ASTROCYTE-MEDIATED HIV-1 TAT NEUROTOXICITY AND ITS MOLECULAR MECHANISMS: ASTROCYTE ACTIVATION AND IMPAIRED NEUROGENESIS

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ABSTRACT

Yan Fan

Astrocyte-mediated HIV-1 Tat neurotoxicity and its molecular mechanisms: astrocyte activation and impaired neurogenesis

Human immunodeficiency virus type 1 (HIV-1) invasion of the central nervous system (CNS) often causes motor and cognitive dysfunction, which is termed HIV-associated neurocognitive disorders (HAND). Although the introduction of combination anti-retroviral therapy (cART) has effectively suppressed viral replication, improved immune function and increased life expectancy among HIV-infected individuals, it has failed to provide complete protection from HAND or to reverse the disease. HIV-1 Tat protein is a major pathogenic factor in HAND. Studies including ours have demonstrated that Tat is taken up by HIV-1 uninfected brain cells and alters the function of these cells, especially astrocytes, neurons and neural progenitor cells. However, the underlying mechanisms are still unclear.

There are two parts to my dissertation research. In the first part, we determined the roles of signal transducer and activator of transcription 3 (STAT3) in Tat-induced glial fibrillary acidic protein (GFAP) transactivation. Astrocytes, the most abundant cells in the brain, not only provide the scaffold support in the brain, but also are essential for maintaining the homeostasis of the brain. GFAP is the specific molecular marker for astrocytes, but it also modulates astrocyte function both physiologically and

pathologically. In this study, we first showed that STAT3 expression and phosphorylation led to significant increases in GFAP transcription and protein expression. Then we determined that Tat expression was associated with increased STAT3 expression and phosphorylation in Tat-expressing astrocytes and HIV-infected astrocytes. In addition, we showed that GFAP, Egr-1 and p300 transcription all showed positive response to STAT3 and its phosphorylation. Moreover, knockdown of STAT3 resulted in significant decreases in Tat-induced GFAP and Egr-1 transcription and protein expression. Taken together, these findings show that STAT3 is involved in and acts upstream of Egr-1 and p300 in the Tat-induced GFAP transactivation cascade and suggest important roles of STAT3 in controlling astrocyte proliferation and activation in the HIV-infected CNS.

In the second part of the dissertation research, we took advantage of the doxycycline-inducible and astrocyte-specific HIV-1 Tat transgenic mice (iTat) and determined the relationship between Tat expression and neurogenesis. Tat expression in astrocytes was associated with detection of fewer neuron progenitor cells (NPC), fewer immature neurons and fewer mature neurons in the dentate gyrus of the hippocampus of the mouse brain. *In vitro* NPC-derived neurosphere assays showed that Tat-containing conditioned media from astrocytes and recombinant Tat protein inhibited NPC proliferation and migration and altered NPC differentiation, while immunodepletion of Tat from Tat-containing conditioned media or heat inactivation of recombinant Tat abrogated those effects. Notch signaling downstream gene Hes1 promoter-driven luciferase reporter gene assay and Western blotting showed that recombinant Tat and Tat-containing conditioned

media activated Hes1 transcription and protein expression, which were abrogated by Tat heat inactivation, immunodepletion, and cysteine mutation at position 30. Moreover, Notch signaling inhibitor DAPT significantly rescued Tat-impaired NPC differentiation *in vitro* and neurogenesis *in vivo*. Taken together, these results show that Tat adversely affects NPC and neurogenesis through Notch signaling and point to the potential of developing Notch signaling inhibitors as HAND therapeutics.

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LIST OF ABBREVIATIONS

ARV	Antiretroviral
AIDS	Acquired immune deficiency syndrome
ANI	Asymptomatic neurocognitive impairment
ARS	Acute retroviral syndrome
AD	Alzheimer's disease
ART	Antiretroviral therapy
BBB	Blood-brain barrier
BMVEC	Brain microvascular endothelial cells
BMP	Bone morphogenetic proteins
cART	Combination antiretroviral therapy
CHARTER	CNS HIV anti-retroviral therapy effects research
CSF	Cerebrospinal fluid
CNS	Central nervous system
CCR5	Chemokine (C-C motif) Receptor 5
CXCR4	C-X-C chemokine receptor type 4
DAPI	4', 6'-diamidino-2-phenylindol
DMEM	Dulbecco's modified eagle's medium
Dox	Doxycycline
ECL	Enhanced chemiluminesence

Egr-1	Early groWTh response protein 1
ER	Endoplasmic reticulum
F12	Ham's F12 medium
F12k	Kaighn’s modification of Ham’s F-12 medium
FIV	Feline immunodeficiency virus
GFP	Green fluorescence protein
GFAP	Glial fibrillary acidic protein
gp120	HIV-1 glycoprotein 120
gp41	HIV-1 glycoprotein 41
HAD	HIV-associated dementia
HAND	HIV-associated neurocognitive disorders
HIV-1	Human immunodeficiency virus type 1
HIV-2	Human immunodeficiency virus type 2
hMR	Human mannose receptor
HRP	Horseradish peroxidase
HTLV-I	Human T cell leukemia virus type I
HTLV-III	Human T cell leukemia virus type III
HIVE	HIV encephalitis
Hes1	Hairy and enhancer of split-1
IF	Immunofluorescence
IL-1β	Interleukin-1 beta
IN	Integrase

LAV	Lymphadenopathy-associated virus
LB	Luria broth
LRP	Low-density lipoprotein receptor-related protein
MS	Multiple sclerosis
MCMD	Minor cognitive and motor disorder
MMP	Matrix metalloproteinase
NSC	Neural stem cells
NPC	Neural progenitor cells
Nef	Negative regulatory factor
NRTI	Nucleotide reverse transcriptase inhibitors
NNRTI	Non-nucleotide reverse transcriptase inhibitors
NP	Neuropsychological
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
PCR	Polymerase chain reaction
PD	Parkinson's disease
PFA	Paraformaldehyde
PAF	Platelet activating factor
PMSF	Phenylmethylsulfonyl fluoride
PI	Protease inhibitors
Rev	Regulator of expression of virion proteins
ROS	Reactive oxygen species
RT	Reverse transcriptase

RT	Room temperature
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SIV	Simian immunodeficiency virus
SOC	Super optimal broth with catabolite repression
STAT3	Signal transduce and activator of transcription 3
TAR	Trans-activation response
Tat	Trans-activator of transcription
TCA	Trichloroacetic acid
TNFα	Tumor necrosis factor-α
UPR	Unfolded protein response
VSV-G	Vesicular stomatitis virus glycoprotein
WB	Western blotting
WT	Wild type

INTRODUCTION

1. HIV-1 and AIDS epidemiology

Human immunodeficiency virus type 1 (HIV-1) is the etiologic agent for acquired immune deficiency syndrome (AIDS), one of the major public health concerns worldwide [2-4]. HIV-1 and human immunodeficiency virus type 2 (HIV-2) are two distinct subtypes. HIV-1, initially termed lymphadenopathy-associated virus (LAV) and human T-lymphotropic virus-III (HTLV-III), is the predominant subtype and contributes to 95% of all infections all over the world, and HIV-2 is mainly seen in a few West African countries [5, 6]. Since the first case of HIV/AIDS was reported in 1981 (Center for Disease Control and Prevention. MMWR, 1981; vol. 30. No. 21. "Pneumocysitis Pneumonia-Los Angeles"), HIV/AIDS has become global epidemic. Based on the 2015 Global AIDS Response Progress Reporting, HIV has infected 38.1 million people and 25.3 million people have died of AIDS-related illnesses since 2000. By the end of 2014, two million people had become newly infected, which was a 35% decline in the number of new infections globally since 2000. Moreover, the latest statistics estimated that 36.9 million people were living with HIV, which is an increase in number from previous years as more people are receiving life-saving combination antiretroviral therapy (cART). cART consists of a minimum of two active antiretroviral drugs from two classes and usually three or more drugs. The major classes of medications in cART include nucleotide reverse transcriptase inhibitors (NRTI), non-nucleotide reverse transcriptase inhibitors (NNRTI), protease inhibitors (PI), or integrase inhibitors, all of which can

effectively interfere with HIV in different stages of infection and replication [7]. As of June 2015, 15.8 million people living with HIV have access to cART, which is an 84% increase from 2010. cART has saved 42% of AIDS-related deaths since the peak in 2004. Despite successes in suppressing HIV replication and increasing the life span of HIV-infected individuals, cART has failed to completely cure the disease or stop the virus transmission. Meanwhile, it has increased the risk of drug side effects, drug resistance, and HIV-associated neurocognitive disorders (HAND). The HIV pandemic remains a devastating crisis with enormous social and economic consequences in the whole world [7].

2. Clinical course and progression of HIV-1 infection

Prior to cART, the clinical course and progression of HIV-1 infection can be described as the progressive destruction of CD4+ lymphocytes. It is mainly divided into three stages: acute infection, clinical latency and AIDS (**Fig. 1**) [8, 9]. Within 2-4 weeks of initial acquisition, HIV infection is termed acute retroviral syndrome (ARS). Up to 90% of HIV-infected individuals develop flu-like syndromes including fever, swollen glands, sore throat, rash, muscle and joint aches and pains, and headache [10, 11]. During this phase, large amounts of HIV-1 are produced by dendritic cells and $CD4^+$ T cells and rapidly carried to the gastrointestinal tract and lymphoid organs [12, 13]. The acute HIV-1 infection leads decreases of CD4+ CCR5+ memory T cells and rapid increases of plasma viral load. During this stage, HIV-infected individuals possess a high risk of transmitting HIV to their partners through sexual contact or needle sharing [12, 14-16]. In

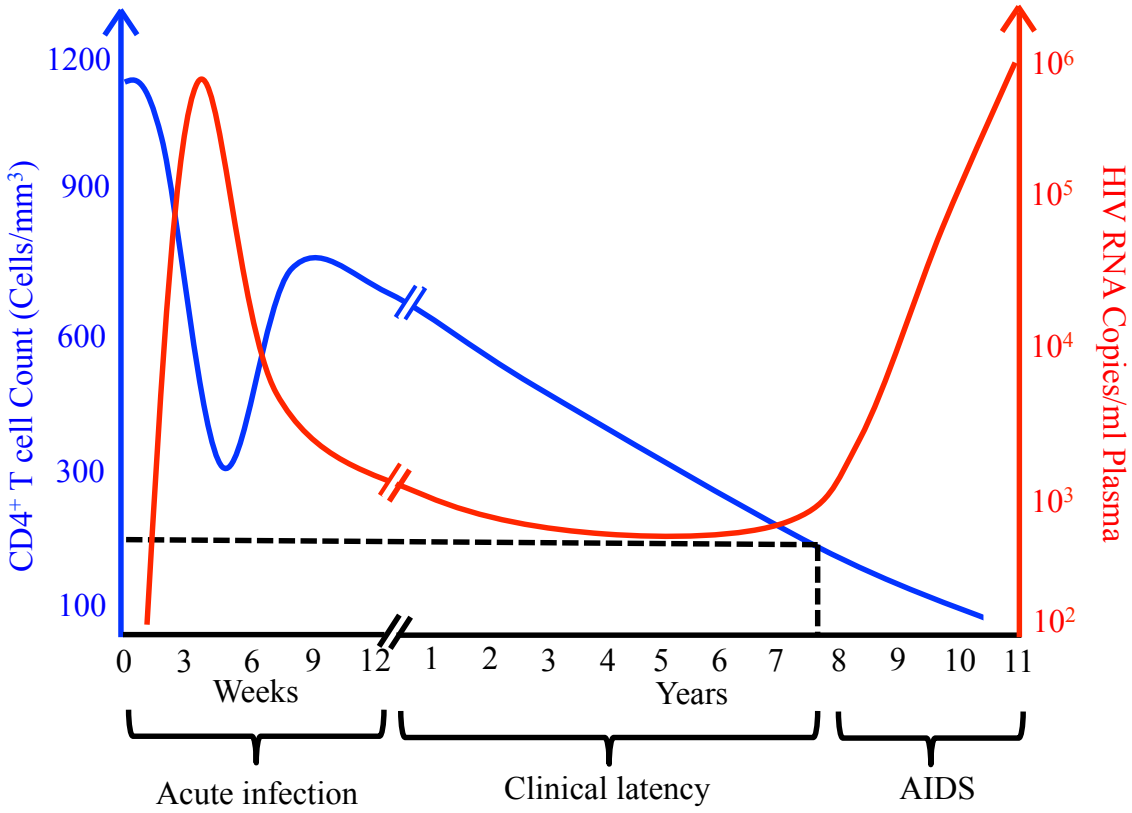
1200
900
600
300
100
CD4+ T cell Count (Cells/mm3)
10^6
10^5
10^4
10^3
10^2
HIV RNA Copies/ml Plasma
0 3 6 9 12
Weeks
1 2 3 4 5 6 7 8 9 10 11
Years
Acute infection
Clinical latency
AIDS

Figure 1. Clinical progression of HIV-1 infection. Clinical progression of HIV-1 infection is demonstrated as changes in patients' CD4+ T cell counts and plasma viral load. X-axis presents the time post primary infection. Y-axis (left) presents patients' CD4+ T cell count per mm^3 in the peripheral blood (blue line) and Y-axis (right) presents HIV RNA copies per ml in the plasma (red line). Clinical stages are indicated at the bottom. Adapted from Pantaleo, G et al. 1993 [9].

accordance with high plasma HIV-1 RNA copies, CD8+ T cell response leads to a decrease in viral load and a rise in CD4+ T cell counts [17, 18]. Eventually, the immune response will bring down the level of viral load to the viral set point and lead patients to enter the clinical latency phase. During the clinical latency stage, the HIV virus continues to replicate at a low level while CD4+ T cell counts gradually decline, and HIV-infected people often experience no apparent or mild symptoms [8, 19]. cART is effective in helping people to live within the latency stage for several decades, while without cART, this stage lasts an average of 10 years. Even within the clinical latency stage or with cART, people are still able to transmit HIV to others. AIDS is defined as the CD4+ T cell counts become lower than 200 cells/mm^3 [8, 20]. In this stage, the immune system is badly damaged and the HIV-infected individuals become vulnerable to opportunistic infection such as infection of bacteria, viruses, fungi, and parasites [8, 21].

3. HIV-1 infection of the central nervous system (CNS)

HIV-1 infection of the CNS occurs within hours of viral exposure in the periphery, which is characterized by the detectable viral load in the cerebrospinal fluid (CSF) and the subcortical structures [22-24]. Introduction of cART has greatly prolonged the life span of HIV-1 infected individuals [25, 26]. Unfortunately, most of the antiretroviral drugs are not able to penetrate the blood-brain barrier (BBB) and suppress HIV replication in the CNS. HAND, particularly the mild form, i.e., minor cognitive and motor disorder (MCMD) has become more prevalent in the era of cART [22, 27]. The primary cell targets of HIV-1 in the CNS are microglia/macrophages and, to a lesser extent, astrocytes.

Neurons are rarely infected by HIV-1, but are mostly affected[28]. The underlying mechanisms of HAND remain incompletely understood.

3.1 HIV-1 entry into the CNS

HIV-1 primarily infects CD4+ cells and macrophages/momocytes in the periphery. Passage of virus across the interface between periphery and the CNS is restricted by BBB. BBB, which consists of brain microvascular endothelial cells (BMVEC) that are tightly adjacent to each other with tight junctions and pericytes that are embedded in the basal membrane of the blood vessel, and surrounded astrocytes that are projected to endothelial cells through glia limitans [29, 30]. Accumulating evidence indicates that HIV-1 is able to traffic across the BBB and gains access to CNS. Several mechanisms have been proposed to explain how HIV-1 enters into the CNS (**Fig. 2**) [31].

3.1.1 "Trojan Horse" hypothesis: HIV-1 is delivered into the CNS by infected peripheral cells.

The Trojan Horse hypothesis is considered the most reasonable model for HIV-1 entry into the CNS (**Fig. 2A-I**). It illustrates that HIV-infected peripheral immune cells, such as CD4+ T cells, monocytes-derived macrophages traffick across the BBB and deliver HIV-1 into the CNS [31-33]. This virus transportation mode, from the periphery to the CNS, was previously described for other retroviruses including visna virus, feline immunodeficiency virus (FIV), simian immunodeficiency virus (SIV) and human T cell leukemia virus type I (HTLV-I) [33]. In addition, infiltration and accumulation of both

CD4+ and CD8+ T cells were discovered in postmortem autopsies brain samples of AIDS patients and SIV-infected rhesus monkeys [34-36]. Moreover, HIV-infected brain cells and their secreted viral proteins could lead to stimulation and secretion of cytokines/chemokines, which result in infiltration of more immune cells and facilitate HIV-1 trafficking through the BBB into the CNS [37].

3.1.2 Other hypotheses: Passage of cell-free HIV-1 virus due to the BBB lesion, brain microvesicular endothelial cell (BMVEC) infection or transcytosis.

HIV-1 direct infection of BMVEC (**Fig. 2A-III**) or HIV-1 viral protein produced by those infected cells, such as Tat and gp120, could both lead to pro-inflammatory responses and activation of BMVEC, which is characterized by secretion of cytokines, such as tumor necrosis factor-α (TNFα) and interleukin-1 β (IL-1β) [38]. Those cytokines and viral proteins in turn cause BMVEC apoptosis, tight junctions impairment and BBB disruption and ultimately result in diffusion of free virus through the BBB lesions [39-41] (**Fig. 2A-II**). Transcytosis, the other possible mechanism, posits that HIV-1 is endocytosed into intracellular vesicles of BMVEC, shuttled through BMVEC layers and released to the other side of BBB [31] (**Fig. 2A-IV**).

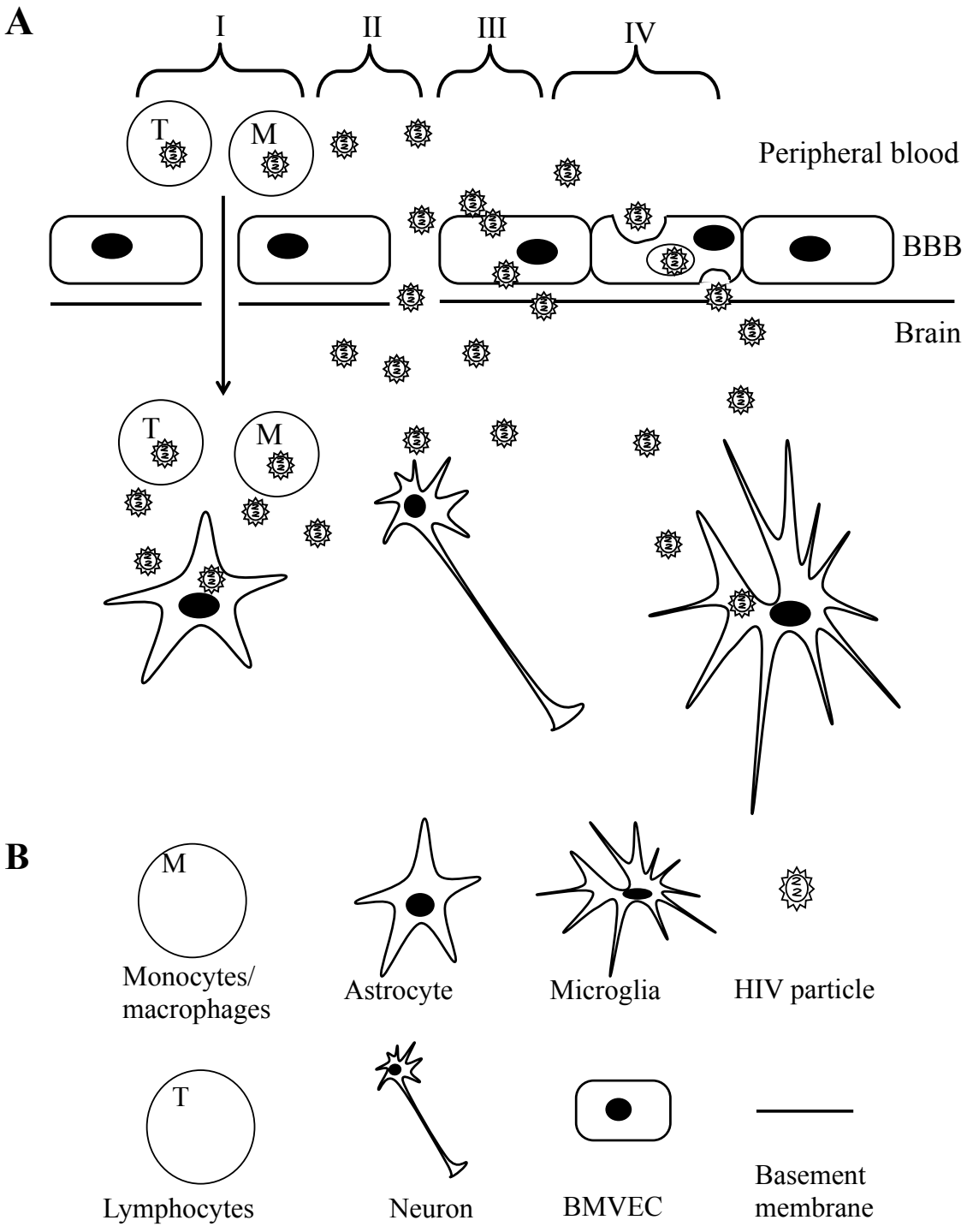
A
I
II
III
IV
T
M
Peripheral blood
BBB
Brain
T
M
B
M
Monocytes/
macrophages
Astrocyte
Microglia
HIV particle
T
Lymphocytes
Neuron
BMVEC
Basement
membrane

Figure 2. Current hypotheses for HIV-1 entry into the CNS. **A.** Illustrations of current hypotheses of HIV-1 entry to the CNS. I. Trojan Horse hypothesis. Virus is brought into the CNS by infiltrated HIV-infected blood cells. II. Cell-free virus is diffused into the CNS through the BBB damage. III. Virus entry CNS by infecting endothelial cells. IV. Virus passes into the CNS through transcytosis of endothelial cells. **B.** Keys for the CNS cells, HIV-1 and basement membrane.

3.2 HIV-1 infection of CNS cells

3.2.1 HIV-1 infection of microglia/macrophages

Microglia are brain-resident macrophages derived from monocytes of hematopoietic origin that migrate from bone marrow into brain [42, 43]. They constitute 10-15% of all cells in the brain and contribute to the pro-inflammatory response and antigen presentation and are constantly scavenging for plaques, damaged neurons and infectious agents [44, 45]. HIV-1 infection of microglia is confirmed both *in vivo* and *in vitro*, by the presence of Env and p24 in HIV-infected patients and productive HIV-1 infection in primary human microglia using both M- and dual-tropic HIV-1 strains [46, 47]. HIV-1 entry of microglia is mediated by receptor CD4 and co-receptor CCR5, CXCR4 or CCR3 [48, 49]. As a primary cell target in the CNS for HIV-1 infection, microglia play a critical role in primary HIV-1 infection of the CNS, support productive HIV-1 infection, impact CNS immune function, and promote the secondary HIV-1 infection of the CNS that contributes to HAND pathogenesis.

3.2.2 HIV-1 infection of astrocytes

Astrocytes are the most abundant cell population in the CNS and constitute more than 70% of the cells in the CNS. They provide scaffold support and are essential for maintaining the homeostasis of the CNS. An estimated 19% of astrocytes are positive for HIV-1 RNA in patients' brains using laser capture micro-dissection together with single-cell multiplex polymerase chain reaction (PCR) [50]. HIV-1 infection of astrocytes has been characterized as a restricted form with abundant expression of HIV early gene

products Tat, Nef and Rev proteins[51-58]. Restricted infection of astrocytes by HIV-1 includes entry blockage of HIV-1 infection and non-productive HIV-1 replication in astrocytes (**Fig. 3**). Firstly, HIV-1 entry requires interaction of viral protein gp120 with main receptor CD4 and co-receptors such as CXCR4 and CCR5 on host cells through fusion [59, 60]. Astrocytes do not have CD4 receptors on the cell surface. They are infected by HIV-1 through a CD4-independent pathway described as human mannose receptor (hMR)-mediated endocytosis [61-64] and recently, our lab has reported that HIV was successfully transferred from HIV-infected CD4+ T cells to astrocytes in a cell–cell contact- and gp120-dependent manner [65]. However, the efficiency of cell-free virus infection of astrocytes is extremely low in most of cases, even though the robust HIV infection of astrocytes caused by the cell-cell contact between astrocytes and HIV-infected CD4+ T cells, viral gene expression is retained in the restricted nature [65]. Virus production and viral structural gene products are limited to a low or even undetectable level soon after HIV-1 is inoculated into astrocytes 7-10 days post infection [57, 66-68]. Persistent infection is illustrated by the presence of early viral protein Nef in the infected astrocytes. HIV-1 Tat protein has also been detected in astrocytes that are latently infected with HIV [69-71].

Several mechanisms have been presented to explain the non-productive HIV-1 infection of astrocytes. Nef, as the negative regulator, reduces the LTR activity in astrocytes and contributes to the restricted virus production in astrocytes [24, 68, 72, 73]. Suppression of viral mRNA exportation to the cytoplasm and lack of structural protein expression due to

severe reduction of Rev activity and abnormal accumulation of Rev in cytoplasm are found in HIV-infected astrocytes [67, 74]. Previously in our lab, we have demonstrated the host protein Src-associated protein of 68 kDa in mitosis (Sam68) impairs Rev function in astrocytes and plays an essential role in HIV-1 Rev nuclear export [75].

In addition, translational defects of Gag, Rev, Nef, and abnormal cleavage of Gag-p24 and Env-gp120 also affect viral production in HIV-infected astrocytes [76, 77]. During latent infection of astrocyte, transient HIV-1 replication can be stimulated by cytokines including NF-κB, TNF-α and IL-1β, as demonstrated by recovery of active reinfection of monocytic or lymphoid cells via co-culturing with latently infected astrocytes [57, 68, 78]. This suggests that latent infection of astrocytes serves as a viral reservoir in the CNS [31, 56, 66].

3.2.3 HIV-1 infection of neuron progenitor cells (NPC)

HIV-1 has been shown to infect nestin-positive neural progenitor cells both in cultures and in autopsy brain sections from pediatric AIDS patients, suggesting that human neural stem cells/NPC can harbor HIV-1, and could be the potential menace contributor of HAND [79-81]. Furthermore, the number of adult NPC was significantly decreased in patients with HAD as compared to the age-matched non-infected or HIV-1-infected non-demented individuals [82]. This decrease may reflect general pathogenic mechanisms arising in the setting of late-stage dementia rather than specific effects of HIV on neurogenesis. But emerging evidence suggests that adult hippocampal neurogenesis is

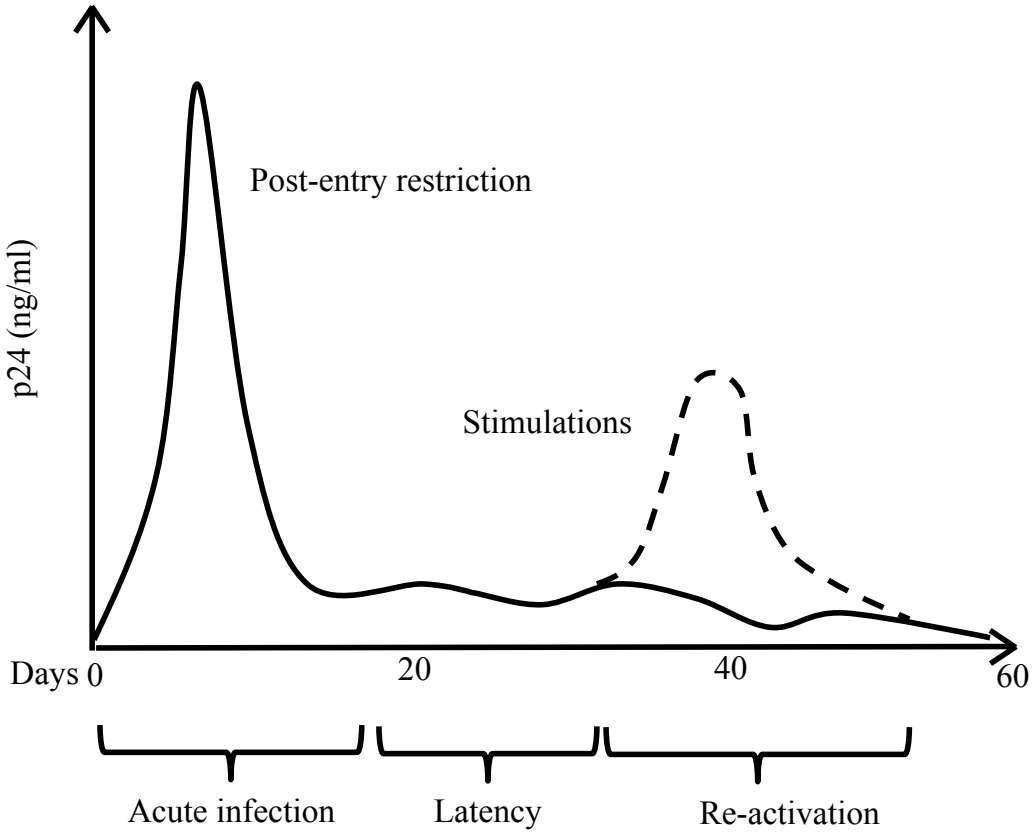
p24 (ng/ml)
Post-entry restriction
Stimulations
Days 0
20
40
60
Acute infection
Latency
Re-activation

Figure 3. Stages of HIV-1 infection of astrocytes. Y-axis shows virus replication, presented by extracellular p24 level (ng/ml). X-axis presents days post infection. Stages of HIV-1 infection are listed at the bottom. Acute infection of astrocytes causes initial peak of HIV-1 production during first 10 days and then virus production is rapidly decreased to a basal level. During HIV-1 latency, addition of stimulatory factors lead to a transient virus re-production. Adapted from Messam and Major 2000 [148].

affected by altered expression of cytokines or groWTh factors by HIV infection and several soluble viral proteins, such as gp120 and Tat [83-85].

3.2.4 HIV-1 infection of other cell types in the CNS (neurons, oligodendrocytes, endothelial cells

There is little evidence that neurons, oligodendrocytes, or endothelial cells are infected by HIV-1. Nevertheless, these cells are known to be dramatically affected during HIV-1 infection of the CNS, and are clearly associated with HAND pathogenesis [31]. Viral proteins and cytokines/chemokines, produced by HIV-infected cells, either directly disrupt the brain homoeostasis and are toxic to the uninfected cells, or are taken up by the uninfected cells and indirectly affect these cell and contribute to CNS abnormality [28, 40, 86, 87].

