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CNS Neural/Glial Progenitors as Targets of HIV-1 and Opiates: Effects on Proliferation and Population Dynamics May Alter Behavior Outcomes.

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

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List of Abbreviations

3-NT	3-nitrotyrosine
AIDS	Acquired immunodeficiency syndrome
ADC	AIDS dementia complex
ANI	Asymptomatic neurocognitive impairment
BBB	Blood brain barrier
cART	Combination antiretroviral therapies
CCR5	C-C chemokine receptor type 5
CNS	Central nervous system
CX3CR1	C-X3-C chemokine receptor 1 (fractalkine receptor)
CXCR4	C-X-C chemokine receptor type 4
DOR	delta-opioid receptor (δ-opioid receptors)
DOX	Doxycycline
FGF	Fibroblast growth factor
GFAP	Glial fibrillary acidic protein
gp120	Glycoprotein 120
GPC	Glial primogenitor cell
GRP	Glial-restricted precursor
HAD	HIV associated dementia
HAND	HIV associated neurocognitive disorder
HIV-1	Human immunodeficiency virus-1
HIVE	HIV encephalopathy
hNPC	human neural progenitor cell

Iba-1	Ionized calcium binding adaptor molecule 1
IGF-1	Insulin like growth factor 1
KOR	kappa-opioid receptor (κ-opioid receptor)
LIF	Leukemia inhibitory factor
MCP-1	Monocyte chemoattractant protein-1 (CCL2)
MCP-5	Monocyte chemoattractant protein-5
MIP-1α	Macrophage inflammatory protein-1α (CCL3)
MIP-1β	macrophage inflammatory protein-1β (CCL4)
MND	Minor neurocognitive disorders
MOR	mu-opioid receptor (μ-opioid receptor)
NO	Nitric oxide
NPC	Neural progenitor cell
NRP	Neural-restricted precursor
Olig2	Oligodendrocyte transcription factor 2
OL	Oligodendroglia
PAF	Platelet-activating factor
RANTES	Regulated upon activation, normal T cell expressed and secreted (CCL5)
SDF-1	Stromal cell derived factor-1
Sox2	SRY (sex determining region Y)-related HMG (high mobility group)-box gene 2
Tat	Transactivator of transcription
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
VEGF	Vascular endothelial growth factor

Abstract

CNS Neural/Glial Progenitors as Targets of HIV-1 and Opiates: Effects on Proliferation and Population Dynamics, May Alter Behavior Outcomes.

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Human immunodeficiency virus (HIV) infected patients with a history of injection opiate abuse have higher incidences of acquired immunodeficiency syndrome (AIDS) and neurological dysfunction. The use of combined anti-retroviral therapy has significantly reduced the prevalence of mortality and progression to AIDS. Due to extended life expectancy, these patients are still at a great risk for HIV-associated neurological disorders and impairment in their later life. Neural progenitor cells (NPCs) play critical roles in brain growth and repair after injury and insult. Pediatric HIV patients whose glial populations are still developing are especially at risk for central nervous system (CNS) damage. Our previous reports suggest that HIV-1 transactivator of transcription (Tat) can directly cause pathology in neural progenitors and oligodendroglia (OLs) (Hauser et al. 2009). Thus, we have hypothesized that NPCs and/or glial progenitors may be targets of HIV proteins ± opiates drugs of abuse.

To determine whether progenitors are targets of HIV-1, a multi-plex assay was performed to assess chemokine/cytokine expression after treatment with viral proteins Tat or glycoprotein 120 (gp120) with/without morphine. Murine striatal progenitors released

increased amount of the beta-chemokines CCL5/regulated upon activation, normal T cell expressed and secreted (RANTES), CCL3/macrophage inflammatory protein-1α (MIP-1α), and CCL4/macrophage inflammatory protein-1β (MIP-1β) after 12 h exposure to HIV-1 Tat, but no to gp120. Secreted factors from Tat-treated progenitors were chemoattractive towards microglia, an effect blocked by 2D7 anti- C-C chemokine receptor type 5 (CCR5) antibody pre-treatment. Tat and opiates have interactive effects on astroglial chemokine secretion, but this interaction did not occur in progenitors.

We also examined effects of Tat and morphine on proliferation and lineage progression of NPCs *in vitro* and *in vivo*. *In vitro*, Tat and morphine independently reduced the proliferation and population of $Sox2^+$ and $Olig2^+$ cells in the absence of cell death. The interactive effects of morphine and either Tat or supernatant from HIV-1_{SF162} infected monocytes varied depending on outcome measure and time of exposure, but interactive effects occurred primarily on proliferation. In rare instances, viable human progenitors were associated with p24 immunolabeling suggesting that progenitors may be infected, a concept that is still controversial. To investigate effects of Tat and morphine on NPCs *in vivo*, we used a mouse model in which HIV-1 Tat_{1-86} is conditionally expressed in astroglia. *In vivo* results in neonatal striata were similar to those in cell cultures.

We extended the experiments into adult mice with inducing Tat expression for 3 month and the effect of sexes was examined in these animals. Intriguingly, males showed more Tat-induced impairment in behavioral tests (rotarod, grip strength, light-dark box) than females. Tat^+ males also showed a greater reduction in the proportion of $NeuN^+$ cells and NeuN immunoreactivity in the striatum, accompanied by greater microglial activation (3-nitrotyrosine$^+$/Iba-1^+). Unbiased stereological estimation in Nissl staining revealed that

the depletion of NeuN immunoreactivity in these mice was not due to neuron cell death or loss, because the total neuron number in striatum and total striatal volume were not affected by long-term Tat induction. Tat exposure appears to selectively reduce levels of NeuN in living neurons, although the reason is not known. Therefore, both the enhanced microglial reactivity and depletion of NeuN levels in males may help to explain sex-specific behavioral outcomes. $Sox2^+$ and $Olig2^+$ cells showed equivalent reduction in their population in both sexes.

Overall, our findings show that CNS progenitors, including both undifferentiated NPCs and glial progenitors, are vulnerable to individual or combined effects of HIV-1 or Tat and opiates. Changes in progenitor dynamics may alter the balance of cell populations in both the developing and adult CNS. We speculate that such changes may contribute to the behavioral abnormalities that we observed in Tat^+ mice and which appear to model aspects of motor, cognitive and anxiety deficits in HIV-infected patients.

Chapter 1

Introduction to the Neuroscience of HIV

Epidemiology of HIV

Over 1 million individuals in the United States (US) and nearly 33 million people worldwide are estimated to be infected and living with HIV (UNAIDS 2010). An estimated number of people newly infected with HIV in 2009 are 43,000 in the US and 2.6 million worldwide. Injection drug abusers are a major part in this population; 3 million people having the history of injection drugs are living with HIV. The rate of HIV infection along with drug injection are raising fast in developing countries and over 80% of all HIV infections in some countries are estimated to be related to drug use (UNAIDS 2009). Twenty to thirty percent of new HIV infections in the US occur among injection drug abusers annually (CDC 2009). Furthermore, they a have high a risk for faster progression to be acquired immune deficiency syndrome (AIDS), and higher incidence of HIV-related neurocognitive deficit/impairments compared to non-drug abusers (Bell et al. 2002). The population of women living with HIV in US are less than 25% of HIV positive people (CDC 2009), while 51% of people HIV positive population in worldwide are women (UNAIDS 2009), and HIV infections in women appear to be increased with globalization. HIV infection does not exclude children as its victims; about 370,000 children under 15 years old are also living with HIV globally (UNAIDS 2009), around 10,000 children in US were diagnosed as AIDS in US 2009 (CDC 2009). Children are known to be more vulnerable to HIV complication and neuropathogenesis than adults (Civitello 1991; Drotar et al. 1997; Drotar et al. 1999; Lobato et al. 1995; Sharer and Cho 1989; UNAIDS 2009;

Van Rie et al. 2007). Since the introduction of combination antiretroviral therapies (cART) since 1996, survival rates related to HIV infection have dramatically improved in the US and other developed countries. Despite these outstanding improvements in mortality and co-morbidity of HIV, the prevalence of HAND continuously raises and remains as a significant public health concern.

Virology of HIV-1

HIV-1 has a single RNA genome consisting of seven structural elements (LTR, TAR, RRE, PE, SLIP, CRS, and INS), and nine genes (*gag, pol*, *env, tat, rev, nef, vif, vpr,* and *vpu*). Six genes transcript into accessory proteins and nine other genes produce envelope, core, and polymerase of HIV-1 (Kuiken et al. 2008). The viral proteins from these genes are essential for infection and new viral replication, and some of them are known to have significant toxicity to neural cells. In this thesis, we will focus two viral proteins, Tat and gp120, which are well studied in HIV neuropathogenesis.

Firstly, gp120 is a viral envelop protein and plays a critical role in HIV infection. The gene *env* encodes gp160, the precursor of both gp120 and gp41. During HIV replication, furin, one of the host cell's protease, cleaves gp160 into the external gp120 and the transmembrane gp41 prior to viral assembly (Kuiken et al. 2008). For the initiation of HIV infection, gp120 binds the CD4 protein of target cells then the envelope complex undergoes a structural change, exposing the chemokine binding domains of gp120 and allowing them to interact with the target chemokine receptors, C-X-C chemokine receptor type 4 (CXCR4) and/or C-C chemokine receptor type 5 (CCR5), co-receptors for HIV-1. This allows the N-terminal fusion peptide gp41 to penetrate the cell membrane as a last

step of infection (Chan et al. 1997; Chan and Kim 1998; Wyatt and Sodroski 1998). gp120 is also known to give the viral tropism to HIV by the preference of gp120 to bind either CXCR4 or CCR5 to infect specific cells (Chan et al. 1997). For examples, T-tropic strains of HIV-1 use the α-chemokine receptor, CXCR4, for entry to $CD4^+$ T cells (Coakley et al. 2005; Deng et al. 1996; Feng et al. 1996). M-tropic strains use the β-chemokine receptor, CCR5, to infect macrophage/microglia (Coakley et al. 2005). Dual-tropic HIV-1 strains are known to be able to use both CCR5 and CXCR4 as co-receptors for viral entry. As well as, other chemokine receptors, such as CCR2, CCR3 and C-X3-C chemokine receptor 1 (CX3CR1)/fractalkine receptor, have been shown to serve as co-receptor for viral entry (Choe et al. 1996; Garin et al. 2003; Parczewski et al. 2009; Puissant et al. 2003).

Another HIV-1 protein, Tat, refers to trans-activator of transcription. This protein is one of regulatory proteins, which is synthesized at the early and late stage of viral replication. Tat can be released by HIV-infected cells into the extracellular environment, and can be taken up by the surrounding cells (Westendorp et al. 1995). This protein is known to have multiple forms; 86 or 110 amino acid forms are formed from two exons. The first exon contributes to the 1-72 amino acid variant, and the second exon forms the remaining 14 to 32 amino acids (Jeang et al. 1999; Kuiken et al. 2008; Li et al. 2010; Nath et al. 1996). HIV-1 Tat is one of the transactivating proteins regulating in HIV expressions. It is essential for retroviral replication by binding to long terminal repeat (LTR) of HIV to lead massive transcription level of the HIV dsRNA. HIV-1 Tat also is required for efficient HIV genomic transcription. The presence of Tat can induce more than hundred-fold levels of HIV transcription compared to activation of the absence of Tat (Cann et al. 1985). There are five functional domains in Tat protein including N-terminal, cysteine-rich, core, basic,

and C-terminal domains. Before identifying the function of Tat in HIV replication, HIV-1 Tat was firstly recognized as having a unique protein transduction domain (Frankel and Pabo 1988; Green and Loewenstein 1988). This domain in HIV-1 Tat contains specific amino acid sequence, GRKKR, which act as a nuclear localization signal. Tat could enter cells by crossing the cell membrane due to its rich lysine and arginine content of this domain (Morris et al. 2001; Schwarze et al. 2000). In summary, viral envelop protein gp120 and regulatory protein HIV-1 Tat can contribute to AIDS pathogenesis in multiple ways. These include facilitating infection, activating HIV transcription, increasing replication of HIV and dysregulating the host's cytokine expression and immune responses. These proteins can affect the cells that are not infected in secondary manner, by their directly binding to target cells which can induce abnormal multiple signal transductions and morphological changing. Understanding the mechanism of these viral proteins in neurobiology may allow us to have opportunities to control or manage HIV associated neurological defects HIV.

Invasion of HIV into CNS

The central nerve system (CNS) is susceptible to infection by HIV. It has been elusive and controversial when and how HIV invades into brain. Although it is unclear wheather HIV infection remains in the CNS, or is cleared and re-enters in later stage of infection, HIV appears to infiltrate into the CNS with different amount of infection and in different regions of the brain (An et al. 1999; Davis et al. 1992; Gonzalez-Scarano and Martin-Garcia 2005; Shapshak et al. 1999; Smit et al. 2004). The HIV infiltrations in multiple brain regions supports researchers in HIV-neuropathogenesis studies to establish

the various mechanisms that how the virus can infiltrate into CNS. Even there are a lot of mechanisms for HIV infiltration in CNS; we will briefly discuss some of mechanisms which are widely accepted. One of mechanisms, once HIV infect monocyte, macrophage or $CD4^+$ lymphocytes in peripheral system, these infected cells brought the virus into brain by accrossing the blood brain barrier (BBB), because the HIV infected cells can express adhesion molecules on their surface to help the infected cells to attach to brain endothelial cells and lead transendothelial migration into the CNS (Dhawan et al. 1997; Hickey et al. 1991; Hult et al. 2008). Second, HIV infected cells released cytokine/chemokines (de Jong et al. 1996; Nottet et al. 1996; Persidsky et al. 1997), Tat (Hofman et al. 1994; Lafrenie et al. 1996a; Lafrenie et al. 1996b) and gp120 (Annunziata et al. 1998), which can up-regulate adhesion molecules on brain endothelial cells and also alter the permeability of BBB, which allow HIV infiltrate easily into the CNS. Third, as HIV can do transcytosis itself (Hocini and Bomsel 1999), HIV in circulating blood stream firstly infect brain endothelial cells lining besides BBB and then HIV can be released by viral budding from the endothelial cells to infect astrocytes in BBB. Therefore, the process of transcytosis allows HIV passage into the CNS (Banks et al. 2001; Bomsel 1997; Owens et al. 1991). Once the virus enter the CNS by utilizing all these mechanisms, HIV can affect all cell types in CNS to induce neuropathogenesis both in direct and indirect manner. Many of the cell types in CNS, including astrocytes, microglia, neurons, oligodendrocytes, endothelial cells and NPCs, are considered to be either direct or indirect targets of HIV. The former two are considered to be target of HIV infection in CNS (Gonzalez-Scarano and Martin-Garcia 2005). These cells will be discussed in sections that follow.

Interaction of HIV-1 and opiate in neuropathogenesis

Injection drug abuse and HIV-1 are tightly interacted in epidemics mentioned above. HIV-1 is widely spread through injection drug use, because of their risk drug-seeking behaviors, needle sharing and sex-trading for drug purchase. Indeed, injection drug users are at higher risk both for HIV-1 infection and for developing neuropathological complications (Hauser et al. 2006; Hauser et al. 2007; Nath et al. 2000; Nath et al. 2002). Opiates, including heroin, oxycodone, morphine and fentanyl, are known to intrinsically exacerbate the pathogenesis and neurological complications of HIV-1 through direct actions in the CNS (Hauser et al. 2006; Hauser et al. 2007). Opiates majorly act through μ-opioid receptors (MORs), and κ-/δ- opioid receptors play roles in their signaling cascades. All these receptors are G-protein coupled receptors and are known to activate inhibitory G proteins (Al-Hasani and Bruchas 2011). The neurotoxicity of opiates has been well documented in opiate addition studies, and furthermore recent studies suggested that opiate abuse might lead Alzheimer-like changes in brains. These studies have shown that opiate alone can increase the depositions of hyper-phosphorylated tau and beta amyloid in brains of drug abusers; even these patients do not have history of HIV infection (Anthony et al. 2010; Ramage et al. 2005). The interaction of opiate and HIV-1 in the CNS mediated by either indirectly or directly manners in astrocytes (1.), microglia (2.), neurons (3.), oligodendrocytes (4.) and neural progenitor cells (5.) will be demonstrated in followed sections.

1. Astrocytes

Firstly, astrocytes can be infected by HIV-1 with low productive manner, and the infected astrocytes can shed HIV-1 protein Tat and gp120 into extracellular spaces. Additionally these HIV proteins can direct affect neighboring astrocytes and other cells. In a way of bystander effects on neurons, infected or affected astrocytes may fail to provide metabolic support to neurons, show reduced ability to buffer toxic substances from surrounding environment, release neurotoxic molecules, increase inflammation response and spread cell death signals through their gap junction to affect other cells (Eugenin and Berman 2007; Eugenin et al. 2011). As subsets of astrocytes express several types of opioid receptors, including mu-opioid receptor, these cells can be affected by and respond directly to opiate drugs (Hauser et al. 1996; Stiene-Martin et al. 2001). In *in vitro* studies, the combination of HIV-1 Tat and morphine causes an exacerbated loss in intracellular Ca^{2+} homeostasis in astrocytes and increased various cytokines released by affected astrocytes (El-Hage et al. 2008b; El-Hage et al. 2005; El-Hage et al. 2006b) and increased in astrocyte death (Khurdayan et al. 2004). Activated astrogliosis by Tat induction was also detected in transgenic mice and this activation might contribute stress to neurons with co-exposure to opiates (Bruce-Keller et al. 2008; Fitting et al. 2010b; Fitting et al. 2010c). Astroglia can be also influenced by glycoprotein 120 (gp120), this HIV viral protein can increase the levels of intracellular calcium (Codazzi et al. 1995; Codazzi et al. 1996; Holden et al. 1999) and modify gene expression (Galey et al. 2003; Wang et al. 2004) to enhance chemokine/cytokine secretion (Buriani et al. 1999; Fitting et al. 2010b; Kong et al. 1996; Ronaldson and Bendayan 2006; Yeung et al. 1995). However, gp120 relatively induces less chemokine/cytokine secretion than Tat with regional heterogeneity and diversity in

chemokine/cytokine production (Fitting et al. 2010b). In the interaction of opiates and gp120, morphine-induced CCL5/RANTES secretion from astrocytes might be neuroprotective against the HIV protein gp120 (Avdoshina et al. 2010), it still controversial. Interaction of opiates and other viral proteins in responses of astrocytes have been less well investigated, thus opioid-induced changes in astrocytes function associated with HIV viral proteins may have critical implications for the neuropathogenesis of HIV.

2. Microglia

Microglia are the brain-resident immune cells to be productively infected by HIV-1. These cells are considered as a major reservoir of viral replication in brain, and can massively produce toxic viral proteins, such as Tat, gp120, and Vpr during viral replication. Moreover, various toxic cellular products are released by infected microglia include cytokines such as tumor necrosis factor alpha (TNF-α), interleukins, platelet activating factor, as well as mediators of inflammation arachidonic acid, L-cysteine, glutamate, quinolinic acid, and nitric oxide (Bruce-Keller et al. 2008; Gupta et al. 2010; Persidsky and Gendelman 2003). As these cellular toxins can activate adjacent uninfected microglia and astrocytes, their activation may initiate cascades through the whole brain which may occur chronically in HIV infected patients (Hult et al. 2008). Abnormal microglia activation is prominently detected in various neuropathological diseases including brains from the end stage of HANDs (Kaul et al. 2001; Persidsky and Gendelman 2003; Tyor et al. 1995). Microglia also express most opioid receptors and each receptor can be a target of opiates and may have different responses corresponding to HIV-1. For example, opiate drugs increase HIV-1 replication in microglia through the activation of μ-

opioid receptors (MOR), and can also induce apoptosis in microglia (Singhal et al. 2002). Furthermore, co-exposure to morphine synergistically increased Tat-induced chemokine/cytokine production and microglial activation through CCL5/RANTES- driven amplification of CCL2/MCP-1 (El-Hage et al. 2008b; El-Hage et al. 2006a; El-Hage et al. 2006b), and that may partly explain relatively high incidences of microglial activation, neuropathology and cognitive disturbance among HIV patients who abuse opiates (Anthony and Bell 2008; Arango et al. 2004; Bell et al. 2006; Bouwman et al. 1998; Dougherty et al. 2002) et al. 2008). Otherwise, the activation of κ-opioid receptor (KOPr) activation is known to reduce viral replication in microglia (Chao et al. 1996). These facts implicate that the interaction of HIV-1 and opiates underlay complicate mechanism in HIV neuropathogenesis.

3. Neurons

It is generally accepted that neurons are defiantly direct and major target of viral proteins, such as such as gp120 (Dreyer et al. 1990), Tat (Haughey et al. 1999; Sabatier et al. 1991), and Vpr (Piller et al. 1998). These viral proteins are considered as major contributing factors to developing neuropathogenesis. Even neurons that are not infected with HIV are also considered as targets of indirect or bystander inflammations and toxicants released by infected microglia and astrocytes, mentioned above. Infected astrocytes and microglia may produce inconsistent amounts of individual viral proteins (Brack-Werner 1999), each HIV-1 protein released from infected cells is directly toxic to neurons (Mattson et al. 2005). Various studies in the neurotoxicity of HIV-1 viral proteins suggests that opiate exposure exacerbates the toxic effects of HIV-1 Tat and gp120 in

neurons, and this interaction of HIV-1 and opiates can induce cell death in neurons, as well as in glial cells (Gurwell et al. 2001; Hu et al. 2005; Khurdayan et al. 2004). Interestingly, opiate exposure seems to reduce the threshold for neurotoxicity (Zou et al. 2011), but opiates make neurons be more susceptible to toxic insults (Hauser et al. 2005; Nath et al. 2002). Moreover, the interactions also results in the destabilization of Ca^{2+} in astrocytes and induce them to release proinflammatory cytokine/chemokines like monocyte chemoattractant protein-1 (MCP-1), monocyte chemoattractant protein-5 (MCP-5), and regulated upon activation, normal T-cell expressed, and secreted (RANTES) (El-Hage et al. 2005), which can significantly affect the functional integrity of neuronal synapse and morphology of neurons (Fitting et al. 2010c). How interactions between opiate drug abuse and HIV-1 toxicity affect the relationships between microglia, astrocytes and neurons is necessary to understand the mechanisms of HIV-1 pathogenesis in the CNS.

4. Oligodendrocytes

The oligodendrocytes are the myelin producing cell in CNS, responsible for proper conduction of axonal signals. Even they have significant contributions for proper function of CNS and the specific vulnerability to many neuropathologic disease, these cells have been underestimated and have a short history in HIV neuropathogenesis compare to neurons. Generally autopsy studies in HIV infected brains detected demyelination, oligodendrocyte cell death and white matter abnormality although the exact mechanism is unknown (Petratos and Gonzales 2000; Richardson-Burns et al. 2002). At least, HIV-1 protein Tat seems to have more direct toxicity to oligodendrocytes than gp120 has by changes of cell morphology (Fitting et al. 2008; Hauser et al. 2009). Oligodendrocytes

express most of opioid receptors including MOR, κ-opioid receptor (KOR), and δ-opioid receptor (DOR), which play important roles in proliferation, differentiation and maturation of oligodendrocytes (Hahn et al. 2010a; Hayashi et al. 2004; Persson et al. 2003). However, the condition of opiate drugs act by mimicking the endogenous opioid system can enhance toxicity of HIV-1 to oligodendrocytes. Previous *in vitro* and *in vivo* data from our laboratory strongly indicated that Tat and morphine significantly induce stress in oligodendrocytes without cell death *in vitro*, but the combined exposure of Tat and morphine synergistically increase oligodendrocyte stress and cell death *in vivo* (Hauser et al. 2009) with their morphological changes. Oligodendrocytes might be more sensitive to co-exposure of HIV-1 than neurons are. For examples, Tat induction through 7 to 10 days significantly increase the proportion of caspase-3 activity, as an indication of stress rather than cell death, in $NeuN^+$ cells. While the Tat induction in the same period increase TUNEL staining in $O4^+$ and APC^+ cells, which indicates cell death (reviewed by Bruce-Keller et al. 2008; Hauser et al. 2009). Therefore, opiate drugs in HIV neuropathogenesis is also likely to be complicatedly involved in the development and survivals of oligodendrocytes.

5. Neural Progenitor cells (NPCs)

The definition of neural progenitor cells (NPCs) in this thesis is the early (primitive) stage of cells having the capacity of proliferation and the multi-potential ability to generate various neural lineages of mature brain cells including neurons, astrocytes, and oligodendrocytes. The NPCs are abundant in different distinct regions of the brain during embryonic and postnatal development, while the adult brain contains these NPCs in limited

neurogenic regions including the subgranular zone (SGZ) in the dentate gyrus, the subventricular zone (SVZ) in the lateral walls of the lateral ventricles, and the olfactory bulb (Ming and Song 2005). In the normal adult brain, NPCs is known to be involved in learning and memory and neural turnover in the hippocampal dentate gyrus and olfactory bulb. However, accumulating evidence suggest that NPCs exist in numerous regions of the brain including the neocortex, spinal cord, tegmentum, substantia nigra, amygdala, and brain stem, as well as in the hippocampal dentate gyrus and olfactory bulb (Ming and Song 2005). The functional significance of the NPCs in these regions is incompletely understood; however, they may play critical roles in damage repair following any injury or insult as well as in learning and memory formation (Brazel et al. 2003; Gage 2000; Gage et al. 1998; Gould 2007; Kempermann and Gage 2000; Ming and Song 2011; Nait-Oumesmar et al. 2008; Romanko et al. 2004).

The procedure of NPCs turning into matured neural cells mainly requires the multiple steps including proliferation, migration, differentiation, survival, and integration of the newly formed matured neural cells into the pre-existed environmental cells in the brain (reviewed Trapp et al. 1997). These multiple steps of NPCs are known to be affected by various endogenous factors (Yoneyama et al. 2011). Although neurological deficits due to brain injury/insults or neuropathogenesis are mainly caused by loss of synaptic integrity, malfunction or loss/death of neurons, the disruption of endogenous neuro/gliogenesis from NPCs might contribute to neurological deficits by hindering the recovery from brain injury/insults. Notably, cumulative reports indicate that the properties of NPCs in HIV neuropathogenesis can be affected by cytokine/chemokines, neurotransmitters, and reactive oxygen/nitrogen species (ROS), which are released by HIV-1 affected neurons as well as

by activated macrophages, microglia, and astrocytes (Whitney et al. 2009; Yoneyama et al. 2011), mentioned above.

Although the critical roles of these cells is in maintaining and repairing of mammalian CNS system, the underlying mechanism of NPCs in HIV neuropathogensis is still elusive. The RNA/DNA of HIV-1 was detected in regions of neurogenesis in human adult and pediatric brain, and *in vitro* studies suggested the prolonged HIV-1 infection of NPCs, it is still controversial whether NPCs can be infected by HIV or not (reviewed Das and Basu 2011; Mishra et al. 2010; Rothenaigner et al. 2007; Schwartz et al. 2007). Moreover, the limited availability and purity in human primary NPCs make numerous difficulties in studies of HIV neurobiology. Since NPCs express opioid receptors, MOR, KOR and DOR (Buch et al. 2007; Hahn et al. 2010b; Hauser et al. 2009; Knapp et al. 2009), these receptors play important roles in proliferation, differentiation and survival of NPCs through endogenous opioids and also make these cells become targets of opiates and their antagonists. Besides, various cytokine/chemokine receptors, including CXCR4 and CCR5, are also expressed on surface of NPCs (Kao and Price 2004; Li et al. 2011; Li and Ransohoff 2008; Tran and Miller 2005), which allow NPCs can be direct and indirect targets of HIV-1. As the indirect way HIV-1 affects NPCs, there have been some reports that cytokine/chemokines released by HIV-1 infected macrophage/microglia affect proliferation and differentiation of NPCs (Peng et al. 2011; Peng et al. 2008) and reductions of NPCs in hippocampus paralleled increased HIV-1-infected microglial activation in mice model of HIV encephalopathy (HIVE) (Poluektova et al. 2005). A few studies indicated that the direct effect of HIV-1 Tat and gp120 can attenuate the proliferation and differentiation of NPCs and exposure to these proteins might lead cell

death in NPCs (Mishra et al. 2010; Okamoto et al. 2007; Tran et al. 2005). Moreover, NPCs may be preferentially vulnerable to HIV-1 infection or chronic opiate exposure because Tat or morphine alone or in combination caused significant increases in NPC cell death *in vitro* and *in vivo* (Buch et al. 2007; Khurdayan et al. 2004). However, most of studies in NPCs with HIV-1 or their viral proteins were only concentrated on reduction in proliferation of NPCs or decreased differentiation into neurons rather than any other cells in brain. Questions still remain, such as what specific stage of NPCs are affected by HIV-1, how HIV-1 affects the properties of NPCs and what population can be generated after the HIV-1 affect NPCs etc. The detail of studies in NPCs under the condition of HIV and opiates are required in HIV neuropathogenesis.

NPCs are relatively less investigated in HIV neuropathogenesis compared to other major neural cells in brain, even these cells might have a significant potential to contribute the recovery or marinating of normal CNS functions during HIV-1 invasion in CNS. Furthermore, NPCs are likely to have different responses from HIV-1 or its viral effects on mature glia as well as neurons. This may critically contribute the facts that pediatric patients frequently present with early and with more pathological forms of neuroAIDS (Drotar et al. 1997; Lobato et al. 1995; Van Rie et al. 2007), since these patients have abundant NPCs which are still developing in their CNS. Thus, opiate-induced changes in functions of NPCs may have critical implications for the pathogenesis of HIV.

Taken altogether, the studies in this article will show that NPCs in striatum can be major target of HIV-1 Tat proteins with opiates exposures by altering their chemokines/cytokines secretions, proliferation and population in specific stage of

progenitors and these potential consequences may result in changing behavior of animal model with sex-specific manner.

Chapter 2

β-Chemokine Production by Neural and Glial Progenitor Cells is Enhanced by HIV-1 Tat: Effects on Microglial Migration.

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Introduction

Human immunodeficiency virus (HIV) infection within the central nervous system (CNS) causes a variety of neuropathological changes. Neurons are not generally thought to be infected by HIV, although latent neuron infection has been reported (Torres-Munoz et al. 2001; Trillo-Pazos et al. 2003). Neuropathology is instead mediated by direct neurotoxic actions of released viral proteins, or secondarily, through toxic effects orchestrated by glial cells (Brack-Werner 1999; Gendelman et al. 1994; Hauser et al. 2007; Kaul et al. 2001; Kramer-Hammerle et al. 2005b; Persidsky and Gendelman 2003). HIV-infected macrophages/microglia reaching the brain create a reservoir of viral infection, and lay the groundwork for inflammation leading to neuropathology and cognitive changes. Although there is little evidence that macroglial cells *in vivo* are productively infected by HIV (Brack-Werner 1999; Gorry et al. 2003; Kramer-Hammerle et al. 2005b), activation of astroglia by viral proteins, or by substances released from reactive microglia, can amplify brain inflammation and neurotoxic sequelae, and also promote infiltration of infected

monocytes from the periphery. Thus, HIV neuropathology results from collective effects of viral proteins and inflammatory mediators on several cell types.

Astroglia from humans and rodents secrete chemokine/cytokines in response to HIV-1 transactivator of transcription (Tat) protein (Conant et al. 1998; El-Hage et al. 2005; Kutsch et al. 2000; McManus et al. 2000; Nath et al. 1999; Rappaport et al. 1999). We have shown that Tat-induced $[Ca^{2+}]_i$ responses mediate (CC motif chemokine ligand) 2/monocyte chemoattractant protein-1 (MCP-1), CCL5/regulated upon activation, normal T cell expressed and secreted (RANTES) and interleukin-6 (IL-6) release, resulting in downstream signaling through necrosis factor kappa B-dependent pathways (El-Hage et al. 2008b; El-Hage et al. 2005). Concurrent exposure to morphine exacerbates Tat-induced chemokine/cytokine production and microglial activation through CCL5/RANTES-driven amplification of CCL2/MCP-1 (Bruce-Keller et al. 2008; El-Hage et al. 2008a; El-Hage et al. 2006a; El-Hage et al. 2006b), an observation that may partly explain relatively high incidences of microglial activation, neuropathology and cognitive disturbance among HIV patients who abuse opiates (Anthony and Bell 2008; Arango et al. 2004; Bell et al. 2006; Bouwman et al. 1998; Dougherty et al. 2002). Astroglia are also sensitive to glycoprotein 120 (gp120), which can elevate $[Ca^{2+}]_i$ (Codazzi et al. 1996; Holden et al. 1999), and alter gene expression (Galey et al. 2003; Wang et al. 2004) leading to chemokine/cytokine secretion (Buriani et al. 1999; Kong et al. 1996; Ronaldson and Bendayan 2006; Yeung et al. 1995), with some evidence for exacerbation by opiates (Mahajan et al. 2005). In our hands, Tat generally elicits a much higher chemokine and cytokine response than gp120, and we observe regional heterogeneity in secretion by astroglia (Fitting et al. 2010c).

Responses of astroglia to other HIV-1 proteins have been less well studied (Kramer-Hammerle et al. 2005a; Lehmann et al. 2006).

We were intrigued by the possibility that less differentiated CNS cells, in addition to microglia and astroglia, might contribute to inflammation. This would parallel situations in other tissues. Unstimulated bone marrow or cord-derived mesenchymal stem cells secrete a spectrum of chemokine/cytokines and growth factors, including multiple fibroblast growth factors (FGFs), interleukins, insulin like growth factor 1 (IGF-1), leukemia inhibitory factor (LIF), CCL2/MCP-1, CCL3/macrophage inflammatory protein-1α (MIP-1α), and CCL4/macrophage inflammatory protein-1β (MIP-1β), stromal cell derived factor-1 (SDF-1), and vascular endothelial growth factor (VEGF) (Chen et al. 2008; Croitoru-Lamoury et al. 2007; Liu and Hwang 2005; Rafei et al. 2008; Schinkothe et al. 2008; Wagner et al. 2007). As mesenchymal stem cells differentiate, the balance of factors released varies with cell fate (Kilroy et al. 2007; Molloy et al. 2009). Neural progenitor cells (NPCs), which derive from undifferentiated neuroepithelial cells, are a self-renewing and multipotential source of neurons and macroglial cells. Common markers for NPCs include the intermediate filament nestin and the transcription factor Sox2 (sex determining region of Y (SRY)-related HMG-box gene 2). As NPCs continue to differentiate, they become largely restricted to either neuronal or glial fates. Differentiating glial-restricted progenitors (GPCs) express markers typical of oligodendrocytes (e.g. Olig1, Olig2, Sox10, myelin proteins) or astroglia (e.g. GFAP, EAAT2). $Nestin^{+}$ and $Sox2^{+}$ cells continue to be found in the mature CNS, although in more restricted germinal zones (Komitova & Eriksson 2004, Ellis *et al.* 2004). There is evidence that neurally-restricted progenitors may have a secretory role. For example, human NPCs expressing nestin and

A2B5 release IP-10/CXCL10 and MCP-1/CCL2 after exposure to TNF-α (Sheng et al. 2005). NPCs also secrete neurotrophins and other growth regulators (Benoit et al. 2001; Llado et al. 2004; Shingo et al. 2001; Taupin et al. 2000), and transplantation of stem cells and/or NPCs increases their own survival (autocrine effects), as well as promoting neuron survival after injury (paracrine effects) (Chang et al. 2003; Llado et al. 2004).

