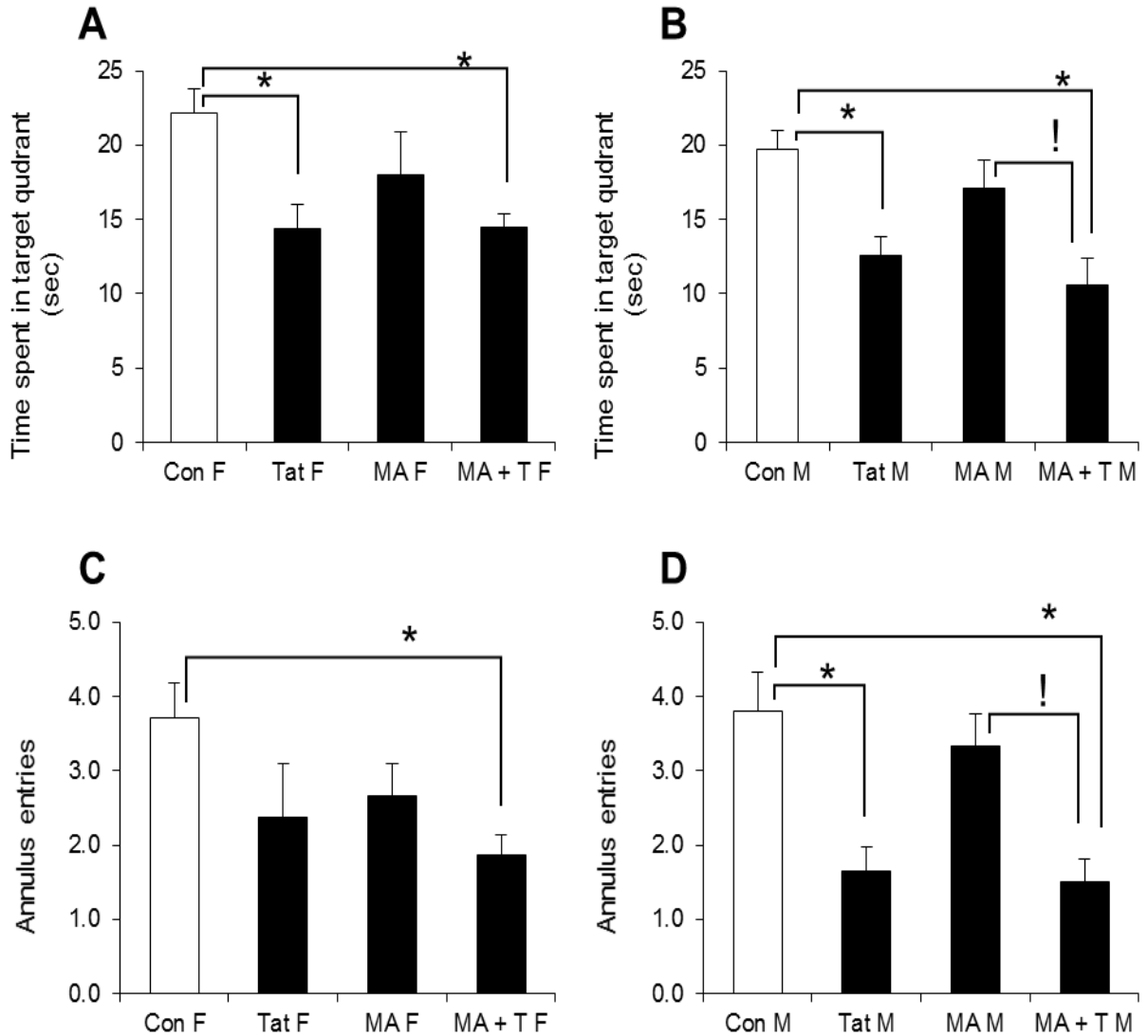


Figure 47: METH treated HIV-1 Tat mice showed impaired spatial learning and memory in Morris water maze: (A, B) 6 month old wild type mice and HIV-1 Tat mice were administered either PBS or METH for 3 weeks and tested on Morris water maze. Each mice was given 60 sec probe trial and time spent in target quadrant (A, B) and total number of annulus entries (platform crossings) (C, D) were calculated. Time spent in target quadrant were significantly lower in HIV-1 Tat mice and HIV-1 Tat mice that were administered METH in females (A) and males (B) compared to WT controls. Total number of annulus entries were decreased in HIV-1 Tat mice and HIV-1 Tat mice that were administered METH in females (C) and males (D) compared to WT controls. There was no statistical difference between sexes (female vs. male) as calculated through ANOVA. * indicates p-value < 0.05 compared to WT controls; # denotes p-value < 0.05 compared to HIV-1 Tat mice; ! denotes p-value < 0.05 compared METH administered mice.

5.2.6 Effect of HIV-1 Tat and METH on expression of synaptic proteins

We wanted to determine the mechanisms that are associated with deficits in learning and memory with HIV-1 Tat and METH. Mice were humanely euthanized and brain to body ratio was calculated (Fig. 48A, B). There was no significant difference in the brain weights between different treatment groups in both sexes.

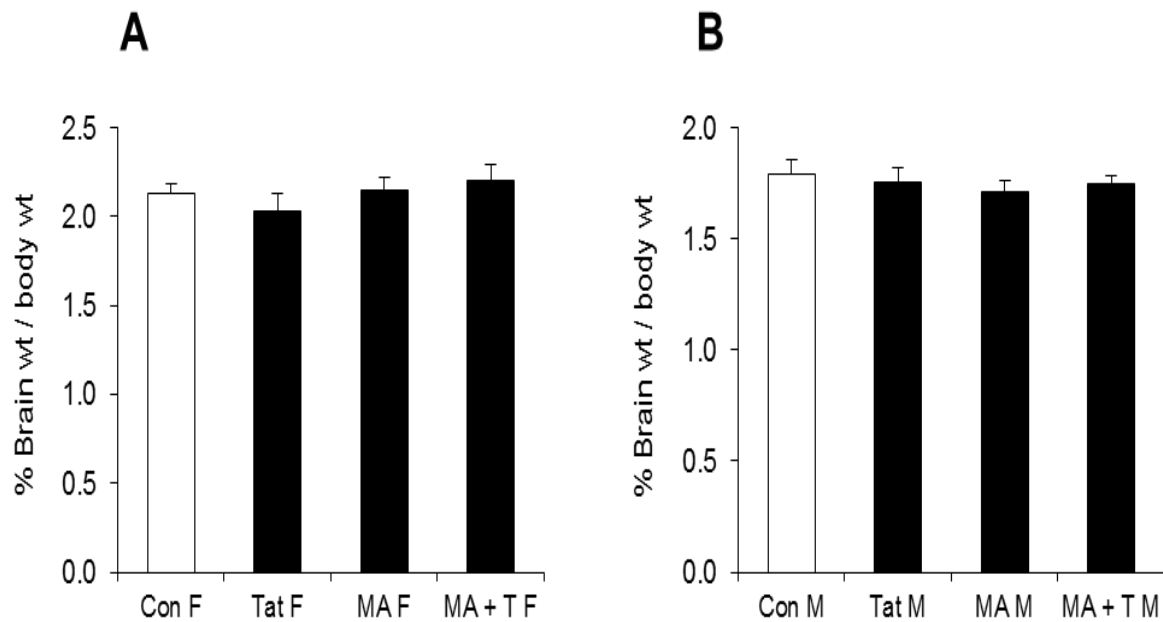


Figure 48: Effect of HIV-1 Tat and METH on brain to body weight ratio: (A, B) 6 month old wild type mice and HIV-1 Tat mice were administered either PBS or METH for 4 weeks. Brain weight and body weight of the mice were taken at euthanasia. HIV-1 Tat induction and METH treatment did not affect brain weight to body weight ratio between different treatment groups in females (A) and males (B).

Milder forms of neurocognitive impairments mediated by HIV-1 result in changes to synapse organization without neuronal death (Everall, Heaton et al. 1999). Therefore, we sought to determine the expression of various synaptic proteins in response to HIV-1 Tat and METH. Synaptic proteins are involved in transmission of synapse and also serve as scaffolds. They are involved in the consolidation of short term memory events into long term memory events (Schmitt, Tanimoto et al. 2009). There are different types of synaptic proteins with diverse functions, classified into pre-synaptic and post-synaptic proteins. Therefore, we performed western blot to determine the effect of HIV-1 Tat and METH on expression of various synaptic proteins, including two pre-synaptic proteins (synapsin1, synaptophysin) and three post-synaptic proteins (PSD-95, Arg3.1 and shank).