4. HIV/neuroAIDS

4.1 HIV/neuroAIDS epidemiology

HIV-1 infection of the CNS occurs within hours of viral acquisition in the periphery [23, 24] andcauses neurological symptoms that include motor and cognitive dysfunction [88]. These syndromes have collectively been termed HIV/neuroAIDS. And the neurological disorders associated with HIV/neuroAIDS are termed HAND, which ranges from asymptomatic neurocognitive impairment (ANI) to MCMD and to the most severe but rare HIV-associated dementia (HAD) [26, 88-90]. In the pre-cART era HAD was a

common complication of AIDS. An estimated of 25% infected adults and more than half of infected children develop HAD [91]. However, the introduction of cART in the mid-1990's led to reduced viral replication, improved immune function, decreased the incidence of HAD, but the prevalence of HAND has actually risen, in the form of MCMD, due to the increasing number of infected subjects and increased life expectancy [92, 93]. A cross-sectional study by the CNS HIV anti-retroviral therapy effects research (CHARTER) reported 52% neuropsychological (NP) patients out of 1555 HIV-infected adults recruited across the United States. NP is one of the criteria for diagnosing HAND. Specifically for HAND diagnoses, 33% out of 1555 HIV-infected adults had ANI, 12% MCMD and 2% HAD [94, 95]. It is clear that cART has failed to prevent, or reverse HAND, but to an extent, increase the prevalence of HAND at the present time [5]. Thus, new approaches to prevention and treatment of HAND is undoubtedly warranted and urgently needed.

4.2 Neuropathology of HIV/neuroAIDS

HIV-1 infection of the CNS causes clinical neurological symptoms that include impaired short-term memory, reduced mental concentration, weakness, slowness of locomotor activity and depression accompanied by behavioral issues like personality disorders, lethargy and social withdrawal [96]. During pre-AIDS stage, there is no evidence that HIV encephalitis (HIVE) or CNS opportunistic infection of lymphoma is present. However, increase in infiltration of CD8+ T cells and CD20+ B cells are present in the brain parenchyma. It suggests that these lymphoid cells may be responsible for

controlling the initial viral infection in the CNS [97, 98]. In addition, subtle gliosis, microglial activation, and mild axonal damage are reported during early infection [99, 100]. As HIV-infected individuals progress into symptomatic stage, the most significant pathologies include emergence of HIVE, opportunistic infections and/or primary CNS lymphomas, presence of widespread reactive astrocytosis, activated resident microglia, cortical neuroinflammation, and synaptic and neuronal loss [90, 101-104]. During HAD, the most severe form of cognitive impairment, symptoms and signs include tremor, gait ataxia, loss of fine motor movement, mental slowing, forgetfulness, poor concentration and behavioral abnormalities [105]. Rather than viral replication or viral load, the best correlate of AIDS dementia is the degree of monocyte infiltration and the level of microglial activation in the brain [106].

4.3 Mechanisms of HIV/neuroAIDS

At the cellular level, the primary cell targets for HIV infection are microglia/macrophages and, to a lesser extent, astrocytes [51, 52, 64, 68, 107, 108]. However, neurons that are mostly affected in the brain of HIV-infected individuals are rarely infected. Therefore, a number of indirect mechanisms have been proposed for HIV/neuroAIDS pathogenesis. The proposed mechanisms could generally be grouped into three main categories: improper immune activation of microglia/macrophages, soluble factors of both viral and host origins from microglia/macrophages and astrocytes, and astrocyte dysfunction. All three categories of the mechanisms may be integrated to function in the HIV/neuroAIDS pathogenesis. First of all, microglia/macrophages

activation which facilitates infiltration of immune cells into the CNS have been widely proposed to be the driving force during the progressing of HAND [109]. Both infected and uninfected activated microglia have been suggested as pathogenic factors of HAND. Microglia activation induces neuronal damage due to release of potentially neurotoxic levels of oxidative radicals, nitric oxide and/or the cytokines, such as TNF-α and IL-1β [110-113]. Secondly, soluble viral proteins, such as gp120, Nef, Tat and Vpr, have been shown be able to directly damage the BBB, exhibit toxicity to neurons, lead to inflammation, induce oxidative stress, and impact intracellular Ca^{2+} homeostasis [114-117]. Lastly, astrocytes dysfunction, caused by reactive astrocytes, also termed astrocytosis, leads to disruption of homeostasis of the CNS, especially the intracellular glutamate concentration. Glutamate is the major excitatory neurotransmitter in the CNS. Glutamate receptor-mediated neurotoxicity is described not only in HAND, but also in Alzheimer's dementia [118]. HIV-1 induces expression of cytokines and chemokines in astrocytes such as MCP-1, IL-1β, IL-6, RANTES, and CXCL10 [37, 119-121], which could in turn recruit more monocyte/macrophages and lymphocytes into the CNS. As the major compartment of BBB, astrocyte dysfunction also leads to disruption of BBB and result in vulnerablility of the CNS to opportunistic infections and other neurotoxic agents [122].

5. HIV-1 Tat protein

HIV-1 is a single-stranded RNA virus, and the RNA genome is about 9 kb. It encodes 9 open reading frames for 15 proteins. Six of them are non-structural proteins, termed

accessory proteins [123]. HIV-1 Tat protein stands for "Trans-Activator of Transcription". It is the accessory protein encoded by the *tat* gene in HIV-1, which consists of 2 exons: the first exon encodes 72 amino acids, and the second one encodes additional 14 to 31 amino acids depending on the viral strain [124]. The primary function of HIV-1 Tat is to promote HIV-1 transcription via transactivation of HIV-1 LTR promotor. Tat binds with TAR, an RNA stem-loop structure located 3' to the LTR transcription start site. In binding to TAR, Tat recruits cyclin T1 and CDK9, and the positive transcription elongation complex (P-TEFb) to the LTR promotor. Then, cyclin T1-associated CDK9 phosphorylates the C-terminal domain of RNA polymerase II, which transitions from a non-processive state into a processive state to produce full-length HIV transcripts [125, 126].

There are five distinct functional domains within Tat protein (**Fig. 4**). N-terminal domain, also known as acidic domain, has 13 amino acids. Cysteine-rich domain, from amino acids 22 to 37, contains seven cysteines that are highly conserved between different isolates of HIV-1. In this region, disulfide bonds are formed intra-molecularly to form monomeric molecules of Tat protein, which is functionally active in cells. Individual mutation in six of the seven cysteines abolishes Tat function [127]. Core domain, from amino acids 40-48, contains the low-density lipoprotein receptor-related proteins (LRP)

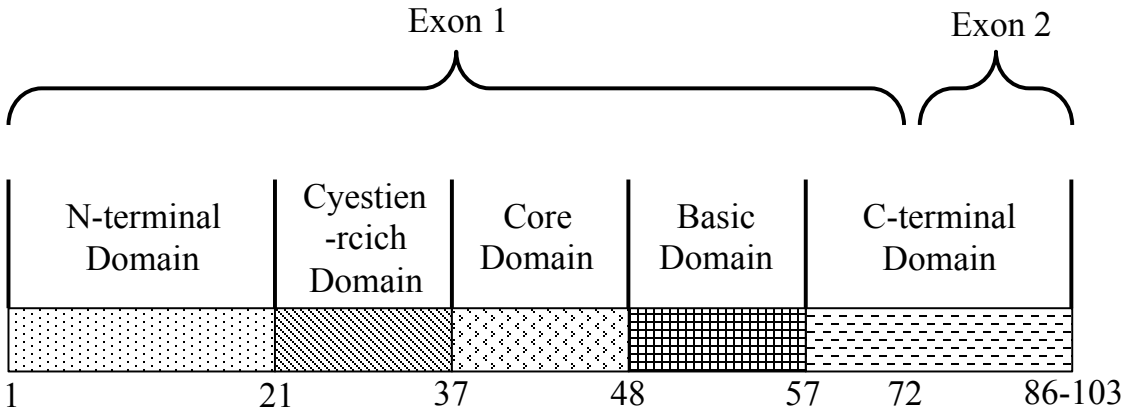
Exon 1
Exon 2
N-terminal Domain
Cyestien -rcich Domain
Core Domain
Basic Domain
C-terminal Domain
1
21
37
48
57
72
86-103

Figure 4. Tat structural and functional domains. Tat consists of five essential domains, which are N-terminal domain, cysteine-rich domain, core domain, basic domain and C-terminal domain. Important residues in each domain are labeled at the bottom.

binding site, which mediates efficient uptake of Tat into neurons [128, 129]. Basic domain, from 49-57, contains a basic RKKRRQRRR motif and is important in TAR RNA-binding properties to Tat, and is critical for Tat nuclear transportation and cellular uptake [130-132]. Mutation in basic domain leads to abolish Tat transactivation activity on LTR promoter [133, 134]. C-terminal domains of the HIV-1 Tat, contain the highly conserved tri-peptide sequence Arg-Gly-Asp, known as RDG sequence, which is important in cell adhesion signal for binding of Tat to cellular integrin [135]. Tat not only plays important roles in productive HIV-1 replication, but also acts as a major pathogenic factor in HIV-associated pathogenesis [136].

5.1 Tat protein secretion and uptake

A substantial amount of Tat protein (0.1-10 ng/ml) is detected in the serum of HIV positive individuals, suggesting that Tat is able to exit cells [137]. Tat protein exit cells in the intact form and does not require cell apoptosis [137, 138]. The autocrine loops in Tat-producing cells are disrupted by specific anti-Tat antibodies. This provides the evidence to support the release of the fully bioactive Tat from cells [139]. Tat can also be released from cells via cell death and turnover of the HIV-infected cells [140]. Evidence has accumulated that extracellular Tat may directly affect HIV replication both *in vitro* and *in vivo*. Specific anti-Tat antibodies have been reported to be inversely correlated with survival of HIV-1 patients [141]. Anti-Tat antibodies have been found to effectively inhibit viral replication in cultures, which may be due to the neutralization of the extracellular Tat [142]

Several studies including ours have demonstrated that Tat protein or Tat basic peptides can be taken up by cells in cultures leading to transactivation of HIV LTR [129, 143, 144]. The basic domain of Tat is critical for Tat protein transduction. It not only allows Tat to crosses the plasma membrane, but also facilitates other proteins to be delivered into the cells [145, 146]. The possible mechanism is that the local invagination of the plasma membrane, caused by the ionic interaction between the side chains of Tat basic domain with the negatively charged phospholipid head of the membrane, leads to reorganization of the lipid bilayer and the penetration of the Tat basic domain and its associated proteins [146, 147]. We have previously demonstrated that Tat enters neurons by specific interaction with LRP on neurons and disrupts the metabolic balance of LRP ligands, suggesting the adverse impact of Tat in HIV-associated neurodegenerative disease [129].

5.3 Function of Tat in HAND

HIV-1 Tat is detected in the CNS of AIDS patients with encephalitis and is positively correlated between the levels of Tat transcripts and HIV-associated encephalitis [148, 149]. Direct exposure of neurons to Tat causes neurotoxicity [150-152]. A trace amount of extracellular Tat, ranging from 1 μM to 0.1 mM is sufficient to cause acute neuron apoptosis, loss of neuronal integrity and neuroexitatory property [153-155]. Expression of Tat in mouse brain causes neuropathologies similar to those noted in the brain of AIDS patients [1]. Intracerebral injection of the basic domain of Tat leads to neuronal loss, inflammation, and reactive astrocytosis [156]. Besides the acute Tat neurotoxicity, Tat is

considered to be the major pathogenic factor in HAND due to indirect interaction of Tat between several brain cells, such as microglia/macrophages, BMVEC, astrocytes and neurons in many different ways [157-159].

5.3.1 Tat and microglia/macrophages

Microglia are an important cell type in the pathophysiology of HIV-1 infection. Microglia/macrophages support HIV-1 replication and are capable of affecting other brain cells. Activated HIV-infected microglia/macrophages secrete high levels of cytokines and bioactive metabolites, such as TNF-α, matrix metalloproteinase (MMP), platelet activating factor (PAF), and arachidonic acid [160-163]. TNF-α and PAF are elevated in the CNS of patients with HIV-1-associated dementia and contribute to neuronal apoptosis via glutamate-mediated neuron excitotoxicity [164, 165]. Arachidonic acid has been reported to inhibit glutamatergic synapses in astrocytes. An elevated level of extracellular glutamate is toxic to neurons [166, 167]. MMP is detected in the cerebrospinal fluid (CSF) of HIV-infected individuals. MMP activity is associated with an increased count of white blood cells in the CNS and an elevated CSF-to-serum albumin ratio, which regarded as the marker of BBB leakage [168, 169].

5.3.2 Tat and BMVEC

The integrity of the BBB is critical for normal brain function. BMVEC as the major compartment of BBB are affected by HIV-1 Tat in different ways. Firstly, treatment of BMVEC with HIV-1 Tat leads to release of nitric oxide (NO). NO released from

activated cultured BMVEC induces signaling for apoptosis in thymocytes [40]. Secondly, HIV-1 Tat protein increases the permeability of BMVEC by decreasing the production of occluding via MMP [170]. Tight junctions are critical structural and functional elements to secure BMVEC in the form of the BBB. Expression of the first exon of Tat in BMVEC both *in vitro* and *in vivo*, alters expression of claudin-5, the most important component of the tight junction, suggesting disturbance of the BBB integrity contributes to HIV trafficking into the brain [171]. In addition, Tat increases intracellular reactive oxygen species (ROS) level, which in turn phosphorylates vascular endothelial (VE)-cadherin complex and increases the permeability of BMVEC [172].

5.3.3 Tat and astrocyte dysfunction

Astrocytes play an important role in maintaining homeostasis for neuronal functionality and are involved in the progression and outcome of many neuropathological conditions. Evidence has accumulated that astrocytes are significant contributors to HAND by modulating the microenvironment in the CNS: (1) HIV evasion into the CNS at the interface of BBB [173]; (2) secretion of cytokines/chemokines to attract cell infiltrates into the CNS and facilitate HIV spread among those cells in the CNS [66, 174, 175], (3) astrocyte activation, also termed astrocytosis and dysfunction induce glutamate metabolism, production of neurotoxins and cytokines/chemokines by astrocytes cause neuronal injury [176-180].

Astrocytosis is one of the hallmarks of HAND and is defined as increases in the number and size of astrocytes and elevated expression of GFAP [176, 181]. As an astrocyte marker, GFAP expression is developmentally and pathophysiologically regulated [182-184]. GFAP forms extensive networks that maintain mechanical strength and shape of the astrocytes and provide dynamic platforms for the organization of the cytoplasm at both structural and functional level [185, 186]. Specifically, GFAP is involved in astrocyte volume regulation [187], glial scar formation [188], and anchoring glutamate transporters to the plasma membrane to facilitate neurotransmitter recycling [189]. Besides, GFAP plays a critical role in neuron-glia interaction and CNS morphogenesis [99, 190]. Recent findings suggest GFAP involvement in the long-term maintenance of the brain architecture, proper function of the BBB, and modulation of some neuronal functions [99, 191, 192]. Higher levels of GFAP lead to pre-mature death [193, 194]. Several studies including ours demonstrated that HIV-1 Tat alone is sufficient to lead to astrocytosis and up-regulation of GFAP, which result in astrocyte-mediated neurotoxicity [195-199]. Our studies have demonstrated that Tat-induced GFAP plays an essential role in astrocyte-mediated Tat neurotoxicity [200]. We have also shown that Tat-induced GFAP accumulation and aggregation leads to unfold protein response (UPR) and endoplasmic reticulum stress (ERS), which in turn causes lysosomal exocytosis and compromised neuronal integrity and survival (manuscript submitted). This process is abolished when GFAP is completely knocked out. These findings support the notion that astrocyte, especially the specific molecular marker GFAP play important roles in Tat-induced neurotoxicity and HIV/neuroAIDS.

5.3.4 Dysregulation of cytokines and chemokines by Tat

As previously introduced, HIV-1 Tat promotes secretion of cytokines and chemokines from the CNS residential cells such as neurons, astrocytes, oligodendrocytes, microglia/macrophages, and infiltrated immune cells [201, 202]. HIV-1 Tat protein has been reported to activate TNF-α, TGF-β, MCP-1, IL-1β, IL-6, RANTES, and CXCL10 [37, 119-121]. TNF-α induces apoptosis in neuronal cells via the apoptotic pathway [203]. Besides, neurotoxic effects of TNF-α may also be due in part to its ability to inhibit glutamate uptake by astrocytes, which in turn results in excitotoxic concentrations of extracellular glutamate [204]. IL-6 is involved not only in the immune response, but also in physiological systems in the CNS, such as neurogenesis. It influences both neurons and glial cells in a wide array of injury models [205]. Increase of *in vitro* IL-6 decreases the differentiation of NPC into neurons but promotes astrogliogenesis especially by transactivation of GFAP expression [206, 207]. So IL-6 dysregulation is involved in HAND through breakdown of BBB and abnormal neurogenesis. The MCP-1 level in the CSF is dramatically increased by Tat and is correlated positively with the severity of dementia in patients with HIV encephalitis [37]. MCP-1 released by Tat has also been shown to increase the permeability of the BBB and facilitate transmigration of monocytes across the BBB [208]. Mutation in MCP-1 has been associated with a 50% reduction in the risk for HIV-1 infection. However, once a patient is infected with HIV-1, the mutation in the MCP-1 allele results in accelerated disease progression and increased risk of dementia [209]. The overexpression of CXCL10 has been observed in several

neurodegenerative diseases including multiple sclerosis (MS), Parkinson's disease (PD) HIV-associated dementia, and Alzheimer's disease (AD) [210, 211]. CXCL10 elicits apoptosis in fetal neurons by elevating intracellular calcium levels [212].

5.3.5 Inducing of oxidative stress by Tat

Oxidative stress is defined as a shift of the antioxidant systems towards the pro-oxidant systems, modulated by various kinds of reactive oxygen species (ROS), which leads to oxidation of proteins and DNA, peroxidation of lipids, and ultimately cell death [213]. Oxidative stress has been demonstrated in the brain and the CSF of HIV-demented patients. The peroxidation product has been found predominantly in neurons, glial cells, and BMVEC in the brains of patients with HIV encephalitis and macaques with SIV encephalitis [214]. Both gp120 and Tat are responsible for induction of oxidative stress during HIV-1 infection of the brain [215]. Tat has been reported to directly interact with neuronal receptors, stimulate production of ROS and trigger the oxidative stress-dependent apoptotic cascades [216]. Striatal injections of Tat causes an increase in protein and lipid peroxidation and astrogliosis [217]. Tat- induced free radical synthase and pro-oxidant production, such as nitric oxide (NO), may be toxic not only to neurons but also to other cells in the brain [218].

6. Neurogenesis

6.1 Neurogenesis

Development of the CNS starts from neural stem cells (NSC) and then proceeds to different linage-restricted NPC, and eventually give rise to the three major cell types: neurons, astrocytes and oligodendrocytes. This process is generally defined as neurogenesis, namely generation of new neurons. It is a highly regulated developmental process, and it includes proliferation and fate specification of NPC such as migration, differentiation, and maturation. This process continues throughout adult life, also known as adult neurogenesis, in the subventricular zone of the lateral ventricle to generate interneurons in the olfactory bulb and in the subgranular zone of the dentate gyrus to generate granule cells in the hippocampus [219-222]. Adult neurogenesis in the hippocampus is involved in specific cognitive functions, such as learning and memory and maintenance of the cognitive functions and is regulated by physiological and pathological stimuli [221, 223-226]

6.2 Signaling pathways in modulating neurogenesis

Neurogenesis generally occurs under normal physiological conditions and is significantly induced after injury as a self-protective mechanism [227]. All components of adult neurogenesis, including NSC and/or NPC proliferation, migration, differentiation, and fate determination, are tightly regulated via both extrinsic environmental influences (pathological) and intrinsic signaling pathways (physiological), such as Wnt, BMP, Shh, Stat3, and Notch [228]. These pathways dynamically maintain proliferation and fate

commitment of the local stem cell population by modulating the activation of a distinct set of transcription factors, which in turn trigger the transcription of neural fate-associated genes. Wnt signaling is a principal regulator of adult hippocampal neurogenesis. Activation of Wnt signaling has been shown to induce NPC proliferation, promote neuronal and astroglial differentiation, but it suppresses oligodendroglial differentiation. HIV-1 Tat inhibits Wnt/β-catenin signaling in astrocytes [229]. Hippocampal astrocytes play an important role in SGZ neurogenesis via promoting the neuronal differentiation. Inhibition of Wnt in astrocytes may lead to blockade of the neurogenic activity of astrocytes, which results in decrease of neurogenesis in SGZ [230, 231]. Bone morphogenetic proteins (BMP) are involved in many development processes. In the brain, BMP itself promote dorsal neural formation [232] and it crosstalk with several other pathways, such as STAT3, Wnt and Shh to induce astrocyte specification but inhibit oligodendrocyte specification [233]. STAT3 stabilizes the astrocyte phenotype via promoting expression of astrocytes specific marker GFAP. A similar phenomenon is observed in Notch signaling, which crosstalk with STAT3 via Hes and promote astrocytes differentiation [234, 235]. Besides, Notch itself typically down-regulates preneuronal gene expression and inhibits neuron differentiation [236].

6.3 HIV-1 and neurogenesis

Impaired neurogenesis has been noted in the hippocampus of HIV-infected individuals, SIV-infected macaques, and severe combined immunodeficient mice injected with HIV-infected human microglia/macrophages [81, 237-239]. Several putative mechanisms are

proposed for HIV-impaired neurogenesis. HIV infection of microglia/macrophages and astrocytes alters expression of cytokines or groWTh factors [84, 206, 240-244], several of which are known to affect adult hippocampal neurogenesis. HIV may have effects on NPC through direct infection [80, 245-247]. HIV has been shown to inhibit proliferation and promote quiescence of NPC [81, 85] and NPC migration [248, 249]. HIV gp120 expression has been shown to inhibit NPC proliferation *in vitro* and in the hippocampal dentate gyrus of gp120-expressing transgenic mice [85]. HIV-1 Tat protein has also been shown to inhibit NPC proliferation and differentiation *in vitro* [83, 250]. Given the recent finding that Tat is persistently expressed in the brain in the era of cART [70], it is imperative to determine effects of Tat expression in neurogenesis and its contribution to HIV-associated MCMD.

7. Summary of the background and our hypothesis

HIV-1 infection of the CNS often leads to HAND ranging from MCMD to HAD [26, 88-90]. The introduction of cART in the mid-1990's led to reduced viral replication, improved immune function and increased life expectancy among HIV-infected individuals [92, 93]. Unfortunately, it has failed to provide complete protection from HAND or to reverse the disease in most of the cases [93]. HAND affects over 50% of HIV-infected individuals in the era of cART, which leads to neurological impairment and neurocognitive dysfunction affecting individuals daily lives, work performance, care needs, and increasing economic burden. HIV-1 Tat protein is a major pathogenic factor in HIV-associated neuropathogenesis [251]. Tat protein is secreted from HIV-infected

cells and taken up by uninfected cells [138, 252]. Accumulating evidence suggests that Tat adversely affects the CNS in both direct and indirect manners. Astrocytes have been shown to play important roles in HIV neuropathogenesis and HIV-1 infection of astrocytes has been documented both *in vivo* and *in vitro* [253, 254]. Astrocyte activation (astrocytosis), characterized by up-regulated GFAP, is responsible for disturbed glutamate metabolism, production of neurotoxins and cytokines/chemokines and neuronal injury [176, 179, 255]. Our previous studies have shown that Tat alone is capable of activating GFAP expression and inducing astrocytosis involving sequential activation of early groWTh response protein 1 (Egr-1) and p300. In this study, we determined the roles of STAT3 in Tat-induced GFAP transactivation. Moreover, despite the progress made in the past few years, our understanding of the underlying mechanisms is still limited to the deleterious effects on mature cells. Until recently, neurogenesis has shown to be affected by HIV, suggesting that NSC/NPC harbor HIV-1, which could be the potential menace contributor of HAND [79-81]. Thus, we determined whether and how Tat would affect NPC proliferation and differentiation using iTat mouse model. Our hypothesis is interactions between HIV-1 Tat protein and astrocytes/NPC contribute to HIV-1/neuroAIDS.

MATERIALS AND METHODS

MATERIALS

Media and supplements

Dulbecco's Modified Eagle's Medium (DMEM), Ham's F12 Medium (F12) and Kaighn's modification of Ham's F-12 medium (F12k) were purchased from Lonza (Walkersville, MD). Neurobasal-A Medium and Penicillin-streptomycin-glutamine and trypsin were purchased from Gibco (Grand Island, NY). Fetal bovine serum was purchased from Hyclone (Logan, UT). Ampicilin sodium salt and Kanamycine sulfate were purchased from United States Biological (Swampscott, MD). The bacteria culture media were prepared as described below. Luria broth (LB) media were made of 10 g/L Bacto tryptone and 5 g/L Bacto yeast extract (BD Biosciences, San Jose, CA) plus 5 g/L NaCl. Solid LB culture plates were made with complete LB medium with the addition of 15 g/L Bacto agar (BD Biosciences). Super optimal broth with catabolite repression (SOC) was purchased from Life technologies (Grand Island, NY). Working concentrations of antibiotics were 100 μg/ml ampicillin and 50 μg/ml kanamycin.

Antibodies

Rabbit anti-STAT3 (sc-482) (WB 1:100), mouse anti-phosphorylated STAT3 (p-STAT3) (sc-8059) (WB 1:100), rabbit anti-Egr-1 (sc-110) (WB 1:100) rabbit anti-Myc (sc-789) (WB 1:1000), mouse anti-MAP2 (sc-32971) (IF 1:200), goat anti-DCX (sc-8066) (WB 1:100, IF 1:200) and mouse anti-Hes1 (sc-166410) (WB 1:100) were purchased from

Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-GFAP (G3893) (WB 1:1000, IF 1:200) and mouse anti-β-actin (A1978) (WB 1:2000) were purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO). Rat anti-BrdU (OBT0030G, Bio-Rad), rabbit anti-GFAP (Z0334, Dako) (IF 1:500) mouse anti- NeuN (MAB377, Millipore, CA), Sheep anti-mouse IgG-horseradish peroxidase (HRP) (NA931V) and donkey anti-rabbit IgG-HRP (NA9340V) (WB 1:3000) were purchased from GE Healthcare (Little Chalfont, Buckinghamshire, UK). Donkey anti-rat Alexa-Fluor-555 (A21434), rabbit anti-goat Alexa-Fluor-555 (A21431), goat anti-mouse Alexa-Fluor-555 (A21422), goat anti-mouse Alexa-Fluor-488 (A11001), and goat anti-rabbit Alexa-Fluor 488 (A11034) (IF 1: 1000) were purchased from Molecular Probes (Eugene, Oregon, USA).

Reagents

Enhanced chemiluminesence (ECL) reagents for Western blotting detection were made in house. Protease inhibitor cocktail set V were purchased from Roche (Indianapolis, IN). T4 DNA polymerase, T4 DNA ligase, calf intestinal phosphatase (CIP), and all restriction endonucleases were from New England Biolabs (Beverly, MA). Poly-L-lysine, Yeast mannan, dextran sulfate, and 4',6'-diamidino-2-phenylindol (DAPI) were purchased from Sigma. All other chemicals were from Fisher (LaGrange, KY).

Biotechnology systems

The expand high fidelity PCR system, Gel and PCR clean-up kit and firefly luciferase

assay system were purchased from Promega (Madison, WI). The Bio-Rad DC protein assay kit was from BioRad Laboratories (Hercules, CA).

METHODS

Cells and cell cultures

Cell lines

Human embryonic kidney cell line 293T, human astrocytoma U373.MG and neuroblastoma cell line SH-SY5Y were purchased from American Tissue Culture Collection (ATCC) (Manassas, VA).

Primary cells

Mouse primary astrocytes were isolated from 18.5-day-old embryonic brain tissue of the Tat-inducible transgenic mice described previously [1, 196, 256]. Human primary fetal astrocytes were isolated, similarly to mouse primary astrocytes, from aborted 16-week fetal tissues (Advanced Biosciences Resources, Alameda, CA). Mouse primary NPCs were isolated from neonatal C57BL/6J mouse pups on p1.

Competent cells for cloning

GC5^{TM} chemically competent *E. coli* was purchased from GeneChoice (Frederick, MD).

Cell cultures

293T and U373.MG were maintained in DMEM. SH-SY5Y were maintained in F12 and DMEM (1:1 mix). Mouse and human primary astrocytes were maintained in F12K. All the culture mediums were supplemented with 10% fetal bovine serum (FBS) and 100 μg/ml Penicillin-streptomycin and 2 mM glutamine Mouse primary NPCs were maintained in Neurobasal-A supplemented with 1% B27, 20 ng/ml EGF, 20 ng/ml bFGF, 50 U/ml penicillin, and 50 μg/ml streptomycin. All cells were cultured at 37℃ with 5% CO_2.

Animal and tissues

Mouse strains

Wild-type C57BL/6J mice (WT) were purchased from the Jackson Laboratory (Bar Harhor, Maine, USA). Doxycycline (Dox)-inducible brain-specific HIV-1 Tat transgenic mouse model **(**iTat**)** mouse model was described previously [79], GFAP knockout mice (GFAP-) was kindly provided by Dr. Messing, University of Wisconsin-Madison. iTat/GFAP- mouse was generated by cross mating of GFAP- mice [99, 257] with iTat mice. nestin-GFP mice was kindly provided by Dr. Jinhui Chen, Indiana University School of medicine [258]. iTat/nestin-GFP mouse was generated by cross mating of nestin-GFP mice with iTat mice.

Mouse treatments and tissues

To induce Tat expression, four-week-old iTat or iTat/GFAP- mice were intraperitoneally injected with Dox (80 mg/kg/day in PBS with pH2.8) for 7 days. As control, age and

gender matched WT mice were intraperitoneally injected with Dox (80 mg/kg/day in PBS with pH2.8) for 7 days or age and gender matched iTat or iTat/GFAP- mice were intraperitoneally injected with pH-matched and equal volume of PBS.

For proliferating study, four-week old nestin-GFP mice and iTat/nestin-GFP mice were intraperitoneally injected with Dox (80 mg/kg/day in ddH_2O with pH2.8) for 7 days and on day 5 of Dox injection, BrdU (50 mg/kg/day in ddH_2O) was injected together with Dox for 3 days. Post-final injection for 24 hr, mice brains were collected, sectioned for floating staining. For neurogenesis study, eight-week-old WT mice and iTat mice were intraperitoneally injected with Dox (80 mg/kg/day in ddH_2O with pH2.8) for 7 days, and on day 5 of Dox injection, BrdU (50 mg/kg/day in ddH_2O) was injected together with Dox for 3 days and injected alone for 4 more days. Post-final injection for 25 days, mice brains were collected, sectioned for floating staining. For neurogenesis study with Notch inhibitor, eight-week-WT and iTat mice were intraperitoneally injected with or without DAPT (120 mg/kg/day in 5% ethanol/95% corn oil), together with Dox (80 mg/kg/day in ddH_2O with pH2.8) for 7 days, and on day 5 of Dox/DAPT injection, BrdU (50 mg/kg/day in ddH_2O) was injected together with Dox for 3 days and injected alone for 4 more days. Post-final injection for 25 days, mice brains were collected, sectioned for floating staining.