Effects of HIV proteins on NPCs or GPCs are relatively unexplored, and are likely different from effects on mature glia. They may be critically important to pediatric patients, who frequently present with early and with more pathological forms of neuroAIDS (Drotar et al. 1997; Lobato et al. 1995; Van Rie et al. 2007). The majority of these patients are infected at birth, when glial populations are still developing. The present study was designed to determine whether NPC/GPCs from striatum have the capacity to produce chemokines/cytokines, and if so, whether exposure to HIV proteins can modify production, with potential functional consequences.

Materials and Methods

All experimental protocols conformed to local Institutional Animal Care and Use Committee (IACUC) and national (PHS) guidelines on the care and ethical use of animals. Experiments were designed to minimize the total number of animals used and their discomfort.

Progenitor Cell Cultures

The striatum is a region that is particularly vulnerable to both HIV-induced and opiate-induced degenerative changes, and is a region of great interest for the synergistic effects of these two insults. Progenitor cell cultures were prepared from the striatum of embryonic day 15 (E15) mice using modifications of a previously published technique (Khurdayan et al. 2004). In brief, embryos were aseptically removed from the uterus of timed pregnant ICR dams (Charles River, Boston, MA) following cervical dislocation. The striata from 10-12 embryos were dissected from the rest of the cortex, finely minced, and incubated with DNase (0.015 mg/ml, Sigma-Aldrich Co., St. Louis, MO) in Dulbecco's Modified Eagle's Medium without additives (15 min, 37˚C). Tissue was triturated through a 5 ml pipette, centrifuged, and resuspended in 5 ml of defined, progenitor maintenance medium containing 1:1 DMEM/F12 (Invitrogen Corp., Carlsbad, CA) and 10% Knockout™ SR (Invitrogen), a commercial serum replacement product that does not contain serum or growth factors. Additional additives included insulin (25 µg/ml), transferrin (100 µg/ml), progesterone (20 nM), putresceine (60 µM), Na-HCO_3 (6 mM), and sodium selenite (30 nM) (all from Sigma-Aldrich Co.). Resuspended cells were filtered through a 70 µm nylon Cell strainer (BD Falcon, Bedford, MA), counted, centrifuged as above, and resuspended in the appropriate volume of the same medium to obtain desired plating densities. Cells were plated on either poly-L-lysine coated 24 well plates for Multi-plex assay (10^5 total cells per well) or poly-L-lysine coated 15mm glass coverslips for immunostaining ($5x10^4$ cells in 150 µl), and medium was changed after 2 days. Cells were growth at 37˚C, in a humidified atmosphere with 5% CO_2 throughout. At 5 days the medium was refreshed again and cells were treated with Tat_{1-86} (100 nM;

ImmunoDiagnostics, Woburn, MA), and/or gp120 (MN strain; 500 pM; ImmunoDiagnostics), and/or morphine sulphate (500 nM; NIDA Drug Supply System, Rockville, MD), and/or naloxone (1.5 μM; Sigma-Aldrich, St. Louis, MO). Morphine, the major bioactive product of heroin in the brain, is a potent μ-opioid receptor agonist, and naloxone is a broad spectrum opioid receptor antagonist. These are concentrations of HIV proteins and opiates that have elicited functional effects, including increases in chemokine/cytokine secretion, in astroglia, oligodendrocytes, and neural progenitors in our earlier studies (Buch et al. 2007; El-Hage et al. 2005; Hauser et al. 2009; Khurdayan et al. 2004). In studies with naloxone, the inhibitor was applied 30 min prior to other treatments, which were applied simultaneously in pre-warmed medium.

Primary microglial cultures

Primary cultures enriched in microglia were prepared from mixed murine glial cultures using modifications of rat culture protocols (Kong et al. 1997; Levesque et al. 2010). Briefly, meninges and blood vessels were removed from whole brains of 1 day postnatal pups. Tissues were gently triturated in microglia-enrichment media and seeded (four brains or > 5×10^7 cells) in poly-L-lysine-coated 150 cm3 flasks. Microglia-enrichment medium consists of 1 : 1 DMEM/F12 containing 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 μM non-essential amino acids, 50 U/mL penicillin, and 50 μg/mL streptomycin (Invitrogen). Cell suspensions were filtered through nylon mesh with 70 μm pores before plating onto 150 cm^3 flasks. Cultures were maintained at 37˚C in a humidified, 5% CO_2 atmosphere. Medium was replenished every third day after the initial seeding. 10~12 days after seeding, when the glia had formed a confluent

monolayer, microglia were mechanically dislodged and the detached cells were panned on uncoated tissue culture dishes for 1–2 h to remove more adherent cells such as astrocytes and progenitors. Microglia were pelleted at 100 g for 5 min, then resuspended in fresh medium. We confirmed that the primary microglia were > 95% pure by immunostaining with the specific marker, Iba-1 (not shown).

Immunohistochemistry and Quantification

Progenitor cell cultures were immunostained at 5 days post-plating, using cell-specific and lineage-specific markers including the O_4 antibody which detects sulfatide (Sommer and Schachner 1982), and antibodies to glial fibrillary acidic protein (GFAP; 1:2,000, Chemicon/Millipore, Billerica, MA), CD11b (1:100, Millipore, Billerica MA), Olig2 (1:100, Immuno-Biological Laboratories, Minneapolis, MN), Sox2 (1:100, R&D Systems; Millipore) and nestin (1:5,000, Aves Labs, Inc., Tigard, OR). In some experiments, and based upon the Bio-plex data, cells were double-immunostained with CCL5/RANTES (1:100, Santa Cruz Biotechnology Inc., Santa Cruz, CA), CXCR4 (clone 12G5, 1:50, Abcam Inc., Cambridge, MA), and CCR5 (clone 45523, 25 g/ml, R&D Systems; clone 2D7, 1:100, BD Pharmingen TM, San Jose, CA) to examine whether precursors and other cells within the cultures could express particular chemokines and their receptors. In all cases, cells were fixed with 4% paraformaldehyde, and for intracellular markers, cells were permeabilized with 0.1% Triton X-100 in PBS containing 5% normal goat serum for 30 min. Double-staining was performed sequentially, with staining for chemokines and receptors followed by immunostaining for precursor markers. Antibodies were applied overnight at 4˚C, followed by visualization with appropriate fluorescent-

conjugated second antibodies (Molecular Probes/Invitrogen, Carlsbad, CA). Immunostained coverslips were finally incubated with Hoechst 33342 dye (8 min) to identify nuclei (Molecular Probes/Invitrogen), then mounted in ProLong Gold antifade reagent (Molecular Probes/Invitrogen) and dried for 8h in the dark. To assess the proportion of different cell types in the cultures, 350-500 Hoechst$^+$ cells were selected randomly per coverslip and assessed for nestin, Olig2, Sox2, O_4, GFAP, or CD11b immunostaining (N=3-5 different experiments) under oil immersion at 63× using a Zeiss AxioObserver system with an integrated MRM camera system (Carl Zeiss, Inc., Thornwood, NY). At least 100 random Olig2$^+$ cells were assessed for RANTES in similar cultures. Similar staining methods were applied to 10 μm tissue sections from adult mouse striatum.

Immunoblotting

CXCR4 and CCR5 expression was examined in 5-day cultures. Cells were harvested by scraping into ice-cold RIPA buffer (50 mM Tris, pH 7.4; 150 mM NaCl; 1% Nonidet; 0.5% deoxycholic acid; 0.1% SDS; protease inhibitor cocktail, Roche, Indianapolis, IN, USA). Cell lysates were pelleted and stored at −80°C until use. Protein was assayed using the BCA protein assay (Pierce, Rockford, IL, USA). Duplicate samples of 20μg total protein were loaded per well onto 4-20% Tris–HCl Ready Gels (Bio-Rad Laboratories, Hercules, CA) using Precision Plus Protein Dual Color Standards (Bio-Rad; MW range: 10–250 kDa) to monitor protein transfer and molecular weight. Proteins were transferred to Hybond-P PVDF membranes (Amersham Biosciences, Piscataway NJ), probed with a monoclonal antibodies to either CXCR4 (ab2074, 1:1,000, Abcam) or CCR5

(clone 45523, 1:1,000, R&D Systems), and visualized using a chemiluminescent substrate (Super Signal West Femto substrate, Pierce). Protein bands were detected on a Kodak Image Station 440CF and analyzed using 1D software.

Multi-plex Assay

After 12 hrs of treatment, 450 µl of media were harvested from each well and stored at -80˚C. 50 µl of media from each treatment group was analyzed for chemokine/cytokine content using the Bio-Plex 200 System (Bio-Rad) and standard mouse 23-plex assay kits or custom 12-plex assay kits, according to the manufacturer's instruction. Concentrations were determined using Bio-Plex Manager Software (Version 5.0), based upon individual standard curves calculated for each chemokine/cytokine.

Microglial Chemotaxis Assay and CCR5 Antibody Neutralization

An 8.0-µm transwell system (Costar, Corning, NY, USA) was used to measure changes in the motility of BV-2 microglia-derived cells or primary microglia in response to Tat- or gp120-treated glial precursor cells in 24-well plates. BV-2 cells were cultured in T-75 flasks in medium consisting of DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with glucose (27 mM), Na_2HCO_3 (6 mM), 10% defined fetal bovine serum (Hyclone Laboratories, Inc., Logan,UT, USA) and antibiotics. Cells were harvested at 90% confluency using a non-enzymatic cell dissociation solution (Sigma-Aldrich Co.), washed and resuspended in DMEM supplemented with glucose (27 mM), Na_2HCO_3 (6 mM), and 1% fetal bovine serum, and then plated onto transwell inserts (10^5 BV-2 cells per insert). Primary microglia were grown in microglia-enrichment medium and mechanically

harvested as described earlier. They were washed, resuspended and plated onto inserts similarly to BV-2 cells. Prior to the addition of transwell inserts containing microglia or BV-2 cells, 10^6 progenitors were cultured in each well of a poly-L lysine coated 24 well plate for 5 days, then treated with Tat or gp120 ± opiates for 12 h. A transwell insert containing BV-2 cells or microglia was then placed into each well. The transwell inserts were incubated for an additional 3 h and then removed for analysis. Cells remaining on the upper surface of the insert were carefully aspirated, leaving those which had migrated to the surface underneath in place. The insert was then transferred to a clean well containing 225 µL of non-enzymatic cell dissociation solution (Sigma-Aldrich Co.) and incubated for 30 min, at 37˚C, during which time each insert was gently tilted several times to completely detach cells. The inserts were discarded and 75 µL of 4 × Lysis Buffer/CyQuant® GR dye solution (Millipore) wereapplied to each well and incubated per manufacturer's instructions. 200 µL of dye/buffer/lysate solution were transferred to a 96-well plate, and the fluorescence intensity of each sample was measured using a PHERAstar FS plate reader (BMG LABTECH Inc. Cary, NC, USA) with a 485 nM excitation filter and 520 nM emission filter. In experiments to assess the role of CCR5 in migration, the BV-2 cells or microglia were incubated with anti-CCR5 (clone 2D7, without sodium azide; BD Pharmingen™) or control IgG (mouse, IgG2a, BD Pharmingen) at a dose of 2 µg per 10^6 cells for 1 h at 4˚C, prior to the placement of the inserts.

Viability Assay

Neural/glial precursor cells were cultured on poly-L-lysine precoated 48 well plates and treated at 5 days after plating with morphine, Tat, gp120, and naloxone either alone or

in combination as in experiments described above. After 12 h, medium containing treatments was replaced with sterile, tissue culture–grade D-phosphate- buffered saline containing the LIVE/DEAD® reagents (Invitrogen; calcein AM, 2 μM and EthD-1, 4 μM) and 5 μg/mL Hoechst 33342. The working solution was added directly to cells and incubated for 15 min, at 37˚C. The fluorescence intensity of each color was measured using a PHERAstar FS plate reader (BMG LABTECH) in n = 8 different cultures using appropriate excitation and emission filters (calcein AM: 495/515 nm; EthD-1: 495/635 nM; Hoechst: 350/510 nM).

Statistical Analysis

Statistical analyses were done by analysis of variance (ANOVA) followed by Bonferroni's *post-hoc* testing using StatSoft software (Statistica, Tulsa, OK).

Results

Progenitor cells from E15 striatum secrete chemokines: Effects of viral proteins and opiates

After 5 days growth in chemically defined medium, the progenitor cell cultures consisted overwhelmingly of a mixture of immature cell types (Fig. 2.1 A), containing both NPCs and GPCs at various states of differentiation. The majority of the cells were immunostained for nestin (82%) and/or Sox2 (44%), markers of relatively undifferentiated CNS progenitors (Ellis et al. 2004; Komitova and Eriksson 2004; Lendahl et al. 1990; Tanaka et al. 2004). Of the nestin-positive cells, 47% also showed Sox2 immunreactivity. Approximately 20% of the cells had entered the oligodendrocyte lineage, as indicated by expression of Olig2 (Ligon et al. 2006; Woodruff et al. 2001). Another ~40% expressed DCX, a microtubule-associated protein found almost exclusively in neuronal precursors. Somewhat surprisingly, very few of the cells expressed the astrocyte marker GFAP (< 3%). Many DCX^+, $Olig2^+$ and $GFAP^+$ cells also were $nestin^+$ or $Sox2^+$, suggesting relatively recent entry into a specific lineage. Less than 0.8% of cells stained with the CD11b microglial marker. As reported previously, over 70% of the $Olig2^+$ cells in these cultures expressed the μ-opioid receptor (MOR) (Hauser et al. 2009). Most cells that were $Olig2^-$ or DCX^- were also MOR^+, indicating that MOR was also expressed in uncommitted precursors (Fig. 2.1 B and C). Thus, multiple types of progenitors are potential targets of MOR agonists and antagonists such as morphine and naloxone.

Progenitors were treated for 12 hr with HIV-1 proteins and/or opiates, after which their conditioned medium was harvested and screened for expression of an array of

chemokines and cytokines significant in the pathobiology of inflammatory disease and/or displaying cross-sensitization to opiates. The 12 hr time-point was chosen because it gave a consistently detectable accumulation of secreted products. Based on our earlier studies with other types of glia (El-Hage et al. 2005), longer time-points can result in higher accumulation, but they also increase the likelihood that autocrine and paracrine effects of the chemokines/cytokines will influence cell secretions. Of the 23 factors initially tested, only 10 were expressed and/or secreted at detectable levels by untreated striatal progenitor. The concentrations of the individual chemokines/cytokines detected after 12 hrs (pg/ml) are given in Table 1. Exposure of progenitors to viral proteins and/or opiates did not change the factors that were secreted, but did affect the accumulated amounts of several. The secretion of β-chemokines CCL3/MIP-1α, CCL4/MIP-1β and CCL5/RANTES were all significantly increased by Tat exposure, but not by gp120 exposure (Fig. 2.2). Unlike prior observations in astrocytes, concurrent exposure to Tat and morphine did not have an interactive effect on chemokine/cytokine release, even though the majority of cells in the cultures expressed MOR. Neither was there an interactive effect of Tat and gp120. CCL2/MCP-1 showed a trend towards a Tat response, but the effect was not significant using ANOVA analysis. It is likely that cells at multiple stages of differentiation contributed to both baseline and Tat induced secretion, as CCL5/RANTES was detected by immunostaining in a subset of both $Sox2^+$ and $Olig2^+$ cells *in vitro* (Fig. 2.3 A–C). CCL5/RANTES staining was also evident in $Sox2^+$ cells *in vivo* (Fig. 2.3 D), but it was quite difficult to detect in $Olig2^+$ cells *in vivo* (not shown), suggesting down-regulation in oligodendroglial precursors in the brain. We also examined expression of the β-chemokine receptors CXCR4 and CCR5, known to be co-receptors for gp120 binding and HIV entry

into cells. Immunohistochemistry showed that both CCR5 and CXCR4 were present on $Olig2^+$ and $Sox2^+$ progenitors, as well as on neighboring $Olig2^-$ and $Sox2^-$ cells. Both receptors were also readily detected on western blots from progenitor cultures (Fig. 2.4 E; 20-μg load), as well as astroglia and striatal homogenates. Major bands were found at ~40 kD, which is the reported molecular weight of both CCR5 and CXCR4.

Progenitor cells induce a chemotactic response in microglial cells

A transwell apparatus was used to test whether secreted factors from progenitors could influence the movement of BV-2 and primary microglial cells. All tests were conducted over a 3-h period. Some cells moved randomly across the membrane during the test period (Fig. 2.5 A–C, medium only). Addition of Tat or gp120 to the medium in the absence of NPC/GPCs did not change the migration of either BV-2 cells or primary microglia (Fig. 2.5 A–C, medium + Tat, medium + gp120). When NPC/GPCs were plated in a 24 well plate and transwell inserts were added after 5 days, secretions from NPC/GPCs did not alter BV-2 or primary microglial migration (Fig. 2.5 A–C, NPC/GPC). However, when the progenitors were pre-treated for 12 h with HIV-1 Tat the result was quite different (Fig. 2.5 A–C, NPC/GPC + Tat). In that case, there was a significant increase in migration of both BV-2 cells and primary microglia across the transwell membrane towards the Tat-treated progenitors. NPC/GPCs treated with gp120 did not elicit a similar enhancement of migration in either type of microglial cell.

As microglia can be attracted by signals from dying cells, we examined cell death using a fluorescent LIVE/DEAD assay kit (Invitrogen). Approximately 10% of the total Hoechst-labeled nuclei belonged to dead cells, but there were no significant differences

among treatments that might account for the additional BV-2 cell migration induced by HIV-1 Tat (n = 8; Fig. 2.6). This suggests that the microglial chemotaxis observed in our experiments is most likely to result from enhanced chemotactic signals released from progenitors stimulated by viral proteins. CCR5 is the common receptor for the b-chemokines that were up-regulated by Tat in progenitor cultures. To further confirm that chemokines up-regulated by Tat were contributing to the enhanced migration of microglial cells, we conducted experiments to neutralize CCR5. Both BV-2 cells and primary microglia were incubated for 1 h with the 2D7 antibody (sodium azide-free), which has been shown by others to be suitable for receptor blockade studies (De Clercq 2009; Jayasuriya et al. 2004; Lee et al. 1999). Figure 2.5 (B) and (C) shows, respectively, that treatment of both BV-2 and primary microglia with the 2D7 monoclonal CCR5 antibody prior to their addition to the transwell insert completely abolished the enhanced migration elicited by NPC/GPCs.

Discussion

HIV-associated neuropathology in the CNS is generally thought to result from the combined effects of direct viral protein toxicity, and inflammatory/toxic conditions propagated by surrounding glial cells. Because of our interests in the effects of co-morbidity between opiate drugs of abuse and HIV exposure in the CNS, we chose to examine the striatum, an area which is both rich in opiate receptors and extremely vulnerable to HIV-induced neuropathology. Our data show that progenitors from embryonic striatum secrete a variety of chemokines/cytokines in culture, and that exposure

to HIV-1 Tat_{1-86} selectively increases secretion of a subset of these. The concept that NPC/GPCs in the normal brain may secrete factors to regulate either their own function or fate, or that of surrounding cells, has been previously established (Benoit et al. 2001; Chang et al. 2003; Shingo et al. 2001; Taupin et al. 2000). Further, it is known that progenitors or stem cells from other regions have this ability. Mesenchymal stem cells, for instance, secrete multiple chemokine/cytokines and growth factors, including multiple FGFs, IGF-1, IL-1β, IL-6, CCL2/MCP-1, CCL3/MIP-1α, CCL4/MIP-1β, SDF-1, VEGF, and others (Chen et al. 2008; Croitoru-Lamoury et al. 2007; Liu and Hwang 2005; Rafei et al. 2008; Schinkothe et al. 2008; Wagner et al. 2007). Importantly, this is the first study to show that secretions from a neural progenitor population have a chemotactic effect on microglial cells, and that these secretions and their effects can be enhanced by exposure to HIV-1 Tat.

A previous study on E15 striatal progenitors grown as proliferating neurospheres (Benoit et al. 2001) examined some of the cytokines that we initially screened. In agreement, we both found a complete lack of IL-3, IL-4 and IL-1α secretion. However, our cells also secreted detectable levels of granulocyte macrophage colony-stimulating factor, IL-6, IL-9 and TNF-α, as well as five other chemokines/cytokines that were not assayed in the previous study. Our cells were grown in monolayers, and assayed at an earlier *in vitro* age using a different methodology, so these differences are perhaps not surprising. There are likely to be additional chemokine/cytokines secreted by striatal progenitors, as our initial screening process used the manufacturer's standard kit which assays for a group of 23 selected analytes.

The progenitor cultures from E15 striatum contain a mixture of cells at different stages of differentiation (Fig. 2.1). Immunocytochemistry showed that most expressed either nestin, Sox2, or both. While 20% expressed Olig2, a transcription factor that indicates commitment to an oligodendroglial fate, there was a complete absence of O_4 staining, suggesting that the Olig2$^+$ cells were still quite immature. Likewise, the 3% of cells that were GFAP$^+$ seemed quite immature based on morphology and variable co-expression of nestin or Olig2. As expected in a striatal progenitor culture, many of the cells were young neural precursors expressing DCX (~40%), but the absence of NeuN reactivity suggested their relative immaturity. Individual chemokines/cytokines appear to be secreted by cells across several stages of development, and there is also variability of expression within specific populations. For example, although most CCL5/RANTES$^+$ cells do not immunostain for Olig2, a subset are Olig2$^+$ (Fig. 2.3); and among Olig2$^+$ cells, only about 50% are CCL5/RANTES$^+$ (data not shown).

Regardless of the stage at which progenitors release these factors, our results show that the production of CCL5/RANTES, CCL3/MIP-1α and CCL4/MIP-1β, is significantly increased following exposure to HIV-1 Tat for 12 hr (Fig. 2.2). In contrast, exposure to gp120 did not affect levels of any of the chemokines/cytokines. It is unlikely that the very small populations of microglia (< 0.8%) or immature astroglia (3%) are responsible for either baseline or Tat-induced chemokine/cytokine secretion, especially as MIP-1α and MIP-1β were not been detected in our other studies. The earlier studies also showed that co-exposure to morphine enhanced Tat-induced CCL5/RANTES production by astroglia (El-Hage et al. 2008b; El-Hage et al. 2005). The lack of morphineinduced synergy in the present results also suggests that astroglia are not the primary source.

In striatal astroglia there is a synergistic effect of morphine on Tat-induced secretion of CCL2/MCP-1, CCL5/RANTES and IL-6. The lack of morphine-induced synergy in progenitor cultures is not because of a lack of appropriate receptors as most progenitors express MOR (Fig. 2.1) (Buch et al. 2007; Hauser et al. 2009). However, the signaling pathways leading to synergistic secretory responses may not be functional in immature cells. gp120 did not increase NPC/GPC secretion of the panel of analytes that we examined. This is similar to our observations in mature astroglia fromseveral brain regions (Fitting et al. 2010c), although gp120 from different HIV strains is variably reported to induce TNF-α, IL-1α, IL-1β, and IL-6 in other astroglial preparations (Buriani et al. 1999; Kong et al. 1996; Ronaldson and Bendayan 2006; Yeung et al. 1995). CD4 is the major receptor for gp120 binding and virus entry, and binding is facilitated by expression of CD4 co-receptors such as CCR5 and CXCR4. The strain of gp120 that we used (MN) preferentially uses the CCR5 co-receptor. Progenitors expressed abundant CCR5 (Fig. 2.6), but do not express CD4 and therefore may have limited ability to respond to gp120. Binding of chemokine ligand versus gp120 to a chemokine receptor may have fundamentally different consequences. For example, in a previous study, gp120 IIIb required 'priming' by exposure to soluble CD4 in order to elicit a simple CXCR4-mediated $Ca2^{+}$ response from similar murine progenitors (Tran et al. 2005).

As Tat exposure can clearly enhance chemokine secretion from progenitors, might this have functional consequences? A transwell assay showed that progenitors exposed to HIV-1 Tat for 12 h were able to enhance the directed movement of both BV-2 cells and primary microglia (Fig. 2.5). gp120 did not enhance chemokine production, so it was not surprising that progenitors treated with gp120 did not elicit the same migratory response

(Fig. 2.5). Importantly, blockade of the CCR5 receptor on microglial cells eliminated the enhanced migration (Fig. 2.5 B and C), showing that secretions of the CCR5 ligands RANTES, MIP-1α and MIP-1β, from progenitors have the potential to direct microglial movement. We believe that our data suggest chemotaxis, not just enhanced, random motility, as there is only a modest exchange of medium over the short assay period through the small membrane plating area. The BV-2 cells are plated into a well containing their original culture medium and are moving towards the continually renewed source of chemokines.

What are the potential consequences of chemokine/cytokine secretion by NPCs/GPCs, and the upregulation of β-chemokine production by HIV-1 Tat? These are best discussed considering the two very different contexts of interest – the normal brain and the HIV-infected brain. Ten of the chemokines/cytokines we examined were secreted at detectable levels by untreated progenitors, and several of these are chemoattractants for inflammatory cells. Under normal conditions, attracting monocytes/microglia may not be harmful. They serve diverse roles essential for normal brain function, independent of the innate immune response, which include providing trophic support, regulating neurogenesis and neuron development, and clearing toxic debris (Aarum et al. 2003; Block et al. 2007; Polazzi and Contestabile 2002; Simard et al. 2006; Walton et al. 2006; Whitney et al. 2009; Ziv et al. 2006). During HIV-infection there are different considerations. CCL5/RANTES, CCL3/MIP-1α and CCL4/MIP-1β are all known to act as endogenous suppressors of HIV infection and cell damage due to their ability to block gp120 binding with its CCR5 co-receptor (Catani et al. 2000; Cocchi et al. 1995), and both CCR5 and its ligands are the subject of ongoing investigation as therapeutic targets to reduce viral entry (reviewed in

DeVico and Gallo 2004; Vangelista et al. 2008). Thus, enhanced β-chemokine secretion might afford a degree of protection. However, all three β-chemokines are also implicated in HIV-neuropathology. Depending upon the severity of inflammation they may contribute to an environment that is detrimental to neurons. RANTES is a well known chemoattractant for T-cells, granulocytes, and monocytes, and is thought to recruit HIV-infected monocytes to the brain, either alone or through triggering MCP-1 production (Conant et al. 1998; El-Hage et al. 2005; Kelder et al. 1998; Luo et al. 2003; Schall et al. 1990). CCL3/MIP-1α and CCL4/MIP-1β are routinely produced by activated macrophages and attract monocytes as well as lymphocyte subpopulations including granulocytes and neutrophils (Menten et al. 2002). β-chemokines also stimulate synthesis and release of pro-inflammatory cytokines, are upregulated and expressed by astroglia and microglia in the HIV infected brain (Kelder et al. 1998; Letendre et al. 1999; Schmidtmayerova et al. 1996), and correlate positively with HIV dementia (Letendre et al. 1999). Thus, dysregulated β-chemokine expression by progenitors and glia might potentiate local inflammation, and might enhance local trafficking of HIV-infected cells into the brain. Supporting this concept, multiple indicators of inflammation were reduced after intrastriatal injection of HIV-1 Tat into RANTES/CCL5 deficient mice compared to control (El-Hage et al. 2008a).

The concept that chemokine/cytokine effects may differ in normal versus inflammatory situations is well-illustrated by considering CCL2/MCP-1. While CCL2/MCP-1 can be protective towards isolated neurons (Eugenin et al. 2003; Madrigal et al. 2009; Yao et al. 2009), in the context of HIV-1 infection, the negative effects of attracting infected monocytes and driving inflammation are likely to outweigh direct, neuroprotective effects observed in isolation. The complexity of these issues is clearly

highlighted by disparate results in two studies using mice deficient in CCR2, showing either protection (Eugenin et al. 2003; Madrigal et al. 2009; Yao et al. 2009) in the context of HIV-1 infection, the negative effects of attracting infected monocytes and driving inflammation are likely to outweigh direct, neuroprotective effects observed in isolation. The complexity of these issues is clearly highlighted by disparate results in two studies using mice deficient in CCR2, showing either protection (El-Hage et al. 2006a) or exacerbation (Yao et al. 2009) after intrastriatal Tat injection, depending on timing, region examined, and outcome measure.

Importantly, we found robust expression of CCL5/RANTES associated with some $Sox2^{+}$ cells in the striatum (Fig. 2.3 D), suggesting that secretion of chemokines by NPCs does occur *in vivo*, and in the adult CNS. Progenitors are a relatively small population in the mature brain and will have less influence on overall inflammatory tone than microglia and astroglia. However, we speculate that they may have an important influence on the local milieu, and particularly in situations where progenitor populations are increased, such as regions of active CNS repair, or during development. In the context of HIV, progenitors may particularly contribute to the neuropathology that occurs in young pediatric patients, where glial populations are still developing. HIV-1 associated progressive encephalopathy occurs in a relatively high percentage of pediatric HIV patients, where it develops aggressively and is frequently the earliest clinical manifestation of disease (Drotar et al. 1997; Ensoli et al. 1997; Schwartz and Major 2006; Van Rie et al. 2007). Chemokine production by progenitors might normally be an adaptive process, to encourage the immune surveillance and approval of cells before they make additional contributions to neural or glial populations. Enhanced secretion of chemokines in the context of HIV might

circumvent this adaptive strategy, by attracting HIV-infected microglia/monocytes to NPC/GPCs, which can be infected by HIV (Lawrence et al. 2004; Rothenaigner et al. 2007), and which appear to pass the virus to their neuronal and glial progeny (Lawrence et al. 2004).

In summary, we have shown that HIV-1 Tat increases production b-chemokines by striatal progenitors, resulting in the CCR5-mediated movement of microglial cells. As Tat mRNA and/or protein have been detected in CNS tissue samples from both patients with HIV-1 encephalitis and rhesus macaques infected with a chimeric HIV-SIV (SHIV) virus (Hudson et al. 2000), these findings have significant disease relevance. The effects of enhanced chemokine release will be contextual, because of complex interplay between multiple secretory and responder cell types *in vivo*, as well as temporal issues during normal and disease processes. Emerging evidence suggests that human neural progenitors may be cellular reservoirs of HIV infection (Lawrence et al. 2004; Rothenaigner et al. 2007; Schwartz and Major 2006), although the extent of NPC/GPC infection in the brain remains unclear. The potential contribution of this infected population to the chemokine milieu must also be considered.

Acknowledgements

We acknowledge generous NIH support from R01 DA024461 and P01 DA19398.

Table 2. 1: Chemokines/cytokines secreted by progenitor cells from E15 striatum (pg/ml)

	IL-1β	GM-CSF	TNF-α	IL-6	IL-9
C	<0.85 #	<1.17 #	<0.3 #	1.78±1.06	<20.4 #
M	1.10±0.25	<1.17 #	<0.3 #	<0.72 #	23.21±2.57
T	5.54±1.52	2.43±0.83	0.68±0.28	5.10±2.88	28.85±3.15
G	1.35±0.32	1.64±0.47	0.44±0.09	<0.72 #	27.54±6.53
N	<0.85 #	<1.17 #	1.27±0.21	<0.72 #	<20.4 #
MT	5.76±2.31	1.64±0.47	1.14±0.36	4.81±3.00	26.03±3.15
MG	<0.85 #	1.96±0.79	0.34±0.04	<0.72 #	<20.4 #
MTN	7.46±4.47	2.46±1.29	0.76±0.23	6.72±4.74	23.21±2.57
MTG	6.99±2.77	3.22±0.96	1.20±0.81	3.09±1.06	26.03±3.15
MTGN	9.87±4.02	1.81±0.64	0.51±0.14	1.78±1.61	<20.4 #
MN	<0.85 #	<1.17 #	<0.3 #	<0.72 #	<20.4 #
	KC	RANTES	MIP-1α	MIP-1β	MCP-1
C	6.70±2.42	5.74±0.56	57.86±15.59	135.5±36.09	68.77±26.9
M	6.21±2.52	5.09±0.35	26.94±0.02	98.07±22.08	57.03±24.12
T	25.40±10.81	336±26.8	312.5±25.11	957±102.5	183.6±78.2
G	7.63±2.29	6.34±1.03	119.5±62.8	273±185.1	94.26±38.65
N	6.11±2.39	3.59±0.81	26.95±0.0	75.17±16.64	58.83±23.72
MT	20.61±9.52	292±59.7	307.1±62.06	714.5±127.8	170.15±76.58
MG	5.74±2.03	5.06±1.09	48.63±21.69	94.12±17.35	47.85±16.08
MTN	32.62±15.89	397±105	376.8±104.6	1009.1±194	237.2±131.6
MTG	24.12±9.13	271.7±32.33	321.9±61.6	945±165.9	167.9±75.83
MTGN	24.81±9.51	336.3±28.31	376.0±46.9	1063±116.3	124.8±75.26
MN	5.85±2.58	4.08±0.58	26.94±0.0	66.93±18.06	55.79±21.8

[a]Sample concentration was determined to be below the accuracy limit of the assay software, based on the standard curve generated for the individual chemokine/cytokine. In those cases, we have indicated the lower limit value in pg/mL. For all samples, n = 6.

The following proteins were not routinely detected in control or treated cells:

IL-1α, IL-2, IL-3, IL-4, IL-5, IL-10, IL-12(p40), IL-12(p70), IL-13, IL-17, G-CSF, Eotaxin, IFNγ.

C, control; M, morphine; Tat, transactivator of transcription; G, gp120; N, naloxone.