5.2.1.1 Effect of HIV-1 Tat and METH on synaptophysin

In order to determine the effect of HIV-1 Tat and METH on synaptic plasticity, we examined the expression of synapsin1 and synaptophysin, two important pre-synaptic proteins in different regions of the brain. Synaptophysin, important pre-synaptic protein plays an important role in formation of synapse and vesicle endocytosis. Similar to synapsin1, HIV-1 Tat and METH decreased its expression in parietal cortex. Compared to the individual groups, expression of synaptophysin was significantly affected in HIV-1 Tat mice treated with METH, with a decrease of $41 \pm 4.5\%$ in males and $40 \pm 5.6\%$ in females (Fig. 49A, B). Synaptophysin expression was also decreased in the hippocampus of HIV-1 Tat induced male mice (with or without METH), but not in the METH alone treated mice. Furthermore, synaptophysin expression remained unchanged in female hippocampus and other brain regions of both male and female mice.

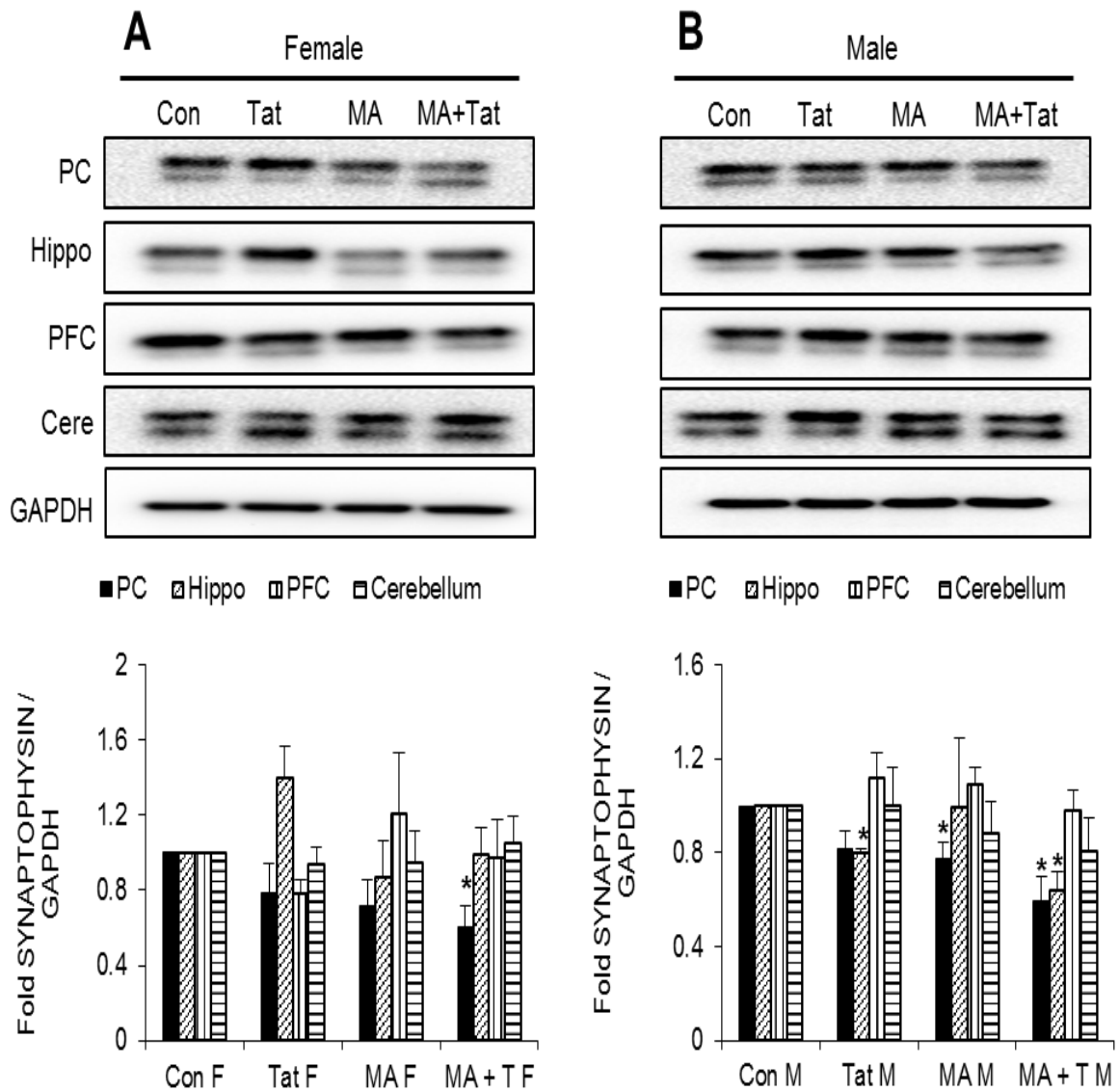


Figure 49: Effect of HIV-1 Tat and METH on the expression of pre-synaptic proteins: (A-B) WT control mice and HIV-1 Tat mice were treated with either PBS or METH. Different brain regions were isolated as described in materials and methods. Similar protein concentration from all the mice in a group were pooled together (n=8-13). (A, B) Expression of synaptophysin was measured in different brain regions through western blot in females (A) and males (B). Results are expressed as mean \pm SE of at least 3 different western blots. Single GAPDH was used as a representative loading control. One-way AVOVA with Tukey's post hoc for multiple comparisons was performed to calculate the statistical significance. * indicates p-value < 0.05 compared to WT controls.

5.2.1.2 Effect of HIV-1 Tat and METH on synapsin1

Synapsin1 accounts for the abundant pre-synaptic protein present in the synaptic vesicles of nerve terminals. It facilitates the release of neurotransmitters that aid in long term potentiation and memory formation. Expression of synapsin1 was decreased in response to HIV-1 Tat induction in parietal cortex of both males and females by $21 \pm 6.3\%$ and $24 \pm 1.8\%$, respectively (Fig. 50A, B). Similar to HIV-1 Tat, METH treatment decreased the expression of synapsin1 in parietal cortex, which was $37 \pm 4.3\%$ and $18 \pm 4.7\%$ in males and females, respectively (Fig. 50A, B). Synapsin1 was also decreased in HIV-1 Tat mice treated with METH in both genders. METH treated female mice showed a significant reduction in the expression of synapsin1 in hippocampus. (Fig 50A). No significant change in the expression of synapsin1 was observed in other brain regions. Furthermore, the expression of synapsin1 was increased in the cerebellum of HIV-1 Tat mice that were given methamphetamine.