Plasmids

cDNA3, GL3-basic and CMV-β-gal plasmids were purchased from Clontech (Mountain View, CA). The constitutively active STAT3 mutant plasmid (STAT3c) was purchased from AddGene (Cambridge, MA). GFAP promoter-driven luciferase reporter GFAP-Luc3 was a gift from Dr. Mark Brenner of University of Alabama at Birmingham, Birmingham, AL. p300 and STAT3 plasmids were gifts from Dr. Cheng-Hee Chang of University of Michigan, Ann Arbor, MI and Dr. Xin-Yuan Fu of Indiana University School of Medicine, Indianapolis, IN, respectively. Tat expression plasmid and p300 promoter-driven luciferase reporter p300-Luc was previously described [75, 259]. Egr-1 expression plasmid was constructed using a standard PCR-based cloning method using mRNA from 293T cells as the template, cDNA3 as the backbone, and primers 5'-GGG GTA CCA TGG CCG CGG CCA AGG CCG AGA TGC-3' and 5'-CGG AAT TCT TAG CAA ATT TCA ATT GTC CTG GG-3' (EcoR I and Kpn I sites underlined). STAT3 promoter-driven luciferase reporter was constructed using genomic DNA from U373.MG as the template, GL3-basic as the backbone, and primers: 5'-CTA GCT AGC GGG GAC TAT CTA AC-3' and 5'-CCC AAG CTT CCC GGG TCC CAG GC-3' (Nhe I and Hind III sites underlined). pHes1-luc was kindly provide by Dr. Raphael Kppan, Washington University [260]. pLTR-Luc was from Drs. R. Jeeninga and B. Berkhout and pE-Tat.BL43.CC, pE-Tat.BL43.CS and pE-Tat.BL43.SS was obtained from Dr. Udaykumar Ranga through the NIH AIDS reagent program.

Bacterial transformation

Plasmids were transformed into GC5^{TM} competent cells according to the manufacturer's directions. Briefly, the GC5^{TM} cells 25 µl were mixed with 0.5-2 µl (50-200 ng) DNA ligation product and incubate on ice for 30 min. The cells were then heat-shocked at 42°C for 45-60 sec, followed by incubation on ice for another 2 min, and addition of 250 µl of SOC medium. The cells were then incubated at 37°C for less than 1 hr with shaking of 225 RPM, and plated on LB plates containing the appropriate antibiotics. For large-scale cultures, transformed cells were growing in LB medium under 37°C for 16 hr with shaking of 225 RPM.

Cell transfections

Transfections were carried out using the standard calcium phosphate precipitation method. Media were replaced 16 hr post-transfection. Transfection efficiency was monitored by GFP expression and was 80%-90% for 293T and 20%-40% for HPA.

Western blotting

Cells were harvested and lysed in RIPA buffer [1 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 1% sodium deoxycholate, 140 mM NaCl, 0.1% sodium deoxycholate, 10 mM Tris HCl, pH 8.0] with protease inhibitor and PMSF (phenylmethylsulfonyl fluoride) (Sigma) for 20 min on ice. Lysates were sonicated on ice and cleared of cell debris by 16000 *g*, 15 min centrifugation and the protein concentration was determined using a

Bio-Rad DC protein assay kit with a BIO-RAD microplate reader. Equal amounts of protein were pre-incubated with 1x SDS loading buffer at 100°C for 10 min. Each sample was loaded onto and separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred onto nitrocellulose membrane and probed with desired primary antibodies and then with appropriate HRP or fluorescence-conjugated secondary antibodies. Chemiluminesence visualization was performed using a homemade ECL system. Florescence visualization was performed using a BIO-RAD Chemi Doc imaging system (BD imager).

Triton soluble (TS) and Triton insoluble fraction (TIS) isolation

For GFAP detection, cells were lysed in a Triton buffer (20mM tris-HCl, ph 7.54, 5mM EDTA, 150Mm NaCl, 1% Triton X-100 plus 1×protease inhibitor cocktail) on ice for 20 min for Triton soluble fraction preparation. Protein concentration was determined using a Bio-Rad DC protein assay kit as described above. The pellets were dissolved in 4×SDS loading buffer (0.2 M Tris-HCl, pH6.8, 8% SDS, 50mM EDTA, 4% β-mercaptethanol) and heated at 60°C for 30 min followed by 100°C for 10 min, and then used for GFAP detection.

RNA isolation and semi-quantitative RT-PCR

Total RNA was isolated using a TRizol Reagent kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Reverse transcription-PCR (RT-PCR) was performed

using a Titan One Tube RT-PCR kit (Boehringer Mannheim, Indianapolis, IN) on a PE Thermocycler 9700 (PE Applied Biosystem, Foster City, CA). The primers for Tat were 5'-GTC GGG ATC CTA ATG GAG CCA GTA GAT CCT-3' and 5'-TGC TTT GAT AGA GAA ACT TGA TGA GTC-3'; the primers for the internal control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were 5'-CTC AGT GTA GCC CAG GAT GC-3' and 5'-ACC ACC ATG GAG AAG GCT GG-3'. The expected sizes for Tat and GAPDH amplification product were 216 and 500 bp, respectively.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay

The neurotoxicity of astrocyte culture supernatants was determined by the MTT assay using mouse primary neurons, or SH-SY5Y. The cells were plated in a 48-well plate at a density of 1 x 10^5 cells/well and cultured for two days. Then, the cells were exposed to culture supernatants (2:1 ratio) collected from astrocytes that were either transfected with plasmids or induced with Dox and continued to culture for 3 days. MTT (5 mg/ml) was added directly to the culture medium to the final concentration of 1 mg/ml, the cultures were incubated at 37°C for 4 hr. The medium was then removed, and the purple crystal precipitates were dissolved in 200 μl acid-isopropanol (44 ml isopropanol plus 6 ml 0.2 N HCl). Aliquots of the acid-isopropanol solvent were transferred into a 96-well plate, and the optical density was taken using a microplate reader at a test wavelength of 490 nm and a reference wavelength of 650 nm and used to represent the relative cell viability.

Preparation of pseudotyped HIV-GFP virus and infection of human primary fetal astrocytes

293T cells were transfected with HIV-GFP and HCMV-G plasmids using the standard calcium phosphate precipitation method. The culture medium was replaced with fresh medium 16 hr post transfection. The cells were cultured for 48 hr. The culture supernatants were collected and removed of cell debris by a brief centrifugation, and used as virus stock. Transfection without pCMV-G plasmid was included as a virus control. Human primary fetal astrocytes were infected with HIV-GFP viruses at multiplicity of infection (MOI = 1) in the presence of 8 μg/ml polybrene at 37°C for 2 hr. The cells were then thoroughly washed with culture medium to remove unbound viruses, cultured for 48 hr, and harvested for protein lysates.

β-galactosidase assay and Luciferase activity assay

Cells were washed twice with ice-cold PBS, lyzed in 1X cell lysis buffer (Promega, Madison. WI), and incubated at room temperature for 5 min. The clear supernatants were collected following a brief centrifugation. For β-galactosidase activity assay, cell extracts (10 μl) was mixed with 3 μl 100X Mg^{2+} solution (0.1 M $MgCl_2$ and 4.5 M mecaptoethanol, 66 μl 4 ng/ml O-nitrophenyl-D-galactopyranoside in 0.1 M sodium phosphate, pH 7.5 and 201 μl 0.1 M sodium phosphate, pH 7.5). The mixture was incubated at 37°C for 30 min, and the optical density at 405 nm was taken using a Bio-Rad iMark microplate reader (Bio-Rad, Hercules, CA). The β-galactosidase activity was then used to normalize the transfection variations among transfections for the subsequent

luciferase reporter gene assay, which was performed using a luciferase assay system (Promega, Madison. WI). The luciferase activity was quantitated using an Opticomp Luminometer (MGM Instruments, Hamden, CT).

Primary mouse NPC proliferation assay

Mouse primary astrocytes were isolated from iTat or WT C57BL/6J mouse, cells were treated with Dox (5 μg/ml) for 3 days and then washed three times with PBS to remove Dox and refilled with NPC culture medium described above. Post-culturing for 24 hr, supernatants were collected. Human primary astrocytes were transfected with pcDNA3 or pTat.Myc. After 16 hr incubation in transfection reagents, cells were washed three times with PBS and refilled with NPC culture medium described above for another 48 hr, and then supernatants were collected. Neurospheres formed by the WT primary mouse NPCs on day 7 were trypsinized into single cells by using TrypLE (GIBICO, OK). The supernatants described above or Tat recombinant proteins were added on the NPCs in 12-well uncoated plates at a density of 2×10^5 cells/well. Neurospheres formed after 7 days. Pictures were taken using Nikon Eclipse Ti microscope. Three random fields under the microscope have been imaged from each triplicated wells of each sample. All the neurospheres in each field have been measured and analyzed by NIS-Elements viewer 4.20 software. The diameter of the neurospheres of smaller than 60 μm were defined as small spheres, and of larger than 60 μm were defined as large spheres. The images were

representative of all the neurospheres in each group. The bar graphs were mean ± SD of triplicates in each group and representative of three independent experiments.

Primary mouse neurosphere migration assay

Neurospheres formed by the WT primary mouse NPCs on day 7 were seeded within the conditioned mediums described above or Tat recombinant proteins on the poly-lysine coated 12-well plates. After 48 hr treatment, thirty neurospheres have been randomly selected and the longest migration distance from central to the migrated edge was measured.

Primary mouse neurosphere differentiation assay

Neurospheres formed by the WT primary mouse NPCs on day 7 were seeded within the conditioned mediums described above or Tat recombinant proteins on the poly-lysine coated coverslips in 24-well plates or in the poly-lysine coated 6 cm plates. After differentiation for 7 days, cells were subjected to immunofluorescence staining or western blotting analysis.

Immunofluorescence staining

To detect MAP-2 and GFAP in the neurospheres, cells were washed with ice-cold PBS at room temperature for 10 min and fixed with 4% paraformldehyde for 30 min, and

followed by permeabilizing with 0.5% Triton-X 100 in PBS for 30 min and blocking with PBS-BB (5% BSA, 1% non-fat milk and 0.3% Triton-X 100 in PBS) for 1 hr. Cells were then incubated in mouse monoclonal α-MAP-2 (Santa Cruz, CA) and rabbit polyclonal α-GFAP (DAKO, CA) antibodies with dilution of 1:200 in PBS-BB at 4°C for overnight. Secondary antibody goat anti-mouse Alexa Fluor 555 and goat anti-rabbit Alexa Fluor 488 (Invitrogen, CA) with dilution of 1:500 in PBS-BB was applied to cells at room temperature for 1 hr. Nuclei were counterstained with 0.1 μg/ml 4', 6-diamidino-2-phenylindole (DAPI). Extensive washes with PBS were performed after each step. Omission of the primary antibody in parallel staining was included as a control to ensure no non-specific staining.

Floating staining

To detect BrdU and NeuN in the mouse brain tissue, thirty μm paraffin sections were cut by cryomicrotome (Leica, IL) throughout the rostrocaudal extent of the hippocampal dentate gyrus of the mouse brain and a one-in-six of series of floating sections were washed in PBS for 5 min for 3 times to remove cryo buffer and O.T.C. Then sections were incubated in 0.2 N HCl at room temperature for 1 hr followed by incubated in 10 mM sodium borate for 10 min. Sections were then blocked with PBS-BB at room temperature for 1 hr followed by incubating with rat α-BrdU (Thermo Scientific, IL) or mouse α-NeuN (Millipore, CA) with dilution of 1:100 in PBS at 4°C for overnight. Secondary antibody goat anti-mouse Alexa Fluor 555 and goat anti-rabbit Alexa Fluor

488 with dilution of 1:500 in PBS-BB was applied at room temperature for 1 hr. Nuclei were counterstained with 0.1 μg/ml 4', 6-diamidino-2-phenylindole (DAPI). Extensive washes with PBS were performed after each step. Omission of the primary antibody in parallel staining was included as a control to ensure no non-specific staining.

Data acquisition and statistical analysis

All values are expressed as mean ± SD from triplicate experiments. Statistical analyses were made using two-tailed Student's t-test and one-way ANOVA. The statistical analyses methods were specified in the figure legends. *p* values were labeled on each statistical graphs. A *p* value of < 0.05 was considered statistically significant, and a *p* value of < 0.01 highly significant. All data were representative of multiple independent experiments.

RESULTS

PART I. ROLE OF STAT3 IN TAT INDUCED GFAP UP-REGULATION

1.1 Tat expression led to increased GFAP expression and aggregation in mouse primary astrocytes

Our previous study shows that Tat alone is sufficient to activate GFAP expression and induce astrocytosis, which involved in astrocyte-mediated neurotoxicity [256, 261]. In this study, we took advantage of our brain-targeted inducible Tat transgenic mice model (iTat) (**Fig. 5**), thus we first confirmed whether Tat expression in the mouse model was sufficient to alter expression of GFAP. Mouse primary astrocytes were isolated from iTat mice and cultured in the absence or presence of doxycycline (Dox) for 3 days. Compare to the control, GFAP RNA level significantly increased in the presence of Dox in mouse primary astrocytes (**Fig. 6A**). GFAP up-regulation has been shown to contribute to the formation of inclusion bodies or the accumulation of Rosenthal fibers, which leads to difficulty of GFAP isolation from cells. To accurately compare the GFAP protein level in mouse primary astrocytes, we apply both triton soluble (TS) and triton insoluble fraction (TIS) to western blot detection. The result showed that GFAP is only detectable in TIS fraction and the expression level is significantly increased in Dox treated mouse primary astrocytes (**Fig. 6B**). We further performed immunocytochemistry staining to investigate the changing of expression pattern of the up-regulated GFAP. The result shows that in the

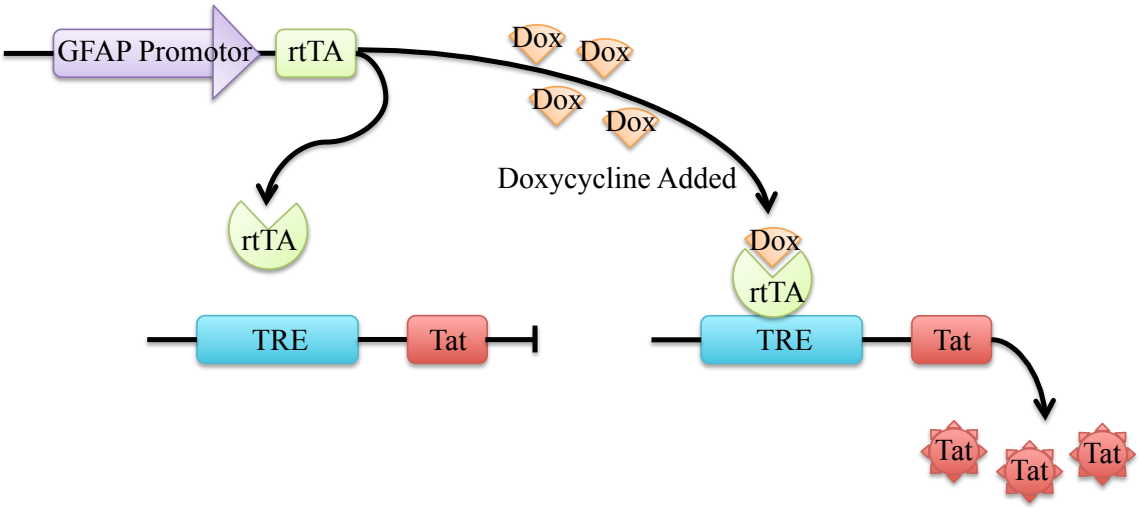
GFAP Promotor
rtTA
Dox
Dox
Dox
Dox
Doxycycline Added
rtTA
Dox
rtTA
TRE
Tat
TRE
Tat
Tat
Tat
Tat

Figure 5. Doxycycline (Dox)-inducible astrocyte-specific HIV-1 Tat transgenic strategy. Dox regulatory element rtTA is under control of GFAP promotor. In the presence of Dox, Tat is expressed under the control of GFAP promotor [1].

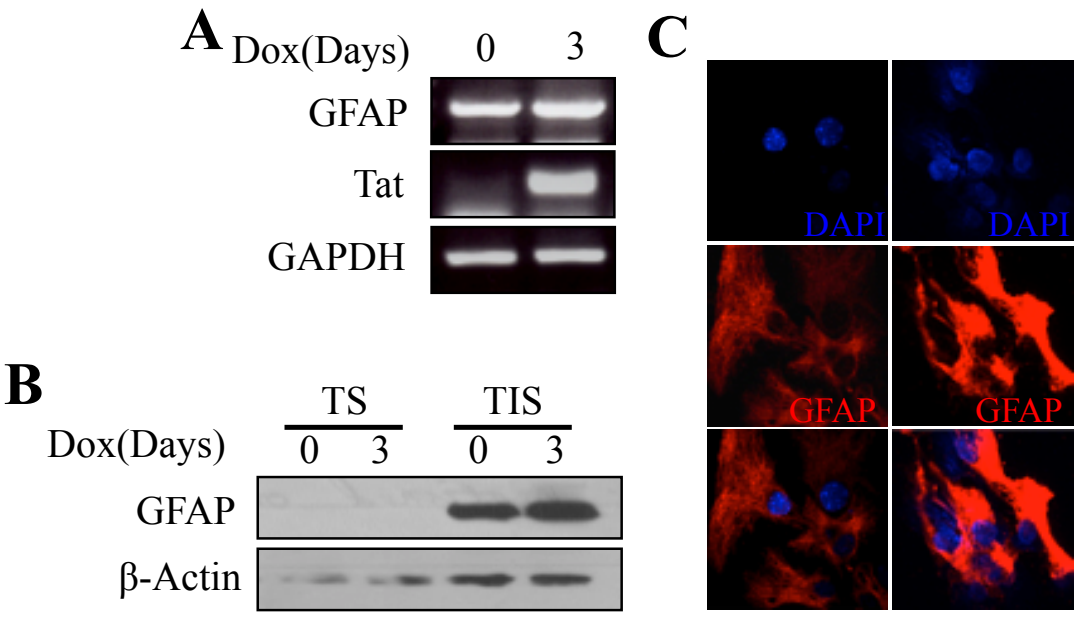
A
Dox(Days)
0
3
GFAP
Tat
GAPDH
B
TS
TIS
Dox(Days)
0
3
0
3
GFAP
β-Actin
C
DAPI
DAPI
GFAP
GFAP

Figure 6. Tat expression alone in mouse primary astrocytes was sufficient to activate GFAP expression and lead to GFAP aggregation. A. Mouse primary astrocytes were isolated from doxycycline (Dox)-inducible brain-specific Tat transgenic mice (iTat) and cultured in the presence of 5 μg/ml Dox for 0 and 3 days. Total RNA was isolated for RT-PCR using Tat- and GFAP-specific primers. GAPDH was included as an internal control. **B.** Cell lysates were separated into Triton X-100-soluble (TS) and insoluble (TIS) fractions and analyzed for GFAP expression by Western blotting. β-actin was included as an equal loading control. **C.** The same cells were immunostained for GFAP expression, followed by microscopic imaging. The cells were counterstained for nuclei using 100 ng/ml DAPI. The data were representative of three independent experiments.

absence of Dox astrocytes spread extendedly, while in the presence of Dox GFAP gather tightly around the nucleus (**Fig. 6C**). These results suggest that in mouse primary astrocytes, Tat alone is sufficient to up-regulate GFAP expression and lead to GFAP aggregation.

1.2 HIV-1 Tat- and HIV-induced GFAP up-regulation in the mouse and human brain.

To avoid the unknown factors caused by the culture system, and to further confirm whether up-regulation of GFAP holds true in vivo, we further tested whether HIV infection in human brain or HIV-1 Tat expression in mouse brain induce GFAP up-regulation *in vivo*. We injected iTat mice with Dox for 7 days. Mouse brain tissues were harvested and subjected to immunofluorescence staining to detect GFAP in the cortex of the brain. GFAP expression showed significantly higher in the brain of Dox injected iTat mouse than in the brain of pH-matched PBS injected iTat mouse (**Fig. 7A**). The similar results were observed in the brain sections of HIV-demented subjects than in the brain of HIV-negative subjects (**Fig. 7B**). The *in vivo* results confirmed that HIV-1 infection, especially HIV-1 Tat was sufficient to cause GFAP up-regulation in the brain.

1.3 HIV-1 Tat expression and GFAP-upregulation in astrocytes led to neurotoxicity

In our previous study, we proposed astrocyte-mediated Tat neurotoxicity, in which the supernatant collected from Tat expressing astrocytes is able to cause neuron death [196]. Here, we further test whether up-regulation of GFAP in astrocytes also led to neuron death.

Supernatants were collected from PBS or Dox treated iTat mouse primary astrocytes, and Tat, GFAP transfected U373 cells, and then added on neuroblastoma cell line SH-SY5Y cells (**Fig. 8A**) or human primary neurons (**Fig. 8B**). MTT assay was performed 3 days after the culturing to analyze cell survival. The data showed significant reduction of MTT activity in the both SH-SY5Y cells and human primary neurons cultured in the supernatants of Tat expressing and GFAP up-regulated astrocytes. The results suggest significant GFAP up-regulation alone is sufficient to induce the astrocyte-mediated neurotoxicity.

1.4 HIV-1 Tat- and HIV-1- induced loss of neuronal dendrites *in vivo.*

To further determine the effect of Tat and HIV on neurons in vivo, iTat mice were injected with Dox for 7 days. Mouse brain tissues were harvested and subjected to immunofluorescence staining to detect MAP2 in the cortex of the brain. MAP2 staining indicated the morphology change of neurons *in vivo*. It showed that obviously neuronal dendrites loss in the brain of Dox injected iTat mouse when compared with the brain of pH-matched PBS injected iTat mouse (**Fig. 9A**). Similar results were observed in the brain sections of HIV-demented subjects compared to the brain of HIV-negative subjects

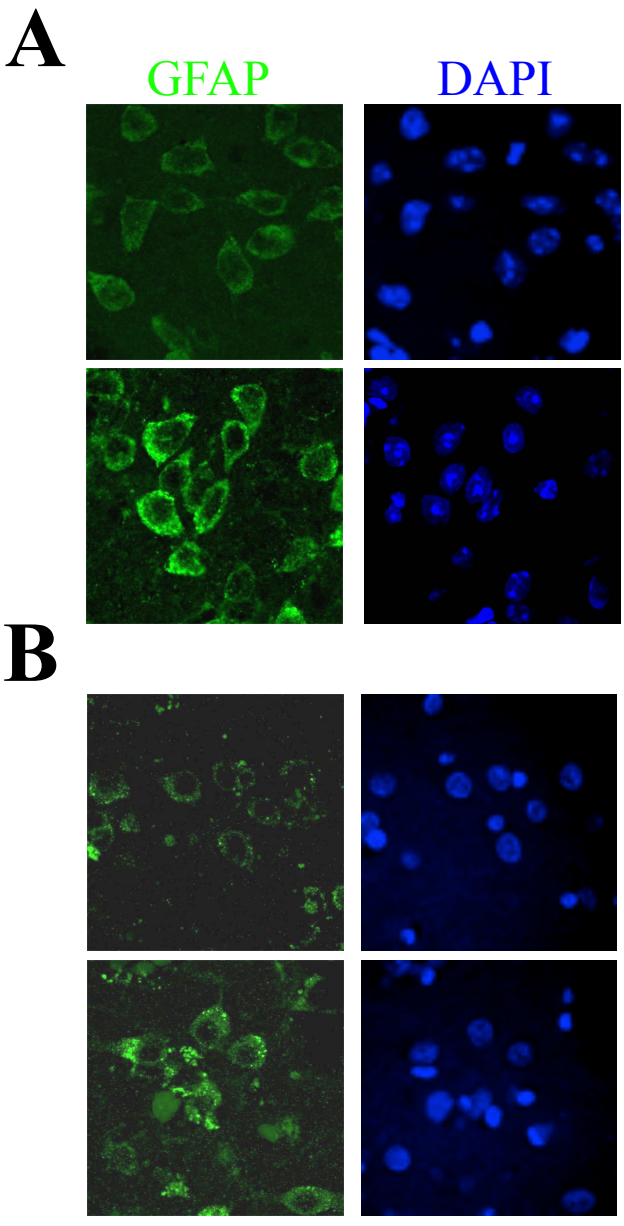
A
GFAP
DAPI
B

Figure 7. Tat expression increased GFAP expression in the mouse and human brain.

A. iTat mice were intraperitoneally injected with Dox (80 mg/kg/day, + Dox) (lower panel) or pH-matched PBS (pH 2.2, - Dox)(upper panel) for 7 days. The brains were collected, sectioned were stained with an antibody for GFAP, **B.** Brain sections of HIV-negative subjects (-HIV, n=3) (upper panel) and HIV-demented subjects (+HIV, n=3) (lower panel) were stained for GFAP, followed by goat anti-rabbit Alexa Fluor 488 and goat anti-rabbit Alexa Fluor 555 The brain sections were then stained with 100 ng/ml DAPI for nuclei. The comparable cortical regions of the brains were imaged. A total of three brain sections from three Dox-treated and three PBS- treated mice were stained. The images were representative of all the stained brain sections in each group.

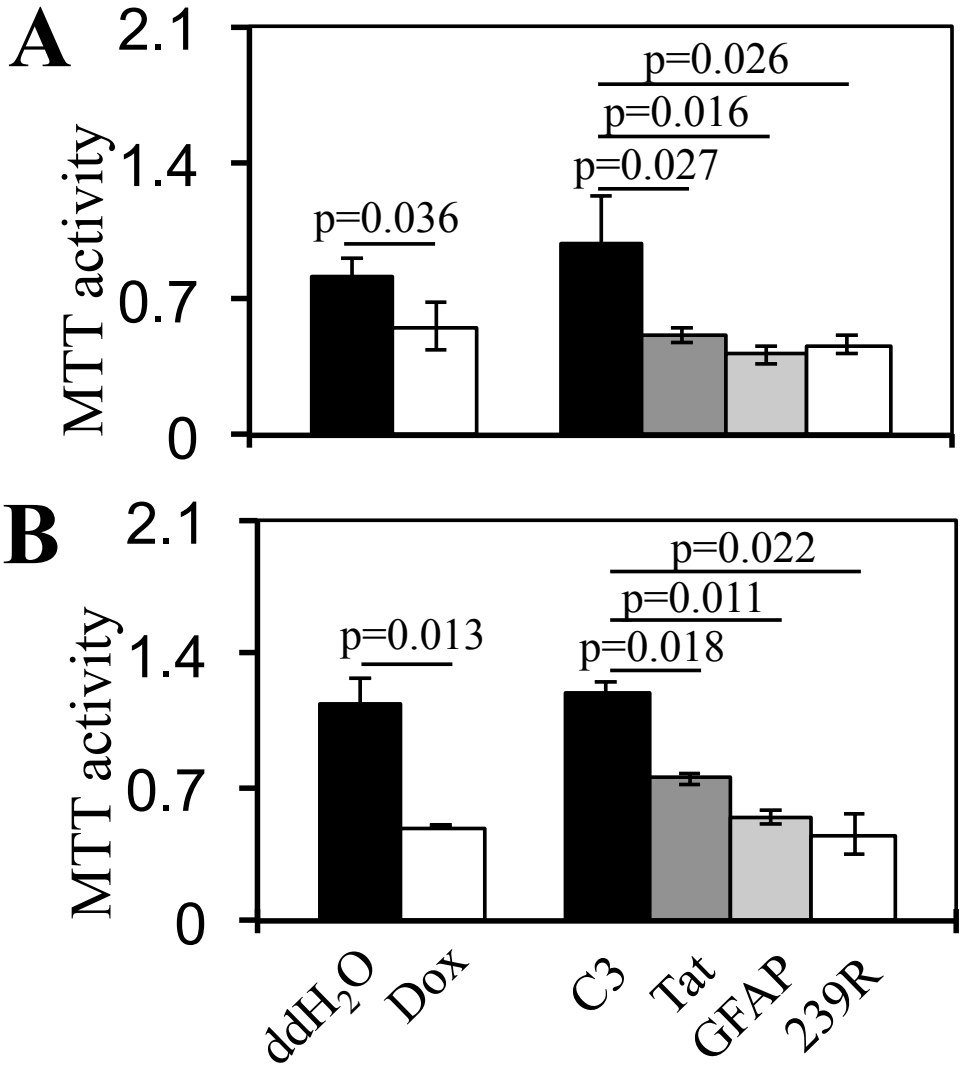
A
2.1
1.4
0.7
0
MTT activity
p=0.026
p=0.016
p=0.027
p=0.036
B
2.1
1.4
0.7
0
MTT activity
p=0.022
p=0.011
p=0.018
p=0.013
ddH2O
Dox
C3
Tat
GFAP
239R

Figure 8. Tat or GFAP expression in astrocytes caused neurotoxicity. iTat primary astrocytes were cultured in the absence or the presence of 5 μg/ml Dox for three days; U373 were transfected with cDNA3 (C3), Tat, GFAP or GFAP mutant 239R expression plasmids and cultured for three days. Culture supernatants were collected and used to determine the neurotoxicity in SH-SY5Y (**A**) and in human primary neurons (**B**) using the MTT assay. The data were mean ± SD of triplicates and representative of three independent experiments. Statistical analyses were performed using one-way ANOVA.