Figure 2.1

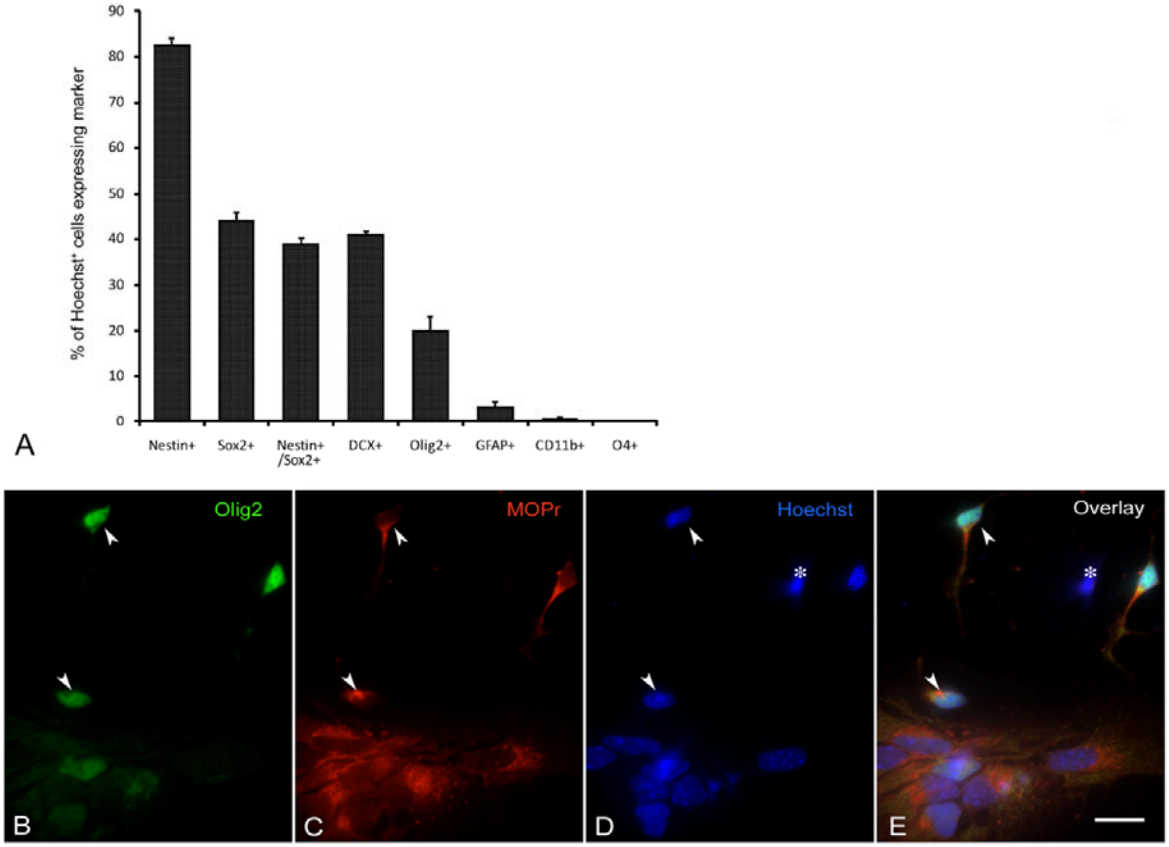
A
% of Hoechst+ cells expressing marker
90
80
70
60
50
40
30
20
10
0
Nestin+
Sox2+
Nestin+ /Sox2+
DCX+
Olig2+
GFAP+
CD11b+
O4+
Olig2
MOPr
Hoechst
Overlay
B
C
D
E

Figure 2.1 Characterization of progenitor cultures from E15 striatum. Cultures were immunostained after 5 days for typical markers of neural and glial precursors, and for MOR. (a) Most cells stained for nestin (> 80%) and/or Sox2 (> 40%), indicating that they were relatively immature precursors. Approximately 40% were DCX^+ and 20% were $Olig2^+$, indicating entry into the path leading towards a neuronal or oligodendrocyte fate, respectively, while less than 3% of cells were $GFAP^+$. Many cells that expressed DCX, Olig2 or GFAP also expressed nestin or Sox2, suggesting very recent entry into a specific lineage. $CD11b^+$ microglia were < 0.8% of the population. None of the cells stained for O4, a marker of more mature OLs. (B–E) Triple labeling shows that most precursors in these cultures, regardless of differentiation state, express MOR (red, B and D). The $Hoechst^+$ nucleus of a cell that is MOR) is indicated by (*) in panel (d). Two $Olig2^+$ cells the express MOR are indicated by arrowheads in each panel. Scale bar = 10 μM.

Figure 2.2

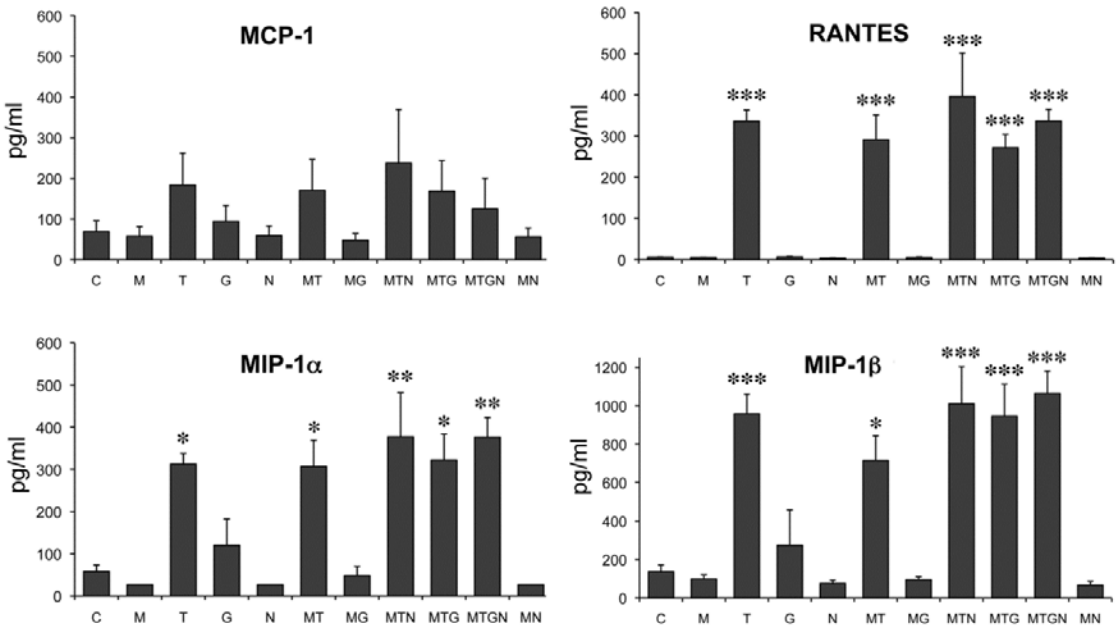

Figure 2.2 Exposure to HIV-1 Tat1-86 increases progenitor secretion of a select group of chemokines. Cells were treated at 5 days after plating with morphine (M), Tat (T), gp120 (G), and naloxone (N) either alone or in combinations as indicated. Of 23 factors tested, 10 were secreted by unstimulated cells (see Table 1). Only RANTES, MIP-1α, and MIP-1β showed a significant change in concentration after 12-h treatment. Cells responded to Tat, but not to gp120. There was no synergistic response of concurrent exposure to either morphine or gp120. MCP-1 showed a similar trend towards a Tat response, but the effect was not significant. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ vs. untreated control cells (C), ANOVA and post-hoc Bonferroni's test. n = 6 independent cultures.

Figure 2.3

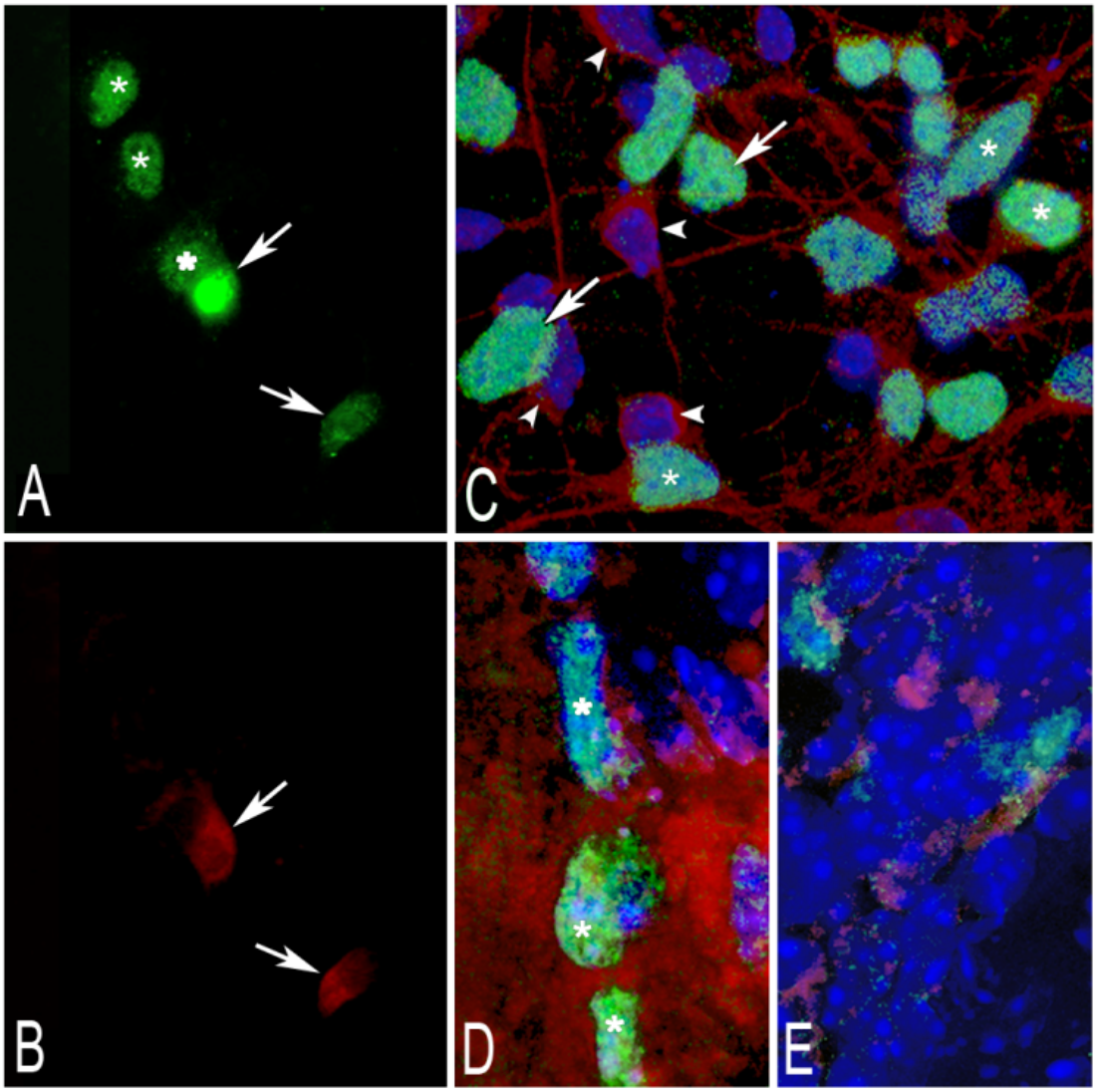

Figure 2.3 Localization of CCL5/RANTES in NPC/GPCs *in vitro* and *in vivo*. Cultured cells were immunostained (A–C) at 5 days *in vitro*. *In vivo* staining (D and E) was done in adult striatum. (A and B) NPC/GPCs were immunostained with an antibody to Olig2 (A) to identify oligodendroglial precursors, and an antibody to CCL5/RANTES (B) to determine which cells are producing this chemokine. Of the five $Olig2^+$ cells in this field, two show CCL5/RANTES expression in their cytoplasm (arrows indicate double-labeled cells). Three other cells that are $Olig2^+$ (asterisks) do not express detectable levels of CCL5/RANTES. For (C–E), Z stacked images (AxioVision software, version 4, Carl Zeiss, Inc.) were taken through the entire depth of the cell layer (C) or section (D and E), then optimized for deconvolution microscopy (AutoQuant X Version 2, Media Cybernetics, Bethesda, MD, USA) in order to better demonstrate sub-cellular details of Sox2 and CCL5/RANTES co-localization. (C) Cells in the same NPC/GPC cultured stained for Sox2 (green) and CCL5/RANTES (red). The $Sox2^+$ cells are a mixture of $CCL5^+$ (asterisks) and CCL5) (arrows). Note also that many $CCL5^+$ cells are Sox2) (arrowheads). Hoechst staining (blue) identifies the nuclei of all cells in the field. (D and E) Co-localization of Sox2 (green) and CCL5/RANTES (red) in the adult mouse striatum *in situ*. Hoechst staining (blue) identifies nuclei of all cells in the field. As CCL5 is a secreted, soluble chemokine, it is difficult to associate specific cell boundaries with CCL5 immunostaining. However, it can be appreciated from panel (D) that $Sox2^+$ cells (asterisks) in the striatum are frequently associated with significant CCL5 immunostaining. The region shown in (E) has a high density of $Sox2^-$ cells, as indicated by the Hoechst staining, and little CCL5 signal.

Figure 2.4

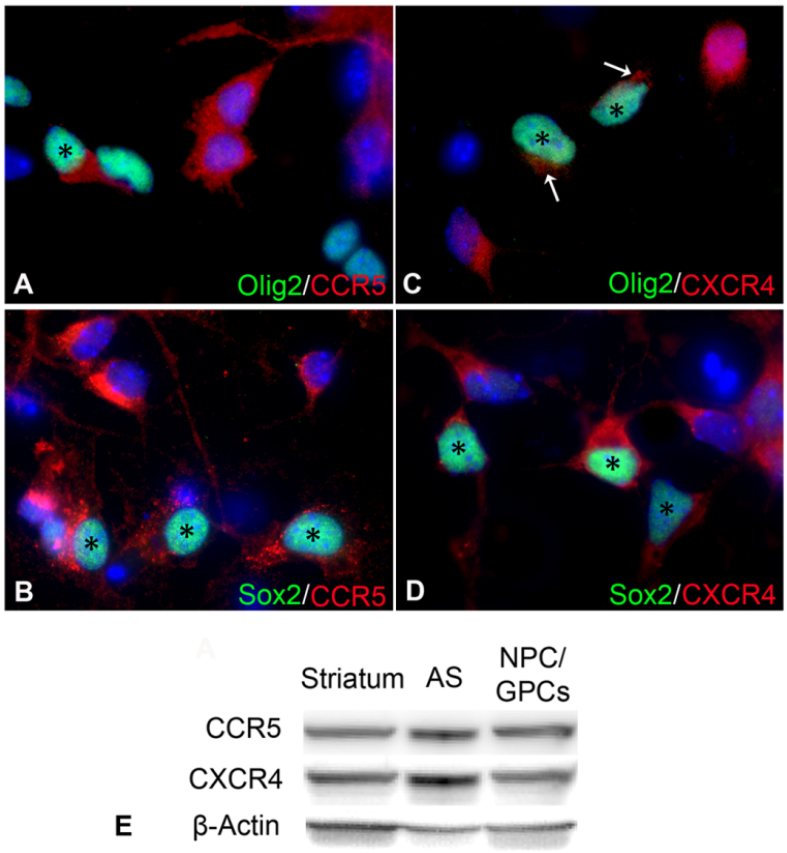

Figure 2.4 NPC/GPCs express both CCR5 and CXCR4 *in vitro*. Olig2^{+} (green) oligodendroglial precursors show both CCR5 (A) and CXCR4 (C) immunoreactivity. The signal for CXCR4 was generally light in these cells, and is indicated by arrows in (C). Undifferentiated Sox2^{+} NPC/GPCs (green) also are immuno-positive for CCR5 (B) and CXCR4 (D). Asterisks in (A–D) indicate double-labeled cells. Hoechst33342 nuclear staining (blue) shows that some cells positive for CCR5 or CXCR4 do not express Olig2 or Sox2. (E) A major band at approximately 40 kDa was noted on immunoblots of NPC/GPCs harvested at 5 DIV using antibodies to both CCR5 and CXCR4. Actin detection is included as a loading control. These bands are also detected in homogenates of cultured astroglia (AS) and whole striatum, preparations that are known to express both receptors.

Figure 2.5

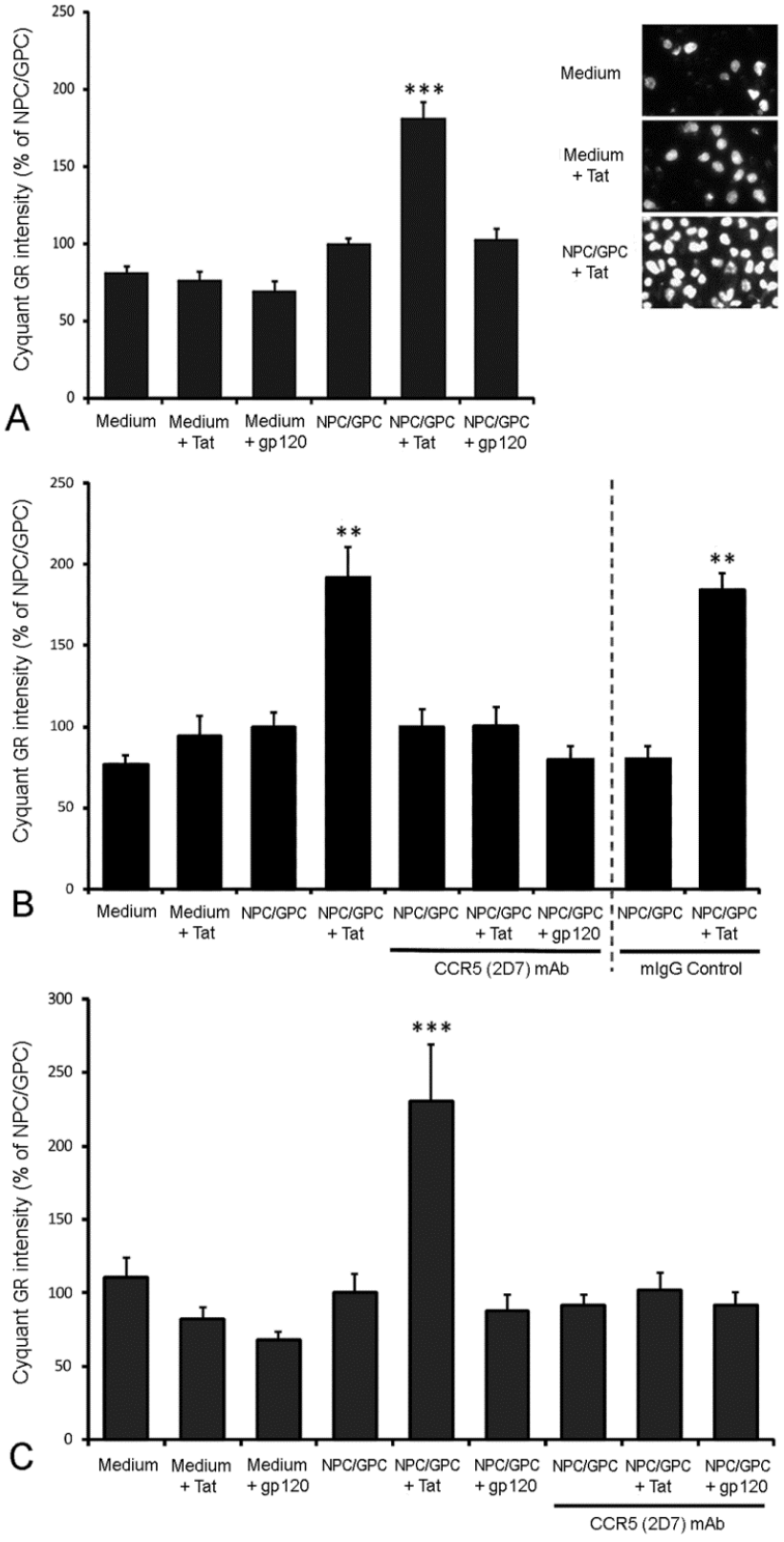
Cyquant GR intensity (% of NPC/GPC)
250
200
150
100
50
0
***
Medium
Medium + Tat
NPC/GPC + Tat
A
Medium
Medium + Tat
Medium + gp120
NPC/GPC
NPC/GPC + Tat
NPC/GPC + gp120
B
**
**
NPC/GPC + gp120
CCR5 (2D7) mAb
mIgG Control
C
300
***
CCR5 (2D7) mAb

Figure 2.5 Chemotaxis of BV-2 cells and primary microglia in response to NPC/GPCs in transwell cultures. (A) NPC/GPCs grown on a 24-well plate were treated with Tat or gp120 for 12 h prior to the addition of transwell inserts containing BV-2 cells. Transwell inserts were removed after 3 h, and cells crossing the insert membrane were labeled with CyQuant® GR dye and quantified on a PHERAstar FS plate reader with appropriate filters. Migration of BV-2 cells was not affected when either Tat or gp120 alone was added to the medium. Untreated NPC/GPCs also had no effect on BV-2 migration. However, the number of BV-2 cells moving across the transwell membrane was significantly enhanced when NPC/GPCs were pre-treated with Tat for 12 h (***$p < 0.001$ vs. all other groups; n = 6 independent cultures). Pre-treatment of NPC/GPCs with gp120 did not affect BV-2 migration. The inset image shows Hoechst staining of BV-2 cells, and illustrates the effect of NPC/GPC treated with Tat to enhance migration. (B) Antibody neutralization of CCR5 on BV-2 cells significantly attenuated Tat-enhanced migration. BV-2 cells on transwell inserts were preincubated with either 2D7 anti-CCR5 monoclonal antibody or control IgG2a for 1 h at 4˚C, then placed into wells containing treated or untreated NPC/GPCs. Incubation with 2D7 antibody, but not control IgG2a, reduced BV-2 cell migration in the NPC/GPC + Tat treatment group back to basal levels. This suggests that the enhanced microglial migration caused by secretions from Tat-treated NPC/GPC is entirely mediated by CCR5-signaling. Antibody neutralization did not reduce migration in other treatment groups to below basal levels. (**$p < 0.01$ vs. all other groups; n = 6 independent cultures). (C) Studies in (A) and (B) were repeated on primary murine microglia with similar results. Migration of primary microglia was significantly enhanced by the presence of NPC/GPCs pre-treated with Tat, although there was no effect of gp120 pre-treatment. Incubation with

2D7 antibody reduced migration in the NPC/GPC + Tat treatment group back to basal levels, but did not affect migration in other treatment groups. (***$p < 0.001$ vs. all other group; $n = 4$ independent cultures).

Figure 2.6

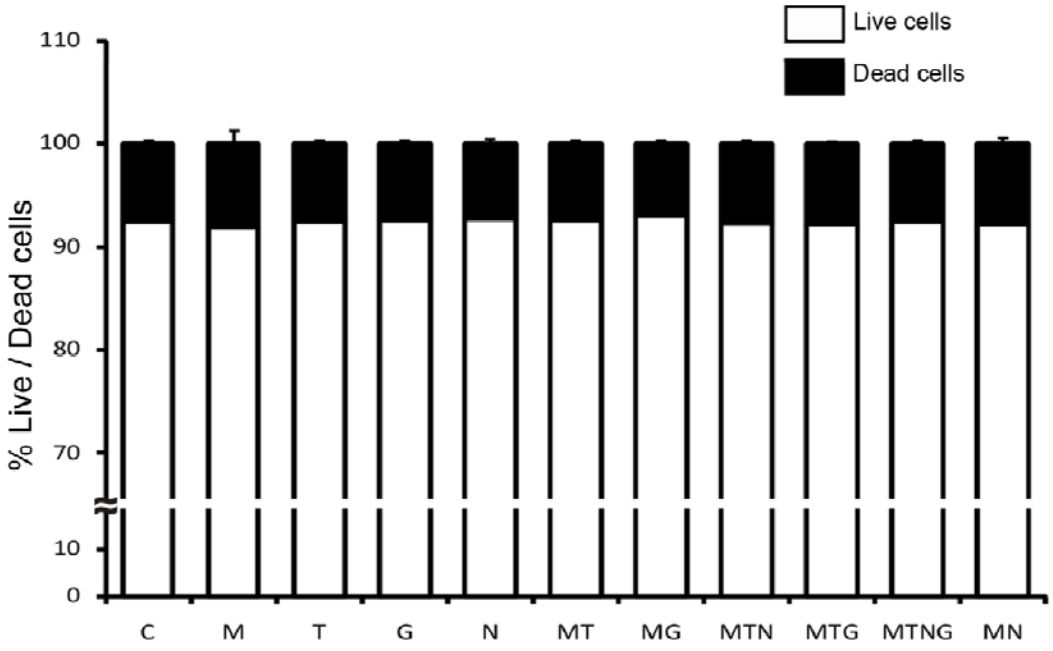

Figure 2.6 A fluorescent viability assay reveals that 12-h exposure to HIV-1 Tat_{1-86} or gp120 alone or in combination with opiates did not increase the rate of NPC/GPC cell death *in vitro*. Cells were treated at 5 days after plating with morphine (M), Tat (T), gp120 (G), naloxone (N) either alone or in the combinations indicated. There was no significant increase in cell death in any treatment group. n = 8 independent cultures

Chapter 3

HIV-1 Alters Neural and Glial Progenitor Cell Dynamics in the CNS: Coordinated Response to Opiates During Maturation

(This chapter was submitted as a paper to Glia and is accepted pending revision.

Introduction

Morbidity and mortality due to human immunodeficiency virus (HIV-1) infection has significantly decreased since the introduction of combined anti-retroviral therapy (cART). cART treatment has also changed the landscape of central nervous system (CNS) disorders. Fewer patients exhibit HIV associated dementia (HAD), but there is increased prevalence of asymptomatic and mild-to-moderate HIV-associated neurocognitive disorders (HAND) among patients with absence of peripheral viremia (Cysique et al. 2004; Gonzalez-Scarano and Martin-Garcia 2005; Gonzalez et al. 2002; Sacktor 2002; Simioni et al. 2010). Factors contributing to CNS pathology and dysfunction include an increased life expectancy of HIV-1 patients receiving cART, and potential toxic effects of long-term anti-retroviral exposure (Apostolova et al. 2011; Clifford 2002). In addition, many HIV therapeutics do not readily penetrate the blood brain barrier, leaving a reservoir of HIV in the CNS.

Microglia are the principal source of active HIV infection in the brain; astroglia can also be infected but tend not to produce new virus (Gonzalez-Scarano and Martin-Garcia 2005; Jordan et al. 1991; Kaul 2008). Several studies suggest that CNS neural progenitor cells (NPCs), the undifferentiated precursors of both neurons and glia, are also susceptible to

HIV-1 infection (Lawrence et al. 2004; Rothenaigner et al. 2007; Schwartz et al. 2007), but the extent that this contributes to the spectrum of CNS disorders remains unclear. Even if NPCs are not readily infected, they may be affected either by HIV-1 proteins directly (Buch et al. 2007; Hahn et al. 2010b; Krathwohl and Kaiser 2004b; Lee et al. 2011; Mishra et al. 2010; Okamoto et al. 2007; Peng et al. 2008), or secondarily by changes in the extracellular milieu. NPCs are widely distributed in germinative zones within the developing CNS. Neurons in most brain regions are formed prenatally, but production of astroglia and oligodendroglia continues into the postnatal period in both rodents and humans (Chan et al. 2002; Geha et al. 2010; Lee et al. 2000; Skoff 1990; Skoff and Knapp 1991; Suh et al. 2007). NPCs also are present in the adult CNS. While neurogenesis occurs in specific regions, including the subgranular zone in the dentate gyrus of the hippocampus and the subventricular zone of the lateral ventricles (Doetsch et al. 1997; Eriksson et al. 1998; Kaplan and Hinds 1977; Kornack and Rakic 1999), glial progenitors are found throughout the adult CNS. There is a slow turnover of glial cells throughout life (Goldman and Sim 2005; Imamoto and Leblond 1978; Kornack and Rakic 1999; Kornack and Rakic 2001; Liu et al. 2004; Messier et al. 1958; Sturrock 1979), and more active gliogenesis occurs during situations of injury/disease (Burns et al. 2009; Rola et al. 2006; Romanko et al. 2004). Glial progenitors constitute 1-to-4% of the cells in normal adult brains (Picard-Riera et al. 2004; Reyners et al. 1986).

To test the hypothesis that HIV-1 infection can affect the dynamics of cell production during development, we used stage-specific markers to follow changes in the proliferation rate and populations of NPCs (nestin^{+} and Sox2^{+} [SRY (sex determining region Y)-related HMG (high mobility group)-box gene2]) and their glial progeny both in culture and in the

intact brain during the perinatal period. Opioid receptors are present and functional on NPCs and glial progenitors in adult and neonatal rodent and human CNS (Buch et al. 2007; Hauser et al. 2009; Persson et al. 2003; Reznikov et al. 1999; Rius et al. 1991; Tripathi et al. 2008). Since co-exposure to opiate drugs of abuse enhances HIV-related deficits in the CNS in patients and experimental models (Anthony and Bell 2008; Bell et al. 2006; Burdo et al. 2006; Byrd et al. 2011; Hauser et al. 2007; Kopnisky et al. 2007), we also examined interactive opiate effects. HIV-1 Tat was used to model HIV exposure both *in vitro* and *in vivo*. We also assessed progenitor responses in a more clinically relevant model by exposing human NPCs (hNPCs) to supernatant from HIV-infected monocytes ± opiates. Results showed that HIV-1 Tat or morphine alone reduced NPC and glial progenitor proliferation and/or altered the population dynamics of murine progenitors both *in vitro* and *in vivo*. In certain subsets of progenitors, co-exposure to morphine accelerated the timing and extent of Tat effects. Similarly, the proliferation of hNPCs was reduced by exposure to Tat or R5 HIV-1_{SF162}, and there was an interactive opiate-virus effect. Findings support the concept that HIV ± opiates can affect the expansion of specific CNS progenitor populations. We speculate that this might alter the balance of glia and neurons in the CNS of pediatric HIV patients. Since NPCs and glial progenitors in adult share certain characteristics with those found in neonatal brains, our findings might also relate to adult HIV patients. At either age, an altered progenitor pool is predicted to adversely affect CNS function.

Materials and Methods

Experimental protocols conformed to Virginia Commonwealth University Institutional Animal Care and Use Committee and national (PHS) guidelines on the care and ethical use of animals. Experiments were designed to minimize the total number of animals used and their discomfort.

Murine neural progenitor cell cultures

Murine NPCs were cultured from striata of embryonic day 15 (E15) mice (ICR strain; Charles River, Boston, MA) using modifications of a published technique (Khurdayan et al. 2004). In brief, embryos were harvested aseptically, and striata from 10 to 12 embryos were dissected from the cortex. Striata were dissected out, finely minced and incubated with DNase (0.015 mg/mL, Sigma-Aldrich Co., St. Louis, MO) in Dulbecco's Modified Eagle's Medium (DMEM) (15 min, 37ºC). Tissue was triturated, centrifuged (5 min at 1000 rpm), and resuspended in 5 mL of defined, NPC maintenance medium consisting of 1:1 DMEM/F12 (Invitrogen Corp., Carlsbad, CA), 10% Knockout® SR (Invitrogen; a serum replacement product), insulin (25 μg/mL), transferrin (100 μg/mL), progesterone (20 nM), putresceine (60 μM), Na-HCO_3 (6 mM), and sodium selenite (30 nM) (all from Sigma-Aldrich). Cells were filtered through a 70-μm nylon cell strainer (BD Falcon, Bedford, MA), counted, centrifuged as above, and resuspended in an appropriate volume of the same medium to obtain desired plating densities. A total of 5×10^4 cells were plated on poly-L-lysine coated coverslips, and medium was refreshed every 2-3 d until treatment started. Cells were grown at 37ºC in a humidified atmosphere of 5% CO_2. After 5

d, the medium was replaced and cells were treated with Tat_{1-86} (100 nM; ImmunoDiagnostics, Woburn, MA), ± morphine sulphate (500 nM; NIDA Drug Supply System, Rockville, MD), ± naloxone (1.5 μM; Sigma-Aldrich) for 12 h, 24 h, or 48 h. Morphine, the major bioactive product of heroin in the brain, is a potent μ-opioid receptor (MOR) agonist; naloxone is a broad spectrum opioid receptor antagonist. These concentrations of Tat_{1-86} and morphine have been established to elicit functional effects in astroglia, oligodendrocytes (OLs), and neural progenitors in our earlier studies (Buch et al. 2007; El-Hage et al. 2005; Hauser et al. 2009; Khurdayan et al. 2004). Naloxone was applied 30 min prior to other treatments, which were added simultaneously in pre-warmed medium. Lastly, 5-bromo-2′-deoxyuridine (BrdU, Sigma-Aldrich) was added to the medium at 12 h prior to harvest (10 μM) to detect proliferating cells in S phase.