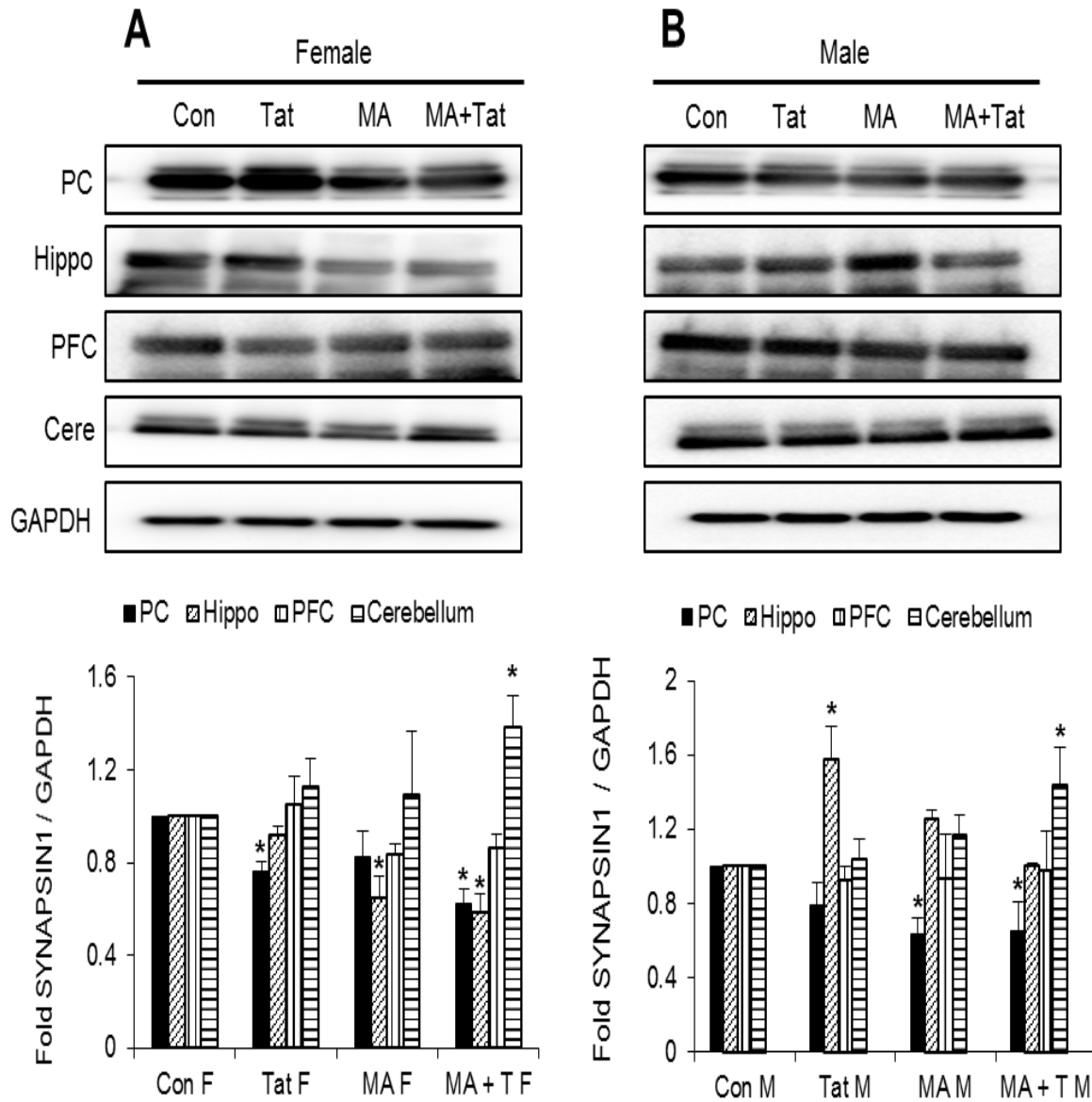


Figure 50: Effect of HIV-1 Tat and METH on the expression of synapsin1: (A-B) WT control mice and HIV-1 Tat mice were treated with either PBS or METH. Different brain regions were isolated as described in materials and methods. Similar protein concentration from all the mice in a group were pooled together (n=8-13). (A, B) Expression of synapsin1 was measured in different brain regions through western blot in females (A) and males (B). Results are expressed as mean \pm SE of at least 3 different western blots. Single GAPDH was used as a representative loading control. One-way AVOVA with Tukey's post hoc for multiple comparisons was performed to calculate the statistical significance. * indicates p-value < 0.05 compared to WT controls.

5.2.7 Effect of HIV-1 Tat and METH on post-synaptic proteins

5.2.7.1 Effect of HIV-1 Tat and METH on PSD-95

Post synaptic density protein 95 (PSD-95) belongs to the family of membrane associated guanylate kinase. It is one of the most important post-synaptic proteins that is involved in synaptic transmission and associated with various forms of memory. It promotes bidirectional synaptic plasticity by interacting with NMDA and AMPA receptors (Beique and Andrade 2003). Previous studies have indicated the key role of PSD-95 in spatial learning, associative learning and fear memory (Migaud, Charlesworth et al. 1998, Nithianantharajah, Komiyama et al. 2013, Rashid, Cole et al. 2014, Fitzgerald, Pinard et al. 2015). Recent studies have indicated the role of HIV-1 and HIV-1 Tat on the expression of PSD-95 on human neuroblastoma and isolated rat hippocampal neurons, respectively (Atluri, Kanthikeel et al. 2013, Hargus and Thayer 2013). Therefore, we determined the expression levels of PSD-95 in response to methamphetamine treatment and/or HIV-1 Tat induction. PSD-95 expression was determined by immunoblot on different brain regions of male and female mice. HIV-1 Tat induction decreased the expression of PSD-95 in prefrontal cortex by $20 \pm 3.0\%$ and METH treatment decreased PSD-95 expression by $21 \pm 4.2\%$ in parietal cortex of male mice (Fig. 51B). PSD-95 expression was not altered by HIV-1 Tat induction or treatment with METH in different brain regions of both male and female mice (Fig. 51A, B). However, expression of PSD-95 was significantly decreased in HIV-1 Tat and METH group of male and female mice in different brain regions, including hippocampus, prefrontal cortex and parietal cortex.

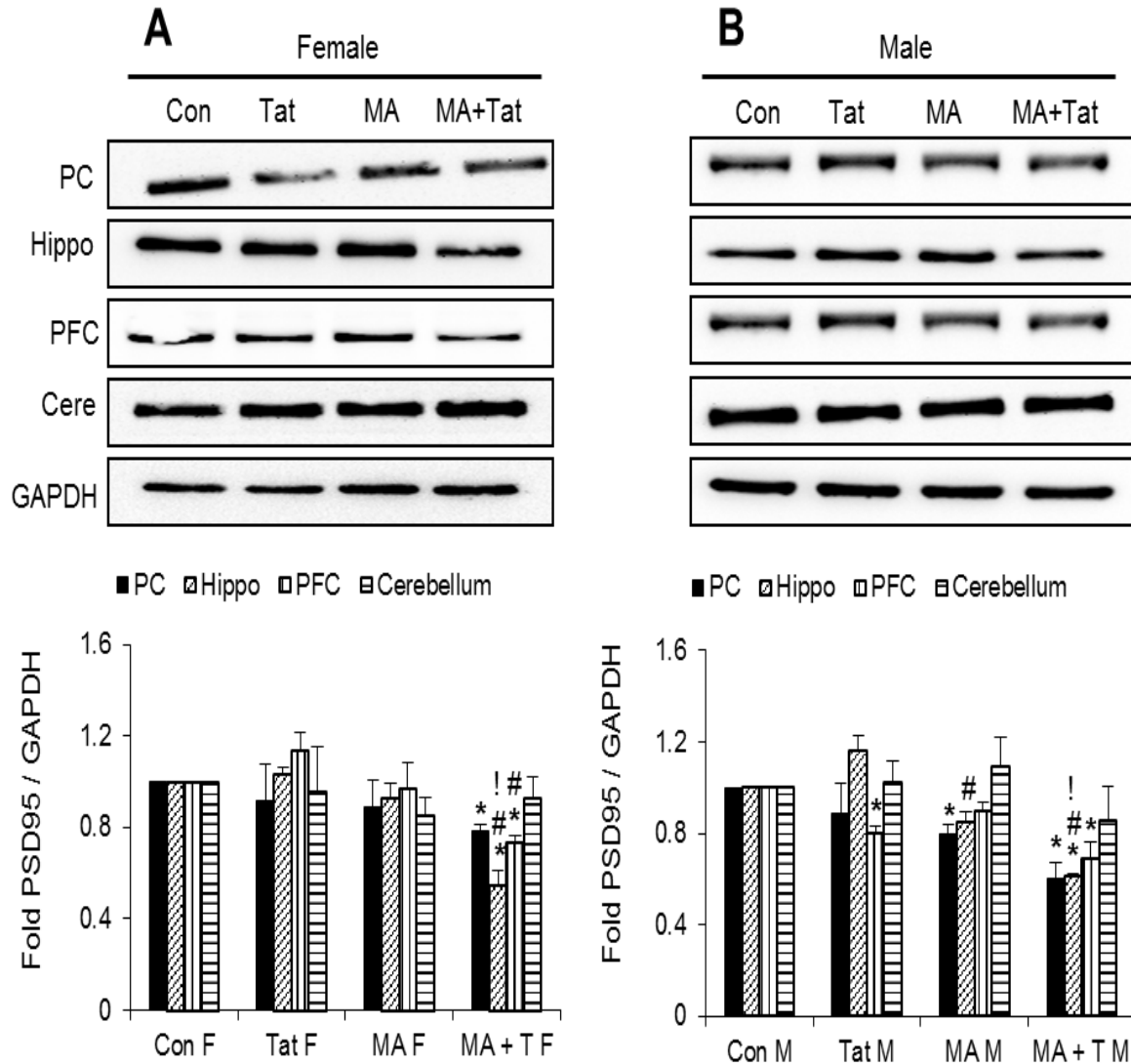


Figure 51: Effect of HIV-1 Tat and METH on the expression of PSD95: (A-B) WT control mice and HIV-1 Tat mice were treated with either PBS or METH. Different brain regions were isolated as described in materials and methods. Similar protein concentration from all the mice in a group were pooled together (n=8-13). (A, B) Expression of PSD95 was measured in different brain regions through western blot in females (A) and males (B). Results are expressed as mean \pm SE of at least 3 different western blots. Single GAPDH was used as a representative loading control. One-way AVOVA with Tukey's post hoc for multiple comparisons was performed to calculate the statistical significance. * indicates p-value < 0.05 compared to WT controls; # denotes p-value < 0.05 compared to HIV-1 Tat mice; ! denotes p-value < 0.05 compared METH administered mice.