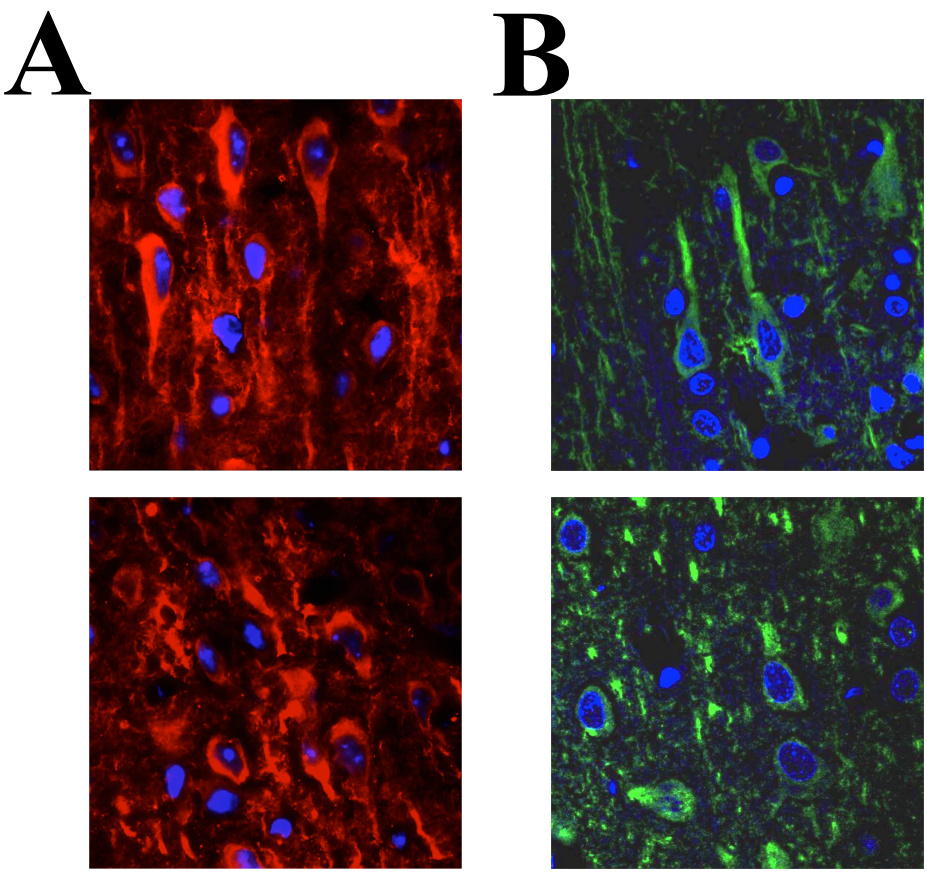
A
B

Figure 9. Loss of neuron dendrites in Tat expressing mouse brain and in HIV-demented human brain. **A.** iTat mice were intraperitoneally injected with Dox (80 mg/kg/day, + Dox) (lower panel) or pH-matched PBS (pH 2.2, - Dox)(upper panel) for 7 days. The brains were collected, sectioned were stained with an antibody for MAP2, goat anti-mouse Alexa Fluor 555. **B.** Brain sections of HIV-negative subjects (-HIV, n=3) (upper panel) and HIV-demented subjects (+HIV, n=3) (lower panel) were stained for MAP2, followed by goat anti-mouse Alexa Fluor 488. The brain sections were then stained with 100 ng/ml DAPI for nuclei. The comparable cortical regions of the brains were imaged. A total of three brain sections from three Dox-treated and three PBS-treated mice were stained. The images were representative of all the stained brain sections in each group.

(**Fig. bB**). The *in vivo* results confirmed that HIV-1 infection, especially HIV-1 Tat was sufficient to impair the neuron integrity in the brain.

1.5 Knockout of GFAP abrogated Tat induced neurotoxicity and Tat-induced neuronal dendrites loss in the mouse brain

To further determine the relationship between GFAP expression and Tat induced neurotoxicity, we generated the GFAP knockout inducible Tat transgenic mice (iTat/GFAP-) by cross breed GFAP knockout mice (GFAP-) with iTat mice. We first isolated primary astrocytes from WT, iTat, and iTat/GFAP- mice. Cells were cultured in absence and in presence of Dox for up to 3 days and harvested for MTT assay. Supernatants from Dox treated WT, iTat, and iTat/GFAP- mouse primary astrocytes were collected and added on the SH-SY5Y cells. Consistent with previous results, significant neuron death was observed in the SH-SY5Y cells cultured in the supernatant collected from Dox treated iTat mouse primary astrocytes, when compared to the PH-matched PBS treated control. Meanwhile, no significant neuron death was observed in the SH-SY5Y cells cultured in Dox treated WT and iTat/GFAP- cells (**Fig. 10A**). Moreover, to investigate whether neuronal dendrites are affected in the brain of Tat expressing iTat/GFAP- mouse, we injected WT, iTat, and iTat/GFAP- mice with Dox for 7 days. Mouse brains were harvested for immunofluorescence staining to detect MAP-2. Consistent with the previous finding, neuronal dendrite loss was observed in Dox treated iTat mouse brain, while no neuronal dendrites loss was observed in the brains of WT and

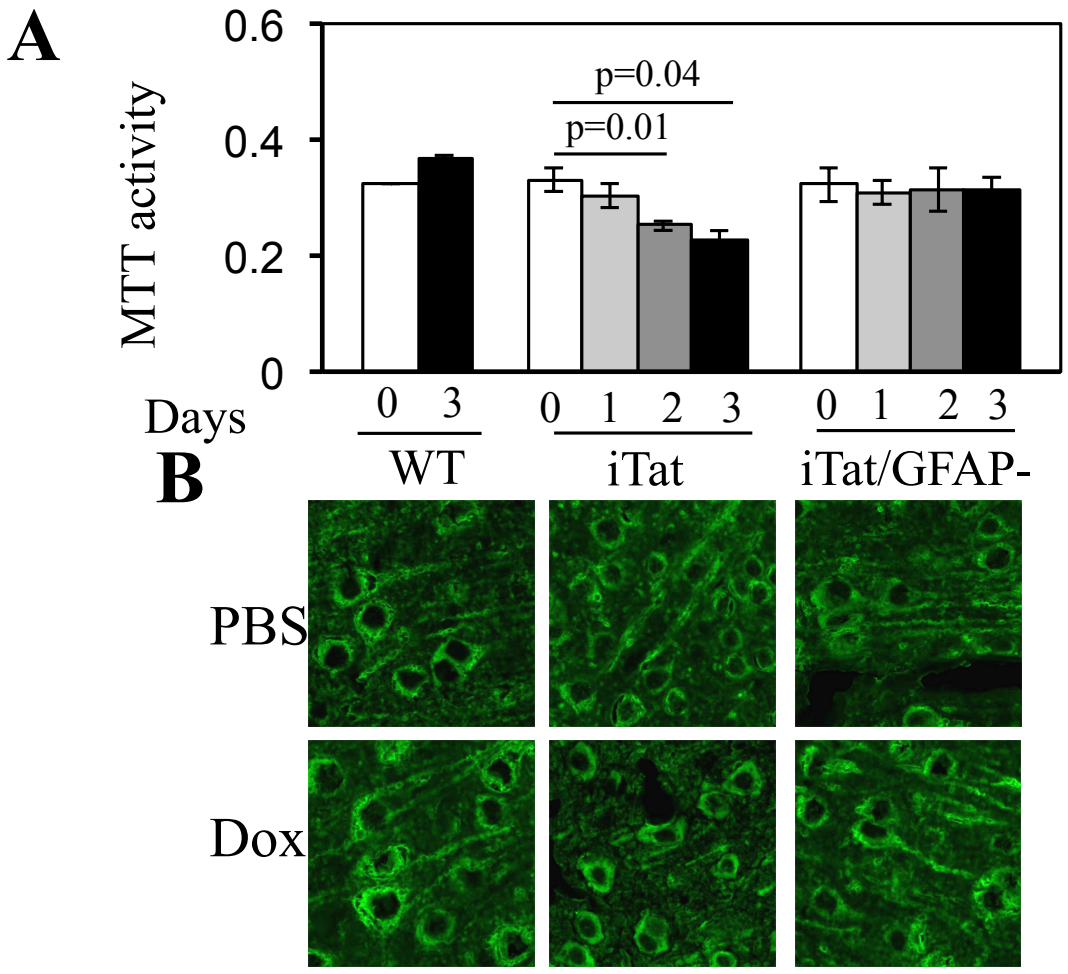
A
0.6
0.4
0.2
0
MTT activity
p=0.04
p=0.01
Days
0 3
0 1 2 3
0 1 2 3
WT
iTat
iTat/GFAP-
B
PBS
Dox

Figure 10. GFAP knockout abolished Tat-induced neurotoxicity and neuron dendrite loss. **A**. Primary astrocytes were isolated from WT, iTat or iTat/GFAP knockout (iTat/GFAP-) mice, cultured in the presence of 5 μg/ml Dox for indicated days, and then the culture supernatants were collected and used to determine the neurotoxicity in SH-SY5Y. The data were mean ± SD of triplicates and representative of three independent experiments. Statistical analyses were performed using one-way ANOVA. **B.** WT, iTat or iTat/GFAP knockout mice (three mice each) were intraperitoneally injected with Dox (80 mg/kg/day) for 0 and 7 days. The brains were collected, sectioned, and stained with an antibody for MAP-2, followed by goat anti-mouse Alexa Fluor 488.

iTat/GFAP- mice (**Fig. 10B**). These results indicated that GFAP plays a critical role in Tat induced neurotoxicity.

1.6 STAT3 and its phosphorylation transactivated GFAP expression

Our previous studies demonstrate that Tat alone is capable of activating glial fibrillary acidic protein (GFAP) expression and inducing astrocytosis involving sequential activation of early groWTh response protein 1 (Egr-1) and p300 [197, 198]. STAT3 has been shown to regulate astrogliogenesis from neuron progenitor cells in the CNS development; it involves external signaling such as IL-6, or internal changes such as epigenetic modifications [234, 262]. Thus, we first determined whether STAT3 expression was sufficient to alter expression of GFAP, the cellular marker of astrocytes. Human primary fetal astrocytes were transfected with a GFAP promoter-driven luciferase reporter and STAT3 expression plasmid or a constitutively active STAT3 mutant, i.e., the phosphorylated STAT3 (STAT3c). Compared to the control, expression of both STAT3 and STAT3c led to significant increases in GFAP-promoter-driven luciferase reporter gene expression (**Fig. 11A**), suggesting that STAT3 expression and its phosphorylation transactivated the GFAP gene transcription. To determine whether the increased transcription would lead to more GFAP protein expression, human primary fetal astrocytes were transfected with STAT3 or STAT3c, GFAP expression was determined by Western blotting. Compared to the control, higher levels of GFAP proteins were detected in the astrocytes transfected with both STAT3 and STAT3c (**Fig. 11B**). These results indicate that STAT3 overexpression and its phosphorylation (activation) in

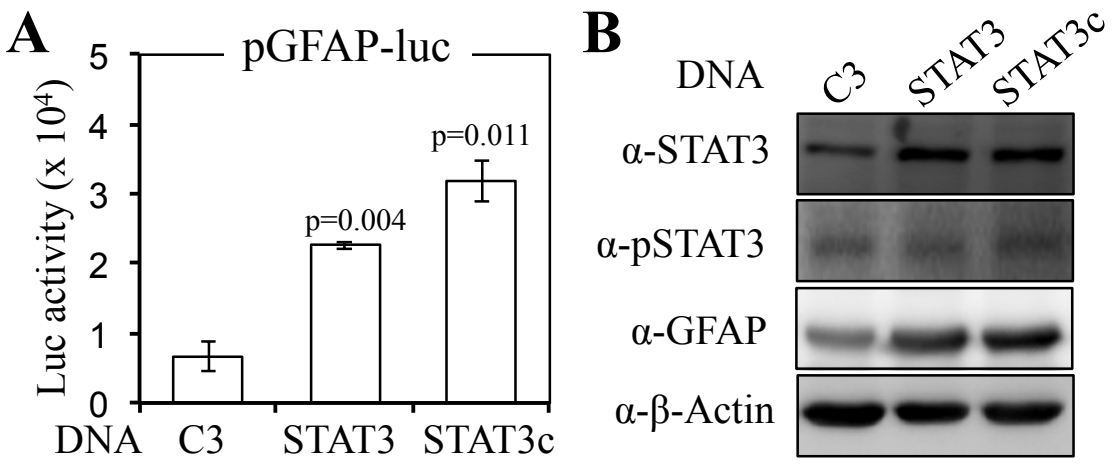
A
pGFAP-luc
Luc activity (x 10^4)
5
4
3
2
1
0
p=0.004
p=0.011
DNA
C3
STAT3
STAT3c
B
DNA
C3
STAT3
STAT3c
α-STAT3
α-pSTAT3
α-GFAP
α-β-Actin

Figure 11. STAT3 expression transactivated GFAP expression in astrocytes. A. Human primary astrocytes were transfected with the GFAP promoter-driven luciferase reporter pGFAP-Luc and STAT3, or STAT3c, cultured for 72 hr and harvested for the luciferase reporter gene assay. cDNA3 (C3) was included as the DNA control; pCMV-βgal was used to normalize for the transfection efficiency variations among all transfections. The data were mean ± SD of triplicates and representative of three independent experiments. Statistical analyses were performed using one-way ANOVA **B.** Human primary astrocytes were transfected cDNA3, STAT3, or STAT3c, cultured for 72 hr and harvested for GFAP, STAT3 and pSTAT3 expression by Western blotting. β-actin was included as a loading control. C3 was included as the DNA control. The data were representative of three independent experiments.

astrocytes is sufficient to transactivate GFAP expression at both mRNA and protein levels.

1.7 Increased STAT3 expression and phosphorylation in Tat and HIV-infected astrocytes

HIV-1 infection of the CNS is associated with astrocytosis, marked by increased GFAP expression [176, 263]. We have shown that HIV-1 Tat directly contributes to the increased GFAP expression and astrocytosis [196, 197]. Next, we determined the relationship between Tat expression and STAT3 expression and its phosphorylation. We first took advantage of the mouse embryonic astrocytes isolated from the astrocyte-specific doxycycline (Dox)-inducible Tat transgenic mice. The mouse primary astrocytes were treated with and without Dox for 3 days and harvested for STAT3 expression and phosphorylation by Western blotting. Compared to the control, Tat expression in Dox-treated astrocytes led to not only increased STAT3 expression but also increased STAT3 phosphorylation (**Fig. 12A**). As expected[196, 197], increased GFAP expression was also detected in Tat-expressing astrocytes. Similarly results were also obtained in Tat-transfected human astrocytoma U373.MG (**Fig. 12B**) and HIV-infected human primary fetal astrocytes (**Fig. 12C**). To determine whether Tat had any effects on STAT3 transcription, human primary fetal astrocytes were transfected with a STAT3 promoter-driven luciferase reporter gene and Tat. STAT3 and STAT3c were also included. Compared to the control, Tat expression led to increased STAT3 promoter-driven

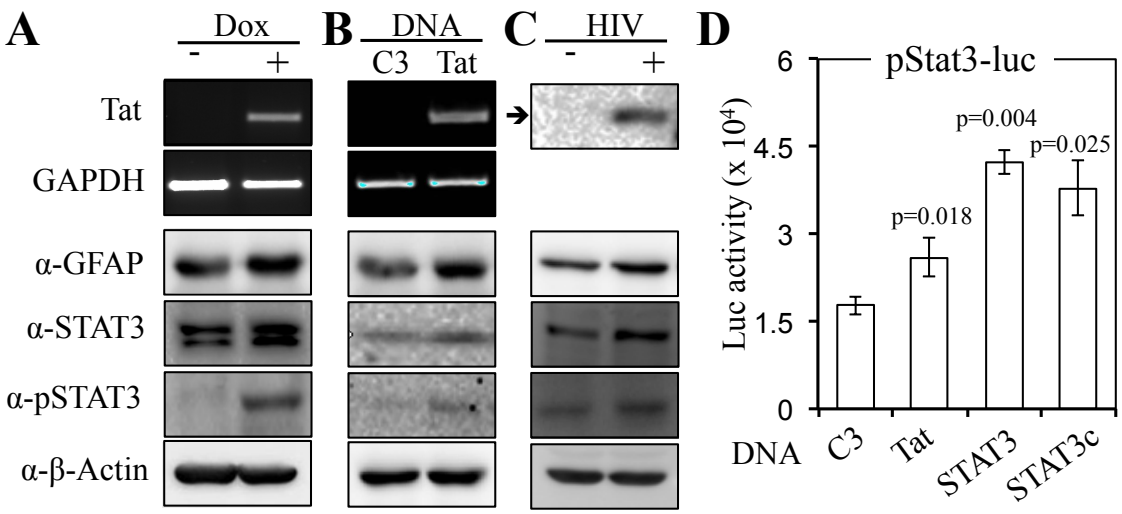
A
Dox
-
+
Tat
GAPDH
α-GFAP
α-STAT3
α-pSTAT3
α-β-Actin
B
DNA
C3
Tat
C
HIV
-
+
D
pStat3-luc
Luc activity (x 10^4)
6
4.5
3
1.5
0
p=0.018
p=0.004
p=0.025
DNA
C3
Tat
STAT3
STAT3c

Figure 12. Tat expression and HIV infection augmented STAT3 expression and phosphorylation in astrocytes. **A.** Mouse embryonic primary astrocytes from iTat mice were treated with and without Dox for 96 hr and harvested for STAT3, pSTAT3, and GFAP expression by Western blotting, or Tat expression by semi-quantitative RT-PCR. β-actin was included as a loading control for Western blotting, GAPDH was included as loading control for RT-PCR. The data were representative of three independent experiments. **B.** U373.MG were transfected with Tat, cultured for 72 hr, and harvested for Western blotting or RT-PCR. The data were representative of three independent experiments. **C.** Human primary fetal astrocytes were infected with VSV-G pseudotyped HIV-GFP virus. Cells were harvested 48 hr post-infection for Western blotting. HIV-GFP viruses without envelope were included as a control. Arrow: Western blotting with an anti-p24 antibody. The data were representative of three independent experiments. **D.** Human primary fetal astrocytes were transfected with the STAT3 promoter-driven luciferase reporter gene pSTAT3-Luc with Tat, STAT3, or STAT3c. Cells were harvested 72 hr post-transfection for the luciferase reporter gene assay. pCMV-β-gal used to normalize the transfection efficiency variations among all transfections. C3 was included as the DNA control (**B & D**). The data were mean ± SD of triplicates and representative of three independent experiments. Statistical analyses were performed using one-way ANOVA.

luciferase reporter gene activity (Fig. 12D). Meanwhile, STAT3 and STAT3c also showed transactivational activity on the STAT3 promoter. These results suggest that Tat expression leads to increased STAT3 transcription, protein expression and phosphorylation.

1.8 STAT3 involvement in the Tat-induced GFAP transactivation cascade

Our previous studies have shown that HIV-1 Tat expression transactivates GFAP expression through up-regulation of Egr-1 and subsequently p300 [197, 198]. Egr-1 has been shown to act downstream of STAT3 [264]. Therefore, we determined whether STAT3 would act upstream of the Tat-induced GFAP transactivation cascade. To this end, human primary fetal astrocytes were transfected with a GFAP promoter-driven luciferase reporter gene with Tat, STAT3, STAT3c, Egr-1, or p300 expression plasmids. Significantly increased the GFAP promoter activities were detected in all transfection (**Fig. 13C**), confirming that Tat, STAT3, Egr-1 and p300 are involved in GFAP transactivation. Similarly, Egr-1 and p300 promoter-driven luciferase reporter genes were also tested along with these plasmids. Expression of Tat, STAT3 and STAT3c all led to significant increases in both Egr-1 and p300 promoter-driven luciferase reporter gene activities (**Fig. 13A & B**). As expected [197], Egr-1 expression transactivated p300 promoter activity (**Fig. 3B**). These results indicate that STAT3 likely functions upstream of the Tat-induced GFAT transactivation cascade.

1.9 Dose-dependent response of GFAP, Egr-1 and p300 promoter activities to STAT3

To further determine the relationship between STAT3 expression and transactivation of the GFAP, Egr-1 and p300 promoter activities, human primary fetal astrocytes were transfected with GFAP, Egr-1 or p300 promoter-driven luciferase reporter gene and increasing amounts of STAT3. GFAP promoter-driven reporter gene activity showed a gradual increase, followed by a gradual decrease when STAT3 was increased (**Fig. 14A**). Meanwhile, the Egr-1 and p300 promoter-driven reporter gene activities exhibited the similar response kinetics (**Fig. 14B & C**). The bell-shape dose response is reminiscent of Tat-induced GFAP activation and the double-edged sword function of GFAP activation and astrocytosis [197, 198]. These results further support the notion that STAT3 acts upstream of Egr-1 and p300 in the Tat-induced GFAP transactivation cascade and suggest that there is a threshold of STAT3 response to Tat expression.

1.10 Knockdown of STAT3 attenuated Tat-induced GFAP transactivation

To further ascertain the importance of STAT3 in Tat-induced GFAP transactivation, human primary fetal astrocytes were transfected with GFAP or Egr-1 promoter-driven luciferase reporter gene, along with Tat expression plasmid, STAT3-sepcific siRNA, or Tat plus STAT3 siRNA. Compared to the control, STAT3 siRNA expression alone led to significant decreases GFAP promoter activities (**Fig. 15A & B**). On the other hand, Tat expression alone activated GFAP and Egr-1 promoter-driven reporter gene expression. Moreover, compared to the Tat expression alone, Tat plus STAT3 siRNA led to

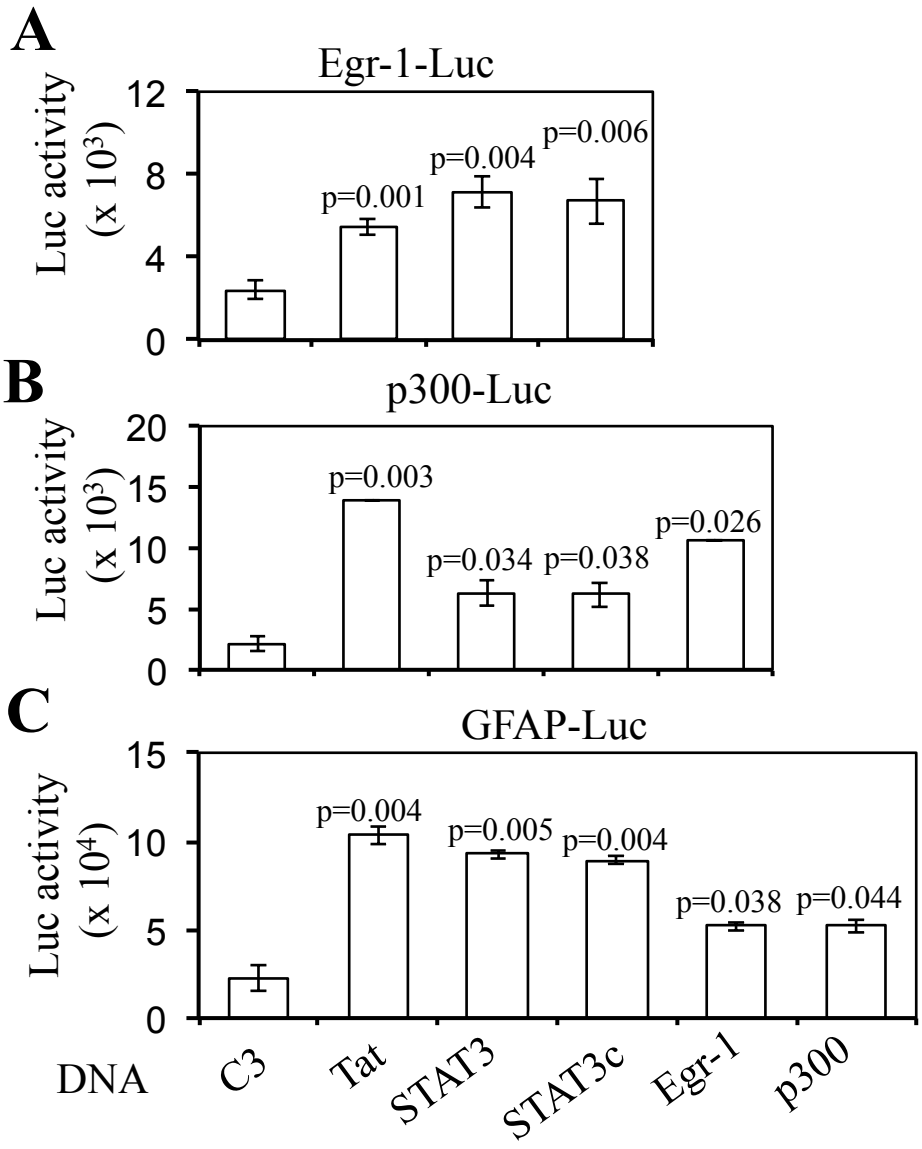
A
Egr-1-Luc
Luc activity (x 10³)
12
8
4
0
p=0.001
p=0.004
p=0.006
B
p300-Luc
Luc activity (x 10³)
20
15
10
5
0
p=0.003
p=0.034
p=0.038
p=0.026
C
GFAP-Luc
Luc activity (x 10⁴)
15
10
5
0
p=0.004
p=0.005
p=0.004
p=0.038
p=0.044
DNA
C3
Tat
STAT3
STAT3c
Egr-1
p300

Figure 13. Interaction of STAT3 with the Tat-Egr-1-p300-GFAP transcriptional cascade. Human primary fetal astrocytes were transfected with GFAP (**A**), Egr-1 (**B**), or p300 (**C**) promoter-driven luciferase reporter gene and Tat, Egr-1, p300, STAT3, or STAT3c expressing plasmid. Cells were harvested 72 hr post-transfection for the luciferase reporter gene assay. C3 was included as the DNA control. pCMV-β-gal used to normalize the transfection efficiency variations among all transfections. The data were mean ± SD of triplicates and representative of three independent experiments. Statistical analyses were performed using one-way ANOVA.

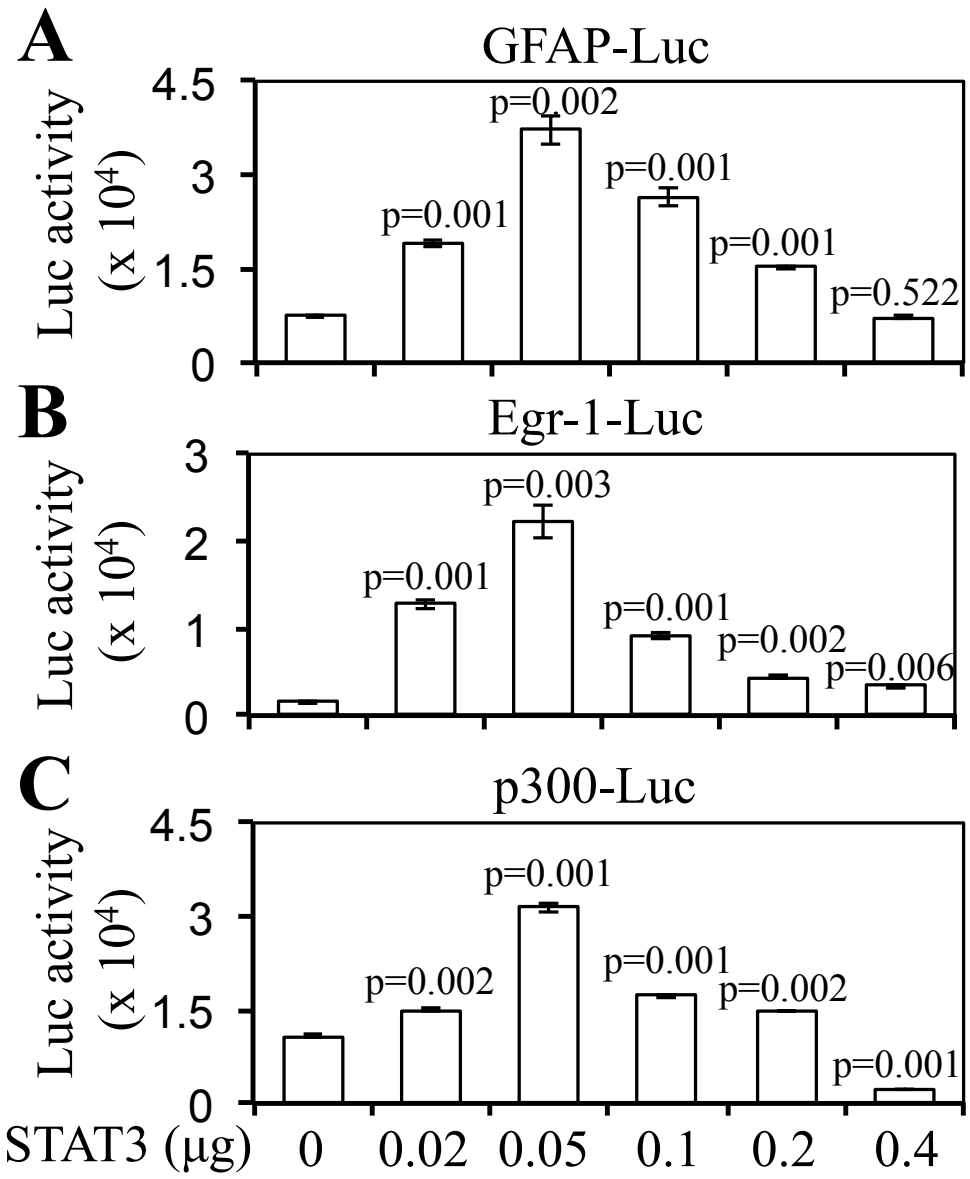
A
GFAP-Luc
Luc activity (x 10^4)
4.5
3
1.5
0
p=0.001
p=0.002
p=0.001
p=0.001
p=0.522
B
Egr-1-Luc
Luc activity (x 10^4)
3
2
1
0
p=0.001
p=0.003
p=0.001
p=0.002
p=0.006
C
p300-Luc
Luc activity (x 10^4)
4.5
3
1.5
0
p=0.002
p=0.001
p=0.001
p=0.002
p=0.001
STAT3 (μg) 0 0.02 0.05 0.1 0.2 0.4

Figure 14. Dose response of GFAP, Egr-1, and p300 transcription to STAT3. Human primary fetal astrocytes were transfected with GFAP (**A**), Egr-1 (**B**), or p300 (**C**) promoter-driven luciferase reporter gene and increasing amount of STAT3 expressing plasmid. Cells were harvested 72 hr post-transfection for the luciferase reporter gene assay. C3 was included as the DNA control. pCMV-β-gal used to normalize the transfection efficiency variations among all transfections. The data were mean ± SD of triplicates and representative of three independent experiments. Statistical analyses were performed using one-way ANOVA.