Opiate delivery to prenatal and postnatal Tat transgenic mice *in vivo*

For *in vivo* studies in perinatal mice, we utilized Tat transgenic mice engineered to express the HIV-1 Tat_{1-86} protein under control of a glial fibrillary acidic protein (GFAP) promoter in a doxycycline-inducible manner. As previously described (Bruce-Keller et al. 2008; Fitting et al. 2010b; Hauser et al. 2009), mice expressing the *tat* gene under control of a *tet* responsive element (TRE) in the pTREX vector (Clonetech, Mountain View, CA) were crossed with mice expressing the reverse tetracycline transactivator (RTTA) driven by a human GFAP promoter. Inducible Tat^+ transgenic mice express both GFAP-RTTA and TRE-Tat genes; Tat^- mice express only the GFAP-RTTA gene. To induce Tat production during the pre- and early postnatal period, pregnant dams were given chow containing doxycycline (Harlan, Indianapolis, IN; 6 mg/g) from gestational day 15-16 until

postnatal day 7. Doxycycline, a water-soluble tetracycline derivative that crosses the placenta and is secreted in breast milk, is reported to reliably induce transgene expression in embryos and postnatally via the *tet*-on system during perinatal development (Fedorov et al. 2001; Perl et al. 2002a; Perl et al. 2002b; Shin et al. 1999). All dams received the same chow, to control for effects of doxycycline unrelated to activation of the transgene in Tat^- pups. Standard PCR procedures were performed on genomic DNA isolated from ear clip samples to confirm the genotype of individual mice. Morphine was delivered by subcutaneous (*s.c.*) injection (NIDA Drug Supply System) at a dose of 2 mg/kg in a 20 µl volume of saline, based on the average daily litter weight. Male, P4 to P7 Tat^+ mice received a morphine injection twice daily at 10:00 am and 6:00 pm. For sham pups, 20 µl of saline was injected *s.c.* twice daily. According to a previous report, 2 mg/kg of morphine *s.c.*, based on the average daily litter weight, gave circulating morphine levels that approximate the range measured in human preterm infants who are given an intermittent or continuous intravenous morphine infusion (Vien et al. 2009). For BrdU labeling *in vivo*, 50 µl of 10 mg/ml BrdU (32.5 mM) was injected *s.c.* in sterile saline twice at 2 h intervals immediately prior to perfusion. Fewer than 5% of the neonatal mice in our studies died, and there was no relationship between morphine-induced toxicity and either genotype or doxycycline administration. For immunohistochemistry studies, pups were deeply anaesthetized with isoflurane (Baxter, Deerfield, IL) prior to perfusion with 4% paraformaldehyde (pH 7.4). After perfusion, brains were removed and post-fixed in fresh fixative overnight. Brains were hemisected, rinsed several times and overnight in 15 ml changes of phosphate-buffered saline, cryopreserved through graded sucrose solutions (10 and 25 %), embedded in Tissue Tek OCT compound (Sacura Finetek, Torrance, CA), and

stored at –80˚C. 10 μm frozen sections containing the striatum were thaw-mounted on SuperFrost Plus slides (VWR Scientific, West Chester, PA) and processed for immunostaining.

Immunohistochemistry and quantification

For *in vitro* studies, NPCs on coverslips were immunolabeled after 12, 24, and 48 h treatment, using antibodies to cell-specific and lineage-specific markers including nestin (1:5000, Aves Labs, Inc., Tigard, OR), Olig2 (oligodendrocyte transcription factor 2; 1:100, Immuno-Biological Laboratories, Minneapolis, MN), and Sox2 (1:200, Millipore, Temecula, CA). Cells were fixed with 4% paraformaldehyde; for intracellular markers, cells were permeabilized with 0.1% Triton X-100 in phosphate-buffered saline containing 1% bovine serum albumin (Sigma-Aldrich) for 30 min. Antibodies were applied overnight at 4ºC, followed by visualization with appropriate fluorescent-conjugated second antibodies (Invitrogen). Double-staining was performed with anti-BrdU (1:200, Abcam, Cambridge, MA) and anti-Sox2 or anti-Olig2 to detect proliferating progenitors. Coverslips were treated with 1 N HCL in PBS to expose incorporated BrdU followed by immunostaining for precursor markers. BrdU antibody was applied overnight at 4ºC, followed by appropriate fluorescent-conjugated second antibodies. Immunolabeled coverslips were finally incubated with Hoechst 33342 dye (0.5 μg/ml, 8 min) to identify nuclei (Molecular Probes/Invitrogen), then mounted in ProLong Gold anti-fade reagent (Molecular Probes/Invitrogen) and dried in the dark. To assess the proportion of different progenitors in the cultures, 300–350 $Hoechst^{+}$ cells were selected randomly per coverslip and assessed for nestin, Sox2, and Olig2 (n = 6) under oil immersion at 63× using a Zeiss

AxioObserver system with integrated MRm camera system (Carl Zeiss, Inc., Thornwood, NY). Proliferation was assessed in the overall NPC population by scoring 300-350 Hoechst$^+$ cells for expression of BrdU. Proliferation was also assessed in each progenitor population by scoring BrdU expression in 200 nestin$^+$, Sox2$^+$, and Olig2$^+$ cells on each coverslip. Similar immunodetection methods were applied to 10 μm tissue sections from neonatal brains. The proportion of different cell types *in vivo* was estimated by averaging the percentage of Sox2$^+$ or Olig2$^+$ cells among 250–350 randomly selected Hoechst$^+$ cells per striatum (left side) in two sections, in a total of n = 6 mice. Proliferation was assessed in the same mice by scoring BrdU expression in 200 cells positive for each progenitor marker in two striatal sections per mouse. Double-immunolabeling was performed to assess whether hNPCs expressed the mu opioid receptor (MOR) and C-C chemokine receptor type 5 (CCR5) *in vitro*. MOR (1:250, Antibodies Incorporated, Davis, CA) and CCR5 (1:200, BD Pharmingen, Mountain View, CA) antibodies were applied to fixed hNPCs and incubated overnight at 4°C, then visualized as described above, prior to labeling with NPC markers and Hoechst 33342. p24 was detected on hNPCs after exposure to HIV-1_{SF162}, with an antibody commonly used to study infective processes (1:50, Dako, Carpinteria, CA) (Eugenin et al. 2011; Hahn et al. 2008; Lawrence et al. 2004). Images were taken under oil immersion at 63× using a Zeiss AxioObserver and integrated MRm camera system.

Viability assay

Mouse NPCs were cultured on poly-L-lysine coated 48 well plates and treated at 5 d after plating with Tat, morphine and naloxone either alone or in combination as in

experiments described above. After 12, 24, and 48 h, medium containing treatments was replaced with sterile, tissue culture–grade D-phosphate-buffered saline containing the final concentration of 4 μM ethidium homodimer-1 (EthD-1) and 5 μg/mL Hoechst 33342. The working solution was added directly to cells and incubated for 15 min, at 37˚C. The intensity of EthD-1 and Hoechst signals was measured using a PHERAstar FS plate reader (BMG LABTECH, Ortenberg, Germany) in n = 8-10 different cultures using appropriate excitation and emission filters (EthD-1: 495/635 nM; Hoechst: 350/510 nM).

RNA Extraction and Reverse transcription PCR

Non-quantitative RT-PCR was performed to confirm expression of Tat mRNA in the striatum of 7 day postnatal pups treated with doxycycline administered through placenta and lactation as described. Pups were deeply anaesthetized with isoflurane prior to perfusion. The striata were rapidly dissected from brains, and stored at -80°C. Total RNA was isolated using TRIzol reagent (Invitrogen). Each RNA sample was cleaned using GenElute^{TM} Mammalian Total RNA kit (Sigma-Aldrich), after RNase-free DNase I treatment to eliminate genomic DNA. cDNA was synthesized from 2 μg of total RNA using the High-Capacity cDNA Archive Kit (Applied Biosystems, Warrington, UK) per manufacturer's instructions. PCR procedures were performed to detect Tat mRNA expression using primers previously described with 18S mRNA as a control (Bruce-Keller et al. 2008; Hauser et al. 2009) Tat1 5′-ATGGAGCCAGTAGATCCTAG-3′, Tat2, 5′-TCATTGCTTTGATAGAGAAACTTG-3′, rTta1 5′-AATCGAAGGTTTAACCCG-3′, rTta2, 5′-TTGATCTTCCAATACGCAACC-3′, 18S1 5'-CACTTGTCCCTCTAAGAAGTTG-3', 18S2 5'-GACAGGATTGACAGATTGATAG-3')

(Chauhan et al. 2003). Final PCR products were separated by electrophoresis using a 1.5% agarose gel, stained with ethidium bromide, and visualized using a Kodak 440CF Image Station (Rochester, NY).

Viral stock preparation

HIV-1_{SF162} viral stock was obtained from Dr. Jay Levy through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, HIV (Cheng-Mayer and Levy 1988). The R5 strain was propagated in a U937 monocyte cell line (ATCC, Chicago, IL). U937 cells were activated with IL-2 (10 U/ml) and phytohaemagglutinin (1 mg/ml) for 2 d in RPMI with 10% fetal calf serum. The peak of infection was monitored by p24 ELISA, and the culture supernatant was collected and passed through a 0.2 μm filter and stored at -80˚C. For FACS analysis, 250 μl of media containing 500 pg virus measured by p24 ELISA was added to hNPCs grown to 75-80% confluence in each well of 6-well plates, with a final volume of 1.5 ml per well. The viral exposure time was 12 h.

Human neural progenitor cell line culture and CCR5 blocking assay

These studies used the human neural progenitor ReNcell VM cell line, derived from 14-wk gestation human ventral mesencephalon (Millipore). This cell line contains over 95% $Sox2^+$ and $nestin^+$ cells, and has the potential to produce dopaminergic neurons (Donato et al. 2007). For all experiments, cells frozen at passage 3 were thawed and expanded on laminin-coated T75 cm^2 tissue culture flasks (Corning) in ReNcell NSC Maintenance Medium (Millipore) supplemented with epidermal growth factor (EGF) (20 ng/ml; Millipore) and basic fibroblast growth factor-2 (FGF-2) (20 ng/ml; Millipore). Three to

four days after plating (i.e., prior to reaching 80% confluence), cells were passaged by detachment with accutase (Millipore), centrifuged at 300 × g for 5 min and the cell pellet resuspended in fresh maintenance media containing EGF and FGF-2. For FACS analysis experiments, cells were replated in laminin-coated 6-well plates at a density of 30,000 cells per well. Three to four days after plating, cells were treated for 12 h with Tat or HIV_{SF162} ± morphine ± naloxone. For the CCR5 blocking experiment, a non-signaling monoclonal antibody (2D7 antibody) to CCR5 (2 μg/ml; BD Pharmingen) (Lee et al. 1999) or mouse IgG (2 μg/ml; BD Pharmingen) were applied 1 h prior to treatment. The hNPCs were treated with BrdU solution (10 μM) 2 h prior to harvesting. For p24 immunolabeling, 5,000 cells per well were replated on laminin-coated coverslips in 24-well plates and treated with HIV-1_{SF162} prior to reaching 80% confluence as described above. All experiments utilized cells from passage 4 to minimize changes in cell characteristics over multiple passages that might influence the results.

FACS analysis of hNPC proliferation and p24 expression

The APC BrdU Flow Kit (BD Pharmingen) was used to determine the proportion of hNPCs in S-phase of the cell cycle. Cells were incubated with BrdU (10 μM) for the final 2 h of a 12 h exposure to Tat or to supernatant from HIV-1_{SF162} infected monocytes ± morphine. The cells were detached using enzyme-free cell dissociation buffer (Sigma-Aldrich) and fixed with BD Cytofix/Cytoperm buffer (containing 4% paraformaldehyde). After permeabilization, cells were treated with DNase to expose BrdU incorporated in DNA. An APC-conjugated anti-BrdU antibody and 7-amino-actinomycin D (7-AAD) were used to determine cell cycle phases (G_o/G_1, S, G_2+M) in all hNPC groups. Fluorescent

signals for APC (Ex: 650 nm; Em: 615 nm) and 7-AAD/DNA complexes (Ex: 546; Em: 647) were detected on a FACSCanto (BD Biosciences, San Jose, CA) and analyzed using the DIVA FACS data program (BD Biosciences). Single and intact hNPCs were first gated by cell area and width. APC-BrdU fluorescence intensity was assessed after gating on approximately 3 x 10^5, 7-AAD$^+$ cells. G_o/G_1 phase cells (APC-BrdU$^-$ and 7-AAD$^+$) were defined using appropriate negative (non-specific IgG followed by APC-conjugated secondary antibody only); and positive (7-AAD only; APC-anti-BrdU antibody only) controls. S-phase cells were defined as being APC-BrdU$^+$ and 7-AAD$^+$.

After 12 h exposure to supernatant from HIV-1_{SF162} infected monocytes, p24 expression on hNPCs was determined by FACS analysis. Cells were fixed and immunolabeled with p24 antibody and Alexa Fluor 488-conjugated second antibody, followed by incubation in 7-AAD. Single and intact hNPCs were gated as above. The intensity of p24 immunolabeling was assessed on approximately 3 x 10^4 intact, 7-AAD$^+$ cells. A single laser (488 nm) and two color fluorescence emission (585/42 band pass; 530/30 band pass) approach was used to account for autofluorescence. Controls included p24 labeling in untreated cells and non-specific IgG followed by second antibody in HIV-treated cells.

Statistical analysis

Significance was assessed by analysis of variance (ANOVA) followed by Duncan's post-hoc testing if indicated, using StatSoft software (Statistica, Tulsa, OK).

Results

Effect of HIV-1 Tat and morphine on murine neural progenitors *in vitro*

To determine whether Tat_{1-86} and morphine affect the maturation of NPCs either independently or interactively, NPCs from embryonic mouse striata were treated with 100 nM Tat_{1-86} ± morphine (500 pM) over a period of 48 h, with BrdU added for the final 12 h. Double-label immunostaining was performed following 12 h, 24 h and 48 h treatments for BrdU and three lineage specific markers, nestin, Sox2, and Olig2. Nestin and Sox2 are markers of uncommitted NPCs (Blumcke et al. 2001; Episkopou 2005; Gu et al. 2002; Nunes et al. 2003; Qu and Shi 2009), while Olig2 is a transcription factor expressed in more differentiated cells that develop largely into oligodendrocytes (OLs) and motor neurons (Masahira et al. 2006; Takebayashi et al. 2002)**.** Treatment with Tat ± morphine for 12 h did not alter either the proportion of $nestin^+$ ($nestin^+$/$Hoechst^+$) or $Olig2^+$ ($Olig2^+$/$Hoechst^+$) cells, or the percentage of $nestin^+$ or $Olig2^+$ cells expressing BrdU (Fig. 3.1 A and D, C and F). There were, however, significant effects on the subset of NPCs expressing Sox2. While neither morphine or Tat alone had any effect, both the percent of proliferating $Sox2^+$/$BrdU^+$ cells, and the total $Sox2^+$ cell population were reduced when cells were exposed concurrently to Tat + morphine (Fig. 3.1 B and E). Since only $Sox2^+$ cells were affected after 12 h, we hypothesized that the sensitivity of NPCs to Tat ± morphine might depend on their stage of differentiation, and that changes in the Sox2 cells might affect their progeny. We extended the experiments to 24 h and 48 h using markers for Olig2 (Fig. 3.2). At both 24 h and 48 h, the total $Olig2^+$ population was reduced by Tat alone with no added effect of morphine (Fig. 3.2 C, D). The proliferating ($Olig2^+$ / $BrdU^+$)

population at 24 h was unaffected by either Tat or morphine alone, but Tat and morphine interacted to reduce BrdU uptake (Fig. 3.2 A). By 48 h, Tat alone, but not morphine alone, had reduced the proliferating, $Olig2^+$ population. Tat and morphine interacted at this time point to further decrease BrdU uptake (Fig. 3.2 B). Thus, interactive effects of Tat and morphine were only observed on the proliferative behavior of $Olig2^+$ cells, while Tat by itself affected cell populations. Naloxone partially reversed the interactive effects of morphine in proliferating cells, as the groups receiving Tat, morphine, and naloxone were not different from control, but also not different from the group co-exposed to Tat and morphine. It also appeared to reduce the overall numbers of $Olig2^+$ cells when given concurrently with Tat and morphine. We conducted the additional experiments described below to understand these findings.

Effect of naloxone on murine progenitors *in vitro*

Although naloxone partly or fully reversed the effects of morphine in most instances, naloxone exposure also decreased the percentage of cells expressing Olig2 when combined with morphine and Tat treatment ("a" in Fig. 3.2 D and E). We thus treated murine NPCs with naloxone alone for 12-48 h and compared the proportion of $Olig2^+$ cells in untreated control and naloxone treated groups. There was no significant reduction in the $Olig2^+$ population (Fig. 3.2 E), suggesting that naloxone by itself is not toxic to $Olig2^+$ cells or their immediate progenitors. Instead, it might interfere with endogenous opioid signals under the specific condition of Tat and morphine treatment. Autocrine opiate signaling pathways are known to play pivotal roles in OL survival (Buch et al. 2007; Hauser et al. 2009; Khurdayan et al. 2004; Knapp et al. 2001), and overall glial cell fate (Gorodinsky et

al. 1995; Kim et al. 2006; Persson et al. 2003). Naloxone might interfere with multiple opioid pathways that modulate proliferation and development of OL lineage specific progenitors under these experimental conditions.

Effect of HIV-1 Tat and morphine on murine neural progenitors: proliferation vs. viability

To determine whether cell death might contribute to the observed reductions in proliferating or total NPCs, we examined the viability of total NPCs at up to 48 h of exposure to Tat ± morphine using an ethidium-based viability assay. There was no significant cell death in response to any treatment at any time period measured by EthD-1 fluorescence intensity (Fig. 3.3).

Effects of HIV-1 Tat and morphine on neural progenitors in striatum

To assess the effect of HIV-1 Tat and morphine on NPCs *in vivo*, we utilized an inducible, tetracycline (tet) "on" system with doxycycline induction. Detection of Tat mRNA and protein in the brains of these mice, including the striatum, was previously reported by our group, (Bruce-Keller et al. 2008; Duncan et al. 2008; Fitting et al. 2010b; Hauser et al. 2009) and also shown here (Fig. 3.4 A-C). Pregnant dams received chow containing doxycycline from gestational day 15-16 through postnatal day 7 to activate the HIV-1 Tat transgene in fetal and neonatal mice. Reverse transcription PCR was performed to detect Tat mRNA expression in cortex and striatum of neonatal mice at P7 (Fig. 3.4 B). Evaluation of PCR band intensities confirmed that doxycycline delivered to dams induced

Tat mRNA expression in the cortex and striatum of Tat^+, but not Tat^- neonatal mice (Fig. 3.4 C).

All mice used in experiments were male. Some mice received morphine s.c. at postnatal days 4–to-7 to assess whether co-exposure to HIV-1 Tat and morphine caused effects on NPCs *in vivo* similar to those seen in culture. There were several significant effects on proliferating cell populations. Morphine exposure and Tat induction independently reduced BrdU uptake in both $Sox2^+$ and $Olig2^+$ cells in the striatum, and in the overall population of striatal NPCs (Fig. 3.4 D). An interactive effect of morphine and induced Tat was seen in the $Sox2^+/BrdU^+$ population and in the overall population of $BrdU^+$ cells (Fig. 3.4 D). We examined whether this might lead to changes in the overall populations of $Sox2^+$ and $Olig2^+$ cells. Morphine and Tat by themselves each reduced the percentage of the population that was $Sox2^+$ or $Olig2^+$ (Fig. 3.4 E); there was no interactive effect between them. Additionally, Tat alone, but not morphine alone, reduced the percentage of the population that was $Olig2^+$; again there was no Tat and morphine interaction.

Effects of HIV-1 Tat, HIV_{SF162}, and morphine on proliferation of a human NPC line

To validate our *in vitro* and *in vivo* results in a more clinically relevant system, we exposed human NPCs to HIV-1 $Tat_{1\text{-}86}$, or to supernatant from U-937 monocytes infected with R5-tropic $HIV\text{-}1_{SF162}$, with or without concurrent morphine. Before conducting experiments, we briefly characterized the hNPC cell line by co-localizing MOR, nestin, and/or Sox2 antigenicity. In accordance with previous reports (Breier et al. 2008), more than 95% of hNPCs expressed nestin and Sox2. Approximately 95% of hNPC also showed

MOR immunoreactivity, suggesting their potential as targets of opiate signaling (Fig. 3.5 A and B). Olig2 immunofluorescence was not detected in hNPCs (not shown). Since we used an R5-tropic HIV strain that preferentially uses C-C chemokine receptor type 5 (CCR5) as a co-receptor for viral entry, we co-localized CCR5 immunoreactivity in Sox2-immunopositive NPCs to verify expression (Fig. 3.5 C).

FACS analysis was performed after 12 h exposure to Tat ± morphine to assess the percentage of hNPCs in S-phase of the cell cycle, as an index of proliferation. We also tested the effect of HIV-1_{SF162} supernatant from infected cells that had been treated ± morphine. Due to their short cell cycle (Breier et al. 2008), hNPCs were treated with BrdU 2 h prior to harvest. FACS analysis showed a significant reduction in S-phase hNPCs in all treatment groups (Fig. 3.5 D). Approximately 47% of control hNPCs incorporated BrdU. Both Tat and morphine independently caused a significant reduction in the proportion of S-phase cells as compared to control, with no synergistic or additive effect of morphine (Fig. 3.5 D, E). Interestingly, hNPCs exposed to HIV-1_{SF162} supernatant by itself also showed significantly reduced proliferation, and there was an additional reduction in their proliferation rate with combined morphine (Fig. 3.5 D, F). HIV-1_{SF162} utilizes CCR5 as a co-receptor for entry, and although the changes in NPC function are not likely due to infection, some effects might be due to viral proteins such as gp120 binding to CCR5 on hNPCs. To examine this question, the specific, non-signaling, CCR5 blocking antibody 2D7 (Lee et al. 1999; Wu et al. 1997) was applied 1 h before exposure to viral supernatant. Compared to treatment with control mouse IgG, 2D7 immunoblockade partially reversed the anti-proliferative effect of HIV-1_{SF162} supernatant (Fig. 3.5 G).

p24 expression in hNPCs by FACS analysis and immunolabeling

hNPC infection by HIV has been sporadically reported by other investigators (Lawrence et al. 2004; Mishra et al. 2010; Schwartz et al. 2007). We assessed hNPCs in the present study for the presence of p24 antigen with an antibody commonly used by other laboratories to study infective processes (Eugenin et al. 2011; Hahn et al. 2008; Lawrence et al. 2004). hNPCs were exposed for 12 h to HIV-1_{SF162} infective supernatant, then fixed and either detached from the plate, stained with 7-AAD, immunolabeled with p24 antibody, and subjected to FACS analysis (Fig. 3.6 A-C), or immunolabeled for fluorescent microscopic analysis of p24 within individual cells (Fig. 3.6 D, E). Immunoreactivity was most often observed in cells that were clearly pyknotic (arrowheads, Fig. 3.6 D, D'). This may represent non-specific binding of antibody to cell debris, or infection and subsequent death of hNPCs; our methods cannot distinguish between these two possibilities. Occasional hNPCs with a normal cellular and nuclear morphology, indicating viability at the time of fixation, were also $p24^+$ (Fig. 3.6 E, E'). For FACS studies, hNPCs were fixed and immunolabeled with the same p24 antibody and Alexa Fluor 488–conjugated second antibody, followed by 7-AAD. Approximately 1 x 10^4 intact, 7-AAD^+ cells were analyzed by a single laser (488 nm), two color fluorescence (585/42 band pass; 530/30 band pass) methodology that permits assessment of autofluorescence. Neither control (Fig. 3.6 B, C) nor experimental (Fig. 3.6 A) groups showed any evidence of autofluorescence. A $p24^+$ signal was detected in 0.34% of the overall population (Fig. 3.6 A). Controls included p24 labeling in untreated cells (Fig. 3.6 B) and non-specific IgG followed by second antibody in HIV-treated cells (Fig. 3.6 C); p24 was detected in 0.1% and 0.001% of the control populations, respectively.

Discussion

NPCs are present throughout ontogeny, including in the adult brain, although the size of the NPC population is substantially reduced with age, as is their distribution and recruitment. We used both murine and human embryonic cultures, as well as an *in vivo* perinatal model, to determine the ability of HIV-1 Tat, or viral supernatant, to dysregulate the proliferation of NPCs, and to affect resulting cell populations in developing systems. Exposure to Tat or supernatant from HIV-1_{SF162}-infected cells both reduced progenitor proliferation, although exposure time and developmental stage were critical variables. Morphine interacted with both Tat and infective supernatant to cause further dysregulation. Results suggest that HIV exposure, and co-exposure to opiates, elicit fundamental changes in NPC function that likely contribute to neurologic impairment in both pediatric and adult patients. Morphine by itself caused changes in the behavior of NPCs in mice exposed during the perinatal period that may be relevant to children exposed to morphine or heroin before birth.

HIV-1 Tat and opiates alter proliferation/population dynamics of murine NPCs *in vitro*

Previous studies have shown effects of HIV-1 Tat on the expansion, viability, and/or defined populations of fetal human or murine NPCs or glially-restricted progenitors in culture, without determining if cells at a particular stage of maturation might be selectively affected (Buch et al. 2007; Khurdayan et al. 2004; Mishra et al. 2010). We were especially interested to test whether opiate exposure might enhance Tat effects, since opiates are a co-

morbid factor that can amplify CNS dysfunction, both in experimental models of HIV (Bokhari et al. 2011; Bruce-Keller et al. 2008; Fitting et al. 2010b; Hauser et al. 2006; Hu et al. 2005; Kumar et al. 2006; Zou et al. 2011) and in patients (Anthony et al. 2005; Bell et al. 2002; Byrd et al. 2011). Studies both *in vitro* and *in vivo* examined the striatum, an area that is rich in opiate receptors and extremely vulnerable to HIV-1-induced neuropathology (Berger and Nath 1997; Mirsattari et al. 1998; Nath et al. 2000; Nath et al. 2002). Murine NPCs derived from E14 striatum were characterized previously as a mixture of progenitors and very immature cells (nestin$^+$, Sox2$^+$, DCX$^+$, Olig2$^+$) (Hahn et al. 2010b). Most cells were actively proliferating and expressed MOR (Fig. 3.5) (Hauser et al. 2009).

We distinguished between cells expressing nestin or Sox2, markers of relatively undifferentiated NPCs, and Olig2, a marker typical of developing OLs (Masahira et al. 2006; Takebayashi et al. 2002). Tat and/or morphine had effects at specific stages of differentiation. As early as 12 h after co-exposure to morphine and Tat, the proliferation rate of Sox2$^+$ cells was reduced (Fig. 3.1 B) and the total Sox2$^+$ population was diminished (Fig. 3.1 E). With increasing time, Olig2$^+$ proliferation was also affected, first by Tat and morphine co-exposure, and then by Tat alone, with combined Tat and morphine leading to an additive effect (Fig. 3.2 A, B). Since Tat by itself did not affect proliferation until 48 h, and did not affect viability (Fig. 3.3), it was surprising that Tat by itself reduced the percentage of total cells that expressed Olig2 at both 24 and 48 h (Fig. 3.2 C, D). A potential explanation that was not explored is that Tat may influence NPC lineage decisions, perhaps stalling cells at the Sox2$^+$ stage, or increasing the production of GFAP$^+$ or NG2$^+$ cells. In keeping with this idea, an increase in the production of GFAP$^+$ cells was noted in human progenitors exposed to HIV-1 (Peng et al. 2008).

Treatment with naloxone partially reversed morphine–Tat interaction in most *in vitro* studies. However, in the Olig2 population studies, treatment with naloxone decreased the percentage of Hoechst$^+$ cells that expressed Olig2 (indicated by "a" in Fig. 3.1 and 2). This effect only occurred for Olig2$^+$ cells, and not for nestin$^+$ or Sox2$^+$ cells. These results were reminiscent of *in vivo* studies with another non-selective opioid receptor antagonist, naltrexone, which was shown to modulate NPC proliferation in adult rodent hippocampus (Holmes and Galea 2002; Ra et al. 2002) and developing rat brain (Schmahl et al. 1989). However, we found that naloxone did not directly affect the Olig2$^+$ population (Fig. 3.2 E). We have previously suggested that naloxone or naltrexone may interfere with endogenous opiate signaling pathways that modify the survival and development of OL lineage cells (Hauser et al. 2009; Knapp et al. 2001; Knapp et al. 1998). The present study suggests a more complex phenomenon, since naloxone reversal was only partial, and particularly impaired in the presence of combined Tat and morphine. CCR5, an HIV co-receptor, has well documented interactions with MOR, leading to bi-directional, heterologous cross-desensitization (Chen et al. 2004; Chen et al. 2007; Suzuki et al. 2002; Szabo et al. 2002; Szabo et al. 2003). Tat is known to elevate the β-chemokine ligands that activate CCR5 (RANTES, MIP-1α, and MIP-1β) in multiple cell types including NPCs (Hahn et al. 2010b), which may interfere with naloxone activity through the above mechanisms.

HIV-1 Tat and opiates alter proliferation/population dynamics of murine NPCs *in vivo*

We used inducible HIV-1 Tat_{1-86} transgenic mice to model Tat and opiate exposure *in vivo*. While all animal models of a human-specific disease have limitations, these mice

have been extensively characterized and shown to exhibit neurologic abnormalities associated with chronic HIV-1 exposure, including activation of microglia and astroglia (Bruce-Keller et al. 2008), dendritic damage and reduced spine density in striatal neurons, abnormal oligodendroglial phenotypes (Fitting et al. 2010b; Hauser et al. 2009), circadian rhythm abnormalities (Duncan et al. 2008), and behavioral changes (Fitting et al, *submitted*). Since our culture experiments used E14 tissue grown for 7 d *in vitro*, we performed *in vivo* studies at a similar time. Results may therefore have particular relevance to pediatric HIV patients, but may also pertain to the smaller population of progenitors in adult brain parenchyma.

We focused on $Sox2^+$ and $Olig2^+$ cells, which are relatively abundant in striata as compared to $nestin^+$ cells. *In vivo* results paralleled the tendency towards a decline in proliferation and progenitor populations seen in cultured cells, but there were notable differences. Of great interest, in mice exposed only to morphine (Tat^- + Morph in Fig. 3.4 D, E), BrdU incorporation was significantly reduced in both $Sox2^+$ and $Olig2^+$ cells, as well as in the overall $Hoechst^+$ cell population. The $Sox2^+$ population was also decreased in this group, although surprisingly the $Olig2^+$ population was stable. Morphine alone did not affect these parameters in cultures. Previous studies of morphine and proliferation have been directed towards understanding the dynamics of neuron production, and it is clear that morphine reduces neuron generation in neonatal (Kornblum et al. 1987; Seatriz and Hammer 1993) and adult rodent brain (Arguello et al. 2008; Eisch et al. 2000; Kornblum et al. 1987). Similar to our results, Kornblum et al. (1987) found that morphine reduced proliferation *in vivo*, but had no effect on tissue dispersed in culture. They postulated an indirect mechanism for morphine-induced changes in progenitor dynamics; our data would

support that concept. Our studies modeled morphine exposure acutely to avoid issues of tolerance and dependence that occur during chronic opiate exposure.

Similar to morphine, the effect of Tat alone was to decrease BrdU uptake into $Sox2^+$, $Olig2^+$, and total cells (Fig. 3.4 D), but in all cases Tat induction caused significantly greater reduction than morphine exposure (Fig. 3.4 D). The greater effect of Tat on BrdU uptake *in vivo* may reflect the longer Tat exposure time (12 d), as BrdU uptake in cultured $Olig2^+$ cells was reduced after 48 h but not earlier. Both $Sox2^+$ and $Olig2^+$ populations were reduced by Tat alone (Fig. 3.4 E), probably due in part to decreased proliferation. As was true in cultures, the only interactive effect between morphine and Tat was on BrdU uptake. This was observed in $Sox2^+$ and total cells, but not in $Olig2^+$ cells, even though co-exposure reduced BrdU uptake into $Olig2^+$ cells in culture. Given the differences in timing and dosing of morphine, these somewhat different responses in culture versus *in vivo* are not surprising.

We consider it likely that the observed effects of Tat, HIV and/or morphine on progenitors will modify the balance of glia and neurons in the CNS, but a different approach, including stereology, is required to determine exactly how mature populations are affected. Glial populations may be preferential targets, since production of both astroglia and oligodendroglia continues well into the postnatal period in both rodents and humans (Altman 1966; Chan et al. 2002; Price 1994; Skoff and Knapp 1991; Skoff et al. 1976; Sturrock 1979; Webster and Astrom 2009). The present studies predict that OLs may be quite vulnerable to HIV-1 Tat, and this has been observed in the striatum of mature, Tat-transgenic mice, especially when the mice are co-exposed to morphine (Hauser et al. 2009).

Tat, HIV-1_{SF162}, and morphine alter proliferation of hNPCs

Since HIV is a human specific disease, the results of our murine models were re-evaluated in a human NPC line generated from 14-wk gestation human ventral mesencephalon. Over 95% of cells are immunopositive for nestin and/or Sox2, and many also expressed MOR and CCR5 (Fig. 3.5 A-C). Tat, HIV-1_{SF162}-infective supernatant, and morphine all individually reduced proliferation after 12 h, with viral supernatant and morphine displaying additive effects (Fig. 3.5). Supernatant and Tat both decreased proliferation to the same extent. In general, these results are in accord with a previous report that 25-100 ng/ml Tat decreased human fetal progenitor proliferation (Mishra et al. 2010). Isolated Tat effects were greater in that study, perhaps reflecting different cell derivations and culture conditions. Gp120 also was reported to reduce human (Okamoto et al. 2007) and mouse progenitor proliferation (Lee et al. 2011). A decrease in young neurons produced in the presence of Tat (Mishra et al. 2010) may also reflect an effect on cell fate, as hypothesized for certain of our findings. Cell heterogeneity may be an important consideration in our results, since all $nestin^+$ or $Sox2^+$ cells do not necessarily express equivalent amounts of functional MOR, and may not uniformly express molecular targets for Tat, such as α v β 5-integrin (Vogel et al. 1993), mannose (Liu et al. 2004) or NMDA receptors (Li et al. 2008), or the low-density lipoprotein receptor-related protein (Liu et al. 2000). The gradually increasing effects of Tat ± morphine during the exposure period may involve a change in receptivity of the cells with development. Interestingly, studies by another group, also using an R5 virus, showed an increase in progenitor production (Peng et al. 2011; Peng et al. 2008). While timing or culture variability might underlie the different outcomes, another critical difference may be the method for assessing

proliferation. BrdU uptake into individual cells after a 2 h pulse gives an immediate picture of DNA synthesis in individual cells. Studies by Peng et al. (2011 and 2008) assayed [^{3}H]-thymidine uptake into the population after 16 h, which reflects a combination of DNA synthesis, division, survival, and further differentiation over time. Those studies demonstrated that exposure to infected supernatant causes re-direction of progenitors towards an astroglial fate, a possibility also suggested by our findings and by others (Mishra et al. 2010). Although concurrent morphine exposure did not alter Tat effects on hNPCs, there was an additive effect of morphine and HIV-1_{SF162} supernatant (Fig. 3.5). Effects of supernatant by itself or with morphine may be due to multiple factors or interactions, since supernatant contains virions, multiple viral proteins, and cytokines/chemokines from infected cells.

HIV-1 has been reported to infect hNPCs (Lawrence et al. 2004; Rothenaigner et al. 2007; Schwartz et al. 2007). We performed FACS analysis and immunohistochemistry after 12 h exposure to infective supernatant to examine whether hNPCs in our system were infected. FACS analyses showed that < 0.04% of intact hNPCs were immuno-positive for p24 (Fig. 3.6 A), and we very occasionally detected cells with a viable morphology that appeared to be $p24^+$ by immunocytochemistry (Fig. 3.6 E, E'). Although our experience does support to the concept that hNPCs can be infected by HIV-1, it was not a common event under our experimental conditions. Most progenitors in the brain appear to respond as bystanders to events triggered by infected cells.