5.2.7.2 Effect of HIV-1 Tat and METH on ARG3.1

Activity regulated cytoskeleton-associated protein (Arg3.1) is an important immediate early gene that is involved in various memory processes, including formation of spatial memory and fear memory (Guzowski, Lyford et al. 2000, Monti, Berteotti et al. 2006). Activated synapses and dendrites contain newly transcribed arg3.1 and this modulates the expression of AMPA receptors (Rial Verde, Lee-Osbourne et al. 2006). HIV-1 Tat induction and METH treatment alone decreased the expression of arg3.1 in parietal cortex of both male and female mice (Fig. 52A, B). However, arg3.1 expression was significantly reduced in HIV-1 Tat mice treated with METH. The decrease in arg3.1 expression was significant in parietal cortex ($31 \pm 8.3\%$ in males and $41 \pm 6.0\%$ in females) and prefrontal cortex ($22 \pm 1.6\%$ in males and $32 \pm 1.8\%$ in females) (Fig. 52A, B).

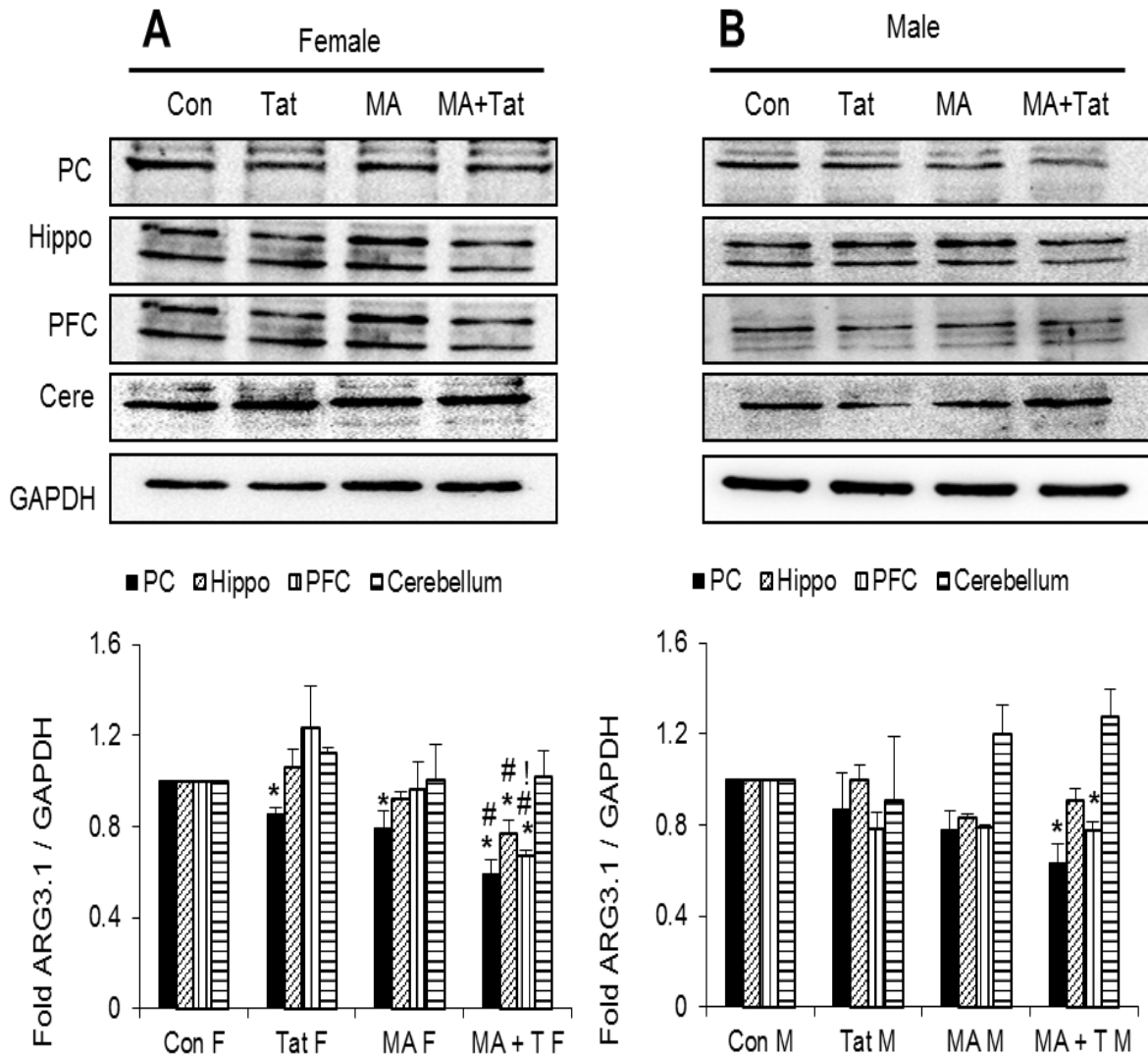


Figure 52: Effect of HIV-1 Tat and METH on the expression of ARG3.1: (A-B) WT control mice and HIV-1 Tat mice were treated with either PBS or METH. Different brain regions were isolated as described in materials and methods. Similar protein concentration from all the mice in a group were pooled together (n=8-13). (A, B) Expression of ARG3.1 was measured in different brain regions through western blot in females (A) and males (B). Results are expressed as mean \pm SE of at least 3 different western blots. Single GAPDH was used as a representative loading control. One-way AVOVA with Tukey's post hoc for multiple comparisons was performed to calculate the statistical significance. * indicates p-value < 0.05 compared to WT controls; # denotes p-value < 0.05 compared to HIV-1 Tat mice; ! denotes p-value < 0.05 compared METH administered mice.

5.2.7.3 Effect of HIV-1 Tat and METH on Shank2

Shank2 is a post-synaptic protein that plays vital role in synaptogenesis. It contains multiple protein binding domains that facilitates the association of different molecules. We evaluated the expression of shank2 in different brain regions by western blot analysis. Expression of shank was significantly altered in the prefrontal cortex region of male and female HIV-1 Tat mice treated with METH ($28 \pm 2.7\%$ in males and $18 \pm 0.9\%$ in females). The decrease in shank 2 expression of HIV-1 Tat mice or methamphetamine treated mice was less than the combination group. Expression of shank2 remained unchanged in other brain regions. However, its expression significantly increased in the cerebellum in the treatment groups of both male and female (Fig. 53A, B).

These results indicate that HIV-1 Tat and METH combination significantly affected the expression of many pre-synaptic and post-synaptic proteins that are responsible for proper synaptic transmission and function.