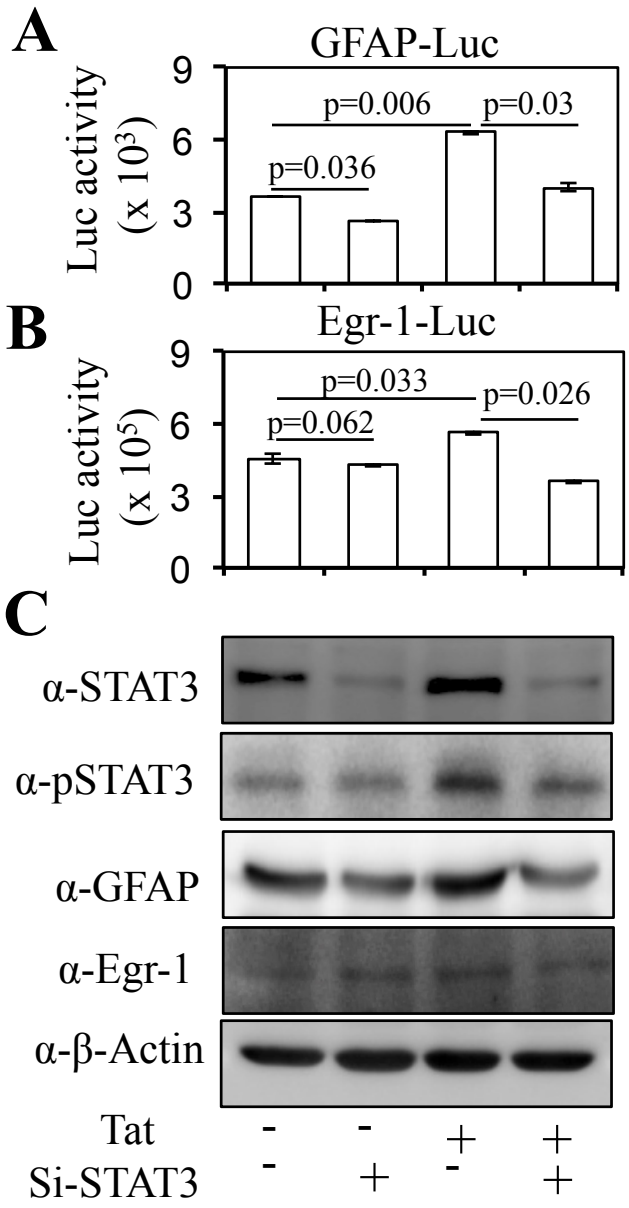
A
GFAP-Luc
Luc activity (x 10³)
9
6
3
0
p=0.006
p=0.03
p=0.036
B
Egr-1-Luc
Luc activity (x 10⁵)
9
6
3
0
p=0.033
p=0.026
p=0.062
C
α-STAT3
α-pSTAT3
α-GFAP
α-Egr-1
α-β-Actin
Tat - - + +
Si-STAT3 - + - +

Figure 15. Attenuation of Tat-induced GFAP and Egr-1 activation by STAT3 knockdown. Human primary fetal astrocytes were transfected with GFAP (**A**) or Egr-1 (**B**) promoter-driven luciferase reporter gene and Tat, p300 siRNA, or Tat plus p300 siRNA. Cells were harvested 72 hr post-transfection for the luciferase reporter gene assay (**A-C**) or Western blotting (**D**). C3 was included as the DNA control (-); control siRNA was included as a control for p300 siRNA (-). pCMV-β-gal used to normalize the transfection efficiency variations among all transfections. β-actin was included as a loading control for Western blotting. The data were mean ± SD of triplicates and representative of three independent experiments. Statistical analyses were performed using one-way ANOVA.

significant decreases in Egr-1 and GFAP promoter activities. Similar results were obtained at the protein levels by Western blotting analysis (**Fig. 15C**). These results provided further evidence that STAT3 is directly involved in Tat-induced GFAP transactivation activation.

Part II. HIV-1 TAT ADVERSLY AFFECTS NEUROGENESIS THROUGH NOTCH SIGNALING

2.1 Impaired neurogenesis in the dentate gyrus of the hippocampus of Tat-expressing mice

Previous studies provide *in vitro* evidence that HIV-1 Tat recombinant protein severely impacts neurogenesis by decreasing the number of Tuj-1 and DCX-positive cells. Using the Doxycycline (Dox)-inducible brain-specific HIV-1 Tat transgenic mouse model (iTat), crossbred with the nestin-GFP mouse to generate iTat/nestin-GFP mouse model, allows us to monitor proliferation, migration, and neurogenesis of nestin-positive neural progenitor cells (NPCs) *in vivo.* We first performed immunofluorescence staining to detect doublecortin (DCX), a well-accepted marker for early events of neurogenesis. Consistent with others' findings, compared to the control, DCX expression in the dentate gyrus of the hippocamous in Tat expressing mouse was obviously lower. At the same time, we observed that nestin-GFP expression in the correlative area of Tat expressing mouse was also dramatically lower than the control (**Fig. 16**). These results indicate that

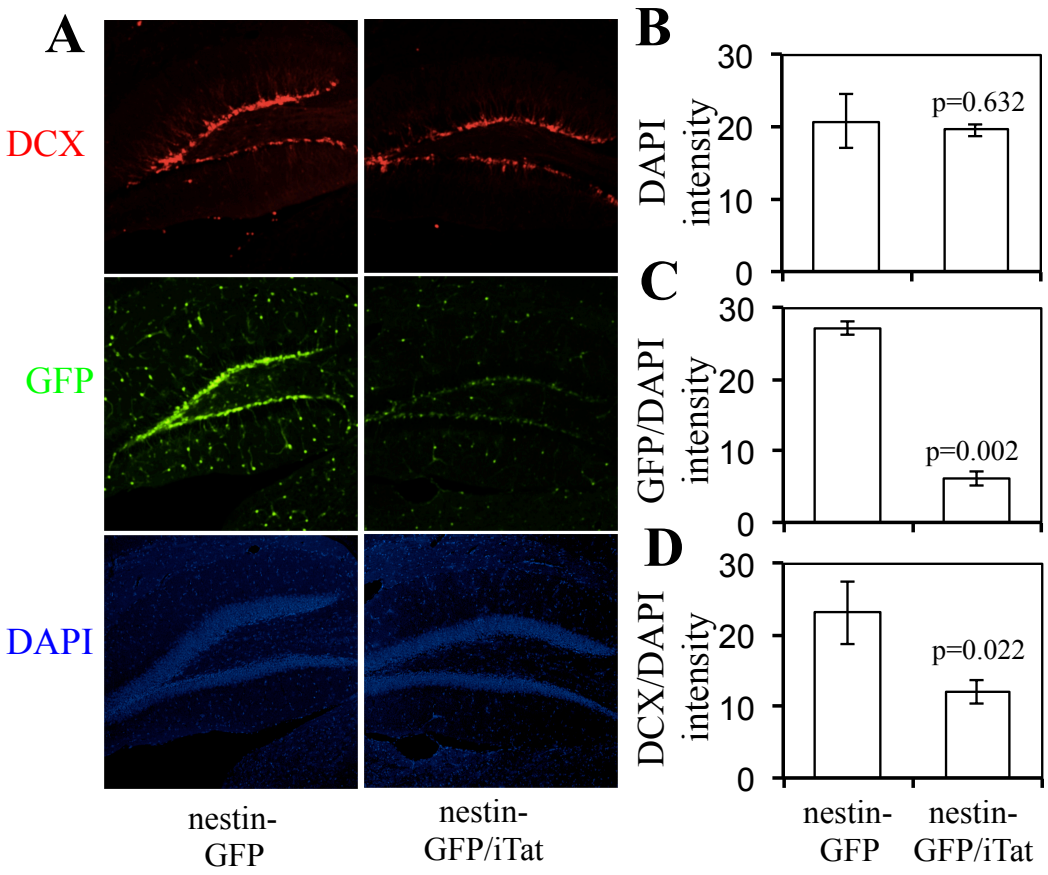
A
DCX
GFP
DAPI
nestin-GFP
nestin-GFP/iTat
B
DAPI intensity
30
20
10
0
p=0.632
C
GFP/DAPI intensity
p=0.002
D
DCX/DAPI intensity
p=0.022
nestin-GFP
nestin-GFP/iTat

Figure 16. Decreases in the staining of NPC marker nestin and pre-neuronal marker DCX in the dentate gyrus of the hippocampus of Tat expressing mouse brain. Four-week-old nestin-GFP mice (upper panel) and iTat/nestin-GFP mice (lower panel) were injected with Dox for 7 days. Post-final injection for 24 hr, mice brains were collected, sectioned and stained with anti-DCX antibody, followed by secondary antibody donkey anti-goat Alexa Fluor 555. The brain sections were then counterstained with DAPI for nuclei. The comparable dentate gyrus of the hippocampus regions of the brains were imaged (**A**). The ImageJ software (NIH) was used to quantify the relative immunofluorescence intensity of each staining (**B-D**). The surrounding background was subtracted. DAPI (**B**) was used as a reference to compare the signal intensity of GFP (**C**), or DCX (**D**). A total of three brain sections from three nestin-GFP and iTat/nestin-GFP mice were stained. The images were representative of all the stained brain sections in each group. The data were mean ± SD of triplicates and representative of three independent experiments. Statistical analyses were performed using two-tailed Student's t-test.

Tat expression in mouse brain leads to the decrease of neurogenesis and the number of nestin-positive cells.

2.2 Decreased NPC proliferation in the dentate gyrus of the hippocampus in the brain of the Tat expressing mouse

Previous studies have shown that HIV-1 Tat recombinant protein reduces the proliferation of NPCs *in vitro*. In this study, we also showed that nestin expression in the dentate gyrus of the hippocampus of the Tat expressing mouse is dramatically lower. In the mouse brain, most of the nestin-positive cells are quiescent. Only those cells in a certain physical condition or in a specific niche are the pluripotent and proliferating NPCs. Not all the proliferating cells are NPCs, but also the different linage restricted progenitor cells or mature cells. To further confirm whether Tat affects NPC proliferation *in vivo*, we took advantage of our iTat/nestin-GFP mouse model. Mice (4 weeks old) were administered Dox, to induce Tat expression in the brain, and BrdU to label the dividing cells (**Fig. 17**). Post injection for 24 h, mouse brain tissues were isolated and subjected to BrdU immunofluorescence staining. BrdU/nestin double positive cells in the dentate gyrus of the hippocampus, which indicate the proliferating NPCs, were counted and analyzed by using stereological methods. Our data showed that HIV-1 Tat in mouse brain sufficiently and significantly decreased dividing NPCs in the dentate gyrus of the hippocampus compared to the nestin-GFP mice that received the same dosage of Dox injection to avoid the side effects caused by Dox in the brain **(Fig. 18)**.

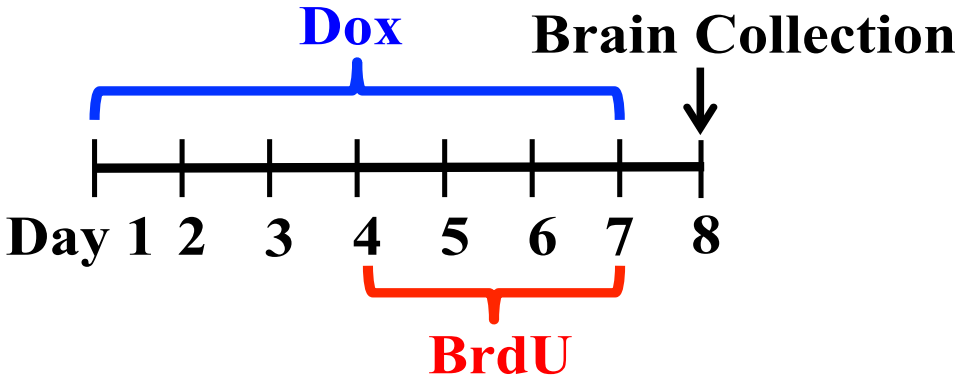
Dox
Brain Collection
Day 1 2 3 4 5 6 7 8
BrdU

Figure 17. The strategy to assess proliferation of NPCs in the hippocampus. Four-week-old nestin-GFP mice and iTat/nestin-GFP mice were intraperitoneally injected with Dox (80 mg/kg/day in ddH_2O with pH2.8) for 7 days and on day 5 of Dox injection, BrdU (50 mg/kg/day in ddH_2O) was injected together with Dox for 3 days. Twney-four hours after the final injection, mice brains were collected.

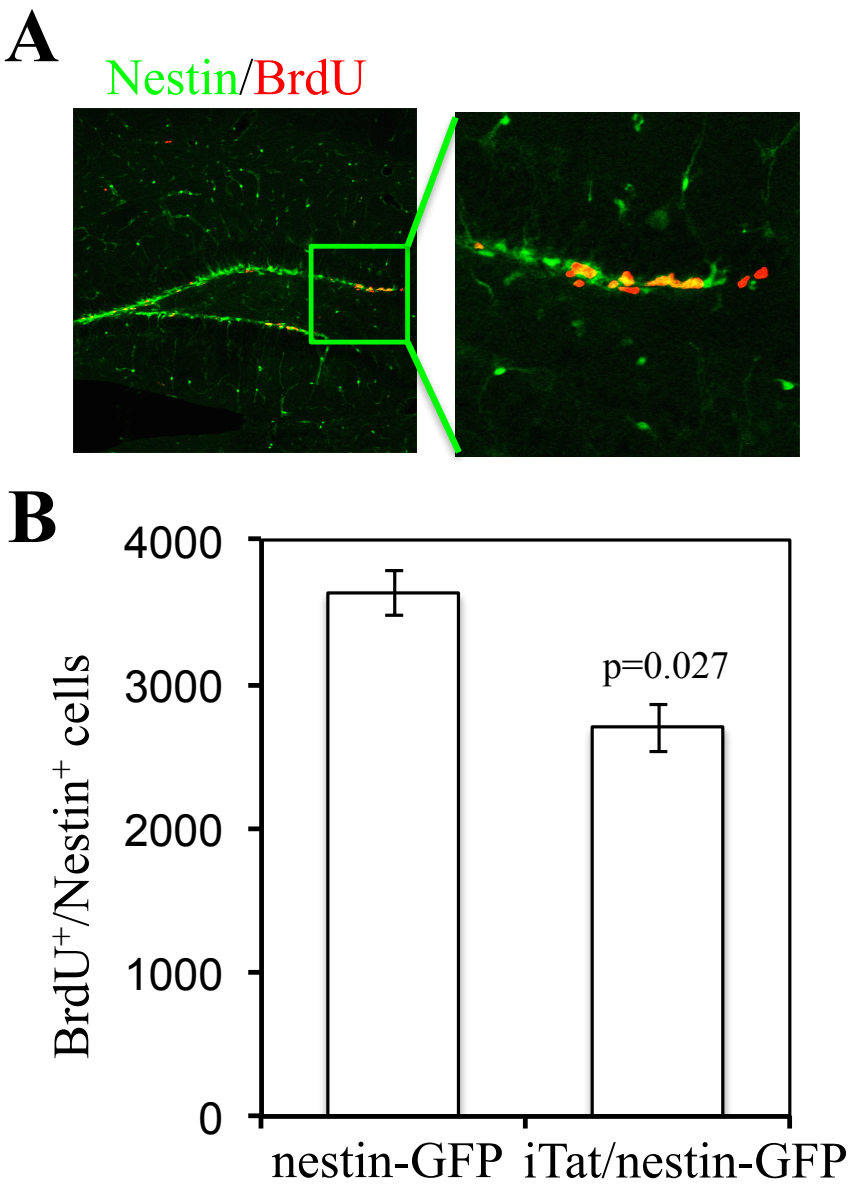
A
Nestin/BrdU
B
4000
3000
2000
1000
0
BrdU+/Nestin+ cells
p=0.027
nestin-GFP
iTat/nestin-GFP

Figure 18. Decreases in the number of BrdU/nestin double positive cells in Tat-expressing mice. Four-week-old nestin-GFP mice and iTat/nestin-GFP mice were injected with Dox and BrdU. Post-final injection for 24 hr, mice brains were collected, sectioned and stained with anti-BrdU antibody, followed by secondary antibody donkey anti-rat Alexa fluor 555. The dentate gyrus of the hippocampus regions of the brains were imaged and magnified by 10X (**A left**) and 80X (**A right**). Stereological method has been used to quantify the number of the BrdU/nestin double positive cells (**B**). A one-in-six of series of sections throughout the rostrocaudal extent of the hippocampal dentate gyrus of three WT and iTat mice brain sections was stained. The images were representative of all the stained brain sections in each group. The data were mean ± SD of triplicates and representative of three independent experiments. Statistical analyses were performed using two-tailed Student's t-test.

2.3 Decreased size of the neurospheres formed by the mouse primary NPC in Tat conditioned medium

In our transgenic mouse model, we express Tat under GFAP promoter, in which Tat is specifically expressed in GFAP expressing cells, such as mature astrocytes. Astrocytes, as the most abundant cell linage in the brain, serve not only as the scaffold-like supportive role in the brain, but are also essential in maintaining the homeostasis of the brain. In our previous studies, we proposed the astrocyte-mediated pathogenesis in HIV-1 infected brain, in which, Tat expressing astrocytes are capable of secreting the unknown factors which causes the HIV-associated neurodegeneration. We next test whether the conditioned medium collected from Tat expressing mouse or human primary astrocytes affect NPC proliferation. Primary neurospheres formed by NPCs were trypsinized into single cells, and the same number of cells were seeded into 12-well uncoated plates and cultured with the conditioned medium collected from Tat expressing iTat mouse primary astrocytes, or Tat transfected human primary astrocytes (**Fig. 19).** Pictures were taken after 7 days while neurospheres formed and the size was relatively stable. Three random fields under the microscope have been imaged from the triplicated wells of each sample, and all the neurospheres in each field have been measured and analyzed. The diameter of the neurospheres varied: those smaller than 60μm were defined as small spheres, while those were larger than 60μm, were defined as large spheres. The results showed that, the percentage of the large spheres in the Tat conditioned medium collected from iTat mouse primary astrocytes, was significantly decreased compared to control, which was cultured within the conditioned medium of non-Tat expressing mouse astrocytes **(Fig. 20A&B).** A

similar result is observed in the neurospheres cultured in the Tat conditioned medium collected from Tat transfected human primary astrocytes (**Fig. 20C&D**). This data suggest that the proliferation of NPCs is affected by the conditioned medium from Tat expressing astrocytes.

2.4 Decreased the size of the neurospheres formed by the mouse primary NPC by Tat recombinant protein

A number of studies have documented that not only Tat is able to secrete from Tat expressing cells, but cytokines/chemokines can also secrete from Tat expressing astrocytes. To further determine whether Tat directly led to decrease proliferation of NPCs, we analyzed the size of the neurospheres formed by mouse primary NPC with or without Tat recombinant protein. Pictures were taken after 7 days while neurospheres formed and the size was relatively stable. Three random fields under the microscope have been imaged from the triplicated wells of each sample, and all the neurospheres in each field have been measured and analyzed. The diameter of the neurospheres varied: those smaller than 60μm were defined as small spheres, while those were larger than 60μm, were defined as large spheres. The results showed that, the percentage of the large spheres cultured with 2 ng/ml or 5 ng/ml Tat recombinant proteins was gradually and significantly decreased compared to control which was cultured without the Tat recombinant protein **(Fig. 21A&B).** This data suggested that the proliferation of NPCs is affected by the conditioned medium from Tat expressing astrocytes.

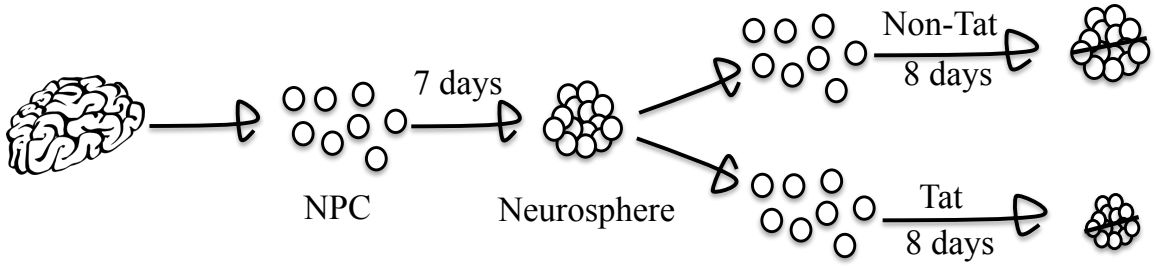
7 days
Non-Tat
8 days
NPC
Neurosphere
Tat
8 days

Figure. 19 The strategy to assess proliferation of NPC *in vitro*. Mouse brain were harvested on P1 and dissected. Neurospheres began to form after 7 days. Spheres were washed with PBS to remove the contaminated cells and trypsinized into single cells. Same number of the NPC single cells were plated into to a 12-well uncoated plate and cultured with the conditioned medium or Tat recombinant protein. Neurospheres reformed after 8 days. Images were taken using a Nikon Eclipse Ti microscope.

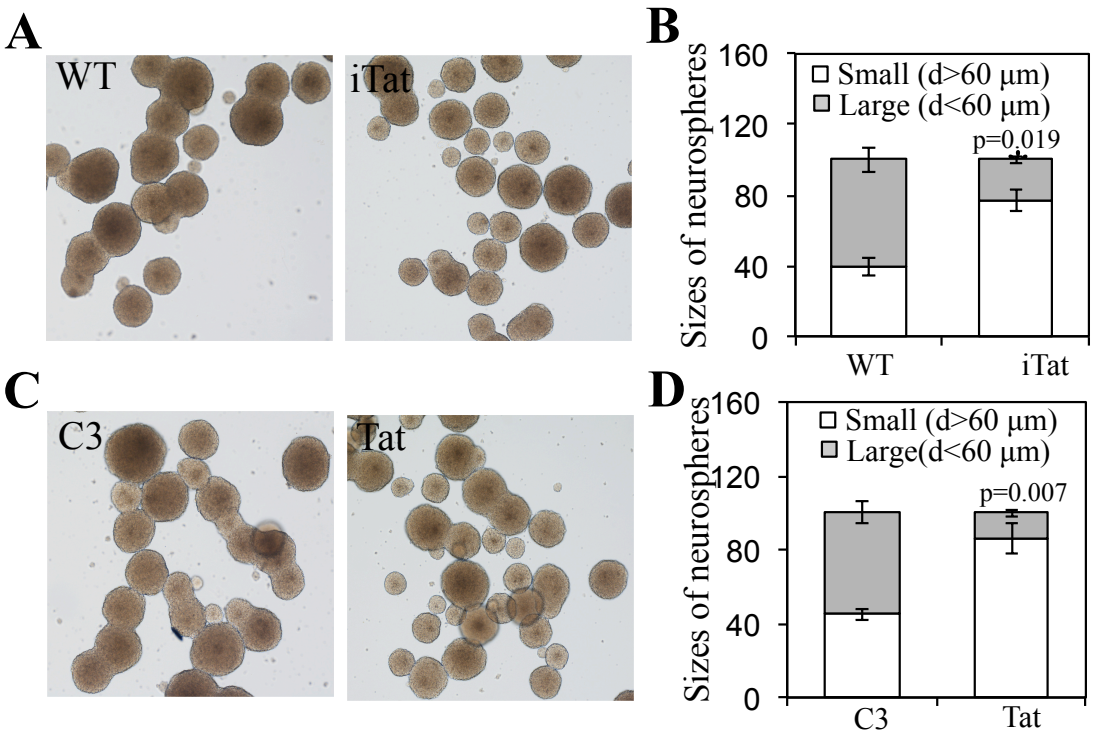
A
WT
iTat
B
Sizes of neurospheres
160
120
80
40
0
Small (d>60 μm)
Large (d<60 μm)
p=0.019
WT
iTat
C
C3
Tat
D
Sizes of neurospheres
160
120
80
40
0
Small (d>60 μm)
Large(d<60 μm)
p=0.007
C3
Tat

Figure 20. Decreases in the size of neurospheres formed by primary NPC in Tat conditioned media or recombinant Tat protein. **A&B.** Wild type and iTat mouse primary astrocytes were cultured in presence of Dox for 3 days. Cells were then washed with PBS three times and refilled with NPC culture medium. Post-culturing for another 24 hr, supernatants were collected and added on wild type mouse primary NPC. **C&D.** Human primary astrocytes were transfected with pcDNA3 or pTat.Myc. Post-transfection for 48 hr, cells were then washed with PBS three times and refilled with NPC culture medium. Post-culturing for another 24 hr, supernatants were collected and added on wild type mouse primary NPC. After 8 days culturing, three random fields under the microscope have been imaged from each triplicated wells of each sample. All the neurospheres in each field have been measured and analyzed. The images and were representative of all the neurospheres in each group. The data were mean ± SD of triplicates and representative of three independent experiments. Statistical analyses were performed using two-tailed Student's t-test.

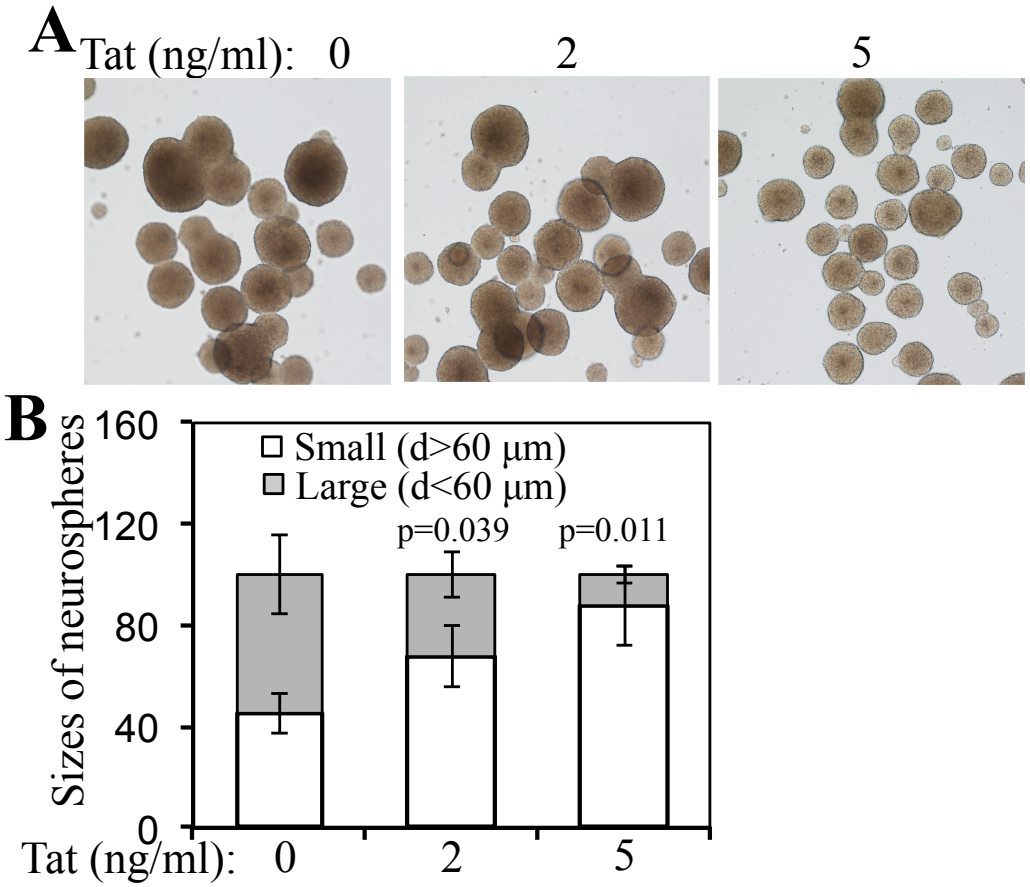
A
Tat (ng/ml): 0
2
5
B
Sizes of neurospheres
160
120
80
40
0
Small (d>60 μm)
Large (d<60 μm)
p=0.039
p=0.011
Tat (ng/ml): 0
2
5

Figure 21. Decreases in the size of neurospheres formed by primary NPC treated with recombinant Tat protein. **A&B.** Indicated amount of Tat recombinant protein were added on wild type mouse primary NPC. After 8 days culturing, three random fields under the microscope have been imaged from each triplicated wells of each sample. All the neurospheres in each field have been measured and analyzed. The images and were representative of all the neurospheres in each group. . The data were mean ± SD of triplicates and representative of three independent experiments. Statistical analyses were performed using two-tailed Student's t-test.

2.5 Decreased migration distance of the neurospheres formed by mouse primary NPC by Tat

To further determine whether the migration was also affected by Tat, primarily formed neurospheres were seeded on the poly-lysine treated 12-well plates and cultured with Tat conditioned medium collected from Tat expressing mouse or human primary astrocytes, or Tat recombinant protein (**Fig. 22**). After 48 hr treatment, thirty neurospheres were randomly selected and the longest migration distance from central to the migrated edge was measured. Compared to the control, the result showed that the migration distance was significantly decreased in the neurospheres cultured in the supernatant of Tat expressing astrocytes (**Fig. 23A-C**). To further investigate whether Tat was directly involved in the decrease of the migration. Tat recombinant protein was applied to the neurospheres. Compared to the control, significant decrease in migration distance was observed in Tat recombinant protein treated neurospheres. The result suggested that Tat alone is sufficient to decrease NPC migration (Fig. 2**3D**).

2.6 Decreased neurogenesis in the dentate gyrus of the hippocampus in the brain of Tat-expressing mouse

To assess neurogenesis *in vivo*, eight-week old iTat mice were administered Dox to induce Tat expression in the brain, and BrdU to label the proliferating cells (**Fig. 24**). Post injection for 25 days, mouse brain tissues were isolated and subjected to immunofluorescence staining to detect BrdU and NeuN. The number of BrdU/NeuN

double-positive cells in the dentate gyrus of the hippocampus, which indicated the new generated neurons from the proliferating NPCs, was counted and analyzed by using stereological methods. Our data showed that HIV-1 Tat expression in the mouse brain sufficiently and significantly decreased the number of the new derived neurons in the dentate gyrus of the hippocampus compared to that of the wild type mice which received the same dosage of Dox and BrdU injection **(Fig. 25A&B)**.

2.7 Increased density of astrocytes in the dentate gyrus of the hippocampus by Tat

One of the hallmarks of brain infection of HIV-1 is astrocytosis, which interpreted as GFAP upregulation and increasing astrocyte proliferation. When we stained the iTat/nesting-GFP mouse brain with GFAP, we observed that, in Tat expressing mouse brain, while nestin expressed in lower level, GFAP expression was obviously higher in the correlating area (**Fig. 26A**). To further assess whether HIV-1 Tat impact astrogliogenesis, we tried to analyze the number of BrdU/GFAP double-positive cells in the dentate gyrus of the hippocampus, which indicated the new generated astrocytes from the proliferating NPCs. But the result showed that none of the GFAP positive cells were stained by BrdU (**Fig. 26B**). In our previous study, we have showed that Tat expression in astrocytes is sufficient to induce astrocytes proliferation, which may result in diluting of BrdU in the new derived astrocytes from NPCs.