Relationship to HIV Pathology in Humans

Prior to the advent of anti-retroviral therapy, the prevalence, rate of onset, and severity of HIV-1-associated CNS disease in children was quite high as compared to adult patients (Mintz 1994; Schwartz et al. 2007; Schwartz and Major 2006). This is not surprising, since much of CNS development occurs after birth, and the immature brains of children or adolescents exposed to HIV are highly vulnerable to pathologic agents. Over-expression of excitatory amino acid receptors during periods of active synapse formation may in particular contribute to excitotoxic injury (Epstein and Gelbard 1999). CNS disease in pediatric HIV patients manifested as either a static or progressive encephalopathy, with classic symptoms that included delay or loss of major motor and mental milestones, as well as atrophy of cortical/subcortical regions and other abnormalities on CT scans (Drotar et al. 1997; Epstein et al. 1988; Epstein et al. 1985; Schwartz and Major 2006; Tahan et al. 2006; Ultmann et al. 1985; Van Rie et al. 2007). In developed countries, the rate of HIV-1 vertical transmission has been reduced to 1-2%, from 40% in 1995 (Buchholz et al. 2010; Van Dyke 2011). Treatment with CNS-penetrating drugs has, by most accounts, reduced the incidence of pediatric and adolescent HIV-associated encephalopathy by ≥ 50%, especially in cases of perinatal acquisition (Patel et al. 2009). It is currently estimated that 10% of HIV^+ children on cART therapy will show signs of CNS disease (Chiriboga et al. 2005). The situation in resource-limited settings is quite different. According to a 2008 estimate, 1,600 children per day become infected with HIV-1 worldwide, 90% of them in sub-Saharan Africa (UNAIDS 2009; UNAIDS 2010). In such regions, mother-to-child transmission of HIV via breastfeeding is common, even in infants not infected at birth, and

HIV-1 infection is still a major source of infant mortality (Becquet et al. 2009; Meyers et al. 2007).

In the context of pediatric HIV, reduced progenitor production and development are likely to have serious consequences. Reduced production of $Sox2^+$ progenitors is expected to impact the populations of neurons and glia that derive from $Sox2^+$ cells. Indeed, we find that immature, $Olig2^+$ OLs are reduced *in vivo*, and others have reported lower neuron production, possibly through altered cyclin D1 and MAPK signaling (Mishra et al. 2010; Okamoto et al. 2007). Although the hypothesis that Tat exposure can alter lineage decisions in uncommitted progenitors remains to be fully tested, a proportional increase in astroglia might enhance interactions between astroglia and HIV-1 infected macrophages/microglia that are deleterious for developing neurons and neural circuitry (Blumberg et al. 1994). Our finding of significant Tat-morphine and HIV-morphine interactive effects is clinically relevant, given the high rate of opiate abuse in HIV patients, and the fact that morphine may initially be used to treat neonatal abstinence syndrome in children of opioid-maintained or non-maintained mothers (Bandstra et al. 2010; Colombini et al. 2008; Ebner et al. 2007; Jansson et al. 2009).

Our results may also be relevant for the adult CNS. If the Tat effects, and morphine-Tat interactions that we observed also occur on adult progenitors, the predicted outcome would be fewer overall NPCs, a tendency towards increased astroglia at the expense of oligodendroglial production, and abnormal neural function. Importantly, neural precursors were reduced in human hippocampal slice cultures by HIV coat proteins and patient CSF (Krathwohl and Kaiser 2004b) and in gp120 transgenic mice (Lee et al. 2011; Mishra et al. 2010; Okamoto et al. 2007). Neural precursors were also reportedly reduced in HIV

patients with neurological deficits as compared to HIV-1 infected individuals without neurological deficits and non-infected controls (Krathwohl and Kaiser 2004b).

Summary

Consistent findings that Tat and HIV reduce NPC production in a variety of model systems that assess maturation are compelling. By disrupting the proliferation of NPCs and their subsequent differentiation into mature glia and neurons, HIV infection may increase the severity of neurological deficits and impairment in pediatric and adolescent patients. Interactions between Tat/HIV and morphine may exacerbate neurologic problems in young HIV patients receiving opiates prescribed for neonatal abstinence syndrome, pain, or other valid reasons, as well as HIV-1^{+} adolescents experimenting with abused opiates. Evidence that some neurologic abnormalities in the pediatric HIV population may be reversible (McCoig et al. 2002) is encouraging from a therapeutic standpoint, and points to the need for full understanding of how HIV and opiates individually and interactively affect CNS progenitors. This is especially true in resource-limited environments, where the spectres of perinatal HIV infection and high rates of pediatric disability/mortality have not been diminished.

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Development Award (DA027374). The authors would especially like to thank Dr. Nazira El-Hage for her help with the viral studies.

Figure 3.1

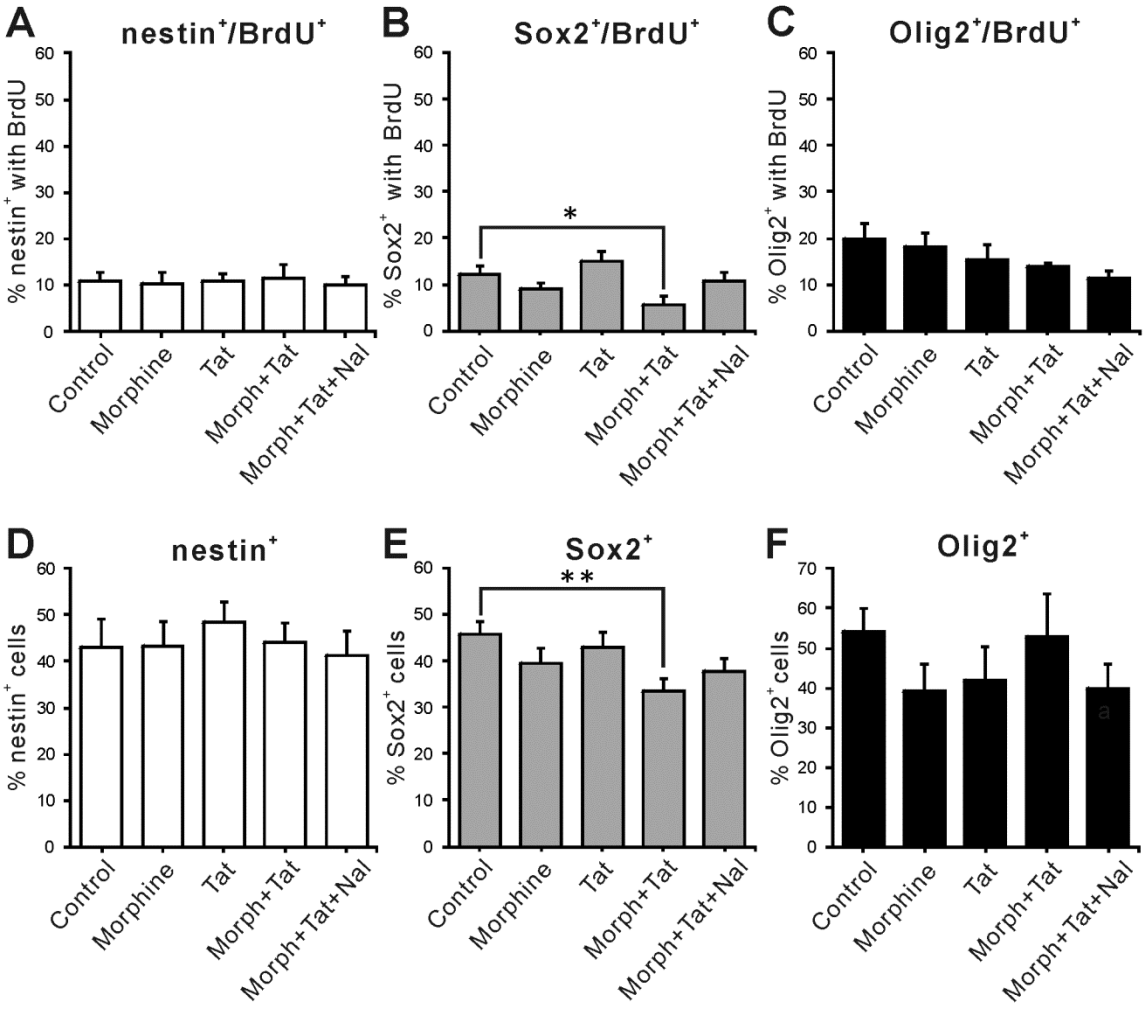

Figure 3.1 Co-exposure to HIV Tat and morphine for 12 h alters the Sox2$^+$ population and proliferation of Sox2$^+$ cells *in vitro*. Neither the nestin$^+$ (A, D) nor the Olig2$^+$ (C, F) populations were affected by any combination of Tat, morphine, or naloxone treatment at 12 h. However, both the percentage of the population that was Sox2$^+$, and the proliferation of cells at this stage of differentiation were decreased in response to Tat and morphine co-exposure (B, E). (* $p<0.05$, ** $p<0.01$, a <0.05 vs. control; with Duncan post-hoc test, n = 4-6, Morph = Morphine (500 nM), Tat (100 nM), Nal = naloxone (1.5 μM)).

Figure 3.2

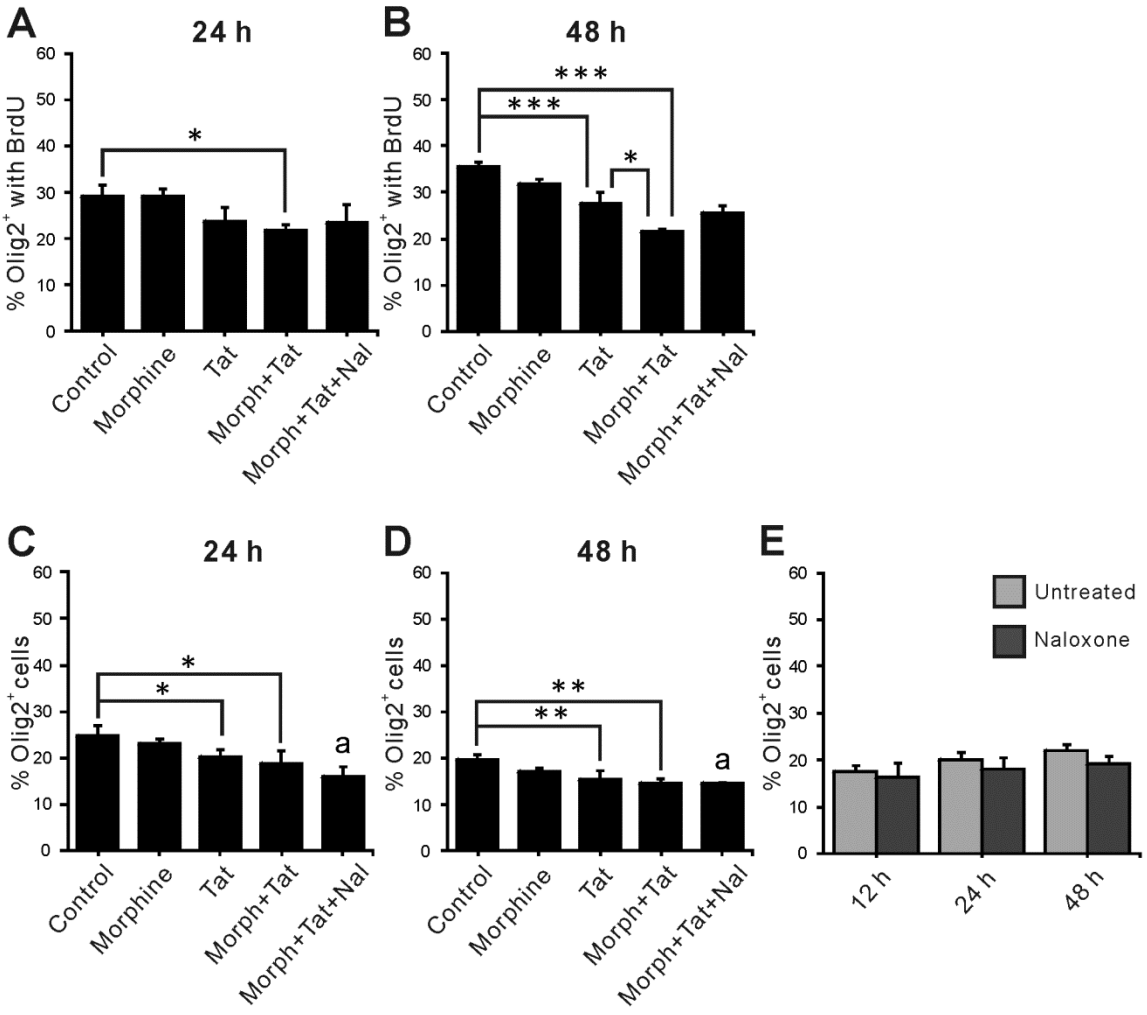
A
24 h
% Olig2+ with BrdU
B
48 h
% Olig2+ with BrdU
C
24 h
% Olig2+ cells
D
48 h
% Olig2+ cells
E
% Olig2+ cells
Untreated
Naloxone
Control
Morphine
Tat
Morph+Tat
Morph+Tat+Nal
12 h
24 h
48 h
a

Figure 3.2 Effects of HIV-1 Tat ± opiates on the population and proliferation of $Olig2^+$ cells, at 24 – 48 h *in vitro*. Interactive effects of morphine and Tat to reduce $Olig2^+$ cell proliferation were observed at both 24 h and 48 h (A and B); Tat by itself also reduced $Olig2^+$ proliferation, but only at the 48 h time point (B). Tat by itself reduced the percent of $Olig2^+$ cells in the overall population, after both 24 h (C) and 48 h (D) of exposure; there were no additional effects of morphine co-exposure. Morphine by itself did not affect populations of $Olig2^+$ cells, or their proliferation indices. Naloxone partially reversed the effect of morphine in both A and B; the percentage of $Olig2^+/BrdU^+$ cells in the Morph+Tat+Nal groups was not different from the control, or from the Morph+Tat treatment group at the same exposure time. However, naloxone did not reverse the interactions between morphine and Tat in C and D (indicated as "a"). This suggested that naloxone might directly affect the $Olig2^+$ population, but this was not the case at 12 - 48 h exposure (E). Morph, Morphine (500 nM); Tat, HIV-1 Tat_{1-86} (100 nM); Nal, naloxone (1.5 μM); * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ^{a} $p < 0.05$ vs. control; ANOVA with Duncan's post-hoc test, n = 4-6.

Figure 3.3

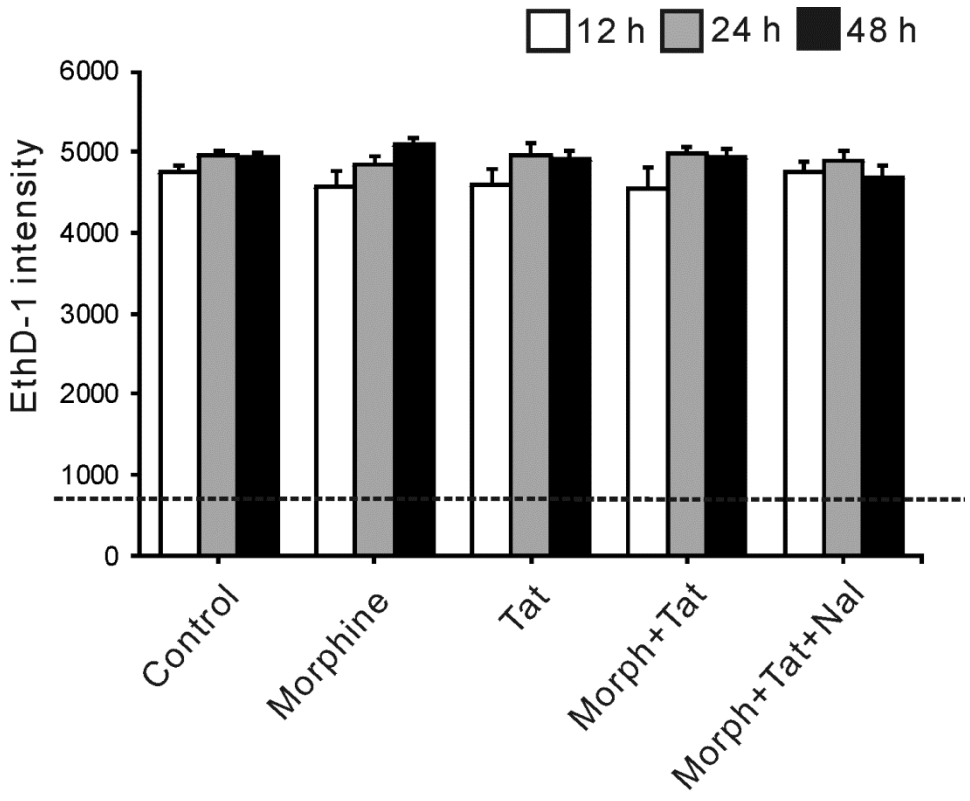

Figure 3.3 Neither Tat nor morphine has a significant effect on NPC death. There is no difference in the ethidium homodimer-1 (EthD-1) fluorescence intensity, measured in arbitrary fluorescence units, between any treatment group at 12-48 h of treatment time, indicating no increase in cell death. The dotted line indicates the signal in empty wells. Morph, Morphine (500 nM); Tat, HIV-1 Tat_{1-86} (100 nM); Nal, naloxone (1.5 μM).

Figure 3.4

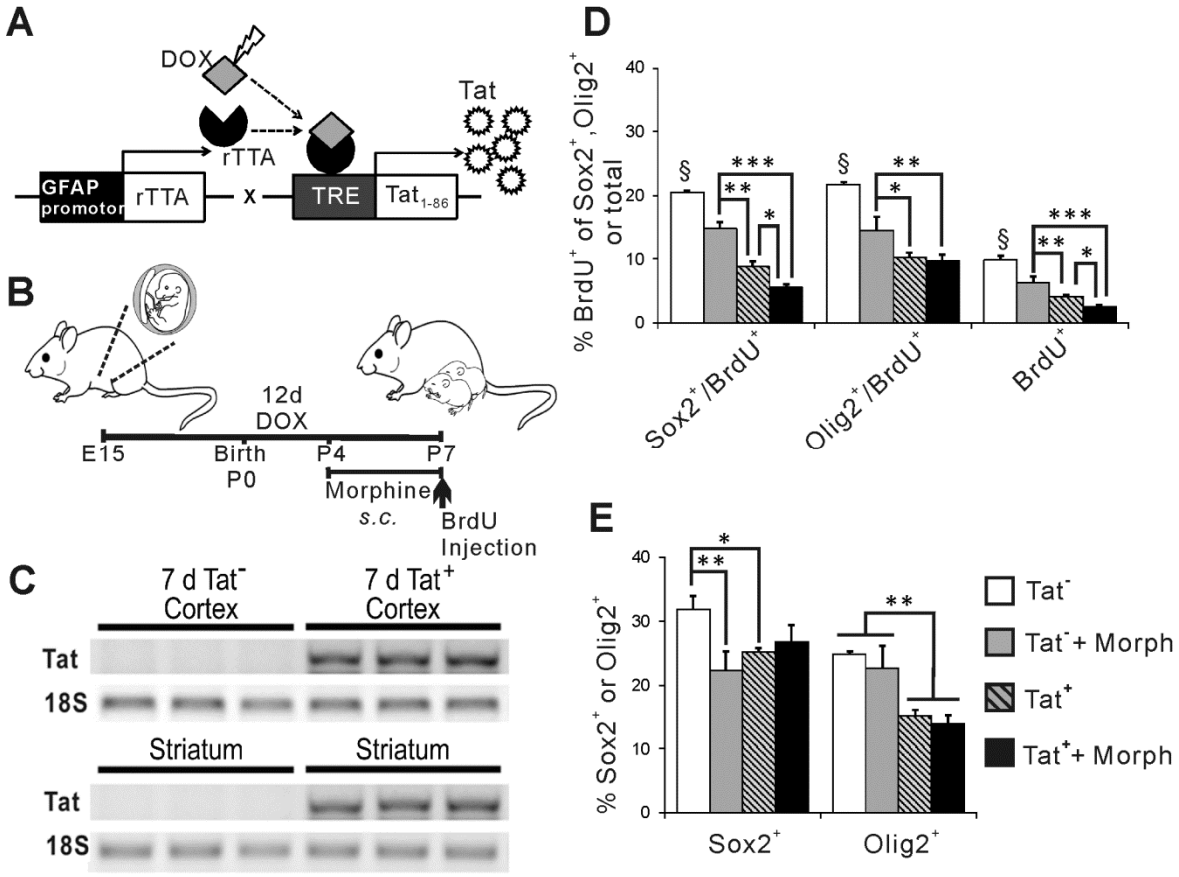
A
DOX
Tat
rTTA
GFAP promotor
rTTA
X
TRE
Tat1-86
B
12d DOX
E15
Birth P0
P4
P7
Morphine s.c.
BrdU Injection
C
7 d Tat- Cortex
7 d Tat+ Cortex
Tat
18S
Striatum
Striatum
Tat
18S
D
% BrdU+ of Sox2+,Olig2+ or total
Sox2+/BrdU+
Olig2+/BrdU+
BrdU+
E
% Sox2+ or Olig2+
Sox2+
Olig2+
Tat-
Tat-+ Morph
Tat+
Tat++ Morph

Figure 3.4 Pre- and neonatal Tat and/or morphine exposure reduces proliferation in the neonatal striatum. HIV Tat_{1-86} was induced via doxycycline delivered sequentially through placenta and lactation (A and B); Tat mRNA transcription was detected by RT-PCR in both cortices and striata of 7 d pups (C). All dams received the same chow so that both Tat^+ and Tat^- pups were exposed to doxycycline. 12 d Tat induction and 4 d of *s.c.* morphine injections significantly reduced overall proliferation ($BrdU^+$), and also reduced proliferation in $Sox2^+$ (undifferentiated progenitors) and $Olig2^+$ cells. Additive effects of Tat and morphine were observed in $Sox2^+/BrdU^+$ cells and in the overall $BrdU^+$ population (D). Changes in progenitor proliferation appear to affect the overall populations of $Sox2^+$ and $Olig2^+$ cells in the neonatal striatum (E). (* $p < 0.05$; ** $p < 0.01$; § $p < 0.05$, Tat^- vs. all other groups, Duncan's post-hoc test, n = 6-8). DOX, doxycycline.

Figure 3.5

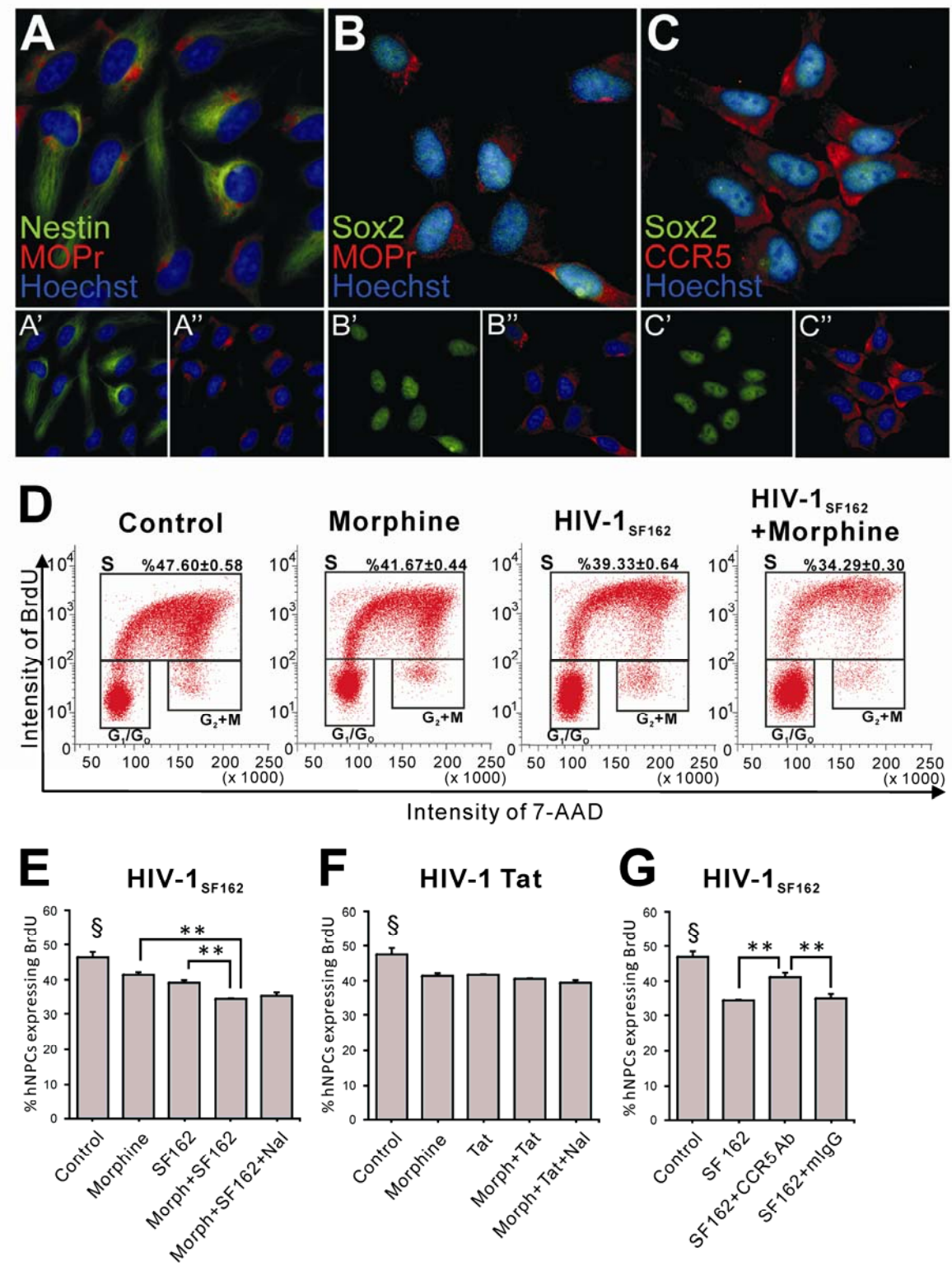
A
Nestin
MOPr
Hoechst
A'
A''
B
Sox2
MOPr
Hoechst
B'
B''
C
Sox2
CCR5
Hoechst
C'
C''
D
Control
Morphine
HIV-1 SF162
HIV-1 SF162 +Morphine
S %47.60±0.58
S %41.67±0.44
S %39.33±0.64
S %34.29±0.30
G1/Go
G2+M
Intensity of BrdU
Intensity of 7-AAD
(x 1000)
E
HIV-1 SF162
F
HIV-1 Tat
G
HIV-1 SF162
% hNPCs expressing BrdU
§
**
Control
Morphine
SF162
Morph+SF162
Morph+SF162+Nal
Tat
Morph+Tat
Morph+Tat+Nal
SF 162
SF162+CCR5 Ab
SF162+mIgG

Figure 3.5 Effect of Tat or HIV-1_{SF162} supernatant ± morphine on proliferation of human ReNcell VM NPCs. Double-immunolabeling confirmed that 95% of hNPC were nestin^{+} and Sox2^{+} positive, and that 95% also showed immunoreactivity for MOR and CCR5 (A-C). The colors representing neural precursor makers are separated in the lower panels (A': nestin (green), nuclei (blue); A'': MOR (red), nuclei (blue); B': Sox2 (green); B'': MOR (red), nuclei (blue); C': Sox2 (green); C'': CCR5 (red), nuclei (blue)). The presence of CCR5 on hNPCs suggested that effectors such as gp120 and -chemokines, released in response to Tat or contained in the viral supernatant, might affect the behavior of hNPCs (C). FACS analyses (D) were performed after 12 h treatment with the Tat, virus, or morphine combinations indicated by the histograms in E-G, with BrdU added during the last 2 h to label S-phase cells. Exposure to supernatant from HIV-1_{SF162}-infected monocytes (E), Tat (F), and morphine (E and F) all decreased the proportion of hNPCs in S-phase compared to control (§ $p < 0.05$, compared to all other groups). BrdU incorporation was additively reduced in cells treated with a combination of HIV-1_{SF162} supernatant and morphine (E). There was no interactive effect of Tat and morphine (F). A CCR5 blocking antibody (2D7) reduced, but did not abolish, the effect of HIV-1_{SF162} supernatant (G), suggesting that BrdU uptake was sensitive to multiple factors in the supernatant, some of which interact with CCR5. (§ $p < 0.05$, compared to all other groups; ** $p < 0.01$, one-way ANOVA, Duncan's post-hoc test, n = 4-7; Morph, Morphine (500 nM); Tat, HIV-1 Tat_{1-86} (100 nM); Nal, naloxone (1.5 μM); SF162, supernatant from U937 monocytes infected with HIV-1_{SF162} (supernatant diluted to 500 pg p24 per well); CCR5 Ab, antibody 2D7 (2 μg/ml); mIgG = monoclonal mouse IgG (2 μg/ml).

Figure 3.6

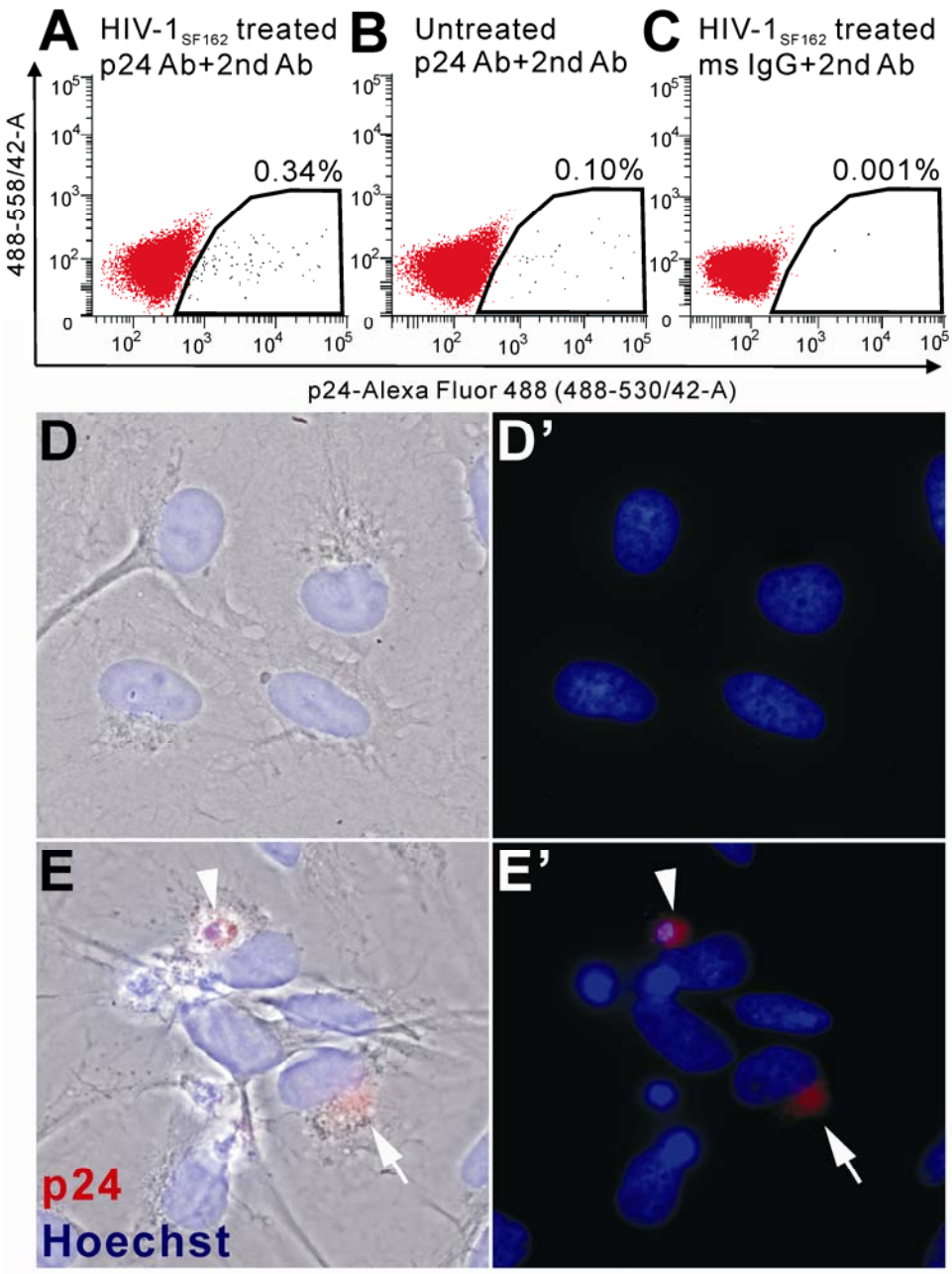
A
HIV-1SF162 treated
p24 Ab+2nd Ab
B
Untreated
p24 Ab+2nd Ab
C
HIV-1SF162 treated
ms IgG+2nd Ab
0.34%
0.10%
0.001%
488-558/42-A
p24-Alexa Fluor 488 (488-530/42-A)
D
D'
E
E'
p24
Hoechst

Figure 3.6 Detection of p24 on hNPCs by FACS and fluorescent microscopy. p24 immunolabeling intensity was assessed on approximately 1 x 10^4, 7-AAD$^+$ cells using a single laser (488 nm) and two color fluorescence emission (585/42 band pass; 530/30 band pass) FACS approach (A-C). A very small percentage (0.33%) of hNPCs exposed to supernatant from HIV_{SF162}-infected monocytes were p24$^+$ (A). Labeling indices in control groups, which included untreated hNPCs immunolabeled for p24 (B) and HIV-treated hNPCs exposed to control IgG and fluorescently-labeled second antibody (C), were 0.1% and 0.001%, respectively, suggesting that a portion of the p24 labeling was specifically related to antigen expression. Autofluorescence (585 > 530 signal) was not detected in any of the samples. FACS diagrams shown are representative of n=3 separate experiments. When individual cells were examined, p24 signal was not detected in untreated hNPCs (D, phase contrast; D', fluorescence). p24 immunoreactivity was associated with two groups of cells in cultures exposed to HIV-1_{SF162} supernatant (E, phase contrast; E', fluorescence). The first group was clearly pyknotic (arrowhead in E and E'). This staining may be non-specific, or may represent infected hNPCs that have subsequently died; our methods cannot distinguish between these possibilities. Other p24$^+$ cells, observed very infrequently, had a viable phenotype (arrow in E and E').