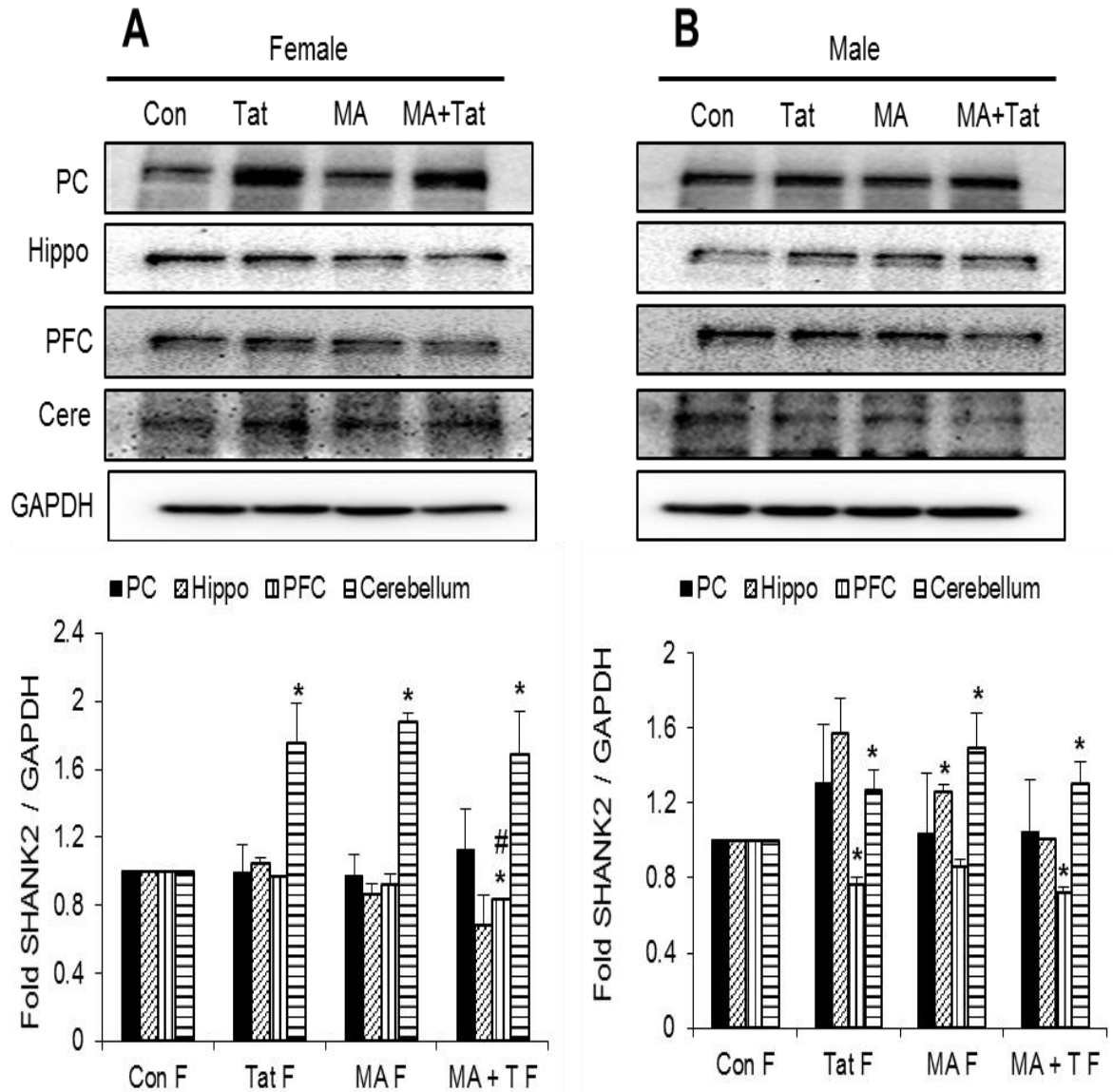


Figure 53: Effect of HIV-1 Tat and METH on the expression of SHANK2: (A-B) WT control mice and HIV-1 Tat mice were treated with either PBS or METH. Different brain regions were isolated as described in materials and methods. Similar protein concentration from all the mice in a group were pooled together (n=8-13). (A, B) Expression of SHANK2 was measured in different brain regions through western blot in females (A) and males (B). Results are expressed as mean \pm SE of at least 3 different western blots. Single GAPDH was used as a representative loading control. One-way AVOVA with Tukey's post hoc for multiple comparisons was performed to calculate the statistical significance. * indicates p-value < 0.05 compared to WT controls; # denotes p-value < 0.05 compared to HIV-1 Tat mice;

5.2.8 Effect of HIV-1 Tat and methamphetamine on expression of neurotrophic factors

Neurotrophic factors perform important roles in synaptic plasticity, neuronal development, survival and proper function (Huang and Reichardt 2001, Sofroniew, Howe et al. 2001, Bramham and Messaoudi 2005). Neurotrophins exert their function by the activation of different classes of receptors, including receptor tyrosine kinases and TNF receptor superfamily. Decrease in the expression of various neurotrophins is associated with various disease states, including Alzheimer's disease (Connor, Young et al. 1997), Parkinson's disease (Nagatsu and Sawada 2007) and HIV infected individuals with encephalitis (Fields, Dumaop et al. 2014). Owing to their role in long term potentiation and memory, neurotrophins are being developed as therapeutic agents. Previous studies have indicated the role of HIV-1 Tat and METH on expression of different neurotrophic factors (Angelucci, Gruber et al. 2007, Darbinian, Darbinyan et al. 2008). In order to determine the combined effect of HIV-1 Tat and METH on the expression of various neurotrophic factors, we performed western blot on different brain regions. We determined the expression of brain-derived neurotrophic factor (BDNF) and ciliary neurotrophic factor (CNTF) in prefrontal cortex, parietal cortex, hippocampus and cerebellum. Expression of BDNF was upregulated in prefrontal cortex and cerebellum of female Tat mice, but not male Tat mice (Fig. 54A, B). However, expression of BDNF remained unchanged in hippocampus and parietal cortex of male and female Tat mice. METH treatment resulted in significant decrease in the expression of BDNF in parietal cortex, hippocampus and prefrontal cortex of female mice and prefrontal cortex and cerebellum of male mice. HIV-1 Tat mice treated with METH showed significant decrease in the expression of BDNF in all the brain regions of both male and female mice. Expression of BDNF was significantly lower in the combination group compared to either HIV-1 Tat or MA groups. We also determined the expression of CNTF in different brain regions of both male and female

mice (Fig. 55A, B). However, no significant differences were observed in any of the treatment groups in both the genders.