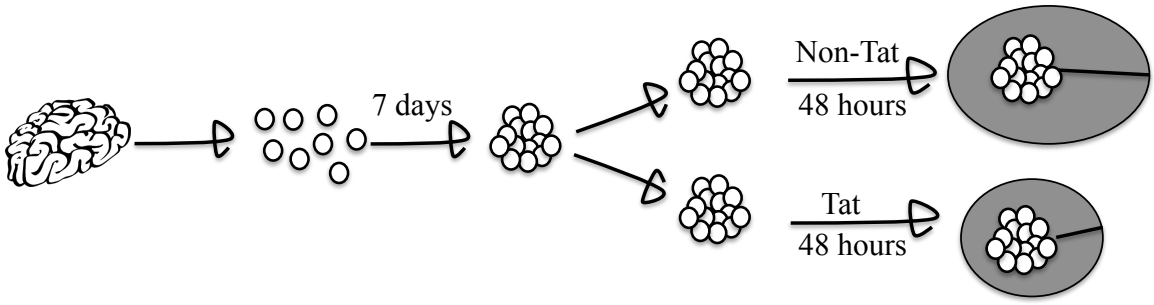
7 days
Non-Tat
48 hours
Tat
48 hours

Figure. 22 The strategy to assess migration of NPC *in vitro*. Mouse brain were harvested on P1 and dissected. Neurospheres began to form after 7 days. Spheres were washed with PBS to remove the contaminated cells and cultured in the poly-lysine treated 12-well plate with the conditioned medium or Tat recombinant protein. After culturing for 48 hr, images were taken using a Nikon Eclipse Ti microscope.

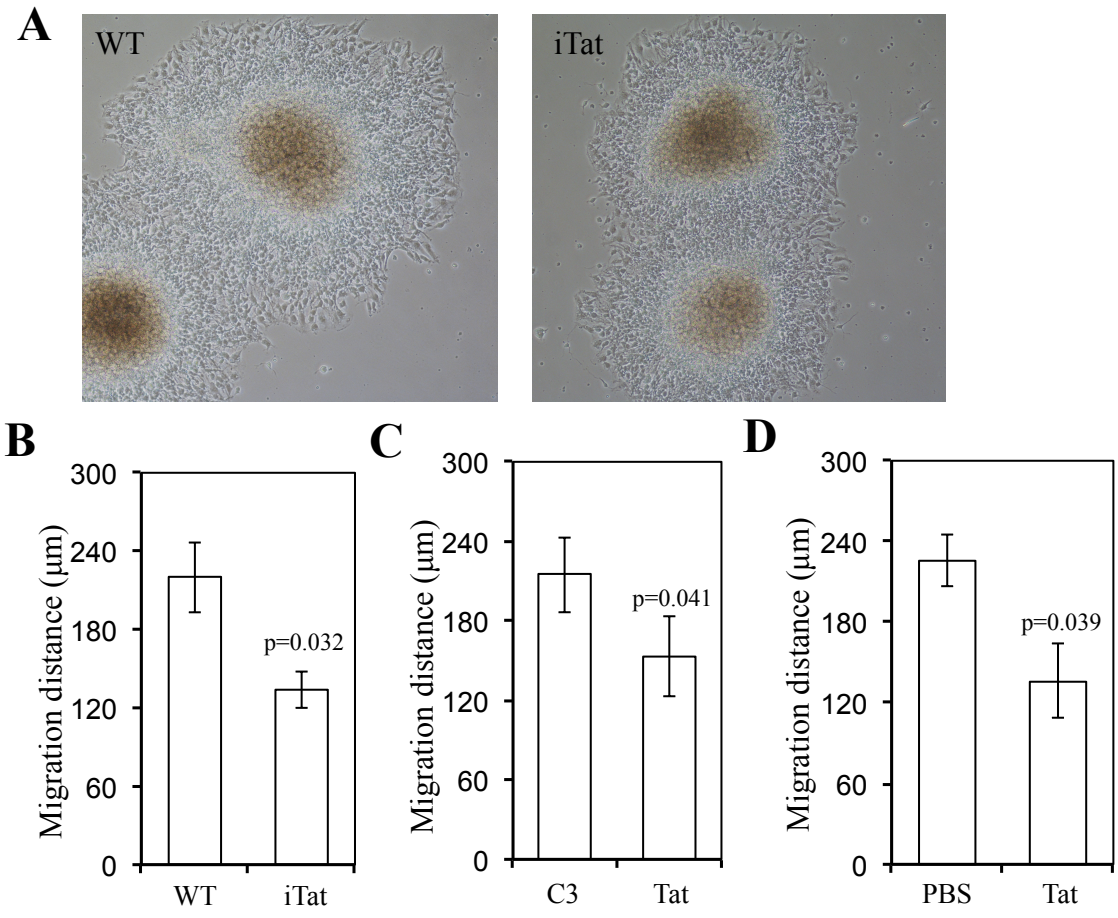
A
WT
iTat
B
Migration distance (μm)
300
240
180
120
60
0
p=0.032
WT
iTat
C
Migration distance (μm)
300
240
180
120
60
0
p=0.041
C3
Tat
D
Migration distance (μm)
300
240
180
120
60
0
p=0.039
PBS
Tat

Figure 23. Decreases migration distance of primary NPC in Tat conditioned media or recombinant Tat protein. **A&B.** WT and iTat mouse primary astrocytes were cultured in presence of Dox for 3 days. Supernatants were collected and added on neurospheres formed by WT mouse primary NPC on day 7. **C.** Human primary astrocytes were transfected with pcDNA3 or pTat.Myc. Supernatants were collected and added on neurospheres formed by WT mouse primary NPC on day 7. **D.** Indicated amount of Tat recombinant protein were added on neurospheres formed by WT mouse primary NPC on day 7. After 48 hr culturing, twenty neurospheres have been imaged from each sample. The longest migration distance of each neurospheres have been measured and analyzed. The images and were representative of all the neurospheres in each group. The data were mean ± SD of triplicates and representative of three independent experiments. Statistical analyses were performed using two-tailed Student's t-test.

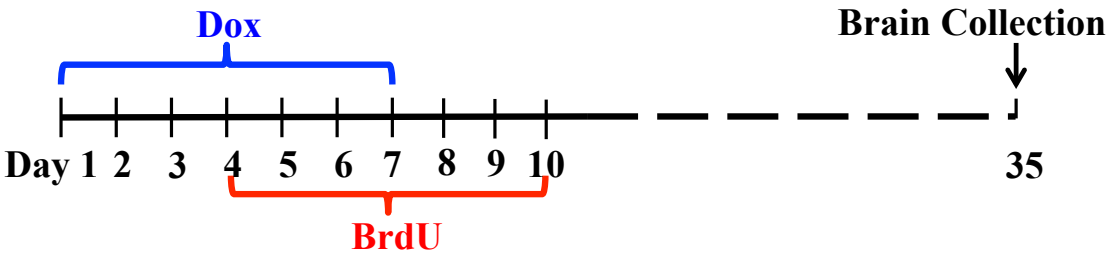
Dox
Brain Collection
Day 1 2 3 4 5 6 7 8 9 10
35
BrdU

Figure 24. The strategy to assess neurogenesis of NPC in the hippocampus. Eight-week old WT mice and iTat mice were intraperitoneally injected with Dox (80 mg/kg/day in ddH_2O with pH 2.8) for 7 days, and on day 5 of Dox injection, BrdU (50 mg/kg/day in ddH_2O) was injected together with Dox for 3 days and injected alone for 4 more days. Twenty-fice days after the final injection, mice brains were collected

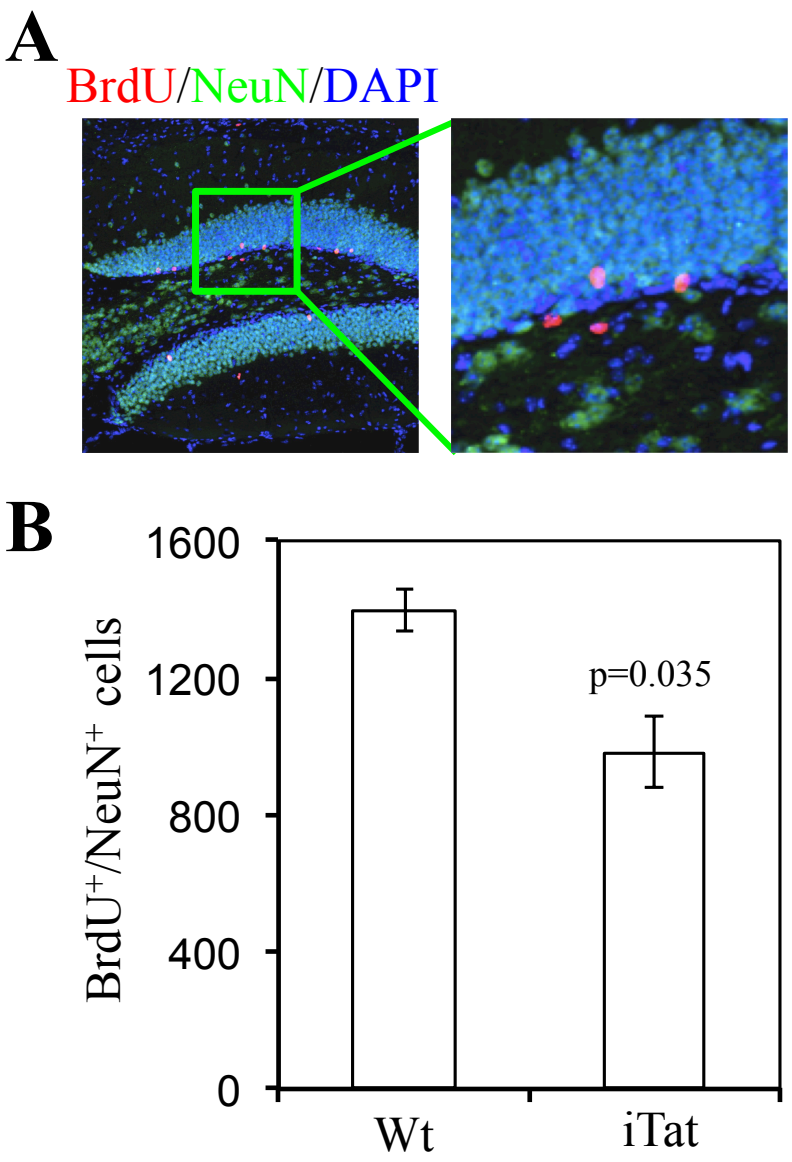
A
BrdU/NeuN/DAPI
B
1600
1200
800
400
0
BrdU+/NeuN+ cells
p=0.035
Wt
iTat

Figure 25. Decreases in the number of BrdU/NeuN double positive cells in Tat-expressing mice. Eight-week-old wild type (WT) mice and iTat mice were injected with Dox and BrdU. Post-final injection for 25 days, mice brains were collected, sectioned and stained with anti-BrdU and anti-NeuN antibody, followed by secondary antibody donkey anti-rat Alexa Fluor 555 and goat anti-mouse Alexa Fluor 488. The dentate gyrus of the hippocampus regions of the brains were imaged and magnified by 20X (**A left**) and 100X (**A right**). Stereological method has been used to quantify the number of the BrdU/NeuN double positive cells (**B**). A one-in-six of series of sections throughout the rostrocaudal extent of the hippocampal dentate gyrus of three WT and iTat mice brain sections was stained. The images were representative of all the stained brain sections in each group. The bar graph was mean ± SD of three whole brains in each group. Statistical analyses were performed using two-tailed Student's t-test.

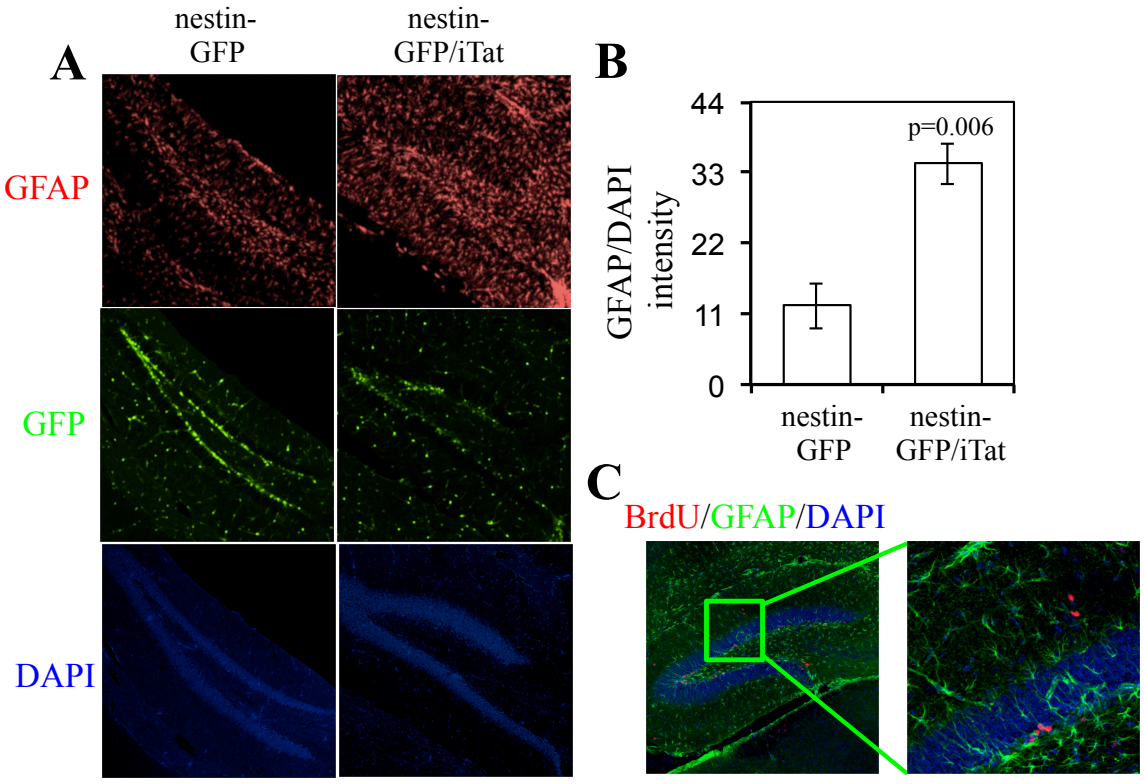
A
nestin-GFP
nestin-GFP/iTat
GFAP
GFP
DAPI
B
GFAP/DAPI intensity
44
33
22
11
0
p=0.006
nestin-GFP
nestin-GFP/iTat
C
BrdU/GFAP/DAPI

Figure 26. Increases the number of astrocytes in the dentate gyrus of the hippocampus in the Tat-expressing mouse brain. A. Four-week-old nestin-GFP mice (upper panel) and iTat/nestin-GFP (lower panel) mice were intraperitoneally injected with Dox for 7 days. Post-final injection for 24 hr, mice brains were collected, sectioned and stained with anti-GFAP antibody, followed by secondary antibody goat anti-rabbit Alexa fluor 555. The brain sections were then counterstained with DAPI for nuclei. The ImageJ software (NIH) was used to quantify the relative immunofluorescence intensity and DAPI was used as a reference to compare the signal intensity of GFAP (**B**). Eight-week-old wild type (WT) mice and iTat mice were injected with Dox and BrdU. Post-final injection for 25 days, mice brains were collected, sectioned and stained with anti-BrdU and anti-GFAP antibody, followed by secondary antibody donkey anti-rat Alexa fluor 555 and goat anti-rabbit Alexa fluor 488. The dentate gyrus of the hippocampus regions of the brains were imaged and magnified by 20X (left panel) and 100X (right panel). A total of three brain sections from three nestin-GFP mice, iTat/nestin-GFP, WT and iTat mice were stained. The images were representative of all the stained brain sections in each group. The data was mean ± SD of three brains in each group. Statistical analyses were performed using two-tailed Student's t-test.

2.8 Decreased neurogenesis and increased astrogliogenesis of NPC in Tat conditioned medium

To further investigate whether HIV-1 Tat impacted neurogenesis and astrogliogenesis, we utilized neurosphere assay, in which neurons and astrocytes were directly derived from NPCs formed neurospheres. Neurospheres were formed from mouse primary NPCs on day 7 and seeded on poly-lysine coated coverslips in 24 well plates and treated with conditioned medium collected from Tat expressing iTat mouse primary astrocytes (**Fig. 27**). After differentiation for 8 days, cells were subjected to immunofluorescence staining to detect the mature neuron marker MAP-2 and the mature astrocytes marker GFAP. The results showed that the neurospheres differentiated within the Tat conditioned medium had lower intensity of MAP-2 staining and higher intensity of GFAP staining (**Fig. 28A**). Our previous study showed that the iTat-conditioned medium is neurotoxic. To further confirm that the lower intensity of MAP-2 staining is not due to Tat-mediated neurotoxicity to the mature neurons, we performed western blotting to detect preneuronal marker DCX in the neurospheres after 2, 4, or 8 days of differentiation. The results showed that DCX expression was decreased on day 4 and day 8, which means that iTat-conditioned medium affected neurons in the preneuronal stage. Meanwhile, the expression of another mature neuron marker, NeuN, is decreased and GFAP showed dramatic increase after differentiation for 4 and 8 days in the iTat-conditioned medium (**Fig. 28B**).

2.9 Decreased neurogenesis and increased astrogliogenesis of NPC by Tat recombinant protein

To further investigate whether Tat alone is sufficient to alter the dynamic balance of neurogenesis and astrogliogenesis, 2 ng/ml or 5 ng/ml Tat recombinant protein was applied to the neurospheres. Compared to the control, in immunofluorescence staining, with 5 ng/ml Tat recombinant protein, MAP-2 expression is lower and GFAP is a slightly higher (**Fig. 29A**). And in western blotting data, in both of the concentration, on day 4 and day 8, NeuN and DCX showed lower expression and with 5 ng/ml Tat, on day 8, GFAP expression showed dramatically higher (**Fig. 29B**).

2.10 Decreased neurogenesis and increased astrogliogenesis of NPC was abrogated by removing Tat

In the previous study, we showed that Tat alone is sufficient to alter the dynamic balance of neurogenesis and astrogliogenesis. Moreover, to test whether Tat is essential in this process, we removed Tat from the conditioned medium collected from Tat transfected human primary astrocytes by immunodepletion as described previously and added on neurospheres for 8 days. Western blotting was preformed to detect NeuN, DCX and GFAP. The result showed that by removing Tat, Tat induced decrease of neurogenesis and increase of astrogliogenesis was abrogated (**Fig. 30A**). Similar results were observed when Tat recombinant protein was heat inactivated (**Fig. 30B**). These results suggested that,

HIV-1 Tat was directly involved in decreasing neurogenesis and increasing astrogliogenesis, which implies that HIV-1 Tat plays an important role in modulating fate determination of NPC differentiation by altering the dynamic balance of neurogenesis and astrogliogenesis.

2.11 Transactivation of Hes1 by HIV-1 Tat

To further investigate the underlying mechanism of how HIV-1 Tat altered the dynamic balance of neurogenesis and astrogliogenesis, we used Tat as bait to perform yeast two-hybrid analyses. The result showed that the majority of Tat-binding proteins shared common EGF-like motifs [265], suggesting a possible binding of Tat to EGF-like motifs. It is known that EGF-like motifs are presented in the extracellular domain of all the Notch isoforms and their respective ligands, and are directly involved in Notch/ligand interactions. Notch signaling plays an essential role in neurogenesis in both the developing brain and the adult brain. Activation of Notch signaling sustains NPC self-renewal, inhibits neurogenesis, and promotes astrogliogenesis (**Fig. 31**). Meanwhile, others have shown that HIV-1 Tat directly interacts with EGF-like repeats on the extracellular domain on Notch proteins, so we further tested whether Tat transactivates the critical molecular downstream pathway of Notch signaling. We cotransfected different amounts of pTat.Myc with pHes1-luc into human primary astrocytes. Hes1 showed significant transactivation when cotransfected with 0.1 and 0.2 μg pTat.Myc.

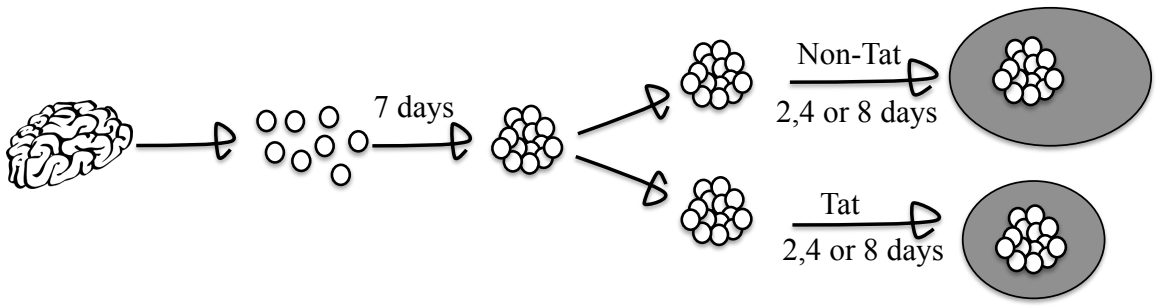
7 days
Non-Tat
2,4 or 8 days
Tat
2,4 or 8 days

Figure 27. The strategy to assess differentiation of NPC *in vitro*. Mouse brain were harvested on P1 and dissected. Neurospheres formed after 7 days. Spheres were washed with PBS to remove the contaminated cells and cultured in the poly-lysine treated coverslips in 24-well plates or poly-lysine treated 6 cm plates with the conditioned medium or Tat recombinant protein. After culturing for 8 days, IF staining were performed and images were taken using a Nikon Eclipse Ti microscope. After culturing for 2, 4, or 8 days, cell lysates were isolated for Western blotting analysis.

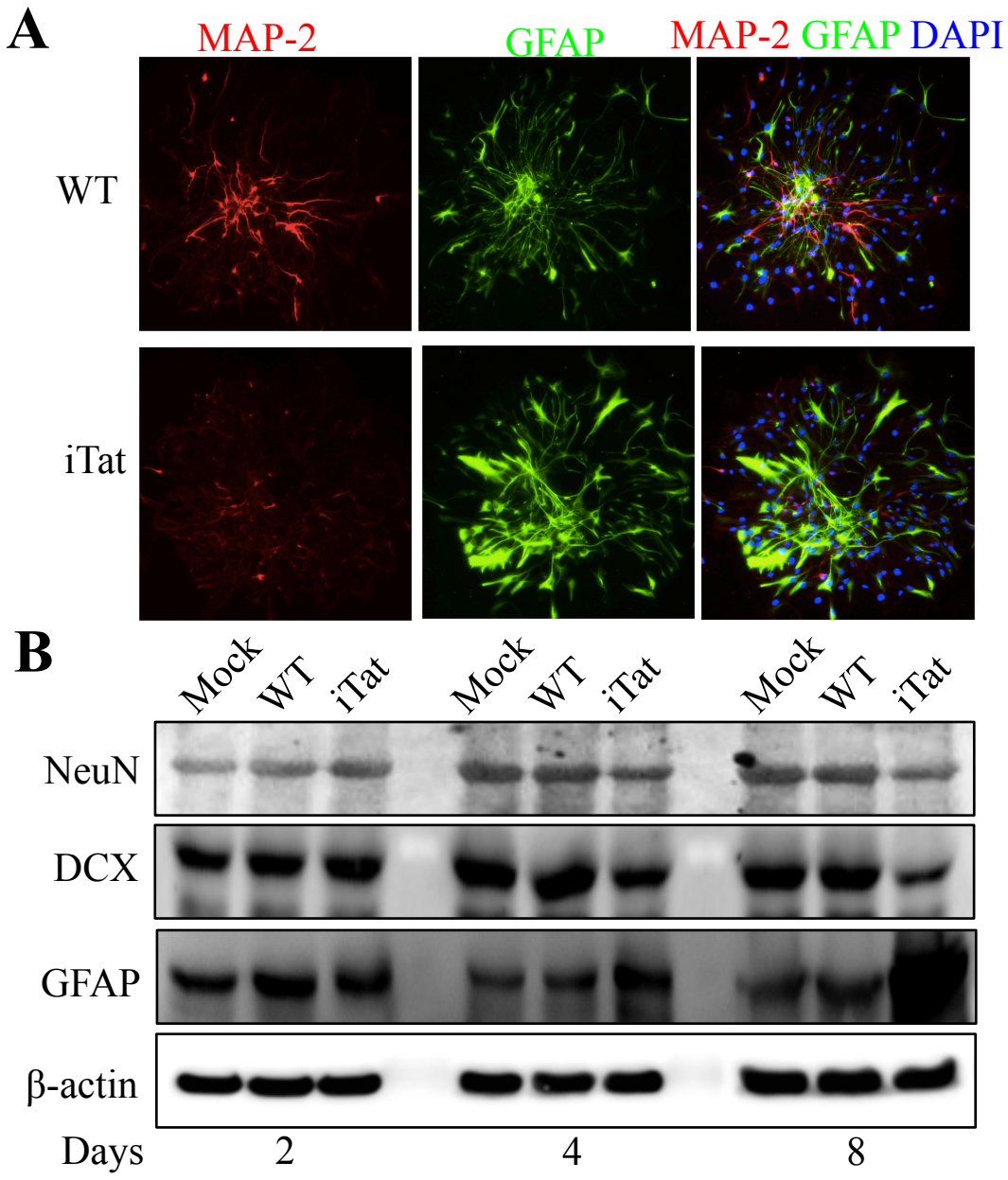
A
MAP-2
GFAP
MAP-2 GFAP DAPI
WT
iTat
B
Mock
WT
iTat
Mock
WT
iTat
Mock
WT
iTat
NeuN
DCX
GFAP
β-actin
Days
2
4
8

Figure 28. Decreases in pre-neuronal marker expression and increases in astrocyte marker expression in NPC by Tat conditioned media. Wild type and iTat mouse primary astrocytes were cultured in presence of Dox for 3 days. Supernatants were collected and added on neurosphere formed on day7 on poly-lysine coated plates. After 8 days differentiation, cells were fixed, and stained with anti-MAP-2 and anti-GFAP antibody, followed by secondary antibody goat anti-mouse Alexa fluor 555 and goat anti-rabbit Alexa fluor 488 (**A**). The images were representative of all the stained neurospheres (n>10) in each group. After 2,4 or 8 days differentiation, total cell lysates were collected and analyzed for NeuN, DCX and GFAP by western blotting. β-actin was included as an internal control (**B**). The data were representative of three independent experiments.

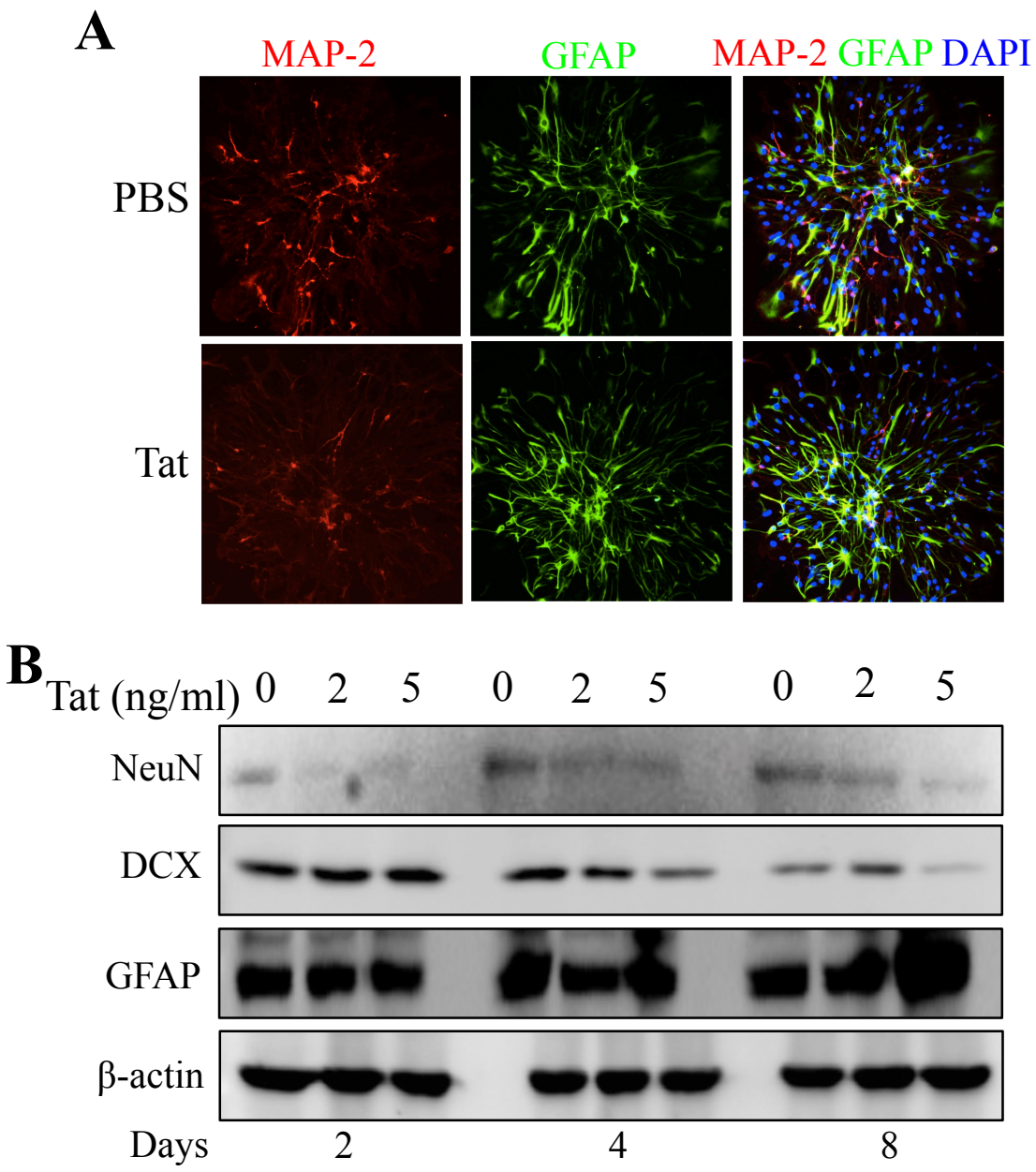
A
MAP-2
GFAP
MAP-2 GFAP DAPI
PBS
Tat
B
Tat (ng/ml) 0 2 5 0 2 5 0 2 5
NeuN
DCX
GFAP
β-actin
Days 2 4 8

Figure 29. Decreases in pre-neuronal marker expression and increases in astrocyte marker expression in NPC by recombinant Tat protein. Indicated amount of Tat recombinant protein were added on neurosphere formed on day7 on poly-lysine coated plates. After 8 days differentiation, cells were fixed, and stained with anti-MAP-2 and anti-GFAP antibody, followed by secondary antibody goat anti-mouse Alexa fluor 555 and goat anti-rabbit Alexa fluor 488 (**A**). The images were representative of all the stained neurospheres (n>10) in each group. After 2,4 or 8 days differentiation, total cell lysates were collected and analyzed for NeuN, DCX and GFAP by western blotting. β-actin was included as an internal control (**B**). The data were representative of three independent experiments.