Figure 3.7

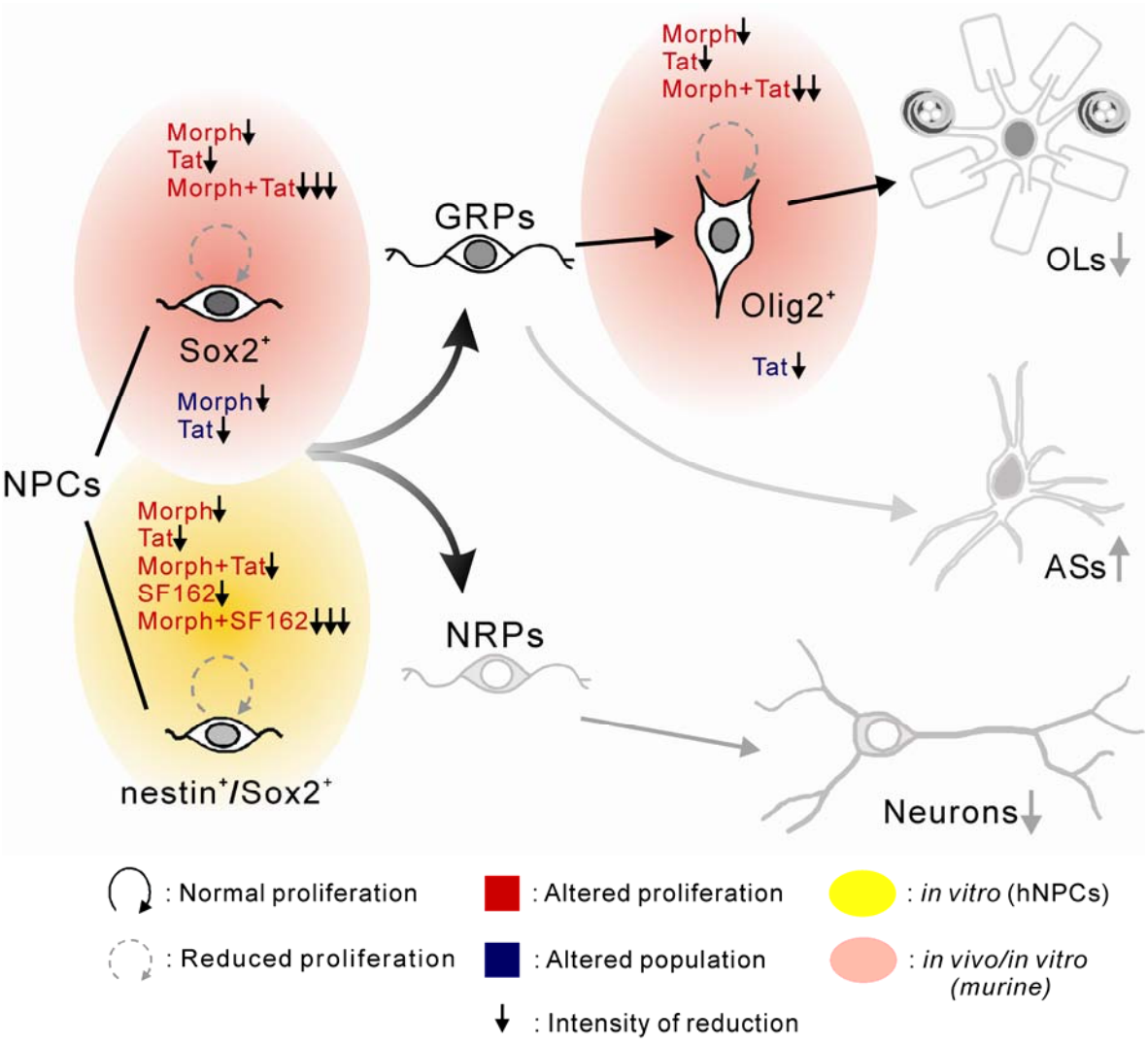
Morph↓
Tat↓
Morph+Tat↓↓↓
Sox2+
Morph↓
Tat↓
NPCs
Morph↓
Tat↓
Morph+Tat↓
SF162↓
Morph+SF162↓↓↓
nestin+/Sox2+
GRPs
NRPs
Morph↓
Tat↓
Morph+Tat↓↓
Olig2+
Tat↓
OLs↓
ASs↑
Neurons↓
: Normal proliferation
: Reduced proliferation
: Altered proliferation
: Altered population
: Intensity of reduction
: in vitro (hNPCs)
: in vivo/in vitro (murine)

Figure 3.7 Overview of the proposed effects of HIV-1_{SF162} and/or morphine on NPCs in the striatum during the perinatal period. Both the intermediate filament nestin and the transcription factor Sox2 identify undifferentiated and self-renewing NPCs in the developing CNS, although nestin and Sox2 expression do not entirely overlap. Sox2 expression is maintained in immature cells destined to become neuroglia (glial-restricted precursors; GRPs) but is lost in immature cells destined to become neurons (neural-restricted precursors; NRPs) (Ellis et al. 2004). GRPs further differentiate into astroglia and oligodendroglia; the transcription factor Olig2 is expressed in precursors destined to become oligodendroglia. Our studies show that HIV-1 Tat, HIV-1_{SF162}, and morphine all affect the proliferation of certain subtypes of progenitors (red lettering). Morphine and Tat reduce the proliferation of $Sox2^+$ progenitors and immature $Olig2^+$ oligodendrocytes in the mouse brain and *in vitro*, and have an additive effect that reduces proliferation even further. Proliferation of human $Sox2^+$/$nestin^+$ NPCs is similarly reduced by Tat, morphine, and infective HIV-1_{SF162} supernatant, and an additive effect between HIV-1_{SF162}, and morphine reduces proliferation further. Reduced NPC and GRP proliferation is associated with changes in cell populations (blue lettering). We specifically measured a reduction in $Sox2^+$ cells with morphine or Tat exposure, and in $Olig2^+$ cells with Tat exposure. Taken as a whole, the findings support a model where the proliferation of both NPCs and GRPs is significantly reduced by morphine or HIV, with interactions that compromise progenitor production even further. While the HIV-1 Tat protein clearly reduces proliferation by itself, effects of HIV supernatant may also reflect other viral proteins such as gp120 (Lee et al. 2011) or numerous secreted factors present in the medium. Reduced proliferation predicts an overall reduction in populations of progenitors, which was observed. Continued effects

on proliferation indices are likely to disturb the overall balance of glia and neurons in the brain, a prediction that remains to be tested. (Note that arrows and cartoons done in grey represent hypothesized outcomes for which we have no direct experimental evidence.)

Chapter 4

Sex Influences the Effects of Long-term HIV-1 Tat Exposure in the Striatum: Do Changes in Inflammation and Cell population Affect Behavior?

Introduction

Epidemiology of neurocognitive disorders in HIV

The introduction of combination antiretroviral therapy (cART) has resulted in dramatic declines in the prevalence of HIV associated dementia (HAD) and mortality of HIV infected individuals, while simultaneously, the overall proportion of HIV-associated neurocognitive disorders (HAND) have increased in these patients (McArthur et al. 2010). For instance, the prevalence of neurolocognitive disorders is more frequent in developing countries (Nakasujja et al. 2005; Wong et al. 2007) and there is a great possibility that these neurocognitive disorders and impairments become the most common types of dementia among young-age people globally (Wright et al. 2008). Some clinical reports suggest that HAND can be diagnosed in HIV infected individuals; even though they have a history of prolonged aviremia with high adherence to cART (Cole et al. 2007). Regardless of cART usage, neurological insults and injury by HIV-1 might continue to occur in some patients (Robertson et al. 2007). HAND can be diagnosed with broad spectrum symptoms ranging from asymptomatic neurocognitive impairment (ANI), to minor neurocognitive disorders (MND), to HIV associated dementia (HAD), because HAND is a progressive disease with different intensity of CNS involvement and symptoms in its development. Approximately,

40-60% of HIV/AIDS patients can be categorized in one of these diagnoses (Ellis et al. 2007; McArthur et al. 2010).

ANI is the mildest form of HAND and 30% of HIV infected individuals under cART belong to this group. These individuals with ANI are more likely to develop the more severe forms of HAND (Ellis et al. 2007). ANI is characterized by a number of criteria, including measurable neurocognitive impairment without recognition by the patients and without impact on day-to-day function. Next, MND is a more severe form of neurolcognitive impairment than ANI, and was often identified in 20 to 30% of HIV infected adults in pre-cART era (Janssen et al. 1989; McArthur et al. 2010; Sacktor et al. 2002). These individuals usually self-report having impaired cognitive or behavioral deficits. Such impairments can include a reduction in attention/concentration, mental slowing, abnormal memory along with other cognitive functions, slowed or uncoordinated movements, apathy, personality change, depression, irritability and mood swing (McArthur et al. 2010). These abnormalities typically can cause mild impairment in work, or negatively affect activities of daily living. MND is known to be associated with shortened survival, reduced adherence with antiretrovirals, as well as worse employability, and increased prevalence of HIV encephalitis in patients from pre-cART period (Cherner et al. 2002; Hinkin et al. 2002; Mayeux et al. 1993; Sacktor et al. 1996). Finally, the prevalence of HAD is rare in cART-treated individuals, approximately 2 to 8% of frequency is still estimated (McArthur et al. 2010).

According to previous clinical studies, the prevalence of neurocognitive disorders in HIV infected patients is high in both those complaining of cognitive impairments (84% of complaining HIV^+ patients) and those without complaints (64% of asymptomatic HIV^+

patients) despite suppression of viremia with cART (Simioni et al. 2010). The high prevalence of neurocognitive disorders and impairments in aviremic, cART-treated individuals suggested that many anti-retroviral medicines may fail to reverse neurological damage, even though cART lengthens the survival (McArthur et al. 2010). Therefore, HAND has become a significant economical and psychological burden to persons living with HIV infection and places excessive damage on the health care system. What was once a uniformly fatal illness is now a chronic disease requiring long-term medical management (Tozzi et al. 2005).

Basal ganglia is especially vulnerable to damage from HIV

The complications of HAND, mentioned above, are tightly associated with neuropathological changes within subcortical regions of the brain, especially the basal ganglia, which also retains the major brunt of the infection in the brain (Berger and Nath 1997). These subcortical changes result in cognitive and motor dysfunction such as mental slowing, memory loss, difficulties in complex tasks, as well as motor disorders (Simioni et al. 2010; Tozzi et al. 2005). Affected individuals can also present with behavioral abnormalities, such as apathy, a decrease in spontaneity, and mood swings. In addition, the incidence of depression and anxiety are more prevalent in HIV infected individuals with HAND (Simioni et al. 2010). The reasons for regional vulnerability of the basal ganglia for the associated neuropathological features of HAND are still elusive. HIV-1 virus was found in the basal ganglia in early infection (Ances and Ellis 2007; Berger and Nath 1997; Kumar et al. 2007). Before the introduction of cART, multinucleated giant cells, microglial nodules, HIV-infected microglial cells, and infiltrating macrophages were often detected in

the basal ganglia compared to other regions within the brain (Navia et al. 1986). These pathological abnormalities were most common within the putamen and caudate in the basal ganglia (Koutsilieri et al. 2001; Navia et al. 1986). Paralleling with abnormatlities in these regions, the atrophy of subcortical brain is often detected in HIV infected individuals in pre-cART era. Even in post-cART era, this pattern of atrophy was also consistent with motor dysfunction found in HIV patients with HAND (Becker et al. 2011; Kuper et al. 2011). Neuroimaging studies in pre- and during cART era have indicated that dopamine transporters and metabolites were abnormal in HAD patients compared with asymptomatic individuals (Berger et al. 1994; Chang et al. 2008; Kramer and Sanger 1990; Wang et al. 2004). Furthermore, the basal ganglia are where HIV proteins Tat and gp120 were prominently detected in patients with HIV encephalitis. The proteins can be detected in glial cells, extracellular matrices, perivascular compartments, and in close proximity to neurons (Hofman et al. 1994; Kruman et al. 1998). These proteins thus have the opportunity to interact with neuronal cell bodies, dendrites, and synaptic terminals (Nath et al. 2000).

Glia associated neuropathogenesis in HIV

The progression of HAND may be associated with chronic inflammation due to low-level viral replication in the CNS because many cART medicines have poor permeability through BBB. Despite suppression of viremia due to cART systemically, the abnormal immune activation of microglia/macrophages triggered by HIV is persistently remained in the CNS (Woods et al. 2009). Productive HIV infection seen within the basal ganglia thought to be due to microglia and macrophages (Everall et al. 1995; Jordan et al.

1991; Speth et al. 2005). Studies with *in situ* polymerase chain reaction and laser capture dissection microscopy have confirmed that productive infection occurs within perivascular macrophages and microglia in brain parenchyma (Williams et al. 2001). Non-productive HIV infection has also been detected in astrocytes (Takahashi et al. 1996; Thompson et al. 2004), and this infection is CD4 independent, because CD4 is not normally expressed on astrocytes (Di Rienzo et al. 1998; Ma et al. 1994). There have been some reports that low numbers of HIV-infected astrocytes might contribute to the disruption of the blood-brain-barrier in HIV infected CNS (Eugenin and Berman 2007; Eugenin et al. 2011). The toxicity of HIV, especially the apoptotic cell signaling molecules, might be amplified by gap junctions in astrocytes, even under the condition of minimal HIV infection in these cells can result in significant damage in CNS, because the disruption of the BBB allows massive numbers of macrophages to infiltrate into CNS (Eugenin and Berman 2007; Eugenin et al. 2011). In addition, robust upregulation of chemokines and cytokines in the basal ganglia also suggests that neuronal dysfunction and injury in progressive HAND may be due to abnormal cytokine production by these astrocytes and microglia (Suzuki et al. 2011; Turchan-Cholewo et al. 2009a; Turchan-Cholewo et al. 2009b).

Replication of HIV in infected microglia/macrophages results in the release of HIV proteins such as gp120 and Tat. In addition the release of viral proteins accompanied by the secession of cellular gene products, including proinflammatory cytokines such as tumor necrosis factor-α (TNF-α), as well as eicosanoids, nitric oxide (NO), platelet-activating factor (PAF), quinolinic acid, and extracellular matrix-degrading proteases (Adamson et al. 1999; Griffin 1997; Heyes et al. 2001; Patton et al. 1998; Smith et al. 2001; Vos et al. 2000; Wesselingh et al. 1997; Yong et al. 1998). gp120 and Tat shed from infected

microglia can bind to and impair the function of neurons and astrocytes, causing them to secrete inflammatory cytokines/chemokines, which can activate adjacent microglia and astrocytes in the early asymptomatic stage of HIV infection. In addition, much evidence points to the role of activated microglia as a chronic source of multiple neurotoxic factors, including TNF-α, interleukin-1β (IL-1β), NO, and reactive oxygen species (ROS), driving progressive neuron damage with opiate exposure (Bruce-Keller et al. 2008; El-Hage et al. 2008b; Turchan-Cholewo et al. 2009a). Furthermore, microglia are known to be chronically activated by a single or chronic exposure to disease proteins, oxidative stress, and cytokines/chemokines, this characteristic of microglia could contribute to HIV neuropathogenesis (reviewed in Lull and Block 2010). Finally, pro-inflammatory factors can generate more reactive astrocytes and chronically activated microglia, which may induce functional damage to adjacent neurons. Taken together, the HIV infected or activated macrophages, microglia, and astroglia up-regulate release of pro-inflammatory cytokines/chemokines, which probably leads damage neurons and circuitry systems and lead HIV related neurological disorder and impairments.

HIV associated neurocognitive disorders in sex differences

The incidence of HIV is higher in men than women (75% men vs. 25% women) in all ethnic groups in the United States and Europe (CDC 2009; UNAIDS 2009). For this reason, it is not surprising that studies of neurocognitive deficits and impairments in HIV are mainly based on studies focusing on men. According to recent reports, 51% of people HIV positive population in worldwide are estimated to be women (UNAIDS 2010). The availability of antiretroviral treatment in women is higher than or equal to that of men in

worldwide (UNAIDS 2010). However, the proportion of HIV-infected individuals receiving antiretroviral therapy was still low in low- and middle-income countries. Only 36% of HIV positive people received antiretroviral therapy in low- and middle-income countries (UNAIDS 2010), compared to 70% of the HIV positive population receiving antiretroviral therapy in the United States (CDC 2009).

There have been some reports that women might be more vulnerable to the development of HIV-associated neurocognitive deficits or impairment than men. However, these studies in some cases have been inconclusive and differ greatly depending on outcome measures. For instance, early studies by the AIDS in Europe Study Group found that the risk of HIV-associated neurocognitive complications (termed as AIDS dementia complex (ADC) at that time) in Europe during pre-cART era were two-times higher in women than men (Chiesi et al. 1996). On the other hand, there also have been reports that the risk of neurocognitive complications among men and women was similar to rates found among men in the United States, Europe and Australia, where availability of cART are well established (Robertson et al. 2004; Wojna et al. 2006), as well as in more resource-limited counties such as Puerto Rico and India (Bouwman et al. 1998; Gupta et al. 2007). However, there have been some concerns that there are numerous immeasurable factors that may make sex studies difficult to assess accurately. For example, globally HIV infected women live under relatively impoverished conditions. This accompanied by low literacy levels, high injection drug abuse rate, poor mental health, cultural barriers to health care services, several relevant genetic risk factors and low social status compared men with HIV, which may significantly influence the diagnosis of neurocognitive disorders in HIV positive women (Basso and Bornstein 2000; Farinpour et al. 2003; Maki and Martin-

Thormeyer 2009). Therefore, additional and controlled studies may be required to address the possibility of sex differences in susceptibility to HIV associated neurocognitive deficits and impairments.

Material and Methods

Animals, husbandry and general procedures

GFAP-driven, doxycycline-inducible HIV-1 IIIB Tat_{1-86} transgenic mice were generated as described in Chapter 3 above. Genotyping was confirmed by PCR and the method is mentioned in Chapter 3 material and methods. In this study, independent sets of 6-8 mice were utilized in each group and included female Tat^-, female Tat^+, male Tat^-, and male Tat^+. Mice were treated with doxycycline in specifically formulated chows (Harlan Laboratories, Inc. Indianapolis, IN) with daily dosages of 6 mg/kg for a period of 3 months. These animals were allowed to reach to chows ad libitum. The doxycycline treatment was started at 3 months of age and preceded until mice were 6 months old. For control groups in grip strength test, open field test and light/dark box test, one cohort of mice received normal chows until they reached to 6 month old.

Accelerating rotarod assay

An accelerating rotarod test, which is sensitive to measures of balance, coordination, and motor control, was used to evaluate motor performance in groups of Tat transgenic mice mentioned above. Rotarod testing began after one week of doxycycline treatment (around 13 weeks old), and was performed once a week (from the first week to

the fourth week). After four rounds of testing within the first month of doxycycline treatment, this behavioral test was paused for one month without removal of doxycycline. This behavioral test was resumed and applied weekly to mice in the third month of doxycycline treatment (from the ninth week to the twelfth week). Independent, age-matched mice were utilized in other behavior tests. The Rotamex-5 treadmill (Columbus Instruments, Columbus, OH) consists of a 3.0-cm diameter cylindrical treadmill connected to a computer-controlled stepper motor-driven drum, which can be programmed to operate in a defined acceleration mode (Rotamex-5, Columbus Instruments). 4 different groups, female Tat^-, female Tat^+, male Tat^- and male Tat^+ mice with doxycycline treatment, were housed 2–4 to a cage and were tested weekly at 13–16 weeks and 21-24 weeks of age. On the test day, mice were moved to the testing room and allowed to habituate for 60 min before testing. All mice in this experiment were naïve to the rotarod apparatus until the first test day at week 13. Each mouse was placed on the rubber covered rods to evaluate rotarod performance. On a given trial, four mice were placed on the cylinder, one mouse in each compartment. The accelerating rotarod was set to accelerate gradually from 1 to 40 rpm, 1 rpm increase per 15 s, for each trial. The starting speed was 1 rpm, and the total time of each trial was 400 s. Acceleration continued until either 40 rpm was reached or the last animal was unable to perform the running response and had fallen to the padded surface. When the animal fell off the rotating drum, individual sensors at the bottom of each separate compartment automatically record the amount of time (in seconds) spent on the treadmill. The time spent on the treadmill was recorded for each successive trial, and a score of one trial per week was analyzed (reviewed Liu et al. 2010).

Forelimb grip strength test

The apparatus is comprised of a push-pull strain digital force gauge, Chatillon® DFE II grip strength meter (AMETEK's Test & Calibration Instruments (TCI), Largo, Florida USA). Mice were housed 2–4 to a cage and were tested at 6 months of age. On the test day, mice were moved to the testing room and allowed to habituate for 60 min before testing. Each mouse was weighed before forelimb grip strength test. A forelimb grip pedestal was used as the grip bar. The mouse was held near the base of its tail and lowered toward the bar until it gripped the bar with both forepaws. The mouse was then gently and steadily pulled directly away from the bar until the bar was released. Peak force disturbance was automatically registered in grams-force (gf) by the apparatus (Grip Strength Meter 2.66, AMETEK's Test & Calibration Instruments (TCI)). Data were automatically recorded, and 5 trials per mouse were given in quick succession. The force of resistance was measured in a single 5-trial session and the strongest measurement was collected as the score (reviewed by Crabbe et al. 2003). The presented scores (gf) in grip strength were normalized by mouse weights (g) for statistically comparing control and all other groups.

Open field test

All groups of transgenic mice, including female Tat^-, female Tat^+, male Tat^- and male Tat^+ mice with/without doxycycline treatment, were housed 2–4 to a cage and were tested at 6 months of aged. On the test day, mice were moved to the testing room and allowed to habituate for 60 min before testing. Prior to the evaluation, animals were habituated to the box for 1 min. Mice were individually placed at the center of a clean open

field apparatus (30 × 30 × 15 cm, divided into nine squares, Med Associates Inc., St. Albans, VT, USA). The observed parameter, number of squares crossed (locomotor activity) was automatically recorded for 10 min (Burger et al. 2005; Ramezani et al. 2011). The data in open field test (percent of total travel distance in 10min, Fig. 4.2 A, Table 4.1 A) indicate that the scores from 6 month old with 3 month doxycycline treatment were normalized by the scores from control age-matched groups without doxycycline treatment.

Light-dark Transition Model of Anxiety

All groups of transgenic mice, including female Tat^-, female Tat^+, male Tat^- and male Tat^+ mice with/without doxycycline treatment were, housed 2–4 to a cage and were tested at 6 months old. On the test day, mice were moved to the testing room and allowed to habituate for 60 min before testing. The light-dark box was adapted from the originally described apparatus (Crawley and Goodwin 1980). Two equally sized compartments (30 cm x 30 cm x 15 cm) were separated by a black plastic partition with an opening in the middle to allow for light-dark transitions (Med Associates Inc., St. Albans, VT, USA). The box was enclosed in a sound attenuating box equipped with overhead lighting and fan ventilation. Each mouse was tested by locating it in the center of the white area, facing to the dark one, and was allowed to explore the novel environment for 10 min. The system was interfaced with Med Associates software (Med Associates Inc.) enabling automatic measurement of activity using a set of 16 infrared beam sensors along the X-Y plane. The observed parameter, number of squares crossed (locomotor activity), was automatically recorded for 10 min (modified from Malmberg-Aiello et al. 2002). The data in light-dark box test (Fig. 4.2 B, Table 4.1 B) indicate that the percent of travel distance in dark side

box from 6 month old with 3 month doxycycline treatment were normalized by the percent of travel distance in dark side box from control age-matched groups without doxycycline treatment.

Immunohistochemistry and quantification

For immunohistochemistry (IHC) studies, mice were deeply anesthetized with isoflurane (Baxter, Deerfield, IL, USA) prior to perfusion with 4% paraformaldehyde (pH 7.4, Sigma-Aldrich Co., St. Louis, MO, USA). After perfusion, brains were removed and post-fixed in fresh fixative overnight. Brains were hemisected, rinsed several times in 15 mL changes of phosphate-buffered saline (PBS) and left overnight in 15 mL of PBS. Brains were then cryopreserved through graded sucrose solutions (20 and 30%), embedded in Tissue Tek OCT compound (Sacura Finetek, Torrance, CA, USA), and stored at –80˚C. 10 μm frozen sections containing the striatum were thaw-mounted on SuperFrost Plus slides (VWR Scientific, West Chester, PA, USA) and processed for immunostaining. The brains from female Tat^-, female Tat^+, male Tat^- and male Tat^+ mice with doxycycline treatment were serially cut and numbered and ordered and stored -80˚C until use. For *in vivo* studies, to assess proportional numbers of specific cells in the striatum, single or double immunostaining was performed on 10 μm frozen brain sections including striatum. Primary antibodies were utilized directed against Iba-1 (1:200, Wako, Osaka, Japan), 3-nitrotyrosine (3-NT, 1:100, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), NeuN (1:200, Millipore, Temecula, CA, USA), Olig2 (1:100, Immuno-Biological Laboratories, Minneapolis, MN, USA), and Sox2 (1:200, R&D Systems, Inc., Minneapolis, MN USA). Tissue sections on slides were rehydrated with 1x PBS (pH 7.4, Invitrogen Corp., Carlsbad,

CA, USA) for 10 min at room temperature. For intracellular markers, tissue sections were permeabilized with 0.2% Triton X-100 in phosphate-buffered saline containing 1% bovine serum albumin (Sigma-Aldrich) for 30 min. For double immunostaining, individual primary antibodies were sequentially applied and incubated overnight at 4ºC in humid chamber; and host-matched fluorescent-conjugated secondary antibodies were applied for visualization 1 hour at room temperature (Invitrogen Corp., Carlsbad, CA, USA). Immunostained tissues were finally incubated with Hoechst 33342 dye (0.5 μg/ml, 8 min, RT) to identify nuclei (Invitrogen), then mounted in ProLong Gold anti-fade reagent (Invitrogen) and dried for 8 h in the dark. To assess the proportion of microglia cells, 300–350 Hoechst$^+$ cells were selected randomly in one side of the striatum to assess the presence of Iba-1 in striata under oil immersion at 63× using a Zeiss AxioObserver system with an integrated MRM camera system (Carl Zeiss, Inc., Thornwood, NY, USA, n = 6-7). To determine the relative activation state of microglia in the striata, an independent group of 200 Iba-1$^+$ cells were randomly selected per striatum and assessed for the 3-NT expression. To verify the proportion of neural progenitors and immature oligodendrocytes in the striata, 300–350 Hoechst$^+$ cells were selected randomly per striatum and assessed for Sox2 and Olig2 expression, respectively (n = 6-7).

NeuN immunoblots and analysis

In vivo NeuN expression was examined by immunoblotting. Striata were harvested and homogenized with T-PER Tissue Protein Extract Reagent (Thermo Scientific, Pittsburgh, PA, USA), including a protease inhibitor cocktail (Roche, Indianapolis, IN, USA). Homogenized tissue lysates were centrifuged; the supernatants were transferred into

new tubes and then stored at -80ºC until use. Protein concentration was measured using the BCA protein assay (Pierce, Rockford, IL). Duplicate samples of 20 μg total protein were loaded per well onto 10% Tris–HCl Ready Gels (Bio-Rad Laboratories, Hercules, CA) using Precision Plus Protein Dual Color Standards (Bio-Rad; MW range: 10–250 kDa) to monitor protein transfer and molecular weight. Proteins were transferred to Hybond-P PVDF membranes (Amersham Biosciences, Piscataway, NJ, USA), probed with mouse monoclonal anti-NeuN (1:1000, Millipore) and anti-GAPDH (1:2500, Abcam, Cambridge, MA, USA). Host-matched IRDye® Infrared Dyes conjugated secondary (LI-COR Biotechnology, Lincoln, Nebraska) were applied to visualize each protein band. Protein bands were detected on an Odyssey® Infrared Imaging System and their intensity were analyzed by Odyssey 2.0 software (LI-COR). The intensities of two NeuN bands in each sample were combined to analyze. To assess changes in NeuN, the combined band intensities were normalized with the intensity of the respective GAPDH band.

Nissl staining

In addition to NeuN immunohistochemistry, we analyzed Nissl stainined tissues in order to estimate the total neuron number in striatum. Nissl staining was performed on 50 μm brain coronal sections were taken serially through the striatum based on stereotaxic coordinates (3rd ed., Academic Press, Orlando, FL, 2008). Sectioning was performed with vibrating blade microtome (Leica VT1200S, Leica, Nussloch, Germany). Sections were prepared in ice-cold PBS (Invitrogen) and each section was stored in 48-well plates by serial order with cryoprotectant solution (25% of glycerin: 25% of ethylene glycol: 50% of 0.1 M phosphate buffer) until use. The Nissl staining was performed as described by

Davenport, H.A. (1960) (Davenport 1960). Briefly, free floating brain sections were mounted on chrom-gelatin coated slides serially and were dehydrated at room temperature until completely air dried. Brain sections were submerged in water for rehydration for 10 min and then underwent 70, 95, and 100% ethanol submersions for dehydration. The brain tissues were stained for 6 min with 0.5% cresyl violet (Sigma–Aldrich Co., St. Louis, MO, USA). After rinsing in water, the sections were submerged in 70% ethanol in 0.34 M acetic acid for 2-3 sec, and then submerged in 95% followed by 100% ethanol for 5 min each. Finally, the sections were dehydrated with xilene for 10 min. The sections were mounted and sealed under coverslips for analysis.

Stereology

For stereology, 50 μm free-floading sections were prepared from fixed brains, as described in IHC section above. After Nissl staining was performed on these brain sections, total numbers of $Nissl^+$ neurons in the striata were estimated using the optical fractionator method (West et al. 1991), with assistance from a computerized stereology system (Stereologer, Systems Planning and Analysis, Alexandria, VA). Every fifth section was selected from the total number of sections through striatum in a systematic-random manner, and 12 sections per sample were generated. The sampling fractions were: (1) section sampling fraction (ssf), the number of sections sampled divided by the total number of sections for each striatum; (2) area sampling fraction (asf), the area of the sampling frame divided by the area of the x-y sampling step; and (3) thickness sampling fraction (tsf), the height of the disector divided by the section thickness. Each reference space was outlined at low power (x5), and cells were counted using a high magnification (x100, oil-

immersion) objective. Nissl^{+} neurons in striata were counted using the optical fractionator method in combination with the disector principle and unbiased counting rules (Mouton 2002). Since Nissl staining detects both neurons and glia in striata, Nissl^{+} neurons were distinguished from glia and identified by their characteristics for unbiased stereological counting. Neurons cells were distinguished by relatively a large oval shape nucleus, the presence of dark, coarsely stained Nissl substance in the cytoplasm, and lightly stained proximal segments of dendritic processes. In contrast, glial cells contain less endoplasmic reticulum and have relatively dark, tight and small nucleus (Fitting et al. 2010a; Sherwood et al. 2006). In counting of neurons, astrocytes and oligodendrocytes, and microglia were excluded without distinguishing among them. A guard volume of 2.0 mm was used during cell counting to avoid sectioning artifacts, including lost caps and uneven section surfaces. Criteria for counting cells required that cells exhibited positive chemical- and immuno-reactivity and morphological features consistent with each cell type. Cells were counted on the left hemispheres. After Hoechst staining was applied on 50 μm free-floading sections, the Cavalieri principle was applied to measure the volume of the striata. The slides were viewed by using a Zeiss AxioObserver system with an integrated Sony 3CCD Exwave HAD camera system (Carl Zeiss, Inc., Thornwood, NY)

Statistical analysis

Linear regression was also performed followed by t-test among groups using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA). Statistical analyses were done by one way and main effect analysis of variance (ANOVA) followed by Duncan's post-hoc testing using Statistica 6.0 software (Statistica, Tulsa, OK).

Results

Accelerating rotarod assay

To examine whether the long-term induction of Tat in transgenic mice affects motor skill in a sex-dependent manner, the accelerating rotarod assay was performed in all groups of mice, female Tat^-, female Tat^+, male Tat^- and male Tat^+. During 3 months of doxycycline exposure, all mice used in this study performed the rotarod test once a week during week 1-4 and 9-12. The accelerating rotarod test is a forced performance test based on a rotating rod with forced motor activity to evaluate basic gait, balance and coordination and duration on rod and maximum speed can be measured (Jones and Roberts 1968). Female Tat^-, female Tat^+, and male Tat^- groups showed the obtaining motor skill by the repeated rotarod tasks and reached their maximum performance at 12 weeks, as measured by an increase in the duration of time spent on the rotarod (Fig. 4.1 A). Three groups of mice maintained balance on the rotarod with the maximum latency, around 300-352 sec (Fig. 4.1 A), and rotation speeds reached the highest rotation speeds of 21-24 rpm before falling by 12 weeks. Interestingly, female Tat^+ mice showed different and slow in obtaining motor skill compared female Tat- group, even they finally gained the motor skill as much as female Tat- group at the end of 12 week doxycycline exposure (* $P<0.05$; linear regression, *t*-test, n = 6, Fig. 4.1 A). In contrast, male Tat^+ mice significantly had more difficulty to maintain balance on the rotarod and/or obtaining motor skill after 12 weeks of doxycycline administration compared to all other groups (Fig. 4.1 A, § $p<0.001$; linear regression, *t*-test, n = 6, Fig. 4.1 A). The maximum time of male Tat^+ group only reached less than 200 sec (Fig. 4.1 A), and their highest rotation speeds in male Tat^+ mice groups

only reached around 14 rpm. In fact, the Tat^+ mice did not show any acquisition of the task, as the slope of their performance measure was not different from 0 (Fig. 4.1 A). The data showed a clear indication of sex differences in the accelerating rotation tasks. Therefore, this data might indicate that motor skill/balance and/or the ability to learn a motor task are more significantly vulnerable to HIV-1 Tat exposure in male than in female.