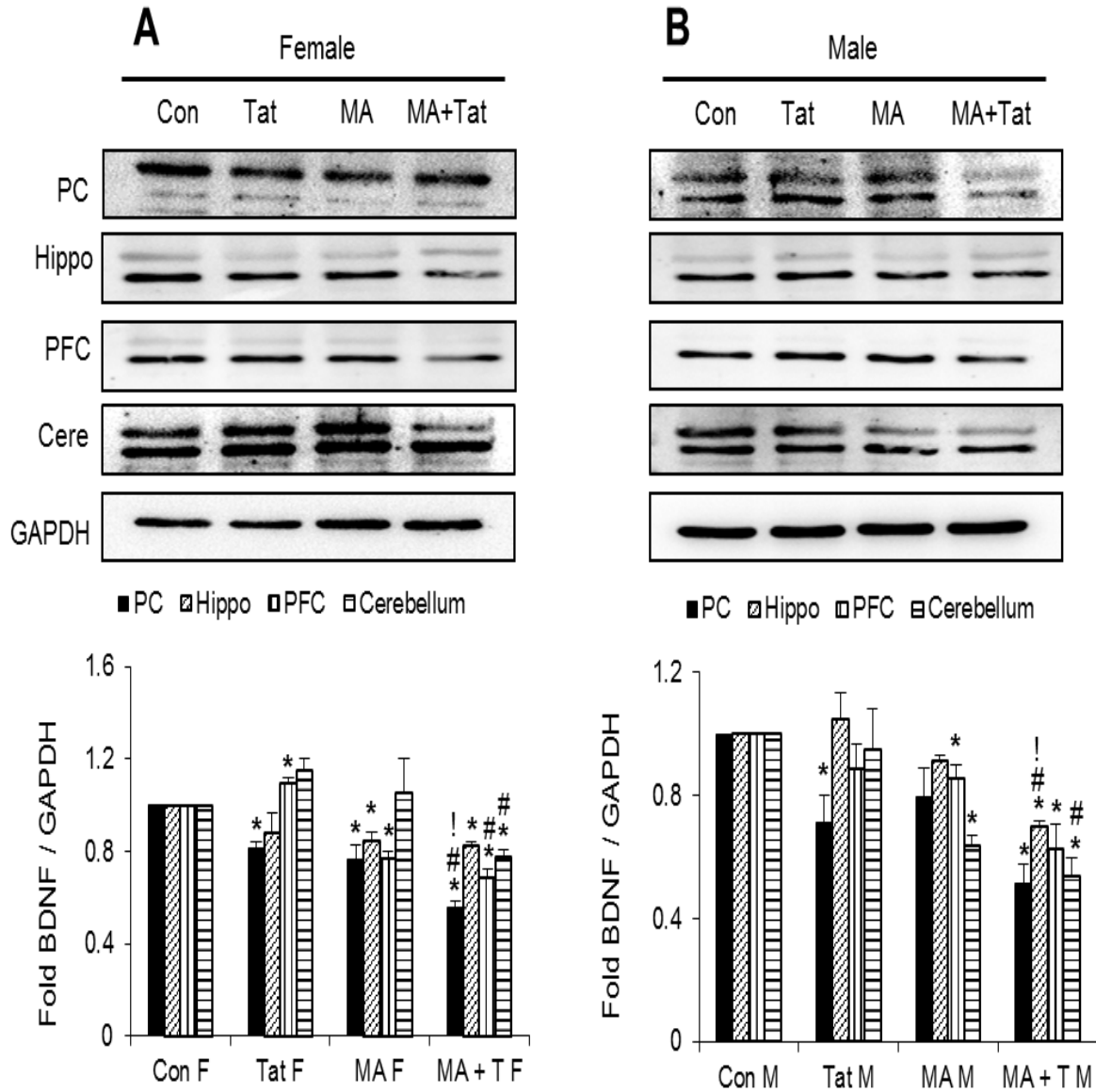


Figure 54: Effect of HIV-1 Tat and METH on the expression of BDNF: (A-B) WT control mice and HIV-1 Tat mice were treated with either PBS or METH. Different brain regions were isolated as described in materials and methods. Similar protein concentration from all the mice in a group were pooled together (n=8-13). (A, B) Expression of BDNF was measured in different brain regions through western blot in females (A) and males (B). Results are expressed as mean \pm SE of at least 3 different western blots. Single GAPDH was used as a representative loading control. One-way ANOVA with Tukey's post hoc for multiple comparisons was performed to calculate the statistical significance. * indicates p-value < 0.05 compared to WT controls; # denotes p-value < 0.05 compared to HIV-1 Tat mice;

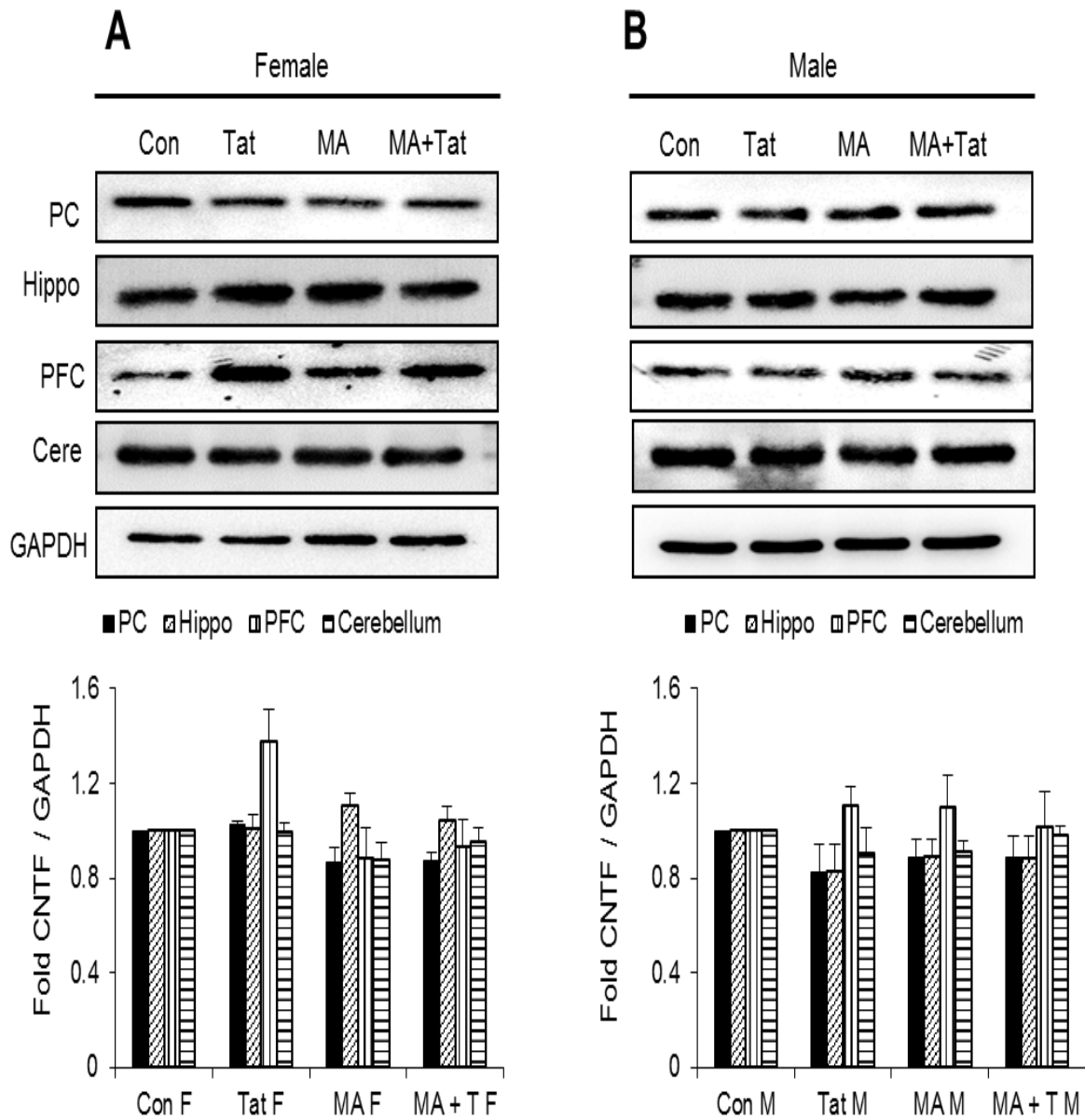


Figure 55: Effect of HIV-1 Tat and METH on the expression of CNTF: (A-B) WT control mice and HIV-1 Tat mice were treated with either PBS or METH. Different brain regions were isolated as described in materials and methods. Similar protein concentration from all the mice in a group were pooled together (n=8-13). (A, B) Expression of CNTF was measured in different brain regions through western blot in females (A) and males (B). Results are expressed as mean \pm SE of at least 3 different western blots. Single GAPDH was used as a representative loading control. One-way AVOVA with Tukey's post hoc for multiple comparisons was performed to calculate the statistical significance.

5.2.9 Effect of HIV-1 Tat and methamphetamine on p-CaMKII expression

CaMKII is highly expressed protein in the brain and is present in abundant quantities in post synaptic density. Activation of CaMKII results in the induction of long term potentiation by its effects on AMPA receptors (Sanhueza and Lisman 2013). Therefore, we measured the effect of HIV-1 Tat and METH on the expression of CaMKII by western blot. We specifically measured the phosphorylated form of CamKII on threonine 286, an active form. As shown in figure Fig 50A and 50B, HIV-1 Tat has significantly decreased the expression of p-CaMKII in parietal cortex, prefrontal cortex and cerebellum of HIV-1 Tat induced male mice, but not female mice (Fig 56B). Furthermore, METH treatment caused a significant decrease in the expression of p-CaMKII in all the brain regions, including hippocampus, prefrontal cortex, parietal cortex and cerebellum. Furthermore, administration of METH to HIV-1 Tat mice showed a significant decrease in all the brain regions of both male and female compared to HIV-1 Tat or METH alone (Fig. 56A, B).