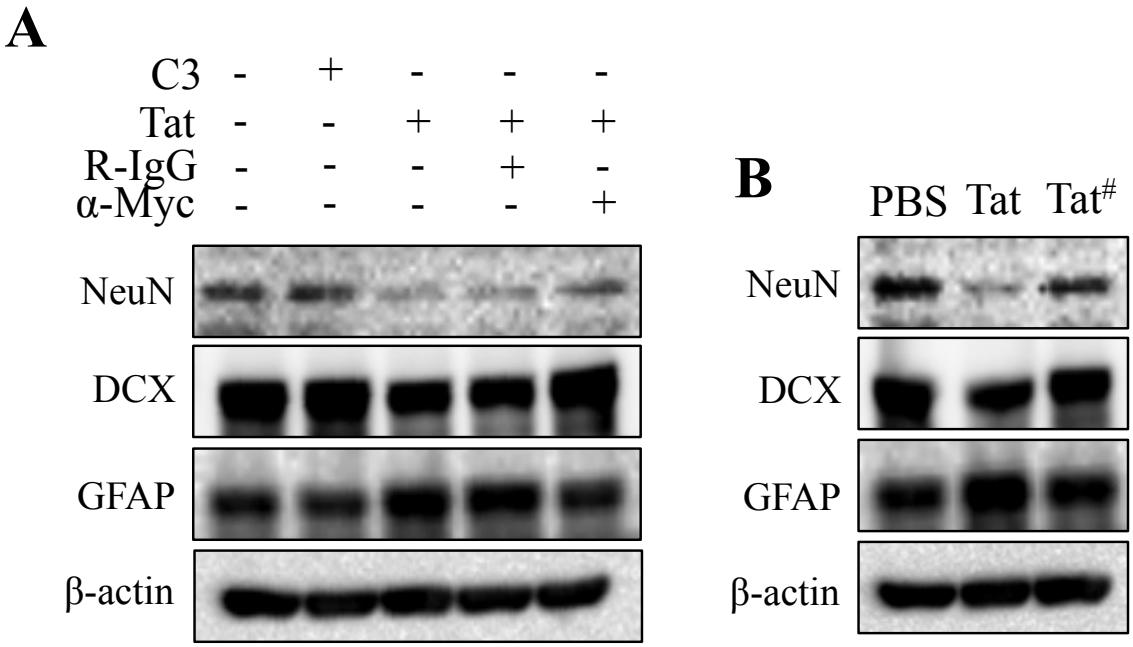
A
C3 - + - - -
Tat - - + + +
R-IgG - - - + -
α-Myc - - - - +
NeuN
DCX
GFAP
β-actin
B
PBS Tat Tat#
NeuN
DCX
GFAP
β-actin

Figure 30. Decreases in pre-neuronal marker expression and increases in astrocyte marker expression in NPC were abrogated by removing Tat. **A.** Human primary astrocytes were transfected with pcDNA3 or pTat.Myc. Post transfection for 72 hr, supernatants were collected and subjected to immunodepletion with anti-Myc. Then the immunodepleted supernatants were added on neuroshperes formed on day7. **B.** Tat recombinant protein were subjected to heat inactivation and added on neuroshperes formed on day7. After 8 days differentiation, total cell lysates were collected and analyzed for NeuN, DCX and GFAP by western blotting. β-actin was included as an internal control. The data were representative of three independent experiments.

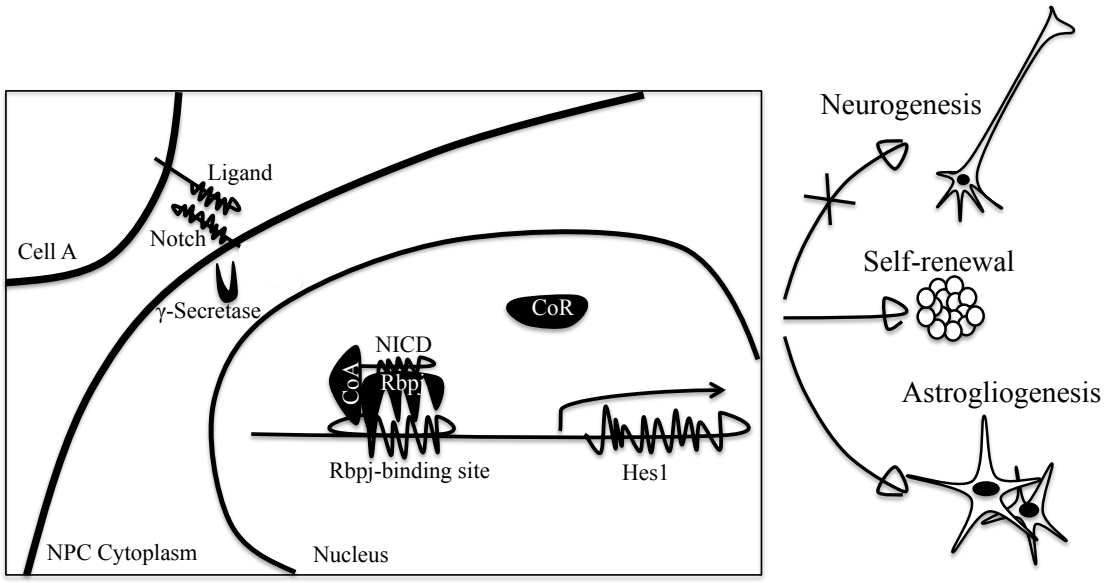
Ligand
Notch
Cell A
γ-Secretase
CoR
NICD
CoA
Rbpj
Rbpj-binding site
Hes1
NPC Cytoplasm
Nucleus
Neurogenesis
Self-renewal
Astrogliogenesis

Figure 31. The role of Notch signaling in neurogenesis. In response the binding of the Notch receptor and the ligand on the neighboring cells, the notch intracellular domain (NICD) is cleaved by γ-secretase, and translocated into the nucleus. NICD binds to the Rbpj binding site on the Hes1 promotor, dissociated with the co-repressor (CoR) and recruit the co-activator (CoA) to transactivate the downstream molecule Hes1 expression. Activation of Notch signaling is sufficient to inhibit neurogenesis, and promote NPC self-renewal and astrogliogenesis.

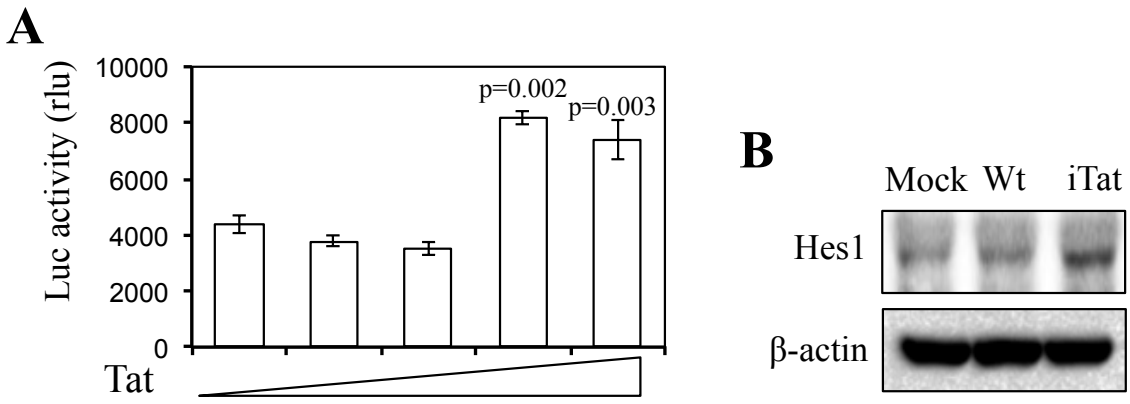
A
Luc activity (rlu)
10000
8000
6000
4000
2000
0
p=0.002
p=0.003
Tat
B
Mock
Wt
iTat
Hes1
β-actin

Figure 32. Hes1 transactivation by Tat. **A.** Human primary astrocytes were transfected with Hes1 promoter-driven luciferase reporter gene along with indicated amount of Tat. Cells were harvested 72 hr post-transfection for luciferase reporter gene assay. C3 was included as the DNA control. pCMV-β-gal was used to normalize the transfection efficiency variations among all transfections. Statistical analysis was performed using C3 as the reference. The data were mean ± SD of triplicates and representative of three independent experiments. Statistical analyses were performed using one-way ANOVA. **B.** Wild type and iTat mouse primary astrocytes were cultured in presence of Dox for 3 days. Supernatants were collected and added on neurosphere formed on day7. Mock, the merely NPC differentiation medium was included as a negative control. After 8 days differentiation, total cell lysates were collected and analyzed for Hes1 by western blotting. β-actin was included as an internal control. The data were representative of three independent experiments.

pTat.Myc transfected (**Fig. 32A**). While Hes1 transactivation by Tat was proved in astrocytes, and NPCs is not be able to be effectively transfected. We further tested Hes1 expression in neurospheres treated with conditioned mediums described above or Tat recombinant protein. First, neurosepheres were treated with the conditioned medium collected from iTat mouse primary astrocytes. After 8 days differentiation, compared to the control, Hes1 protein level showed up-regulation in the neurospheres cultured within the iTat conditioned medium (**Fig. 32B**).

2.12 Transactivation of Hes1 was abrogated by removing Tat

To determine whether Tat is essential in activate Notch signaling by transactivating the downstream molecule Hes1, Hes1 expression levels were detected when Tat was removed or inactivated. Tat conditioned medium was collected from Tat transfected human primary astrocytes, Tat was removed from the conditioned medium by immunodepletion. The immunodepleted medium was then added on the neurospheres. After 8 days culturing, compared to the control, Hes1 up-regulation in the neurospheres was abrogated (**Fig. 33A**). In addition, Tat recombinant protein was inactivated by heating, and then added on the neurosepheres for 8 days culturing. Compared to the control, Hes1 up-regulation in the neurospheres was abrogated with treatment of heat inactivated Tat protein (**Fig. 33B**). The results suggested that Tat alone sufficiently and specifically transactivated Hes1.

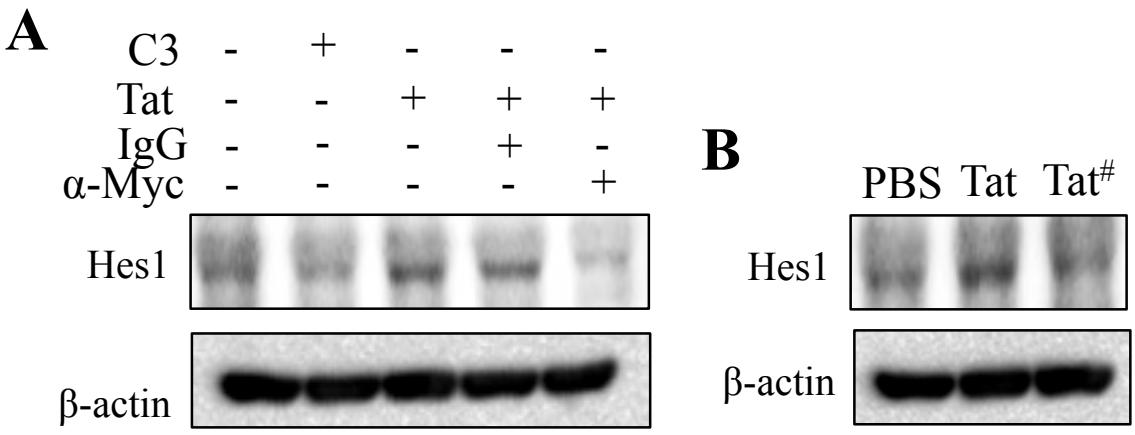
A
C3 - + - - -
Tat - - + + +
IgG - - - + -
α-Myc - - - - +
Hes1
β-actin
B
PBS Tat Tat#
Hes1
β-actin

Figure 33. Hes1 transactivation was abrogated by removing Tat. **A.** Human primary astrocytes were transfected with pcDNA3 or pTat.Myc. Supernatants were collected and subjected to immunodepletion with anti-Myc. Then the immunodepleted supernatants were added on neuroshperes formed on day7. **B.** Tat recombinant protein were subjected to heat inactivation and added on neuroshperes formed on day7. After 8 days differentiation, total cell lysates were collected and analyzed for Hes1 by western blotting. β-actin was included as an internal control. The data were representative of three independent experiments.

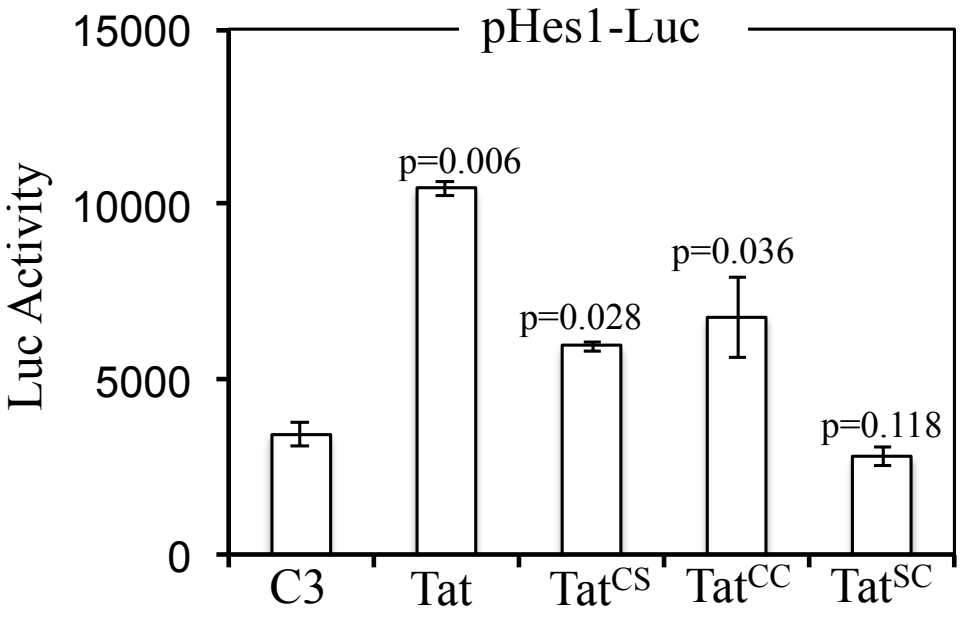
pHes1-Luc
15000
10000
5000
0
Luc Activity
p=0.006
p=0.028
p=0.036
p=0.118
C3
Tat
Tat^CS
Tat^CC
Tat^SC

Figure 34. Tat induced Hes1 transactivation was abrogated by cysteine-rich domain mutation of Tat. Human primary astrocytes were transfected with Hes1 promoter-driven luciferase reporter gene along with pTat.Myc, pC-Tat.BL43.CS, pC-Tat.BL43.CC or pC-Tat.BL43.SC. Cells were harvested 72 hr post-transfection for luciferase reporter gene assay. C3 was included as the DNA control. pCMV-β-gal was used to normalize the transfection efficiency variations among all transfections. Statistical analysis was performed using C3 as the reference. The data were mean ± SD of triplicates and representative of three independent experiments. Statistical analyses were performed using one-way ANOVA.

2.13 Cysteine-rich domain on Tat-induced Hes1 transactivation

Literature indicates that the cysteine-rich domain on Tat plays a critical role in Tat transactivative activity and in directly binding of Tat to EGF-like repeats on Notch receptors [266]. To demonstrate whether cysteine-rich domain on Tat played a directly role in activating Notch downstream signaling, we cotransfected the cysteine-rich domain mutated Tat along with pHes1-luc into human primary astrocytes. Hes1 transactivity was abrogated in the cysteine-rich domain mutant transfected cells compared to cells transfected with the wild type Tat (**Fig. 34**).

2.14 Extracellular soluble Tat, especially cysteine-rich domain on Tat-induced Hes1 transactivation

Since Notch is an extracellular receptor, it receives signals from neighboring cells by binding with ligands exposed on these cells. To investigate whether extracellular soluble Tat interacted with Notch receptor, we detected Hes1 transactivity in human primary astrocytes by adding the supernatant collected from pTat.Myc transfected human primary astrocytes. Ahead of which, we cultured pLTR-luc transfected 293T cells with the supernatant for 24 hr. LTR activity was measured by luciferase assay, and existence of soluble Tat in the supernatant was confirmed by the increased LTR transactivation (**Fig. 35A**). Compared to the control, the Hes1 transactivation was significantly increased in the cells cultured within the supernatant containing wild type soluble Tat. Meanwhile, Hes1

transactivation was not obviously changed in the cells cultured in the supernatant containing cysteine-rich domain mutated soluble Tat (**Fig. 35B**).

2.15 Cysteine-rich domain on Tat-induced decreased neurogenesis and increased astrogliogenesis of NPC

Moreover, to determine whether the cysteine-rich domain on Tat contributed Tat-induced dynamic imbalance of neurogenesis and astrogliogenesis, the supernatants described above were added on neurospheres. After differentiation for 8 days, western blotting was performed to detect Hes1, NeuN, DCX, and GFAP. Compared to the control, Hes1 and GFAP were up-regulated, NeuN and DCX were down-regulated in the neurospheres cultured within the wild type Tat conditioned medium, while the up-regulation of Hes1 and GFAP and the down-regulation of NeuN and DCX were abrogated in the neurospheres cultured within the cysteine-rich domain mutated Tat conditioned medium (**Fig. 36**). These results suggested that HIV-1 Tat, especially the cysteine-rich domain of Tat was directly involved in the Hes1 transactivation and the dynamic imbalance of neurogenesis and astrogliogenesis.

2.16 Notch signaling contributed to Tat-induced decreased neurogenesis and increased astrogliogenesis *in vitro* and decreased neurogenesis *in vivo*

Previously, we showed that HIV-1 Tat contributed to Hes1 transactivation and the dynamic imbalance of neurogenesis and astrogliogenesis. To further determine whether Notch signaling was directly involved in HIV-1 Tat induced dynamic imbalance of neurogenesis and astrogliogenesis, we first performed neurosphere assay by using two inhibitors, in two different principles, to block the Tat induced Notch signaling. DLK1 is one of the best-studied non-canonical Notch ligand, which has been shown as a competitive ligand to inhibit Notch signaling *in vitro [267, 268]*. Another one is a typical γ-secretase inhibitor DAPT [269]. We treated the neurospheres with Tat conditioned medium (**Fig. 37**) or Tat recombinant proteins (**Fig. 38**) together with these inhibitors respectively as indicated in the figure. The Notch activator Jagged-1 was included as the positive control. After 8 days differentiation, western blotting was performed to detect Hes1, NeuN, DCX and GFAP. Compared to the control, we observed that, in the neurospheres, Tat conditioned medium and Tat recombinant protein induced up-regulation of Hes1 and GFAP and down-regulation of NeuN and DCX were abrogated by both of the inhibitors. To further assess whether blocking of Notch reversed the Tat reduced neurogenesis *in vivo*, eight-week-old iTat mice were administered Dox, BrdU and DAPT (**Fig. 39**). Post injection for 25 days, mice brain tissues were isolated and subjected to floating staining to detect BrdU and NeuN. The number of the BrdU/NeuN double positive cells in the dentate gyrus of the hippocampus were counted and analyzed by using stereological methods. Our data showed that, compared to the WT mice that received the

same dosage of Dox and BrdU injection, and consistent with previous result, there is a significantly decreased in number of BrdU/NeuN double positive cells in the dentate gyrus of the hippocampus of the iTat mice. While compared to the WT mice that without DAPT injection, WT mice with DAPT showed no significant difference in number of BrdU/NeuN double positive cells in the dentate gyrus of the hippocampus. Compared to WT mice with or without DAPT, iTat mice with DAPT showed significant decrease in number of BrdU/NeuN double positive cells in the dentate gyrus of the hippocampus, while compared to the iTat mouse without DAPT, it showed significant increase in number of BrdU/NeuN double positive cells in the dentate gyrus of the hippocampus (**Fig. 40**). Both *in vitro* and *in vivo* data indicated that by blocking Notch signaling, HIV-1 Tat induced Hes1 activation and/or the dynamic imbalance of neurogenesis and astrogliogenesis has been reversed in certain level to the normal condition.

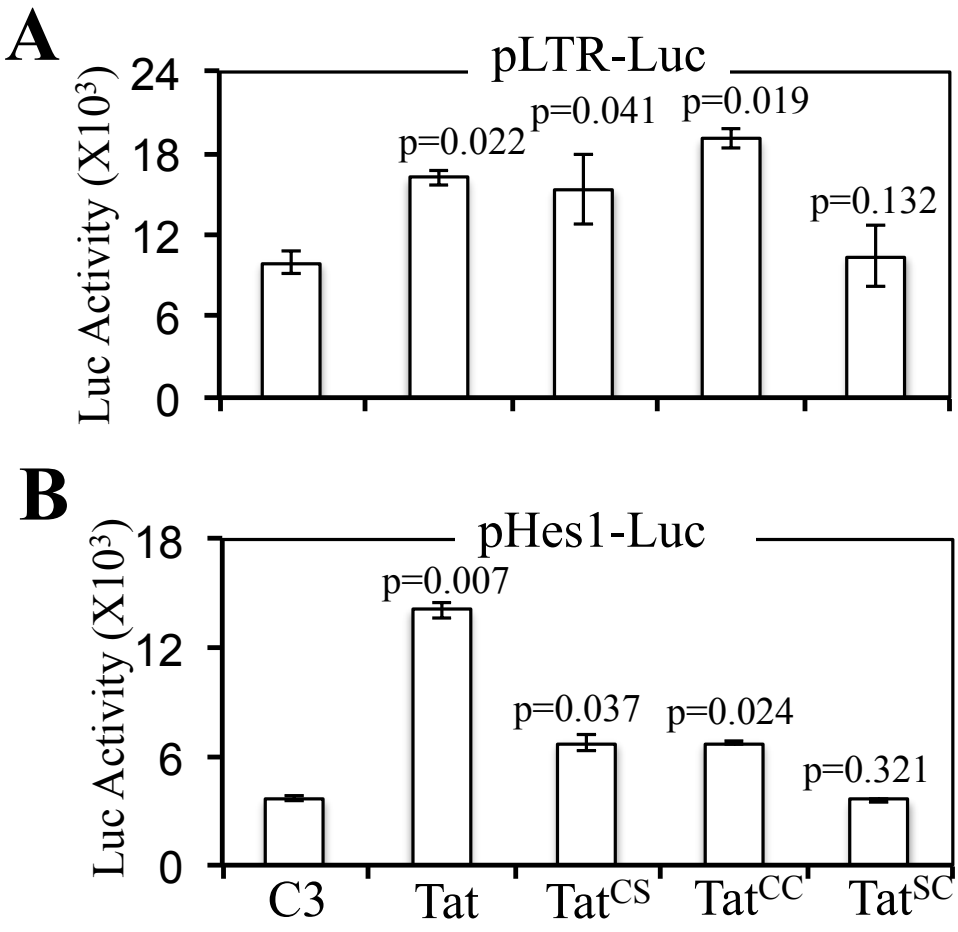
A
pLTR-Luc
Luc Activity (X10³)
24
18
12
6
0
p=0.022
p=0.041
p=0.019
p=0.132
B
pHes1-Luc
Luc Activity (X10³)
18
12
6
0
p=0.007
p=0.037
p=0.024
p=0.321
C3
Tat
TatCS
TatCC
TatSC

Figure 35. Hes1 was transactivated by extracellular soluble Tat and the transactivation was abrogated by cysteine-rich domain mutation of Tat. Human primary astrocytes were transfected with pTat.Myc, pC-Tat.BL43.CS, pC-Tat.BL43.CC or pC-Tat.BL43.SC. Supernatants were collected 72 hr post-transfection and added on LTR promoter-driven luciferase reporter gene transfected 293T cells. Cells were harvested 24 hr post-coculturig for luciferase reporter gene assay (**A**). The same supernatants were added on Hes1 promoter-driven luciferase reporter gene transfected human primary astrocytes. Cells were harvested 24 hr post-coculturig for luciferase reporter gene assay (**B**). The data were mean ± SD of triplicates and representative of three independent experiments. Statistical analyses were performed using one-way ANOVA.

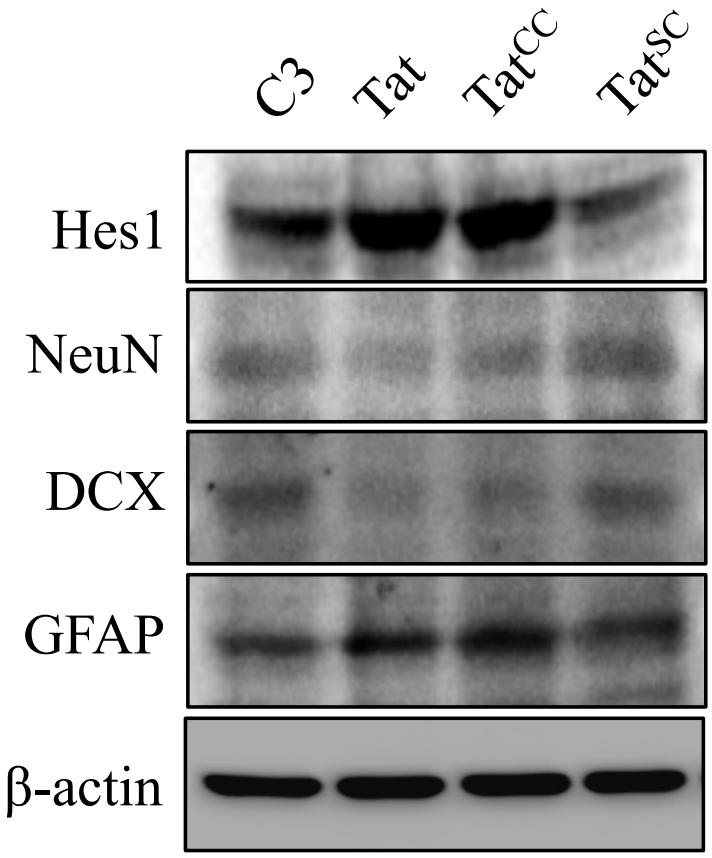
C3
Tat
Tat^CC
Tat^SC
Hes1
NeuN
DCX
GFAP
β-actin

Figure 36. Decreases in pre-neuronal marker expression and increases in astrocyte marker expression in NPC by Tat were abolished by cysteine-rich domain mutation of Tat. Human primary astrocytes were transfected with pTat.Myc, pC-Tat.BL43.CS, pC-Tat.BL43.CC or pC-Tat.BL43.SC. Supernatants were collected 72 hr post-transfection and added on neuroshperes formed on day7. After 8 days differentiation, total cell lysates were collected and analyzed for Hes1 by western blotting. β-actin was included as an internal control. The data were representative of three independent experiments.

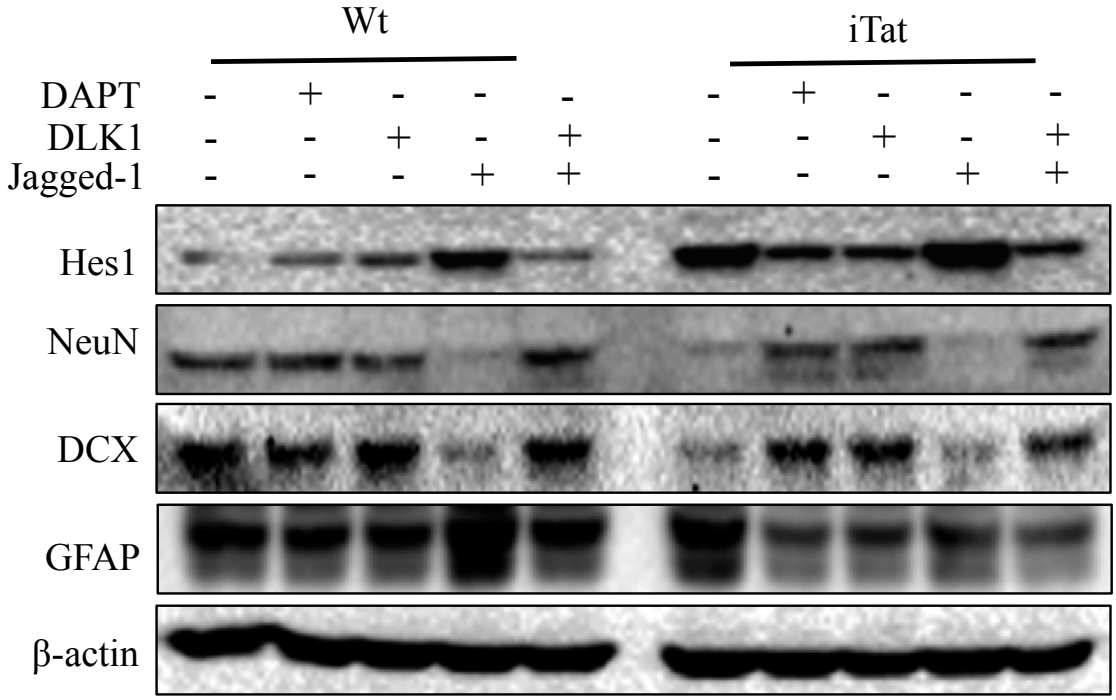
Wt
iTat
DAPT - + - - - - + - - -
DLK1 - - + - + - - + - +
Jagged-1 - - - + + - - - + +
Hes1
NeuN
DCX
GFAP
β-actin

Figure 37. Decreases in pre-neuronal marker expression and increases in astrocyte marker expression in NPC by Tat conditioned media were abrogated by Notch inhibitors. WT and iTat mouse primary astrocytes were cultured in presence of Dox for 3 days. Cells were then washed with PBS three times and refilled with NPC differentiation medium. Post-culturing for another 24 hr, supernatants were collected and added on neurosphere formed on day7 along with Notch inhibitor DLK1 or DAPT.