Forelimb grip strength

Forelimb grip strength test was performed in all 8 groups of mice with/without 3 month doxycycline treatment groups. Reduction or abnormality in grip strength indicates inappropriate neuromuscular functioning or motor deficits in the brain. Since our transgenic mice almost exclusively express HIV-1 Tat_{1-86} in their CNS, the test could be utilized to assess whether long-term induced Tat might cause motor deficits in their CNS that can affect strength of forelimbs in sex specific manner. 4 groups of 6 month old mice without doxycycline exposure showed no significant difference in forelimb grip strength, based on their sex or genotype difference of Tat transgene, after normalizing by their weight (Fig. 4.1 B). Doxycycline exposure to 12 weeks induced significant differences resulted to sex and transgene expression. Importantly, doxycycline by itself did not appear to influence on forelimb grip strength since female Tat^-, female Tat^+ and male Tat^- mice had similar strength. Tat expression significantly reduced grip strength only in female Tat^+ mice (§* $p<0.05$, control male Tat^- vs. male Tat^- with doxycycline treatment, one-way ANOVA, Duncan's post-hoc test, n = 6, Fig. 4.1 B). Male Tat^+ mice also showed the weakest grip strength among the groups with doxycycline treatment (**, $p<0.01$, one-way ANOVA, Duncan's post-hoc test, n = 6, Fig. 4.1 B). This result suggests an interactive

effect of sex and Tat induction, since Tat^+ mice with doxycycline showed significant reduction in forelimb grip strength compared to other groups. Therefore, similar to rotarod test in gaining motor function, males were more affected by HIV-1 protein Tat than females in forelimb grip strength.

Open-field test

To examine whether the locomotor activity during exploration of a novel environment may be affected by long-term induction of Tat in male and/or female mice, an open-field test was performed in all groups of mice including after 3month Tat induction. The open field test and light/dark box test can measure a tendency to explore novel environments. Depressed or anxious animals in this test will show reduced traveling distances in open field if they lose their will to seek novel environment (Cryan and Holmes 2005; Wallace et al. 2008). The distance traveled in 10 min was significantly reduced in both male and female Tat^+ mice, to the same extent after normalization by age-matched groups without doxycycline exposure (** $p<0.01$, *** $p<0.001$, One way ANOVA, Duncan's post-hoc test, n=6-7, Fig. 4.2 A, Table 4.1 A). Interestingly, there was no sex specific effect. Results suggest that long-term exposure to HIV-1 Tat reduced locomotor activity and/or exploring behavior independent of sex.

Light/dark box test

A standard light/dark box test was performed to examine whether HIV-1 Tat expression in mouse CNS induced the anxiety-related behavior in these mice and whether there were sex dependent factors. In rodent behavior studies, increased time spend or travel

distance by freely ambulating in dark side box has been taken as an indication of enhanced anxiety in previous numerous studies with anxiogenic or anxiolytic treatment (Bourin and Hascoet 2003). The Tat induction by doxycycline treatment did not increase the travel distance in dark side of female Tat^-, femalr Tat^+ and male Tat^- mice (Fig. 4.2 B, Table 4.1 B). However, age matched male Tat^+ mice after Tat induction showed significantly increased anxiety-like behavior, since Tat^+ males showed significantly increased travel distance in dark side compared to any other groups. (* $p<0.05$, one-way ANOVA, Duncan's post-hoc test, n = 6, Fig. 4.2 B and C, Table 4.1 B). Since the only the group showed increased travel distance/time on dark side after Tat induction were male Tat^+ mice, male mice may be more vulnerable to the anxiogenic effects of HIV-1n Tat exposure.

Effects of long-term Tat induction on the proportion of microglia and their 3-NT immunoreactivity

Since microglial activation has been detected in CNS from the broad spectrum of HAND patients (Anderson et al. 2003; Glass et al. 1995; Gray et al. 2001; Patton et al. 1996; Tyor et al. 1995), the effects of long-term doxycycline treatment on sex-specific microglial activation was determined in the striata of Tat^- and Tat^+ after 3 month doxycycline treatment. Firstly, the proportion of microglia was quantified in the striata from all groups by performing Iba-1 immunohistochemistry as described in the Materials and Methods. Blinded cell counts revealed that the percent of Iba-1^+ cells in total cells in striata were equivalent in Tat^- males and Tat^+ females (Fig. 4.3 A). However, long-term Tat induction caused significant increases in the proportion of Iba-1^+ cells in both Tat^+ females and Tat^+ males without sex difference. Increases were similar in both male and female Tat^+

mice (** $p<0.01$, *** $p<0.001$, one-way ANOVA, Duncan's post-hoc test, n = 6, Fig. 4.3 A).

We next determined whether microglial activities in male mice have a different level of those of female mice, the extent of Iba-1 and 3-NT co-localization was examined by double labeling immunihistochemistry. Since 3-nitrotyrosine is a product of tyrosine nitration mediated by reactive nitrogen species such as peroxynitrite anion and nitrogen dioxide. Among CNS cells, activated microglia are considered as a major cell mainly releasing reactive nitrogen species. 3-NT immunoreactivity has been used to identify inflammatory cells as a marker of reactive nitrogen species-induced oxidative stress (Ryu and McLarnon 2006; Shavali et al. 2006; Shishehbor and Hazen 2004; Zhang et al. 2005). Tissue sections from mouse brains were processed to detect both Iba-1 and 3-NT immunoreactivity as described in Materials and Methods, and control sections, incubated with secondary antibodies only, were inspected to verify the specificity of the antibodies and to determine the pattern of co-localization. Female and male Tat^- treated with doxycycline did not show significant difference in the percentage of Iba-1^+ cells that expressed 3- NT, suggesting that doxycycline by itself had no effect on this parameter (Fig. 4.3 B). However, long-term Tat-induction significantly increased the proportion of 3-NT immunoreactive microglia in both female Tat^+ and male Tat^+ groups (* $p<0.05$, *** $p<0.001$ one-way ANOVA, Duncan's post-hoc test, n = 6, Fig. 4.3 B). Importantly, there was a significant effect of sex in the proportional reactivity of microglia, since doxycycline-induced male Tat^+ mice had a significantly higher proportion in cells being 3-NT staining within microglia compared with female Tat^+ mice that also received doxycycline (** $p<0.01$ one-way ANOVA, Duncan's post-hoc test, n = 6, Fig. 4.3 B).

Effects of long-term Tat induction on the expression of NeuN in striatum

The NeuN antibody is well known to have high specificity to a neuron-specific nuclear protein and has been widely used to identify the loss or reduced number of neuronal cells in various studies of HIV neuropathogenesis and other situations including normal neuronal cell counting (Dou et al. 2003; Everall et al. 2002; Mullen et al. 1992; Zujovic et al. 2000). It was recently shown that NeuN corresponds to Fox-3 (Feminizing locus on X-3 or hexaribonucleotide-binding protein 3) containing an RNA recognition motif and binds specifically to an RNA element for mRNA splicing (Kim et al. 2009). Fox-3 is one of a family of mammalian homologues of C. elegans Fox-1, a gene involved in sex determination. Fox-1 family consists of Fox-1, Fox-2 and Fox-3, and they also known to have an RNA recognition motif. Even estimated sizes of all these Fox proteins are about 46kDa in size, mouse monoclonal NeuN antibody recognize two bands in 46 and 48kDa size (Mullen et al. 1992) due to alternative splicing Fox-3 pre-mRNA (Kim et al. 2009). To assess the effect that long-term Tat induction might have on the number of neurons in the striatum, the proportional number of $NeuN^+$ cells was counted in striata of age matched female Tat^-, female Tat^+, male Tat^- and male Tat^+ after 3 month doxycycline treatment. In both female and male Tat- mice, the proportion of total ($Hoechst^+$) cells expressing NeuN was same (Fig. 4.4 A). Long-term Tat induction significantly decreased the proportion of $NeuN^+$ cells in both female and male Tat^+ mice compared to their respective controls (***$p<0.001$, one-way ANOVA, Duncan's post-hoc test, n = 6, Fig. 4.4 A). Furthermore, the effect on NeuN was significant in male than in female (**$p<0.001$, one-way ANOVA, Duncan's post-hoc test, n = 6, Fig. 4.4 A). We also confirmed the reduction of NeuN by performing an immunoblot analysis of all groups. NeuN was detected as two bands at

approximately 42-kD and 48-kD (Fig. 4.4 D), as mentioned previous studies (Kim et al. 2009; Mullen et al. 1992). The intensity of NeuN bands significantly reduced only in the tissue lysates from male Tat^+ mice compared to other groups (Fig. 4.4 B and C). Although there was a tendency for reduction in intensities of NeuN bands in the female Tat^+ group; it did not reach statistical significance. Taken together, our results showed that long-term Tat induction significantly reduces the proportion of total NeuN cells in striatum and overall NeuN levels are reduced in male Tat^+ mice but not in female Tat^+ mice.

Stereological analysis of $Nissl^+$ neurons in striatum and striatial volume after long-term Tat induction

Although our results showed a significant reduction in $NeuN^+$ cells in Tat transgenic mice, there have been reports that decreased $NeuN^+$ cells means the depletion of NeuN protein level or loss of protein antigenicity by neuronal injury rather than neuronal cell death or loss (Collombet et al. 2006; Unal-Cevik et al. 2004). We performed unbiased stereological estimation of total neuron number ($Nissl^+$) and the volume in the striata of female Tat^-, female Tat^+, male Tat^- and male Tat^+ mice after 3 months doxycycline treatment. Nissl histochemical staining has been commonly utilized to quantify the number of neurons in all brain regions in numerous stereological studies (Cyr et al. 2005; Fitting et al. 2010a; Fitting et al. 2008; Nielsen et al. 2009). Since Nissl staining in brain can detect both neurons and glia, morphological identification was required between $Nissl^+$ neurons and glia for counting cells. Neurons were recognized by their larger, pale and oval nuclei surrounded by darkly cytoplasmic stained in their Nissl bodies. In contrast, glia, including astrocytes, oligodendrocytes and microglia, were identified with relatively smaller size of

their dark, tight and round nucleus with lack of stained cytoplasm (Fitting et al. 2010a; Sherwood et al. 2006). All Nissl$^+$ glia were eliminated from counting in unbiased stereological estimation of total neuron number by morphological identification mentioned above. The unbiased stereological estimation in total Nissl$^+$ neuron showed that female mice had higher numbers of neurons than males did (* $p<0.05$, two-way ANOVA, Duncan's post hoc test, N=3, Fig. 4.5 A). There was no significant difference in the estimated striatal volume from any groups (Fig. 4.5 B). In summary, female mice naturally had higher numbers of neuron in their striata than did male mice in striata. Striatal volumes were similar and unchanged by Tat expression, suggesting different cell density. Since long-term Tat induction did not affect either neuron number or striatal volume, the loss of NeuN imminoreactivity in striata with Tat induction is likely to be the reduction of NeuN expression rather than loss or death of striatal neurons in striata.

Effects of long-term Tat induction on the population of neural progenitor cells and oligodendrocytes in adult striata

Since one of our main interests has been the effect of HIV-1 Tat t on neural progenitor cells, we also examined the population of Sox2$^+$ cells in adult striata from 4 different groups mentioned above, after 3 month doxycycline treatment. HIV-1 Tat induction caused significantly decreased the populations of Sox2$^+$ cells in the striata of both Tat$^+$ females and Tat$^+$ males, but there were no significant differences due to sex (Fig. 4.6 A, * $p<0.05$; ** $p<0.01$; one-way ANOVA or two-way ANOVA, Duncan's post hoc test, N=6-8). Similarly, Olig2$^+$ oligodendrocytes equally decrease in the striata both Tat$^+$

females and Tat^{+} males (Fig. 4.6 B, * $p<0.05$; ** $p<0.01$; one-way ANOVA or two-way ANOVA, Duncan's post hoc test, N=6-8)

Discussion

cART has dramatically improved survival rates over the past decade, and has decreased the incidence of HIV-1 associated dementia (HAD), but neurocognitive impairments still remain in high prevalence and affect up to 60% of HIV-infected people (Ellis et al. 2007; Wojna et al. 2006) and have shown no decline (Dore et al. 2003; Lawrence and Major 2002; Sacktor et al. 2001). These phenomenon might be the reflection of low blood brain barrier (BBB) permeability of many cART medicines, which may contribute to higher viral loads in the CNS compared to peripheral immune system (Gonzalez-Scarano and Martin-Garcia 2005; Sacktor et al. 2001). Although the prevalence of neurocognitive impairments in HIV infected population was rapidly increased along with acquired immunodeficiency syndrome (AIDS) and HIVE in (Antinori et al. 2007; Grant et al. 1995; Heaton et al. 1995; Woods et al. 2009), but relatively little is known about whether sex can influence the prevalence and characteristics of neurocognitive dysfunction in HIV positive patients.

Whether or not the occurrence of HAND has sex bias in HIV positive patients has not been conclusively determined. Furthermore, it has been quite a challenge to design and develop animal models for studying in development of HAND. Several animal models have been generated for use in understanding the mechanisms in the development of HIV associated neurocognitive deficits. Transgenic mice expressing HIV-1 gp120 in glial cells

showed age-related impairments in open field activity and spatial reference memory at 12 month old (D'Hooge et al. 1999), while 3 month old animal didn't show any changes in their behaviors. Even simian immunodeficiency virus is different from HIV-1, infection of this virus in macaque monkeys showed impairments in various cognitive and neuromotor deficits (Bokhari et al. 2011; Cheney et al. 2008). Intracrebral inoculation of HIV-infected human monocytes in severe combined immunodeficient mice also resulted in cognitive impairment and motor deficits (Avgeropoulos et al. 1998).

Inducible HIV-1 Tat_{1-86} transgenic mice were utilized to determine if sex can influence to develop symptoms of neurocognitive deficit/impairments by HIV-1 Tat protein induction in CNS and to begin to uncover the underlying mechanisms. HIV-1 Tat_{1-86} transgenic mice have been used in numerous studies from our labs for understanding HIV neuropathogenesis. For instance, doxycycline-induced Tat expression induced the activation of microglia and astroglia (Bruce-Keller et al. 2008), which resulted in dendritic abnormality and reductions in spine density of neurons, and structural alterations of OLs in the striatum (Fitting et al. 2010b; Hauser et al. 2009). HIV-1 Tat induction in these mice also resulted in circadian rhythm abnormalities and sensory changes (Duncan et al. 2008). These results in Tat transgenic mice seem to be correlated with neuropathogenesis in HIV patients showing neurocognitive disorders and impairments. Therefore, they are a reasonable animal model to study sex differences in development of HAND-like deficits.

For the diagnosis of neurocognitive disorders and impairments in HIV positive patients, a battery of tests are usually performed to assess cortical and subcortical functions known to be affected by HIV infection. The cognitive field tests typically include executive functions, episodic memory, speed of information processing, motor skills,

attention/working memory, language, and sensoriperception (McArthur et al. 2010; Woods et al. 2009). Individual tests would require a comparison in performance to the mean of a equivalent HIV uninfected group as a control. In terms of assessing subcortical dysfunctions, severe motor impairments, such as chorea, myoclonus, dyskinesia, and dystonia, were not frequently observed in most of HIV positive population (Mirsattari et al. 1998; Woods et al. 2009). Subtle neurocognitive motor abnormalities such as slowed movement and slowed information processing can be frequently detected in older aged adult HIV patients along with other motor impairments (Valcour et al. 2008); even when they were under the cART. Different behavior tests, such as gait velocity (Robertson et al. 2006), handgrip strength (PrayGod et al. 2011; Rabeneck et al. 1998), finger tapping (Heaton et al. 1995), and manual dexterity with grooved pegboard (Carey et al. 2004; Wojna et al. 2006) can be used to detect these subtle symptoms of motor deficits in HIV positive patients. Gait velocity test usually was applied to HIV-1 positive patients in severe stage of HAND, while most of tests are based on the psychomotor speed tests, which are limited to motor skill of the upper body and associated with attention and procedural learning in human subjects, since these tests are often applied to HIV positive patients with subtle motor deficits. These tests are designed to examine the condition of human subjects, and cannot be exactly equated with animal behavior tests. However, the present study shows that some well-characterized animal behavior tests applied to access our HIV-1 Tat transgenic mouse model to detect a spectrum of motor deficits and disorders. The rotarod test/grip strength test were performed to assess subcortical motor deficits, while open-field test and light/dark box test were applied to our animal model to assess the aspect of rodent behavior that has been linked to mental status of humans.

The accelerating rotarod test is a forced performance test based on a rotating rod with forced motor activity to evaluate basic gait, balance and coordination tightly associated with the function of the basal ganglia (Jones and Roberts 1968). The grip strength test is also a forced assessment which is widely used in conjunction with the rotarod test, since a normally coordinated rodent will show a low score with low strength of forelimbs in the basal ganglia affected by neurodegenerative diseases (Prodoehl et al. 2009). Long-term Tat induction resulted in a significant transgene-sex interaction. Male Tat^+ mice had difficulty in obtaining the rotarod motor skill over the course of 12 weeks compared to all other groups. Interestingly, female Tat mice also showed some difficulty to acquire motor skills as shown by the slight, but significant difference in linear regression analysis compared to female Tat^- mice (Fig. 4.1 A). When the results normalized by their own body weight, male Tat^+ mice showed significant reductions in forelimb grip force strength compared to all other groups with doxycycline and were the only group in which doxycycline treatment caused reduced grip strength versus controls (Fig. 4.1 B).

Symptoms of HIV related neurocognitive disorders are likely to be accompanied by abnormal mental health conditions such as anxiety, depression or other mood disorders in HIV infected individuals. Thus, neuropsychological tests may be performed simultaneous to cognitive tests. Therefore, we hypothesized that long-term Tat induction might also induce neuropsychological impairments in transgenic Tat^+ mice in a sex specific manner. The rotarod test and grip strength test are "forced" performance tests, while the open field test and light/dark box test can be used in rodents as qualitative and quantitative measures of general locomotor activity and willingness to explore, an indication of mental or neuropsycological status (Lalonde and Strazielle 2008; Stanford 2007). The open field test

and light/dark box test are based upon the rodent's conflict between an innate aversion to exposed spaces and a tendency to explore novel environments (Cryan and Holmes 2005; Wallace et al. 2008). In the open field test, depressed or anxious animals will show reduced traveling distances in an unrestricted environment, reflecting their loss of exploring new noble environment. In the dark/light box condition, depressed or anxious animals will tend to spend more time in exploring noble environment in the dark side (Bourin and Hascoet 2003). Both behavior tests were only conducted in short time period (5-10 min), so that the animals in these tests do not lose their novelty to new environment by habituation. Interestingly, our results showed that an altered neuropsychological status varied between sexes varied according to the behavioral test being performed. Long-term Tat induction significantly attenuated the distance of exploratory activity in an open field in both female and male mice, with no effect of sex (Fig. 4.2 A) In the light/dark box test, only male Tat^+ transgenic mice showed significantly increased time and activities in the dark versus control (age matched male Tat^+ unexposed to doxycycline). All other groups, including Tat^+ females, were not different from their respective control group without doxycycline administration (Fig. 4.2 B).

All together, our HIV Tat transgenic animal model showed that Tat induction resulted in significant changes in psycho-behavioral status. Open-field exploratory behavior was reduced in both sexes; whereas male mice showed preference to stay in dark conditions, perhaps indicating a sex-specific component of anxiety. Therefore, our results may mean an underlying sex difference in the vulnerability to certain neurocognitive and psychological disorders in HIV infected individuals. It has been difficult to study human

subjects under controlled condition, but our result suggested that sex should be considered as an important variable.

Based on our behavior test results, multiple evaluations were performed in tissues from these mice to determine whether CNS cells were affected by long-term Tat induction in sex specific manner. Such changes might be involved in the development of sex specific behavioral abnormalities. Microglial activity and their inflammation *in viv*o became the first evaluation target in this studies, since previous studies in our laboratory strongly suggested that Tat can induced changed in microglial numbers and reactivity (Bruce-Keller et al. 2008; Fitting et al. 2010c; Gupta et al. 2010; Hahn et al. 2010b; Suzuki et al. 2011). Increased number of microglia and enhanced microglial reactivities significantly increase the inflammatory stress on neurons as direct effects or secondarily through microglial activation (Bruce-Keller et al. 2008; Suzuki et al. 2011; Zou et al. 2011). Data from the present study also showed that the overall proportions of microglia in striata were increased to a similar extent by long-term Tat induction in both sexes. However, when we assessed the reactive properties of microglia by assaying for 3-NT expression we noted that it can be influenced by the sex of animals. Long-term Tat expression appeared to enhance 3-NT expression in microglia significantly in male mice. Dysregulated macrophages/microglia in CNS, which over-produce various proinflammatory cytokines/chemokines, are frequently detected in autopsy studies of the basal ganglia from chronic HIV infection (McArthur et al. 2010; Pereyra et al. 2009). Therefore, our finding that the striata of male Tat^{+} mice have more elevated microglial population with increased 3-NT immunopositivity suggest that the neurons in male mice striata may be even more vulnerable to the stress from microglial reactivity than those in female mice. This implies that a similar, sex-related vulnerability

may occur in the CNS of HIV positive patients. There have been some studies that sex hormones might play an important role in microglial cytokines/chemokines secretion. For example, estrogen may modulate immune responses of microglia by leading micriglia in female mice to release greater anti-inflammatory cytokines than microglia in male mice, even though the increased proportion of microglia were similar in both sexes after exposure to Tat and/or opiates (Bruce-Keller et al. 2007). The underlying mechanism and relationships between microglia activation and sex-specific vulnerability of the CNS are still correlative and further studies are required to show direct, causal links.

Neurons are considered a major target of the HIV-1 Tat protein. Previous *in vivo* studies indicated that certain HIV-1 proteins, including Tat and gp120, are neurotoxic, since not intended secondary/bystandard effects can induce cell loss, low neuronal integrity in synapses, caspase-3 activation, mitochondrial hyperpolarization and dendridic spine loss in parallel with macrophage/microglia activation (Everall et al. 2002; Ratai et al. 2010; White et al. 2011). Due to neuronal injury or loss, subcortical brain atrophy (Castelo et al. 2007; Cohen et al. 2010) can frequently be detected by resonance imaging in HIV infected individuals, even these patients are in the cART. We utilized a NeuN antibody to identify the proportional number of neuronal cells in the striata of transgenic mice, because we thought the loss of neurons could be basis of behavior changes. In our transgenic mice that the proportion of $NeuN^+$ neurons in striata was significantly decreased after 3 month of HIV-1 Tat induction. There was also the effect of sex-Tat interaction to reduction in $NeuN^+$ cell population; male Tat^+ mice proportionally lost more striatal $NeuN^+$ cells than females did under same period of Tat induction. Immunoblotting with NeuN antibody was also

performed to semi-quantitatively compare the expression levels of NeuN protein in striatal lysates from those animals.

As the lysates from male Tat^+ mice striata showed more reduction in the intensity of NeuN bands compared to those from female Tat^+ mice, the pattern of reduction in NeuN immunoreactuvity were quite similar to the proportional counting reuslts. Nearby 50% with the NeuN signal lost in both measurements, this seemed like a tremendous amount of cell loss, and we hypothesized that long-term induction might cause the depletion of NeuN expression in neurons, rather than 50% neuron death in the striata. To assess the hypothesis, unbiased stereological techniques were applied to estimate neuron number in strata and striatal volume all groups. Comparable to previous reports (Cyr et al. 2005; Oorschot 1996), we measured neuron number in the striata to be approximately 1.8 million cells in females and slight, but significantly less in males. Furthermore, prolonged Tat induction didn't affect the total neuron number in the striatum or the striatal volumes in any groups. Thus, in agreement with previous data after 10 days of doxycycline treatment in these transgenic mice, long-term Tat induction did not result in neuron death (Bruce-Keller et al. 2008). Based on stereological measurements, we believed now that the striatal neuron population is not reduced by long-term Tat expression, but that Tat induces neuron damage and interferes with production if NeuN or its antigenesity by unknown mechanism. Although NeuN has been utilized as a neuronal specific marker for over a decade; the NeuN protein was only recently identified as Fox-3, a new member of the Fox-1 gene family of RNA splicing factors (Kim et al. 2009; Kim et al. 2011). Therefore, the underling mechanisms of 1) how induction of Tat causes altered expression of NeuN, or 2) how an altered level of NeuN could affect neurons, 3) how many other environmental cues (i.e.

inflammatory factors from microglia/astrocytes) would be involved in NeuN associated pathogenesis are still required further studies.

Since one of our interests was how HIV-1 can affect NPCs in CNS, especially in striata, we also examined whether long-term induction of HIV-1 Tat alters the population of NPCs and oligodendrocytes in striata. The population of $Sox2^+$ and $Olig2^+$ cells in striatum were significantly decreased by 3 month induced Tat expression in all Tat^+ transgenic mice groups. There was no sex specific manner in the decline of either cell type. According to previous reports, HIV-1 proteins such as Tat (Mishra et al. 2010), gp120 (Okamoto et al. 2007) can alter the proliferation of NPCs both *in vitro* and in the hippocampus and may result in differentiations more toward astrocytes and less toward neurogenesis (Krathwohl and Kaiser 2004a; Tran and Miller 2005). The reduction in NPCs seen here is in line with those results and our results from Chapter 3.in neonatal animal. There have been some suggestions that cytokine/chemokines secreted from microglia/macrophage induced by HIV-1 or its proteins might increased proliferation of NPCs (Gong et al. 2006; Peng et al. 2008), however, these studies had limitation that those experiments were short term condition of exposure and were also *in vitro* on isolated cells. Since long-term induction of Tat in CNS in our transgenic mice resulted in significant increase the activity of microglia, the survival or production of both NPCs and OLs can be secondarily affected by these activated microglia, which would produce not only pro-inflammatory cytokine/chemokines but also nitro-oxidtive stress (Das and Basu 2008; Whitney et al. 2009). The long-period exposure to these multiple factors and HIV-1 Tat might decrease the population of NPCs by inducing fast turn-over of NPC proliferation or promoting quiescence of NPCs, since in preliminary studies, still in progress, we couldn't

detect any proliferation of NPCs in adult striata by BrdU administration. Our experiment suggested that long-term Tat induction in CNS significantly decreases populations of NPCs and OLs as one of evidence of HIV neuropathogenesis. The depletions of NPC and OL populations correlate with altered motor and psychological behavioral test changes in our transgenic mice, but we cannot, of cause, conclude that there is a cause of relationship.

In conclusion, the data in this thesis support the hypothesis that HIV-1 Tat protein, produced by chronic replication of HIV in CNS, could play a significant role in the development of neurocognitive disorders in HIV patients, perhaps by induction of pathological microglial activation in the striatum. Data also show in this model that sex may affect the vulnerability in developing behavioral deficit/impairments with males, being significantly more affected in several standard rodent tests. Our data also suggested that sex-related changes in microglial activity may cause the depletion of NeuN immune reactivity in males, which is more affected than that is in females. In other measures, NPCs and OLs were reduced similarly in Tat^+ males and females. At present, we can only say that depletion of $NeuN^+$ neurons and enhanced microglial reactivity correlate with sex related behavioral changes. A cause-and-effect relationship remains to be established in later studies.

Figure 4.1

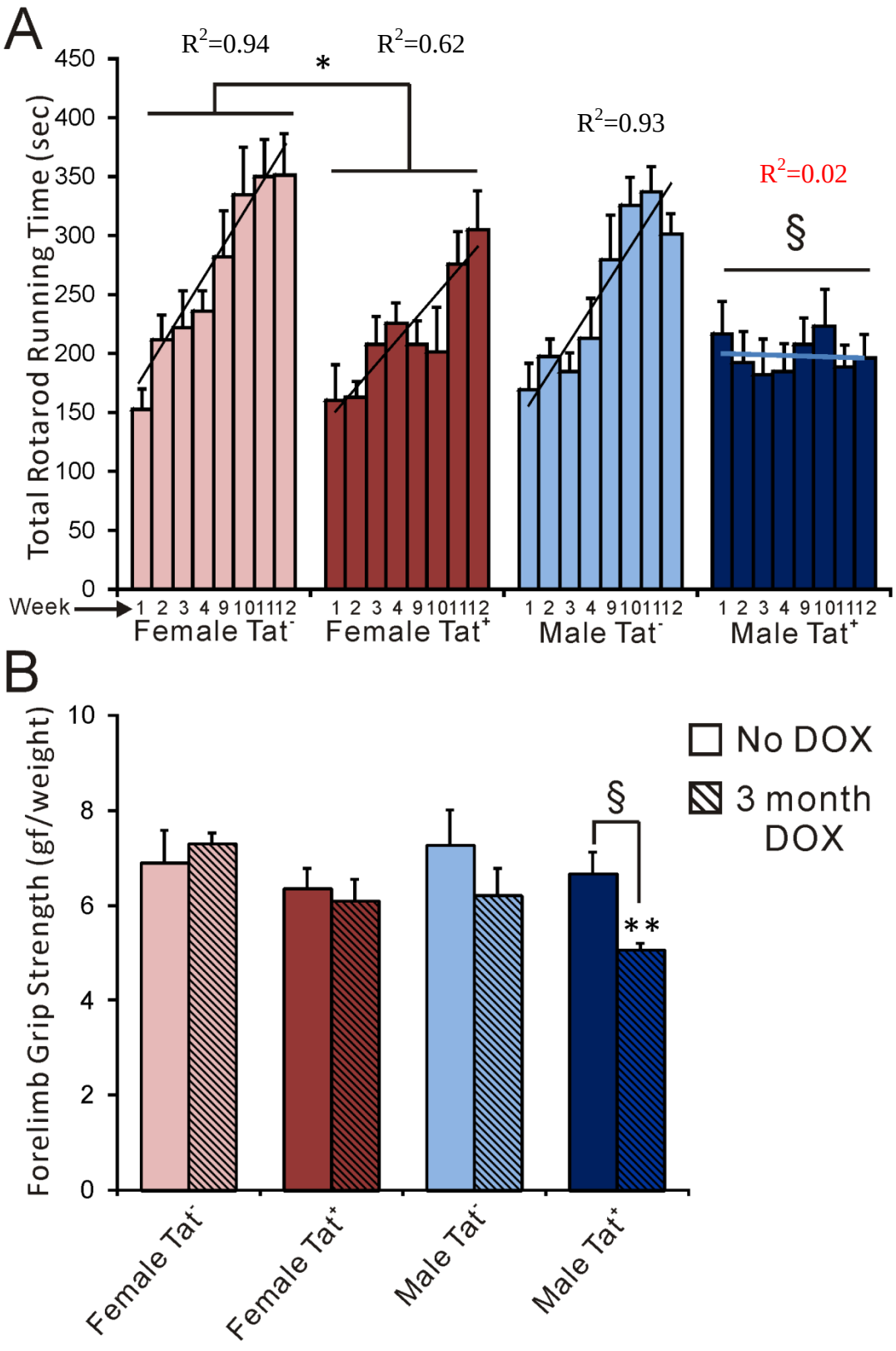
A
R2=0.94
*
R2=0.62
R2=0.93
R2=0.02
§
Total Rotarod Running Time (sec)
450
400
350
300
250
200
150
100
50
0
Week
1 2 3 4 9 10 11 12
Female Tat-
Female Tat+
Male Tat-
Male Tat+
B
Forelimb Grip Strength (gf/weight)
10
8
6
4
2
0
No DOX
3 month DOX
§
**
Female Tat-
Female Tat+
Male Tat-
Male Tat+

Figure 4.1 Long-term Tat expression in transgenic mice revealed a sex difference in Tat effects on motor activity and grip strength. Four different groups (female Tat^+ and Tat^-, male Tat^+ and Tat^-) were fed chow containing doxycycline continuously for 12 wk to induce Tat expression *in vivo*. (A) Rotarod results. There was no difference between these four groups after 1 wk of doxycycline exposure. However, long-term induction of HIV-1 Tat by 3 month doxycycline administration caused significant difficulty in obtaining motor skill in the male Tat^+ group compared to all other groups. (B) Grip strength results. To compare and normalize the effects of long-term Tat induction *in vivo*, a matching set of 4 groups with normal chow feeding were separately prepared. After 12 wk, when all mice were 6 month old, a grip strength test was performed. There was no difference due to sex or genotype without doxycycline exposure. However, the long-term exposure to HIV-1 Tat caused significantly decreased forelimb grip strength in the male Tat^+ group compared to all other groups (§ $p<0.05$; ** $p<0.01$, male Tat^+ vs. all other groups with DOX, Duncan post-hoc test, N=6-8). Based on these behaviors, we suggest that long term Tat exposure affects mice in a sex-specific manner, with effects on male mice that are not observed in female mice. DOX, doxycycline.