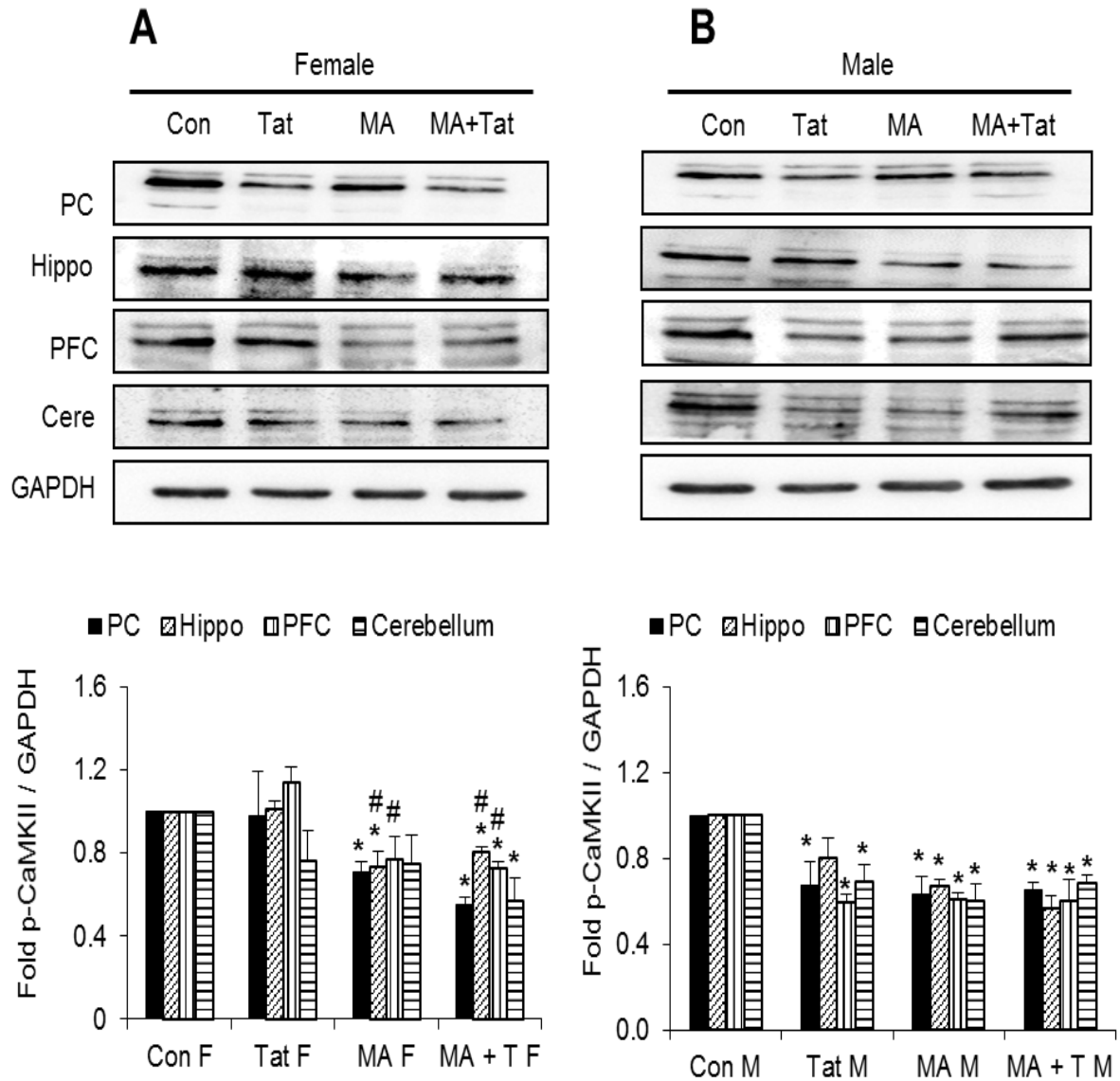


Figure 56: Effect of HIV-1 Tat and METH on the expression of p-CaMKII: (A-D) WT control mice and HIV-1 Tat mice were treated with either PBS or METH. Different brain regions were isolated as described in materials and methods. Similar protein concentration from all the mice in a group were pooled together (n=8-13). (A, B) Expression of p-CaMKII was measured in different brain regions through western blot in females (A) and males (B). Results are expressed as mean \pm SE of at least 3 different western blots. Single GAPDH was used as a representative loading control. One-way AVOVA with Tukey's post hoc for multiple comparisons was performed to calculate the statistical significance. * indicates p-value < 0.05 compared to WT controls; # denotes p-value < 0.05 compared to HIV-1 Tat mice; ! denotes p-value < 0.05 compared METH administered mice.

5.3 Discussion

METH dependence increases the risk for HIV-1 infection and also in the development of associated neuropsychological impairments (Rippeth, Heaton et al. 2004). There is significant decrease in the number of interneurons in the frontal cortex of HIV-1 infected individuals with a history of METH use (Chana, Everall et al. 2006). HIV-1 viral proteins, including HIV-1 Tat and gp120 are known to play an important role in the development of psychological and neurological impairments that are similar to those seen in HIV-1 infected individuals (Carey, Sypek et al. 2012, Hofer, Sanchez et al. 2015, Kesby, Markou et al. 2015, Marks, Paris et al. 2016). In the present study, we wanted to determine the effect of escalating, multiple dose METH regimen in adult HIV-1 Tat transgenic mice that conditionally express HIV-1 Tat in the presence of doxycycline. As previously reported, multiple and escalating doses of METH did not induce hyperthermia (data not shown) and reflected abuse pattern seen in human population (Furtmuller, Arnhold et al. 2003, Hofer, Sanchez et al. 2015). Previous studies by different groups have indicated that HIV-1 Tat induces anxiety. Our results from open field assay and light/dark box are in agreement with those results. However, there were no adverse effects of METH administration in control and Tat transgenic mice. Furthermore, the present treatment has decreased the anxiety levels induced by HIV-1 Tat. Future studies are required to determine the long term effects of HIV-1 Tat and METH on anxiety.

Previous studies using escalating and multiple doses of METH in mice have shown increased latency to escape in Barnes maze and alterations in the prepulse inhibition (Henry, Geyer et al. 2014). Our Y maze results indicated that there is significant difference in the working memory as indicated by the number of spontaneous alternations made in HIV-1 Tat transgenic mice that were treated with METH. Chronic HIV-1 Tat induction did not affect the number of spontaneous alterations compared to control in both male and female. Treatment with METH showed a trend towards decreased number of spontaneous alterations. There was

no difference in the number of spontaneous alterations based on the gender. Results from morris water maze indicated that both HIV-1 Tat and METH affected spatial learning. However, combination of HIV-1 Tat induction and METH treatment resulted in longest times to reach the escape platform during the acquisition trial. There was no significant effect of METH on the time spent in target quadrant and number of platform crossings in probe trial, in both wild type and HIV-1 Tat transgenic mice. These results indicate that METH treatment did not affect the reference memory. Results from Y maze and morris water maze indicate that executive function is more compromised in the combination mice group (HIV-1 Tat and METH). This is in agreement with the previous studies indicating poor working memory in METH users and HIV-seropositive individuals (Stout, Salmon et al. 1995, Gonzalez, Bechara et al. 2007).

Proper synaptic integrity is required for transmission of information and thereby achieving synaptic plasticity. There are different number of synaptic proteins, located either presynaptically or postsynaptically that are responsible for the maintenance of synaptic integrity. We evaluated the expression of synaptophysin and synapsin I as markers of pre-synaptic integrity. PSD-95, Arc and Shank 2 are evaluated as markers of post-synaptic integrity. Synaptophysin is a protein located abundantly in the synaptic vesicle with diverse functions, including regulation of synaptic vesicle endocytosis, synapse formation and long term potentiation (Goodglick and Kane 1990, Janz, Sudhof et al. 1999, Tarsa and Goda 2002). Protein expression analysis indicates that the levels of synaptophysin were affected by HIV-1 Tat and METH in parietal cortex of both males and females. The effect was more pronounced in HIV-1 Tat mice treated with methamphetamine. The results are consistent with previous reports indicating decreased synpatophysin expression in cell culture and animal models of HIV and METH induced brain injury (Kaewsuk, Sae-ung et al. 2009, Maung, Hoefler et al. 2014, Torres and Noel 2014).

Synapsin is a pre-synaptic protein that is present in the synaptic vesicles of nerve terminals. It facilitates the organization of synaptic vesicle clusters and modulates the release of neurotransmitters (Bykhovskaia 2011). HIV-1 infection reduced the expression of synapsin 1 in the synaptosomes and the degree of reduction correlated with the viral load (Gelman and Nguyen 2010). Also, chronic METH administration decreased the expression of synapsin 1 in rats (Wang, Sun et al. 2012). Similar to these findings, our results indicated reduced expression of synapsin 1 in various brain regions of HIV-1 Tat induced animals and animals treated with chronic METH regimen. Decreased expression of synapsin 1 might result in the depletion of synaptic vesicles in the releasable pool and thereby affects synaptic plasticity (Gitler, Takagishi et al. 2004). Future studies are required to determine the effect of HIV-1 Tat and METH on reduced expression of synapsin 1 at excitatory synapses or inhibitory synapses.