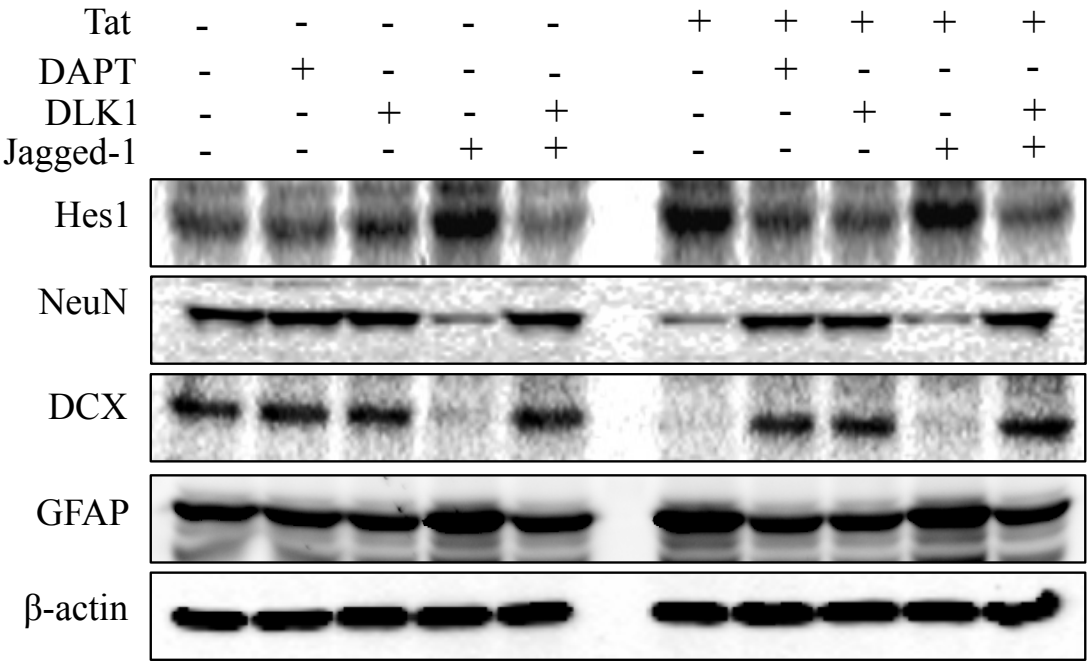
Tat - - - - - + + + + +
DAPT - + - - - - + - - -
DLK1 - - + - + - - + - +
Jagged-1 - - - + + - - - + +
Hes1
NeuN
DCX
GFAP
β-actin

Figure 38. Decreases in pre-neuronal marker expression and increases in astrocyte marker expression in NPC by recombinant Tat protein were abrogated by Notch inhibitors. Neurosphere formed on day7 were cultured in the absence or in the presence of Tat recombinant protein together with or without Notch inhibitor DLK1 or DAPT. Notch activator Jagged-1 was included as a positive control. After 8 days differentiation, total cell lysates were collected and analyzed for NeuN, DCX and GFAP by western blotting. β-actin was included as an internal control. The data were representative of three independent experiments.

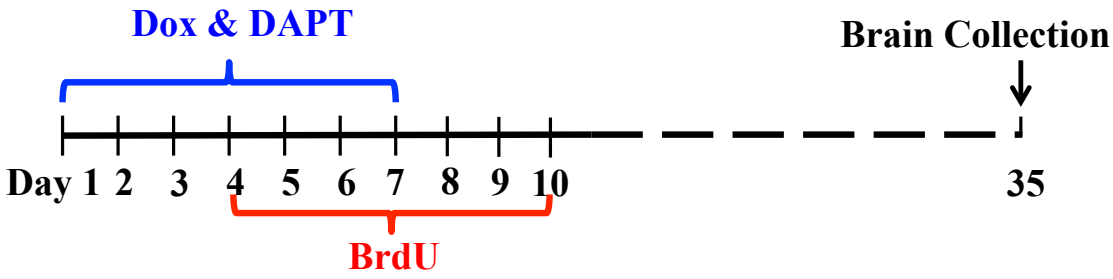
Dox & DAPT
Brain Collection
Day 1 2 3 4 5 6 7 8 9 10
35
BrdU

Figure 39. The strategy to assess the involvement of Notch in Tat impaired neurogenesis. Eight-week old WT mice and iTat mice were intraperitoneally injected with Dox (80 mg/kg/day in ddH_2O with pH 2.8), with or without DAPT (120 mg/kg/day) for 7 days, and on day 5 of Dox/DAPT injection, BrdU (50 mg/kg/day in ddH_2O) was injected together with Dox for 3 days and injected alone for 4 more days. Twenty-five days after the final injection, mice brains were collected.

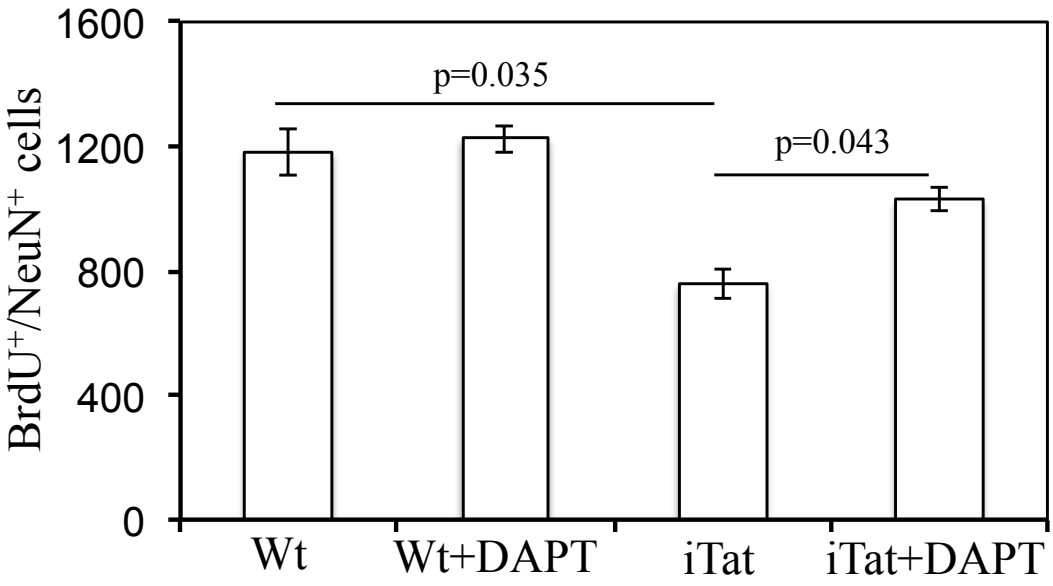
1600
1200
800
400
0
BrdU+/NeuN+ cells
p=0.035
p=0.043
Wt
Wt+DAPT
iTat
iTat+DAPT

Figure 40. Decreases in the number of BrdU/NeuN double-positive cells in the dentate gyrus of the hippocampus of the Tat-expressing mouse brain was reversed by DAPT. Wild type and iTat mice were injected with or without DAPT, and together with Dox, and BrdU. Post-final injection for 25 days, mice brains were collected, sectioned and stained with anti-BrdU and anti-NeuN antibody. Stereological method has been used to quantify the number of the BrdU/NeuN double positive cells in the dentate gyrus of the hippocampus regions of the brains. A one-in-six of series of sections throughout the rostrocaudal extent of the hippocampal dentate gyrus of three WT and iTat mice brain sections was stained. The bar graph was mean ± SD of three whole brains in each group. Statistical analyses were performed using one-way ANOVA.

DISCUSSION

Summary of the results

In the first part of the study, we showed that HIV-1 Tat-induced GFAP up-regulation and aggregation in astrocytes contributed to Tat neurotoxicity (**Fig. 6-10**). We next demonstrated that demonstrated that STAT3 expression and its phosphorylation led to GFAP transactivation and protein expression in astrocytes (**Fig. 11**). We also showed that HIV-1 Tat expression increased STAT3 expression and phosphorylation in astrocytes, which also occurred in HIV-infected astrocytes (**Fig. 12**). Moreover, we provided evidence to suggest that STAT3 likely acted upstream of the Tat-induced GFAP transactivation cascade (**Fig. 13 & 14**). Lastly, we demonstrated that knockdown of STAT3 led to decreases in not only constitutive but also Tat-induced GFAP and Egr-1 transcription and protein expression (**Fig. 15**). Taken together, these findings suggest that STAT3 is involved in Tat interaction with astrocytes and subsequent changes of astrocyte function.

In the second part of our study, we showed that HIV-1 Tat decreased NPC maker nestin and preneuronal marker DCX in the dentate gyrus of the iTat mouse brain (**Fig. 16**). We next provided further evidence that Tat directly contributed to a decrease of NPC proliferation (**Fig. 17-21**) and NPC migration (**Fig. 23**). We also found that neurogenesis was impacted in the dentate gyrus of the hippocampus of the iTat mouse brain (**Fig. 25**), while astrocyte maker GFAP showed dramatically increases in the correlated area (**Fig.**

26). Furthermore, we demonstrated Tat directly contributed to alter the dynamic balance of neurogenesis and astrogliogenesis (**Fig. 28-30**). To further investigate the underlying mechanisms, we connected the effects of Tat on NPC differentiation together with Notch signaling by observing that the majority of Tat-binding proteins shared common EGF-like motifs [265], which presents in the extracellular domains of all the Notch isoforms and their respective ligands, and are directly involved in Notch/ligand interaction. We further demonstrated that HIV-1 Tat is sufficient to activate Hes1, which is the critical molecular downstream pathway of Notch signaling (**Fig. 32&33**). In this process, the cysteine-rich domain of Tat plays an essential role in Hes1 activation and modulating neurogenesis and astrogliogenesis (**Fig. 34-36**). Lastly, we proved that Notch signaling was directly involved in HIV-1 Tat induced dynamic imbalance of neurogenesis and astrogliogenesis both in vitro (**Fig. 37&38**) and in vivo (**Fig. 40**).

Involvement of GFAP dysregulation in astrocyte-mediated Tat neurotoxicity

As an astrocyte marker, GFAP is a type III intermediate filament (IF) protein and is mainly located in cytoskeletal compartments but is secreted [270-273]. Its expression is developmentally and pathophysiologically regulated [see review [182-184]]. The GFAP protein has 432-amino acids of 55 kDa [274, 275] and is considerably conserved among species [275, 276]. Like other cell-type specific IF proteins, GFAP forms extensive networks that maintain the mechanical strength and shape of the astrocytes and provide dynamic platforms for the organization of the cytoplasm at a structural and functional

level [185, 186]. Specifically, GFAP is involved in astrocyte volume regulation [187], glial scar formation [188], and anchoring glutamate transporters to the plasma membrane to facilitate neurotransmitter recycling [189]. Besides, GFAP clearly plays a critical role in neuron-glia interaction and CNS morphogenesis [99, 190, 277, 278]. Recent findings suggest GFAP involvement in the long-term maintenance of the brain architecture, proper function of the blood-brain barrier, and modulation of some neuronal functions [99, 191, 192, 279]. Mutations in GFAP cause Alexander's disease (AxD) [280, 281], while higher levels of GFAP leads to pre-mature death [193, 194]. Interestingly, both cases are pathologically characterized by formation of cytoplasmic GFAP aggregates or inclusions [280, 281] [193, 194]. Similar findings have been noted with other intermediate filament proteins such as keratin and desmin [282-284]. Thus, we evaluated the effects of Tat-activated GFAP expression on the organization of GFAP filaments in astrocytes and found that Tat-activated GFAP expression led to formation of GFAP aggregates and alteration of the intermediate filament network in astrocytes (**Fig. 6&7**). These results provide the very first evidence to support the involvement of GFAP dysregulation in astrocyte-mediated Tat neurotoxicity.

STAT3 as the upstream regulator of Tat-induced GFAP transactivation cascade

STAT3 plays important roles in tumorigenesis and tumor progression [285, 286]. Overexpression of STAT3 inhibits cell apoptosis and promotes cell groWTh and survival [287]. Blockade of JAK-STAT and MAPK signaling decreases cell viability [288]. Egr-1 is downstream of MAPK pathway [289, 290] and the downstream of STAT3 signaling

[264], but there is no evidence that Egr-1 directly interacts with STAT3. Activated JAK kinase phosphorylates and activates Src homology-2 domain-containing tyrosine phosphatase, which in turn activates Ras-mediated signaling include ERK, one component of MAPK, while ERK is known to function upstream of Egr-1 [291, 292]. Egr-1 activation is dependent on ERK but independent of STAT3[293-295]. Meanwhile, others studies have shown that JAK-STAT and MAPK signaling pathways, in which STAT3 and Egr-1 are involved, play a synergistic role in regulating cell groWTh and apoptosis [288]. In this study, we showed that both STAT3 overexpression and phosphorylation transactivated Egr-1 and likely contributed to Tat-induced astrocytosis, the increased GFAP expression and astrocyte proliferation in the context of HIV-1 infection of the CNS.

Proposed model of Tat-induced STAT3-mediated GFAP transactivation cascade

Based on our early studies [1, 196-198, 256] and the current study, we have further modified the following Tat-induced GFAP transactivation cascade (**Fig. 41**). When Tat is expressed in HIV-infected astrocytes, there are two possible outcomes. One begins with Tat release outside the infected cells (1), followed by uptake back into the cells (2), or interaction with cell surface molecules (3). Tat interaction with the cell surface molecules leads to STAT3 phosphorylation and dimerization (4), and subsequent nuclear translocation (5). In the nucleus, STAT3 transactivates Egr-1 (6), Egr-1 transactivates p300 (7), and p300 transactivates GFAP (8). The other outcome begin with Tat nuclear translocation (9), followed by STAT3 transactivation (11) and phosphorylation (12),

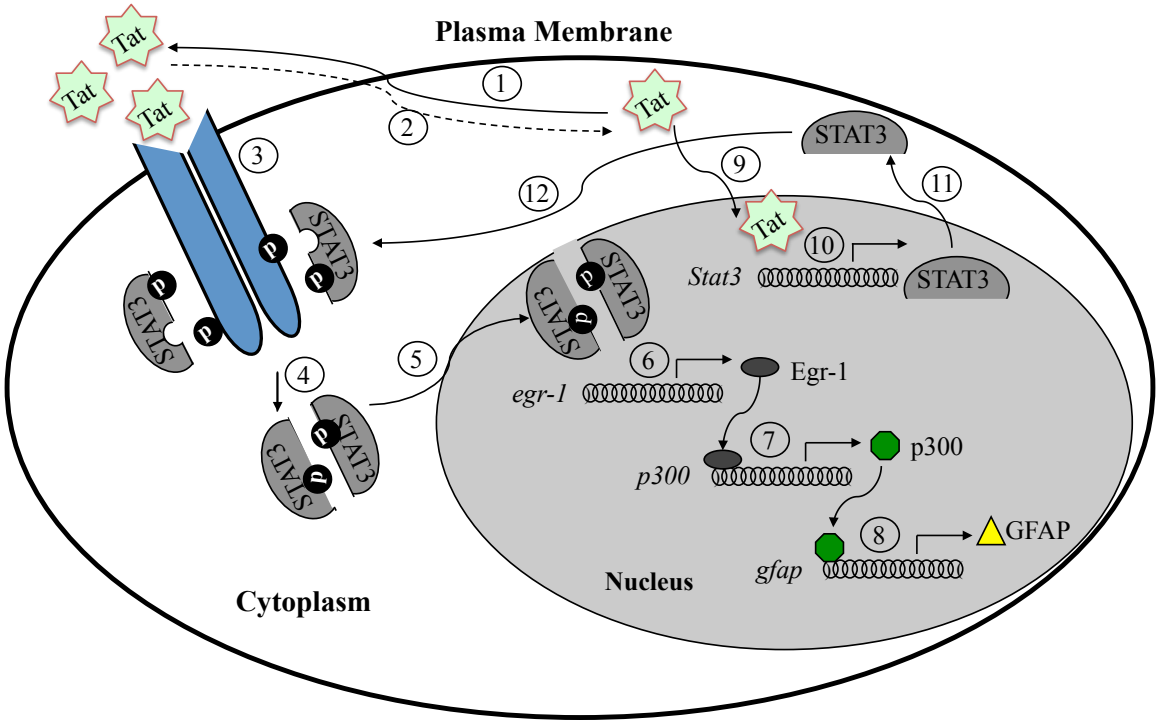

Figure 41. Proposed Tat-induced GFAP transactivation cascade with inclusion of STAT3.

where the first and the second pathways merges and eventually contributes to astrocyte proliferation and increased GFAP expression in the context of HIV infection.

Effects of HIV-1 on neurogenesis

Until recently, imapired neurogenesis has been noted in the hippocampus of HIV-infected individuals, SIV infected macaques, and severe combined immunodeficient mice injected with HIV-infected human microglia/macrophages [81, 237-239]. HIV-1 was believed to infect nestin-positive neural progenitor cells in culture and in autopsy brain sections from pediatric AIDS patients, suggesting that human neural stem/progenitor cells harbor HIV-1, which could be the potential menace contributor of HIV-associated neurodegeneration [79-81]. Further, the number of adult neural progenitor cells significantly decreased in patients with HAD as compared to the age matched non-infected or HIV-1-infected non-demented individuals [82]. Although this decrease may reflect general pathogenic mechanisms arising in the setting of late-stage dementia rather than specific effects of HIV on neurogenesis, emerging evidence suggest that adult hippocampal neurogenesis is affected by altered expression of cytokines or groWTh factors modulated by HIV infection and several soluble viral proteins, such as gp120 and Tat [83-85].

Detection of Tat effects on neurogenesis in vivo

In 2007, to investigate how HIV-1 surface protein gp120 affects NPC proliferation *in vivo,* Okamoto and colleges injected BrdU to gp120 transgenic mouse and counted BrdU-positive cells in the dentate gyrus of the hippocampus as relevance to proliferating NPCs [85]. In the adult brain, NPCs are localized primarily in two key regions of the CNS: the subventricular zone (SVZ), lining the lateral ventricles of the forebrain, and the subgranular zone of the dentate gyrus of the hippocampus [296]. While nestin is a wild-accepted NPC marker, it could be detected in cells located in the whole region of the brain (**Fig. 16, 18A & 26 A, GFP columns**). It is known that most of the nestin positive cells are not counted as NPC because they are either out of the certain microenvironment or remaining quiescent under any circumstances [297]. Also not all the proliferating cells are counted as NPCs because in the developing CNS, NSCs switches to asymmetric division cycles and give rise to lineage-restricted progenitors, such as neural progenitors and glio progenitors [298]. In this case, to improve Okamoto's strategy, in this study, we injected BrdU to iTat/nestin-GFP transgenic mouse and counted the number of BrdU/nesin double-positive cells in the dentate gyrus of the hippocampus as relevant to proliferating NPCs in order to provide refined evidences of how HIV-1 Tat affects NPC proliferation *in vivo*. By using iTat mouse model, in 2012, Hahn and colleges investigated Tat and opiate drug effect on NPC proliferation. In their study, they have shown that none of the nestin positive or nestin/BrdU double-positive cells are affected by Tat or morphine [299]. By looking into the detail, they have focused on the deleterious effect of Tat on embryonic

developmental stage, in which, Sox2 expresses earlier than nestin and plays a dominant role in the early stage.

Effects of Tat on astrogliogenesis

Astrocytes are the most abundant cell linage in the brain, they play not only the scaffold-like supportive role in the brain, but are also essentially in maintaining the homeostasis of the brain [300, 301]. Astrocytes also have been shown to play a crucial role in the initiation and progression of the diseases which show up-regulation and aggregation of astrocytic specific intermediate filament protein GFAP, modification in astrocytic morphology, and dramatic increases of astrocyte proliferation. All of this was termed astrocytosis and which in turn changes astrocytic network connections and consequently leads to destruction of the spatial relationship between astrocytic processes, asymmetric synapses and corticostriatal transmission, and ultimately results in disruption of the brain homeostasis and neuron synaptic integrity [56, 69, 301]. It is generally accepted that astrocytes proliferate during brain development, while the mature astrocytes do not proliferate. Astrocytes regain their proliferative properties under pathological situations [302, 303]. In our previous study, we have shown that expression of HIV-1 Tat in astrocytes is sufficient to up-regulate GFAP and promote astrocyte proliferation, which plays a critical role in HIV-1 mediated neuropathology [196-199]. In this study, by performing immunofluorescence staining, our data showed that, in Tat expressing mouse brain, GFAP staining was dramatically increased in the dentate gyrus of the hippocampus

(**Fig. 26A**). But whether the increased GFAP is due to Tat induced astrogliogenesis is unproved by analyzing the number of BrdU/GFAP double-positive staining (**Fig. 26B**). In iTat mouse brain, with GFAP as the promoter, the NPCs derived mature astrocytes are expressing Tat and rapidly proliferating, so the BrdU was diluted in those cells. To test whether soluble Tat directly affected mature astrocytes proliferation, we treated mature WT mouse primary astrocytes with Tat conditioned media or Tat recombinant protein. By performing MTT proliferation assay, none of Tat conditioned medium or Tat recombinant protein lead to astrocyte proliferation. By performing western blotting, GFAP expression is also not affected directly by adding Tat conditioned medium or Tat recombinant protein (data not shown). Based on the neurosphere assay data, the neurons and astrocytes were directly derived from NPCs. Observing the decrease of the preneuronal marker DCX and the increase of the mature astrocyte marker GFAP, allowed us to concluded that HIV-1 Tat impact the dynamic balance of neurogenesis and astrogliogenesis of NPCs.

Signaling pathways in controlling neurogenesis

All levels of adult neurogenesis, including neural stem cell (NSC) and/or NPC proliferation, migration, differentiation, and fate determination, are tightly regulated via both extrinsic environmental influences (pathological) and intrinsic signaling pathways (physiological), such as Wnt, BMP, Shh, Stat3, and Notch [228]. These pathways dynamically maintain proliferation and fate commitment of the local stem cell population by modulating the activation of a distinct set of transcription factors, which in turn triggers

the transcription of neural fate-associated genes. Wnt signaling is a principal regulator of adult hippocampal neurogenesis. Activation of Wnt signaling has been shown to induce NPC proliferation, promote neuronal and astroglial differentiation, and suppresses oligodendroglial differentiation. In 2012, Henderson showed that HIV-1 Tat inhibits Wnt/β-catenin signaling in astrocytes [229]. As shown in2005, hippocampal astrocytes play an important role in SGZ neurogenesis via promoting the neuronal differentiation. Inhibition of Wnt in astrocytes may leads to blockade of the neurogenic activity of astrocytes, which results in a decrease of neurogenesis in SGZ [230, 231]. Bone morphogenetic proteins (BMPs) are involved in many development processes. In the brain, BMP itself promotes dorsal neural formation [232] and it crosstalks with several other pathways, such as Stat3, Wnt and Shh to induce astrocyte specification but inhibit oligodendrocyte specification [233]. Stat3 stabilizes the astrocyte phenotype via promotion of astrocytes specific marker, GFAP, expression. A similar phenomena is observed in Notch signaling, where crosstalk with Stat3 via Hes and promotes astrocyte differentiation [234, 235]. Otherwise, Notch itself typically down-regulates preneuronal gene expression and inhibits neuron differentiation [236].

HIV-1 Tat function as a Notch ligand

Employing the yeast two-hybrid assay, we have shown that the majority of Tat-binding proteins shared common EGF-like motifs [265]. By using the same assay, others have shown that HIV-1 Tat protein was directly interacting with the EGF-like repeats of Notch

through the cysteine-rich domain on Tat [266, 304]. HIV-1 Tat is one of the viral soluble factors secreted from HIV-infected microglia/macrophages and astrocytes in the CNS [116, 138]. In the current case, soluble Tat protein is either taken up by the uninfected cells to act as a transcriptional factor and elicit the intracellular cascades, or it may work as an active ligand to bind directly to the EGF-like repeats on the extracellular domain of the Notch receptor. This was demonstrated by transactivation of Hes1 in both transfected Tat and in Tat conditioned medium (**Fig. 32-35**). In previous reports, others demonstrated that the EGF-like protein DLK1 competes with the canonical ligands, such as Jagged-1 or DLL4 for Notch receptor binding and decreases Notch activity [267, 268]. To further demonstrate that soluble Tat worked as an active ligand to directly activate Notch signaling and whether Notch was directly involved in Tat induced dynamic imbalance of neurogenesis and astrogliogenesis. The result of the neurosphere assay indicated that, by blocking Notch signaling with DLK1 protein, Tat induced Hes1 activation and Tat induced dynamic imbalance of neurogenesis and astrogliogenesis were abolished (**Fig. 37&38**). Taken together, this study provided *in vivo* evidence that HIV-1 Tat directly decreased NPC proliferation and impacted NPC differentiation by interrupting the dynamic imbalance of neurogenesis and astrogliogenesis. Moreover, this is the first time we demonstrated the underlying mechanism that Notch signaling was directly involved in the HIV-1 Tat induced dynamic imbalance of neurogenesis and astrogliogenesis.

PERSPECTIVES

HIV-1 infection of the central nervous system occurs early in the course of infection and often leads to cognitive, motor, and neurobehavioral dysfunction [305-309]. In the era of cART, MCMD has become increasingly prevalent among the HIV-infected population [26-28]. Several putative mechanisms are proposed for HIV-impaired neurodegeneration. HIV infection of microglia/macrophages and astrocytes alters expression of cytokines or groWTh factors [84, 206, 240-244], and soluble viral proteins, such as gp120, Nef and Tat, are known to lead to neuronal and astrocytic dysfunction. However, none of the findings have successfully led to effective therapies to reduce, prevent, or reverse MCMD. Thus, a better understanding of the cellular and molecular mechanisms for HIV-impaired neurodegeneration is needed.

Role of STAT3 in HIV-1/Tat impaired neurogenesis

Adult neurogenesis, including NSC and/or NPC proliferation, migration, differentiation, and fate determination, are tightly regulated via both extrinsic environmental influences (pathological) and intrinsic signaling pathways (physiological), such as Wnt, BMP, Shh, STAT3, and Notch [228]. These pathways dynamically maintain proliferation and fate commitment of the local stem cell population by modulating the activation of a distinct set of transcription factors, which in turn trigger the transcription of neural fate-associated genes. Activation of STAT3 is reported to be able to induce astrocyte differentiation from neuron progenitor cells (NPCs) by promote GFAP expression through p300 bridged

synergistic signaling [234, 262, 310, 311]. It is known that HIV-1 Tat induces IL-6, which binds with IL-6 receptor (IL6-R) and gp130 to activate the downstream janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway, especially STAT3 [312]. In the current study, we demonstrated that both STAT3 and its phosphorylation are induced by HIV-1 Tat, which result in GFAP activation in mature astrocytes. As noted in 2005, hippocampal astrocytes play an important role in SGZ neurogenesis via promoting the neuronal differentiation. The direct or indirect role of STAT3 mediated HIV-1/Tat impaired neurogenesis can further be investigated. For the *in vivo* study, iTat/nestin-GFP mice administered with/without JAK/STAT3 inhibitor could be used to assess the interaction between Tat-induced STAT3 and NPC proliferation. Meanwhile, the iTat mice administered with/without JAK/STAT3 inhibitor could be used to assess the interaction between Tat-induced STAT3 and NPC neurogenesis. Similarly to the current study, the *in vitro* neurosphere assay could be recruited to determine the role of Tat-induced STAT3 on NPC proliferation, migration and differentiation.

Role of GFAP in HIV/Tat impaired neurogenesis

It is noted that GFAP expresses not only in the mature astrocytes, but also in the hippocampal progenitors, which have a radial process spanning the entire granule cell layer and ramify in the inner molecular layer [313-315]. GFAP-expressing progenitors are considered to be the principal source of constitutive neurogenesis in the adult mouse forebrain. Knockout of GFAP in dividing progenitors in the adult mouse subependymal and subgranular zones abolished the generation of neuroblasts and new neurons in the

olfactory bulb and the hippocampal dentate gyrus [314]. In addition, lack of immature neurons in the dentate gyrus was also observed in the mouse model of Alexander disease (AxD), with dominant gain of function mutations in GFAP, which is characterized by cytoplasmic protein aggregates known as Rosenthal fibers along with variable degrees of leukodystrophy and intellectual disability [316], In contrast, a significant increase of neurogenesis and astrogliogenesis were observed after coculturing of NPC with GFAP-/- Vimintin -/- astrocytes or grafted NPC in the GFAP-/- Vimintin -/- transgenic mouse [317]. Taken together, GFAP expression in NPC and GFAP in astrocytes both play important roles in controlling NPC differentiation. In our previous study, GFAP up-regulation and aggregation is one of the hallmarks of HIV-associated brain pathogenesis. In further study, we would take advantage of the iTat/ GFAP- mouse model. NPC proliferation and neurogenesis could be assessed and compared to the wild type or iTat mouse to illustrate the role of GFAP in HIV-1/Tat impaired neurogenesis.

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Therapies to reduce, prevent, or reverse GFAP-mediated Tat neurotoxicity

The previous studies showed that HIV-1 Tat induced neurotoxicity was diminished in iTat/GFAP- mice (**Fig. 10**), suggesting an essential role of GFAP in Tat neurotoxicity and HIV-1 associated neurological disorders. But the neurotoxic factor mediated by Tat/GFAP neurotoxicity is still unspecified. In our previous study, we demonstrated that HIV-1 Tat induces ER stress via GFAP upregulation and aggregation. Based on this finding, the chemical chaperon 4-PBA, the ER stress inhibitor, have been tested to effectively revers the GFAP mediated Tat neurotoxicity both *in vitro* and *in vivo*. Moreover, to determine

the neurotoxic factors, proteomic analysis was performed to analyze the difference between the supernatants from wild type and the supernatants from Tat mouse primary astrocytes. The results indicated that the lysosome exocytosis has been elevated by Tat blocking lysosomal exocytosis via vacuolin-1 and inhibiting the lysosomal hydrolytic enzyme cathepsin B via Z-AM-FZK have been tested to abolish the astrocyte-mediated Tat neurotoxicity in vitro respectively. In our previous and the present study, blocking, mutation or knockdown each step of the Tat-GFAP cascade, for example, STAT3, Egr-1 or p300 can specifically attenuate the Tat-induced GFAP transactivation [197-199]. In the second part of the dissertation, blocking of Notch signaling by DLK1 or DAPT *in vitro* and DAPT *in vivo* reverse the Tat-impaired neurogenesis. Each step could be the potential target to reduce, prevent or reverse the HIV-1 Tat mediated neurodegeneration and which needs further effort to clarify and specify.

Roles of HIV-1 Tat in brain aging

By 2015, an estimated a half of all HIV-positive individuals will be older than 50 due to the introduction of HAART [92]. HIV positive individuals are showing associated age-related disorders at much younger ages: stroke, heart attack, arthritis, cancer and cognitive problems [318]. HIV-positive individuals lose brain functional abilities 15 to 20 years prematurely probably because of either HIV infection itself or the treatments used to control it [319]. Aging-related decline in neurogenesis in both the dentate gyrus and SVZ is the result of a dramatic decline in the division of progenitor cells [318]. In the current study, we showed the similar phenomenon induced by HIV-1 Tat suggesting the

correlation between Tat and aging-associated cognitive decline. To investigate whether Tat accelerates brain aging, the age matched wild type mice and iTat mice will be compared for several markers for brain aging, such as learning and memory function, plaque and tangle formation, mitochondrial function.

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