Figure 4.2

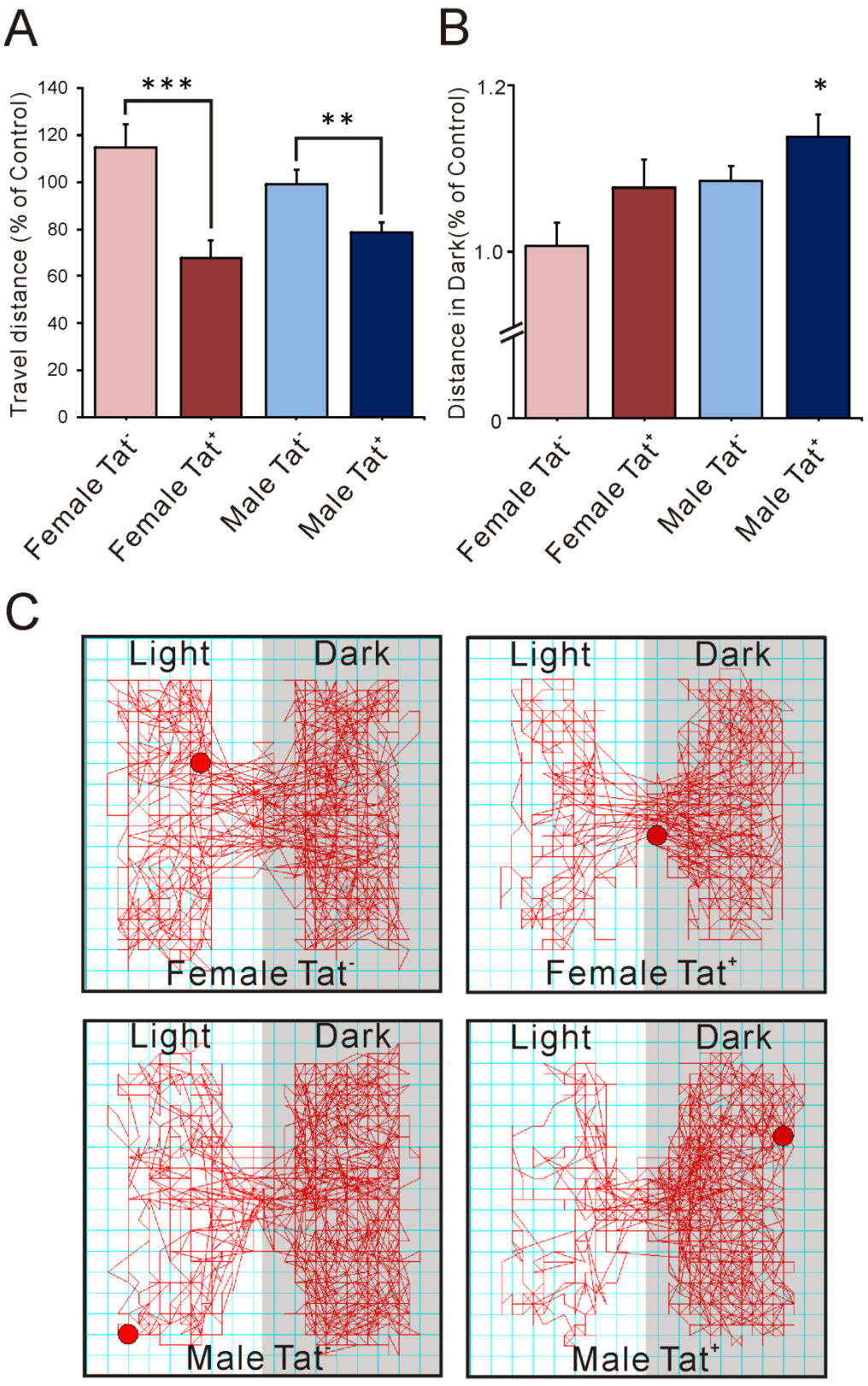
A
B
C
Travel distance (% of Control)
Distance in Dark(% of Control)
***
**
*
Female Tat-
Female Tat+
Male Tat-
Male Tat+
Light
Dark

Figure 4.2 The effect of long-term Tat exposure on locomotor activity and anxiogenic-like behavior of adult transgenic mice. Open-field tests and light/dark box tests were applied to two sets of 4 different groups (female Tat^+ and Tat^-, male Tat^+ and Tat^-, with/without doxycycline treatment) were tested at 6 month old. A matching set of 4 groups with feeding normal chow at 3 month old were separately prepared and tested to normalize the data from other groups mentioned above. (A) Open field test results. There was a difference in locomotor activity due to sex, even in groups that did not receive doxycycline treatment. Long-term Tat induction significantly reduced locomotor activity in both sexes. (B) Light/dark box results. Long-term Tat exposure selectively induced anxiogenic-like effects in the male Tat^+ group compared to all other groups. (C) Representative diagrams of light/dark box results. Tat^+ males showed significantly increased travel distance in dark side compared to any other groups. Based on these behaviors, we suggest that long term Tat exposure affects mice in a sex-specific manner. By most measures, male mice are adversely affected by Tat induction in comparison to female mice. (* $p<0.05$; **§** $p<0.05$, female Tat^- vs. all other groups, Duncan's post hoc test, N=6-8.) DOX, doxycycline.

Figure 4.3

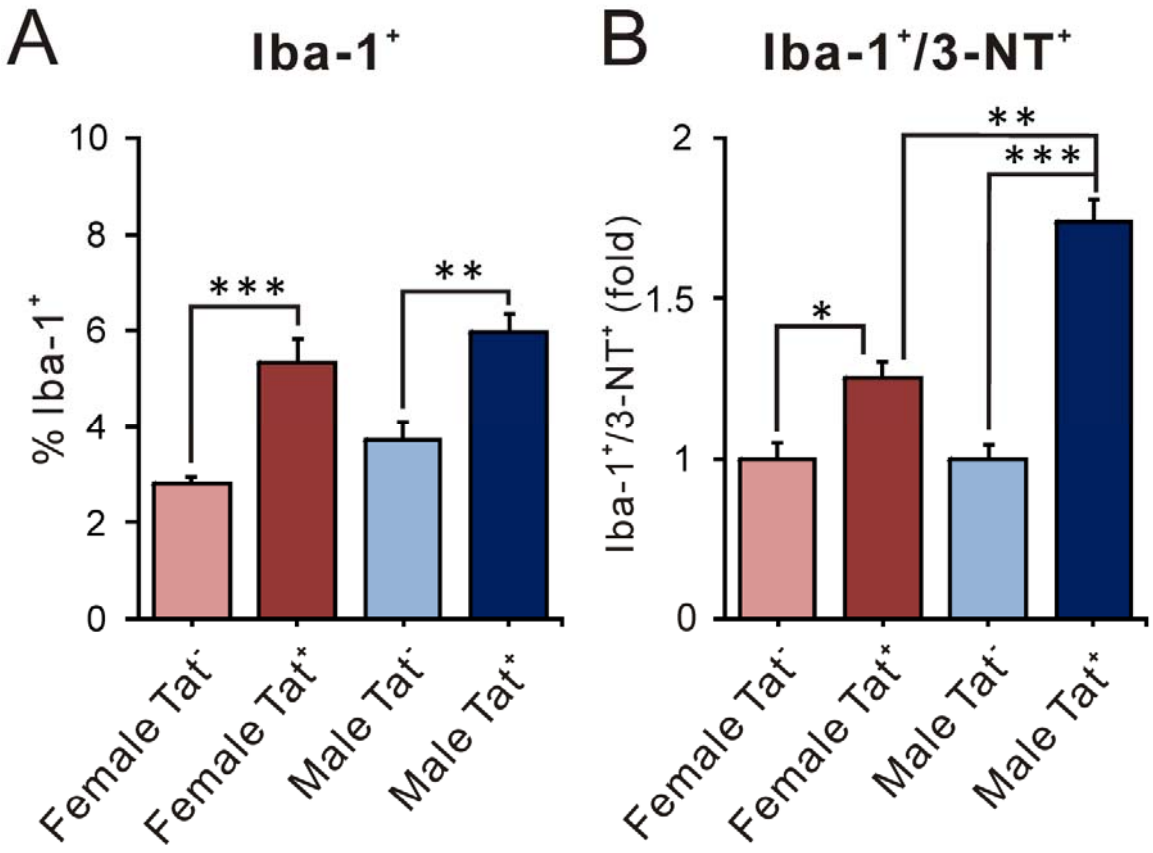
A
Iba-1+
% Iba-1+
10
8
6
4
2
0
***
**
Female Tat-
Female Tat+
Male Tat-
Male Tat+
B
Iba-1+/3-NT+
Iba-1+/3-NT+ (fold)
2
1.5
1
0
*
**
***
Female Tat-
Female Tat+
Male Tat-
Male Tat+

Figure 4.3 Long-term Tat expression in transgenic mice revealed a sex difference in Tat effects on microglial populations and their activities in adult striatum. The brains were harvested from four different groups (female Tat^+ and Tat^-, male Tat^+ and Tat^-), with 12 wk Tat induction by doxycycline *in vivo*. The effects of long-term Tat exposure on the microglial population in adult striata were examined by multiple immunohistochemistry. There was a significantly increased population of Iba-1^+ cells in both male and female Tat^+ groups compared to their Tat^- control (A). Although there was no sex difference in Tat effect on Iba-1^+ cells, long-term exposure to HIV-1 Tat did induce a significant increase in the activity of microglia in the male Tat^+ group compared to all other groups, based on the expression of 3-nitrotyrosine (3-NT^+) (B). Based on these results, we suggest that long term Tat exposure affects mice in a sex-specific manner, with effects on male mice that are not observed in female mice. (* $p<0.05$; ** $p<0.01$; *** $p<0.001$; § $p<0.05$, female Tat^- vs. all other groups, Duncan's post hoc test, N=6-7.) DOX, doxycycline.

Figure 4.4

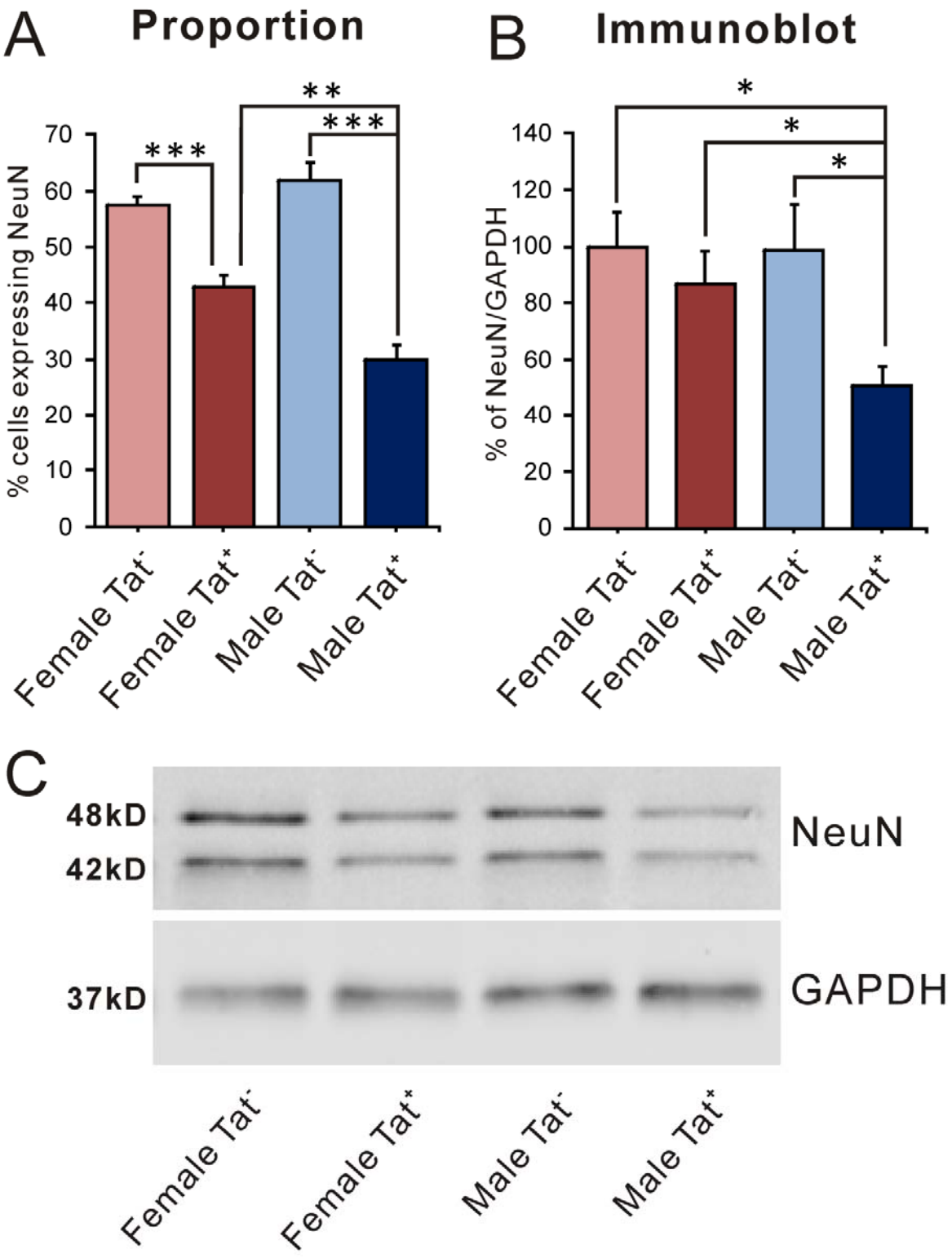
A
Proportion
% cells expressing NeuN
70
60
50
40
30
20
10
0
***
**
***
Female Tat-
Female Tat+
Male Tat-
Male Tat+
B
Immunoblot
% of NeuN/GAPDH
140
120
100
80
60
40
20
0
*
*
*
Female Tat-
Female Tat+
Male Tat-
Male Tat+
C
48kD
42kD
NeuN
37kD
GAPDH
Female Tat-
Female Tat+
Male Tat-
Male Tat+

Figure 4.4 Effects of long-term Tat induction on the population of $NeuN^+$ cells and NeuN immunoreactivity in adult striatum. NeuN immunohistochemistry were performed on the brain sections including striata from 4 different groups (female Tat^+ and Tat^-, male Tat^+ and Tat^-) with doxycycline treatment at 6 month old for $NeuN^+$ cell counting. HIV-1 Tat induction for 12 wk caused significantly decreased the population of $NeuN^+$ cells in the striata of both female and male Tat^+ mice. There was a significant sex effect, as the effect in Tat^+ males was significantly greater than in all other groups (A). Immunoblots were performed with NeuN antibody to measuring any alteration in the level of NeuN in the striata after long-term Tat induction. Striata were dissected and harvested from independently prepared 4 different groups (female Tat^+ and Tat^-, male Tat^+ and Tat^- with DOX treatment at 6 months old) for immunobolting. The intensity of bands was significantly decreased by Tat exposure only in tissue lysates from male Tat^+ mice (B, C). (* $p<0.05$; ** $p<0.01$; *** $p<0.001$, one-way ANOVA or two-way ANOVA, Duncan's post hoc test, N=6-8.) DOX, doxycycline.

Figure 4.5

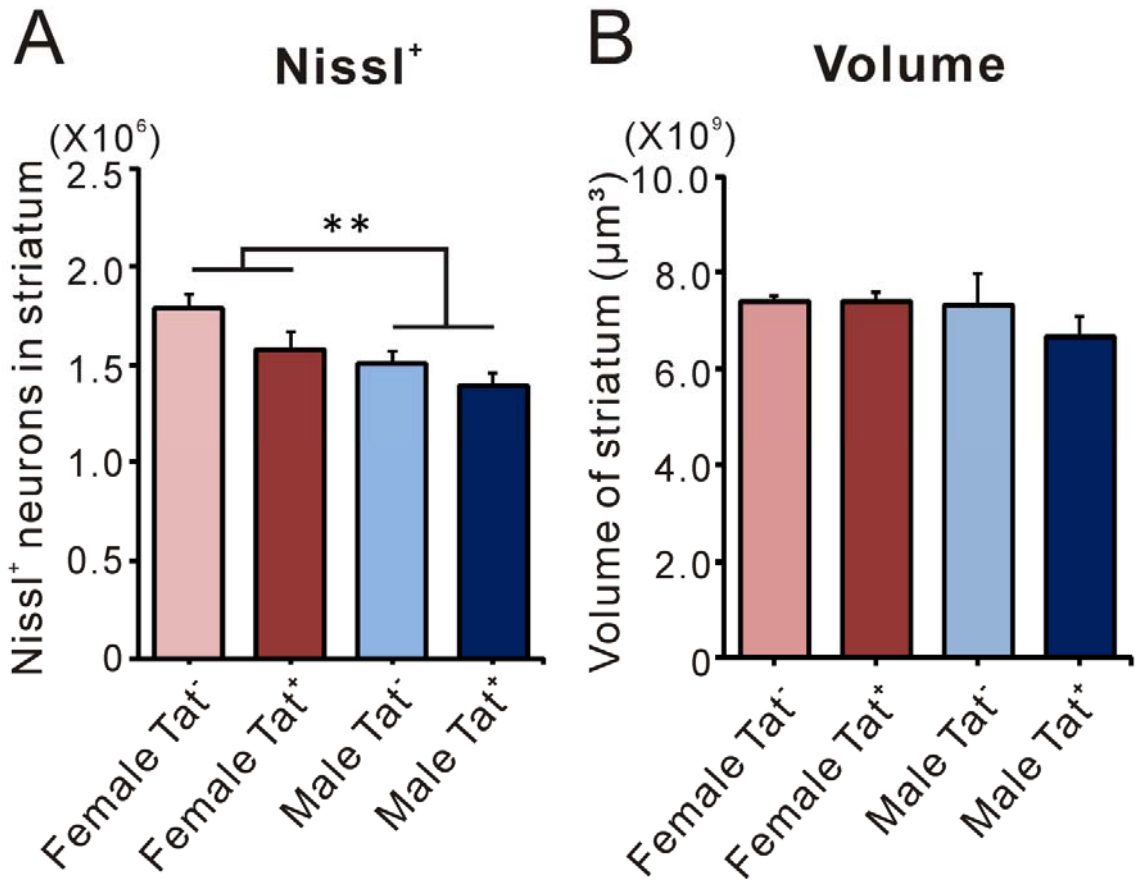

Figure 4.5 Effects of long-term Tat induction on total number of Nissl$^+$ neurons in transgenic mouse striata and the volume of striata. The brain sections including striata from 4 different groups (female Tat$^+$ and Tat$^-$, male Tat$^+$ and Tat$^-$) with doxycycline treatment at 6 month old for stereological estimation of Nissl$^+$ neuron numbers. Unbiased stereological estimates showed that total neuron number (Nissl$^+$) were higher in female mice than in male mice; there was no effect of Tat on Nissl$^+$ neurons (A). Furthermore, doxycycline induced Tat didn't alter the estimated striatal volume in any group (B). Based on these results, we suggest that long term Tat exposure might affect expression level of NeuN in the neurons of male Tat$^+$ mice, without actual loss of neurons or a change in volume of the striatum. (* $p<0.05$, two-way ANOVA, Duncan's post hoc test, N=3.) DOX, doxycycline.

Figure 4.6

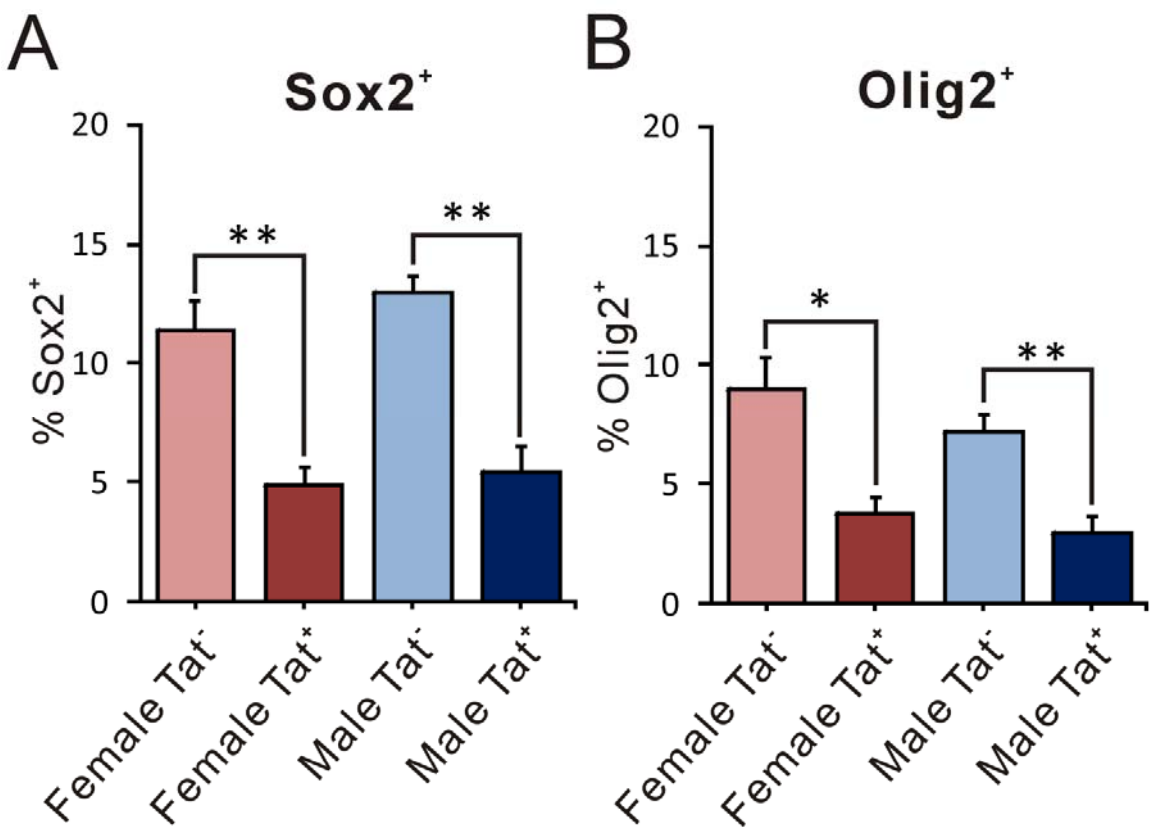

Figure 4.6 Long-term effect of Tat expression in transgenic mice on the population of neural progenitor cells and oligodendrocyts in adult striata. HIV-1 Tat induction by 12 wk doxycycline administration caused significantly decreased populations of $Sox2^+$ cells in the striata of both Tat^+ males and Tat^+ females, but there were no significant differences due to sex (A, * $p<0.05$; ** $p<0.01$; one-way ANOVA or two-way ANOVA, Duncan's post hoc test, N=6-8). Similarly, $Olig2^+$ oligodendrocytes equally decrease in the striata both both Tat^+ females and Tat^+ males (B, * $p<0.05$; ** $p<0.01$; one-way ANOVA or two-way ANOVA, Duncan's post hoc test, N=6-8). DOX, doxycycline.

Table 4.1 Measurements of activity in open field and light/dark box tests

A.

Open field test	Travel distance (cm/10min ± SE)	
	6 month old No DOX	6 month old 3 month DOX
Female Tat$^-$	1904.68±334.36	2184.96±183.83
Female Tat$^+$	2484.53±116.25	1682.55±190.38
Male Tat$^-$	2045.54±165.53	2027.74±128.80
Male Tat$^+$	2152.74±199.09	1689.94± 94.54

B.

Light/Dark test	% of Travel distance in dark side (cm/10min ± SE)	
	6 month old No DOX	6 month old 3 month DOX
Female Tat$^-$	79.77 ± 12.03	80.28 ± 5.63
Female Tat$^+$	77.49 ± 7.57	83.44 ± 6.48
Male Tat$^-$	71.71 ± 8.68	77.82 ± 3.01
Male Tat$^+$	71.39 ± 4.06	81.26 ± 4.69

DOX, doxycycline.

Chapter 5

Final Conclusions

The introduction of cART has dramatically improved survival rates in the HIV/AIDS population; however, neurocognitive impairments in these patients still remain in high prevalence. 60% of HIV-infected people are estimated to be affected by HIV related neurocognitive disorders (Ellis et al. 2007; Wojna et al. 2006) and the prevalence of HAND has shown no decline, even with cART treatment (Dore et al. 2003; Lawrence and Major 2002; Sacktor et al. 2001). Low blood brain barrier (BBB) permeability of many cART medicines might contribute to these phenomenon, so that higher viral loads still remained in the CNS compared to peripheral tissues where cART is quite effective in decreasing viral load (Gonzalez-Scarano and Martin-Garcia 2005; Sacktor et al. 2001). Approximately, 30% of HIV positive patients were infected through the use of contaminated needles during injection (UNAIDS 2009). HIV-positive, opiate abusers have high a risk for faster progression to acquired immune deficiency syndrome (AIDS), as well as higher incidence of subtle form of neurocognitive disorders compared to non-drug abusers, especially since misuse of drugs leads them to decrease their adherence to cART medications (Bell et al. 2006; Bell et al. 2002; Byrd et al. 2011). HIV-associated neuropathology in the CNS is considered as a combined and complex result generated from the direct toxicity of multiple viral proteins on neurons as well as by the indirect effects of pro-inflammatory factors or nitro-oxidative stress propagated by surrounding glial cells (Brack-Werner 1999; Gendelman et al. 1994; Hauser et al. 2007; Kaul et al. 2001; Kramer-Hammerle et al. 2005b; Persidsky and Gendelman 2003). NPCs exist both in the pediatric

and adult brain, and the size of their population is relatively small compared to other CNS cell populations. NPCs are primitive, undifferentiated cells having the capacity of proliferation and the multi-potential ability to generate various neural lineages of mature brain cells including neurons, astrocytes, and oligodendrocytes. The size of the NPC population is substantially reduced with age, as is their distribution and recruitment. The effect of HIV-1 ± opiates on NPCs has been less well studied compared to other cells, partially because their small populations and transient characteristics make their study difficult. We have hypothesized that NPCs may be targets of HIV proteins ± opioid drugs of abuse and that exposure of HIV ± opiates may alter the balance of cells in the CNS that derive from NPCs.

In Chapter 2, we have shown that HIV-1 Tat_{1-86} selectively increases the production of β-chemokines, CCL3/MIP-1α, CCL4/MIP-1β and CCL5/RANTES from striatal progenitors using a multi-plex cytokine/chemokine assay. Because of our interests in the effects of co-morbidity between opiate drugs of abuse and HIV exposure in the CNS, we chose to examine the striatum, an area which is both rich in opiate receptors and extremely vulnerable to HIV-induced neuropathology. The chemokines upregulated by Tat also induced a chemotactic migratory response of both primary microglial cells and the BV-2 mouse cell line. Furthermore, we showed that these effects were mediated by CCR5 by using antibody blockade, as expected with β-chemokines. Even though immunohistochemistry on NPCs showed that they were immunopositive to MOR and CCR5/CXCR4, there was not any synergistic effect of opiate interaction or toxicity of gp120 alone in these experiments. In addition, the survival rate of NPCs was not affected by any treatments. These findings are significantly relevant to HIV related neuropathology,

as Tat mRNA and/or protein have been detected in CNS tissue samples from patients infected with HIV (Hudson et al. 2000; Wiley et al. 1996). Furthermore, some recent reports suggested that human neural progenitors may be infected by HIV (Lawrence et al. 2004; Rothenaigner et al. 2007; Schwartz and Major 2006), although the extent of NPC infection in the brain is still controversial. The potential contribution of this infected population to the chemokine secretions and their effects require further studies. Importantly, our results show that Tat can affect the function of NPCs in the absence of infection. In this chapter, we examined two critical components of NPC function, their communication with other cells through secreted factors and their survival. We suggested that secretions of β-chemokines by NPCs might be useful in promoting microglial surveillance of newly formed NPCs and clearing toxic debris. Other potential effects might to provide trophic support or to regulate microglial proliferation and differentiation, although we have no evidence for these. Other critical NPC/GPC behaviors including proliferation and lineage decision are examined in other chapters.

In Chapter 3, to investigate the effect of HIV-1 on proliferation of NPCs in developing systems, we firstly examined the effect of Tat ± opiates on murine striatal progenitors *in vitro*. Individually, Tat and morphine did not affect the proliferation of either $Sox2^+$ or $Olig2^+$ cells *in vitro* at 12 h, although the combination of Tat and morphine interactively reduced the total population of $Sox2^+$, and $Sox2^+$ proliferation index. At the later time points, Tat alone reduced the proliferation and population of $Olig2^+$ cells, although interactive effect of Tat + morphine was seen on its proliferating cells. By using doxycycline-inducible Tat transgenic mice, *in vivo* experiments were performed in perinatal model as a next step. Tat and morphine independently both reduced the

proliferation of $Sox2^+$ and $Olig2^+$ cells. An interactive effect was seen only on $Sox2^+$ proliferation. The $Sox2^+$ population was reduced by both morphine and Tat, while the $Olig2^+$ population was reduced by only by Tat. We did not observed any interaction between Tat and morphine on the cell populations we hypothesis that these might be measured at later time points. Since HIV is a human specific virus, we also examined the effect of supernatant from HIV-1_{SF162}-infected cells or Tat ± morphine on proliferating hNPCs. The proliferation of human $Sox2^+$/$nestin^+$ NPCs is similarly reduced by Tat, morphine, and infective HIV-1_{SF162} supernatant, and an additive effect between HIV-1_{SF162} and morphine reduces proliferation further. The additive effect of HIV supernatant and morphine in reducing hNPC proliferation, which was not seen in Tat + morphine treatment, may reflect the interaction between multiple factors in HIV supernatant including other viral proteins such as gp120 (Lee et al. 2011) as well as numerous secreted or reactive factors present in the medium. Previously, we mentioned that NPCs may be a target of HIV infection. To examine this, we imaged the HIV-1_{SF162} supernatant treated NPCs with p24 immunohistochemistry, and our finding supports the possibility of low level hNPC infection. Taken together, our results show that the effects of HIV-1 Tat or HIV-1_{SF162} supernatant and morphine significantly reduce the proliferation of NPC/GRPs, and interactions among them can compromise both murine and human progenitor production even further. These findings suggest the possibility that HIV infected pediatric patients, who have developing brains with significant number if NPCs and immature glia, likely will develop neuropathological complications due to HIV infection alone, and the complications maybe worse if they are exposed to opiates.

In Chapter 4, we extend our interest into the effect of long-term Tat exposure on animal behavior, since chronic low levels of HIV replication in CNS may play a significant role in the development of neurocognitive disorders in HIV patients. We utilized several standard rodent tests to examine the effect of long-term Tat induction in animal model. Interestingly, male Tat^+ mice showed overall greater deficits than all other groups. Doxycycline-induced male Tat^+ mice had decreased rotarod performance and forelimb grip strength, and they also showed increased anxiety-like behavior as compared to doxycycline-induced female Tat^+ mice and control groups. There was no sex related difference in open field locomotor activity. In parallel to these behavioral tests, we performed correlative experiments to assess Tat effects on certain cell populations. We determined that microglial activation was more enhanced in striatum of induced Tat^+ males compared to that of induced Tat^+ females, although their microglial number was increased to the same extent. NeuN levels in striatum were also more reduced in male Tat^+ mice by Tat induction as compared to all other groups. Interestingly, we determined that the reduction in NeuN immunoreactivity was not consequent to loss or death of neurons in striatum, suggesting down-regulation of NeuN expression in living neurons. Since we were also interested in the effect of Tat on NPCs, we also examined the populations of NPCs and OLs by immunostaining for Sox2 and Olig2. The reduction in their populations was similar in doxycycline-induced Tat^+ males and females. These data suggest that sex related behavioral changes may be modulated by enhanced microglial reactivity, which may also cause the depletion of NeuN level in neurons and the reductions in populations of NPCs and OLs in striata. However, a cause-and-effect relationship remains to be established in later studies. Overall, our data suggest that HIV patients may be vulnerable to developing

neurocognitive disorders in a sex dependent manner, making sex an important variable in human studies.

In conclusion, one of most important findings in our studies is that NPCs are major targets of HIV-1 viral protein Tat and that certain effects of Tat are interactively affected by concurrent morphine exposure. Most of our studies are directly related to neonatal/perinatal systems, which is relevant to the neuropathogenesis in pediatric HIV population. The role of NPCs is well studied in stem cells, memory formation and repairing CNS after injury/insults, however, the importance of them has been understudied in HIV-induced pathology in the CNS. Even though neurons in most CNS are formed prenatally, but production of gliogenesis continues into the postnatal and adolescence period (Chan et al. 2002; Lee et al. 2000; Skoff 1990; Skoff and Knapp 1991). Because of this reason, the CNS of younger HIV patients have and require relatively large populations of NPCs compared to adult HIV patients. Furthermore, these pediatric/adolescent patients have a great possibility that their CNS could be exposed to opiates. This might appear through the use of opiates as pain control medication through their lives, for examples, during surgery. It might also occur during experimentation with drug abuse. Our data strongly suggested that certain major functions of NPCs are sensitive to both HIV-1 and opiate exposure and are vulnerable to the interactive effect between HIV-1 and opiates. There are presently few options for non-opiate, pain-control medications in HIV pediatric patients does not existed yet, therefore, the development of the new non-opiate, pain-control medications might be of great clinical benefit to these pediatric patients, for example, toll-like receptor (TLR) antagonists. Some studies suggested that opiates can induce glial activation mediated by TLR to lead neuropathic pain, and this glial activation can be alleviate by TLR antagonists

to reduce the neuropathic pain (Hutchinson et al. 2007). Another important finding in our studies is that NPC populations existing in the adult CNS can be vulnerable to long-term HIV exposure. Our data from an *in vivo* animal model also indicate that behavioral outcomes might be affected by long-term HIV-1 exposure to CNS in sex-specific manner. HIV-1 Tat transgenic mouse model, that we have, females show less cognitive and motor deficits along with less severity in neuropathogenesis compared to males. Interestingly, changes in $Sox2^{+}$ NPCs and $Olig2^{+}$ oligodendrocytes do not correlate to sex-related behavioral changes, but increased microglia activation and depletion in NeuN level do show a correlation. Even though a cause-and-effect relationship has not been shown here, and is required to be established in the future, we cautiously hypothesize that these sex differences in the phenotype of sub-population of microglia cells may reflect an effect of sex hormones, such as estrogen, on microglial activation induced by HIV-1 or viral proteins. Some i*n vitro* studies suggested that pretreatment with estrogen can attenuate microglial activation, mediated by mitogen activated protein kinase and NF kappa B pathways, to reduce superoxide/nitrite release, phagocytic activity, and TNF-α secretion from microglia (Bruce-Keller et al. 2001). Since there have been few studies in adult NPCs under the long-term condition of HIV infection, further studies are needed to show how HIV effects on NPCs relate to overall CNS pathology in HIV patients, as well as to the behavioral deficits/abnormality in HAND, and the influence of sex should also be considered in future studies.

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Vita

Yun Kyung Hahn was born in Seoul, Republic of Korea, on September, 15, 1978. She graduated from Chang-Duck Girl's High School in Seoul, Republic of Korea in 1997. Her education continued at Sook-Myung Women's University in Seoul, Republic of Korea where she earned her Bachelor of Science in Biological Sciences. After her graduation Sook-Myung Women's University in 2001 and she began her graduate education; earning a Master of Science in Biological Sciences from the Department of Biological Sciences at Seoul National University in Seoul, Republic of Korea in 2004. The following winter Yun Kyung came to United States as an Exchange Researcher to extend her career and was hired by Department of Health Sciences in University of Kentucky at Lexington. She transitioned into the Ph.D. program in the Department of Anatomy and Neurobiology, University of Kentucky in the fall of 2005 and joined the laboratory of Dr. Pamela E. Knapp in the fall of 2006. She transferred into the Department of Anatomy and Neurobiology at Virginia Commonwealth University, Richmond, VA to follow her professor in the fall of 2007. As a graduate student, Yun Kyung was given the opportunity to present her research at the Society for Neuroscience meeting, American Society for Neurochemistry meeting, Central Virginia Chapter Society for Neuroscience as well as various institutional presentations. She earned awards as a winner of fall poster presentation day at Central Virginia Chapter of the Society for Neuroscience 2011, Richmond, VA and as a winner of Young Investigator Educational Enhancement (YIEE) at American Society for Neurochemistry 2012 meeting.

Manuscripts resulting from Yun Kyung's work at Virginia Commonwealth University:

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