Owing to its role in CNS development and function, we have also determined the expression of PSD-95 as a marker for post-synaptic plasticity. PSD95 expression was unaltered in most brain regions upon HIV-1 Tat induction or treatment with METH. Significant difference in the expression of PSD-95 was observed in the combination group of both the genders in different brain regions. Decrease in the expression of PSD-95 decreases the ability of glutamate receptors to connect with the intracellular signaling pathways (Sheng 2001). This could be partly responsible for decreased long term potentiation that was previously observed in mice expressing HIV-1 Tat protein or treated with amphetamines (Xu, Ma et al. 2010, Fitting, Ignatowska-Jankowska et al. 2013).

Arc is an important post-synaptic protein that is involved in learning and memory (Czerniawski, Ree et al. 2011). Increased expression of Arc is associated with activity of neurons responsible for storing and processing information (Guzowski, McNaughton et al. 1999). Furthermore, Arc knockout mice have been shown to perform poorly in water maze task (Plath, Ohana et al. 2006). Determination of Arc by western blotting indicated that HIV-1 Tat and METH have significantly decreased its expression in the parietal cortex of both genders,

but not in the other brain regions. There was further decrease in the expression of Arc in HIV-1 Tat mice treated with METH in parietal cortex and prefrontal cortex. Our findings concur previous reports, indicating decreased expression of Arc in different brain regions of mice after treatment with METH (Bowyer, Pogge et al. 2007, Cheng, Hsu et al. 2015). Atluri et al. reported a decrease in the expression of Arc in the HIV-1 infected human astrocytes (Atluri, Kanthikeel et al. 2013). The expression of Shank 2 was affected by METH administration and HIV-1 Tat induction in different brain regions of male and female mice.

Different types of neurotrophic factors exist in the brain with diverse functions on neuronal plasticity, survival and development (Huang and Reichardt 2001). Altered expression of neurotrophic factors are associated with neurological disorders, including HIV-1 (Albrecht, Garcia et al. 2006, Fields, Dumaop et al. 2014). Results of western blot analysis indicated that HIV-1 Tat does not affect the levels of BDNF whereas METH significantly decreased the expression of BDNF in different brain regions. However, previous studies have showed that METH induces the expression of BDNF in multiple brain regions in rat (Galinato, Orio et al. 2015, Garcia-Cabrerizo and Garcia-Fuster 2016). A plausible explanation for this could be a shorter duration of METH administration (Braun, Herring et al. 2011) or METH administered to postnatal mice (Grace, Schaefer et al. 2008), as opposed to the long-term METH treatment protocol that we have employed. The increase in the levels of BDNF can be a compensatory mechanism to overcome the neurotoxic effects of METH. A significant decrease in the expression of BDNF with METH treatment and HIV-1 Tat induction could be responsible for increased spatial memory deficits in these mice. Therefore, administration of BDNF can be used as a therapeutic approach to counteract the memory deficits associated with HIV-1 and METH. There were no significant differences in the expression of CNTF in any of the brain regions of both genders.

Previous studies have indicated the vital role of CaMKII to promote memory formation by strengthening the synapse. It is involved in the process of long term potentiation by

modulating the expression and conductance of AMPA and glutamate receptors (Benke, Luthi et al. 1998, Lisman, Schulman et al. 2002). Activation of enzyme catalytic site occurs through phosphorylation of Threonine 286 (Yang and Schulman 1999). We, therefore, examined the expression of CaMKII as a marker of synaptic plasticity and strength. The expression of p-CaMKII was found to be significantly lower in HIV-1 Tat induced mice and mice treated with METH. Previous studies performed on primary rat cortical neurons and in SIV infected monkeys have shown a decrease in the expression levels of p-CaMKII (Gupta, Kelly et al. 2010, Akay, Cooper et al. 2014). Furthermore, acute and chronic METH administration have been shown to affect the expression of CaMKII in rat (Akiyama and Suemaru 2000). The decrease in the expression levels of CamKII might affect the function of synapsin 1 and thereby affecting the vesicle number in the releasable pool (Greengard, Valtorta et al. 1993).

In conclusion, administration of METH to HIV-1 Tat transgenic mice exacerbated the deficits in spatial learning and memory characterized by decreased spontaneous alternations in Y maze and increased latency time to reach the escape platform in Morris water maze. We correlated the changes in learning by measuring various synaptic markers and neurotropic factors that contribute to neuroplasticity and memory formation. HIV-1 Tat transgenic mice that were treated with METH showed significant decrease in the expression of synaptic markers and neurotropic factors. Future studies will be directed to identify the molecular mechanisms that are involved in the altered expression of synaptic markers and neurotropic factors by HIV-1 Tat and METH. Furthermore, future studies will also focus on the inflammation markers, structural changes of neurons and use of therapeutic agent to overcome the cognitive deficits mediated by HIV-1 Tat and METH.

CHAPTER 6

SUMMARY AND FUTURE DIRECTIONS

The incidence of HIV associated neurocognitive disorders have decreased over the past 2 decades with the initiation of cART. However, the prevalence of less severe forms neurocognitive disorders have increased at the same time. This presents a difficulty in performing day to day activities among HIV infected individuals and also a huge economic burden on the society. Since, HIV cannot infect neurons, neurotoxicity mediated by HIV-1 could be a result of direct infection of astrocytes and microglia or release of soluble mediators that in turn acts on cells of CNS origin to produce toxicity. Among the released soluble mediators is HIV-1 Tat that plays an important role in promoting neurotoxicity. This neurotoxicity is further exacerbated in individuals who abuse illicit drugs.

The present study was undertaken to determine the role of HIV-1 in the production of pro-inflammatory cytokines from astrocytes. HIV-1 Tat mediated time dependent increase in the expression of IL-6, IL-8 and CCL5 at the level of mRNA and protein. Upregulation of these cytokines involved different upstream signaling molecules and transcription factors. These upstream signaling molecules include PI3K/Akt and MAPKs such as p38 and JNK. These cytokines involve the activation of NF- κ B and AP-1 transcription factors. The involvement of various signaling molecules were verified by the use of specific inhibitors and siRNA. We have also shown that HIV-1 Tat mediated induction of IL-6 is exacerbated by methamphetamine administration and involves overlapping NF- κ B and PI3K/Akt signaling pathways.

We also undertook an in vivo study to determine the effect of HIV-1 Tat and METH on various behavior paradigms. Effect of HIV-1 Tat and METH on ambulation and anxiety were determined by employing open field assay and light/dark box assay. Furthermore, the combined effect of HIV-1 Tat and METH on neurocognition was correlated to changes in the expression levels of various synaptic proteins, neurotrophic factors and secondary calcium messenger.

6.1 Conclusions and future directions

This study provides novel evidence to elucidate the extensive involvement of pro-inflammatory cytokines, and role of synaptic proteins and neurotrophic factors in exposure to METH and HIV-1 Tat. Following future investigations are required to further explain the role of HIV-1 Tat and METH.

1. The intersection between the molecular events evoked by METH and HIV-1 Tat and their functional outcomes, such as expression of ER stress and oxidative stress, are required for comprehensive understanding of their role.
2. Future studies are required to understand the effects of HIV-1 Tat and METH on inflammation markers, synaptic changes in neurons and use of therapeutic agent to overcome the cognitive deficits mediated by HIV-1 Tat and METH.

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VITA

Anantha Ram Nookala was born on February 24, 1986 in Rayachoti, India. He was educated in public schools in Hyderabad, India. He attended R.G.R. Siddhanthi College of pharmacy, affiliated to Jawaharlal Nehru technological university at Secunderabad, India. He attained his bachelor's degree in pharmaceutical sciences with distinction in 2010. He joined interdisciplinary Ph.D program in the department of Pharmacology and Toxicology at University of Missouri Kansas City in the Fall of 2011 to pursue doctoral degree. His major was Pharmacology and toxicology and co-discipline was pharmaceutical sciences. He maintained a GPA of 3.99/4.00 with an excellent performance in course work. His keen interest in research helped him to publish 5 research articles, of which he served as first author in 3 publications. During his tenure as a graduate student at UMKC, he was bestowed with several honors and awards, including, Dean's scholar award, Best student of the year award, Preparing future faculty award and two best poster presentation awards. He presented his work at several national and international meetings and also presented his work as invited speaker at Society on Neuroimmune Pharmacology (2014).