HIV-TAT PROTEIN MEDIATION OF NEUROAIDS CENTRAL NERVOUS SYSTEM PATHOLOGY AND BEHAVIORAL DEFICITS

A dissertation presented
by

Amanda Natalie Carey

to
The Department of Psychology

In partial fulfillment of the requirements for the degree of
Doctor of Philosophy
In the field of
Psychology

Northeastern University
Boston, Massachusetts
June, 2010
HIV-TAT PROTEIN MEDIATION OF NEUROAIDS CENTRAL NERVOUS SYSTEM PATHOLOGY AND BEHAVIORAL DEFICITS

by

Amanda Natalie Carey

ABSTRACT OF DISSERTATION

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Psychology in the Graduate School of Arts and Sciences of Northeastern University, June, 2010
ABSTRACT

This dissertation investigated the role of HIV-Tat protein in mediating behavioral
dysfunction and neurodegeneration associated with NeuroAIDS. NeuroAIDS is the syndrome
consisting of neuropsychological manifestations and central nervous system pathology resulting
from HIV-1 infection. The syndrome is characterized by the progressive loss of cognitive and
motor function, and is also linked to neuropsychological disturbances that include changes in
mood, “personality”, and drug abuse. Research suggests that the cognitive and
neuropsychological dysfunction may have a common etiological and biological basis; it is
theorized that the release of toxic viral proteins from HIV-infected cells is a key mechanism by
which HIV-1 indirectly and adversely affects neurons. HIV-1 Tat viral protein has been
implicated in the pathogenesis of HIV-1 neurological complications, but direct behavioral
demonstrations are limited. The central hypothesis tested in this thesis is that HIV-Tat
protein, when expressed in brain, mediates behavioral dysfunction and
neurodegeneration in an exposure-dependent manner. Four studies tested this hypothesis
using the GT-tg bigenic mouse. The GT-tg mouse possesses an inducible and brain-selective
tat gene that codes for Tat protein. These mice were uniquely suited for this study, as the
expression of Tat protein is under the control of the researcher through the administration of
Doxycycline (Dox). The dosage and number of days mice are administered Dox may be varied
in order to control the amount and the length of time of Tat expression.

The first study tested if Tat was sufficient to impair learning and memory processes in
Tat-induced GT-tg mice compared to the C57Bl/6J parent strain of mice and/or the uninduced
GT-tg littermates. The Barnes maze was used to assess spatial learning and memory. The GT-
tg mice induced to express Tat demonstrated longer latencies to find an escape hole, committed
more total, reference, and working memory errors, and favored a less efficient search strategy
during acquisition compared to their uninduced GT-tg littermates, suggesting impaired spatial learning. Tat-induced mice also demonstrated poor probe trial performance, suggesting impaired spatial memory. Furthermore, reversal learning was impaired in Tat-induced mice, as the mice learned a new escape location slower than uninduced littermates. Tat-induced mice also demonstrated deficiencies in novel object recognition (NOR), a behavioral model for a different type of learning and memory performance than the Barnes maze. Tat-induced mice displayed long-lasting (i.e., up to one month) NOR deficits. Furthermore, NOR impairment was dependent on the dose and duration of Dox exposure, suggesting that the amount and duration of exposure to Tat progressively mediated deficits. The results of the Barnes maze and NOR testing provide evidence that Tat protein may mediate cognitive deficits seen in HIV-infected individuals and may be responsible for some of the behavioral pathology seen in HAD.

The next study tested the hypothesis that Tat can result in increased anxiety depending on the extent of Tat protein expression. Uninduced and Tat-induced GT-tg mice were initially tested in the open field test for anxiety-like behavior 48 h and one week after the completion of Tat induction to determine if any increases in anxiety were transient or persistent. Increases in anxiety-like behavior were apparent in mice induced to express Tat compared to controls, but mice administered the maximum induction regimen (Dox, 100 mg/kg, 7 days) were noted to have a more complex behavioral profile. These data were confirmed in the light-dark box. Mice induced to express Tat using various Dox induction regimens were also tested in the elevated plus maze. Interestingly, Tat-induced mice demonstrated behavioral changes in the elevated plus maze that were suggestive not of anxiety, but of behavioral disinhibition, a behavior commonly observed in patients suffering from dementia. Together, this battery of tests was used to characterize the behavioral profile of the transgenic mice induced to express varying amounts of Tat for different lengths of time. These studies demonstrated the complex
relationship between Tat and behavioral dysfunction that could potentially mimic the progression of the mood disorders and cognitive decline in the disease process.

The third study evaluated the ability of Tat to modulate the rewarding and sensitizing effects of cocaine. Once expressed, Tat produced sensitization to the locomotor effects of acutely administered cocaine, as the locomotor effects induced by a single injection of cocaine were significantly potentiated, a response typically seen only after repeated exposure to cocaine. Furthermore, mice expressing Tat showed a greatly potentiated cocaine-conditioned place preference response as compared to C57Bl/6J or uninduced mice, suggesting that brain expression of Tat protein enhanced the rewarding effects of cocaine. The magnitude of this effect was dependent on the dose and duration of Dox administered and the corresponding Tat induction. We also confirmed that the potentiated preference response was not a transient event, and became extinct at the same rate as that of uninduced littermates. Experiments further demonstrated the effects of Tat on an established reward state, with the induction of Tat protein significantly potentiating the effect of an additional exposure to place conditioning over both prior preference and the response of mice that were uninduced. Together, these experiments suggest that induction of Tat protein can potentiate reward in subjects with no previous experience with cocaine and in drug non-naïve subjects. Finally, we demonstrated that after extinction of cocaine preference in untreated GT-tg mice, induction of Tat protein directly resulted in the reinstatement of cocaine-seeking behavior.

The final study tested the hypotheses that Tat protein expression in the central nervous system is associated with decreases in gray matter density, white matter microstructural changes, and histological abnormalities in brain areas known to be affected in NeuroAIDS patients. In the study, brains were excised from Tat-induced, uninduced, and C57Bl/6J mice for ex vivo anatomical histology-grade magnetic resonance imaging using a 9.4 Tesla Varian magnet. Reductions in gray matter density in the amygdala, amygdala-hippocampal area,
piriform, perirhinal, and entorhinal cortices were found in the brains of 5 day Tat-induced mice compared to Dox-treated C57Bl/6J mice. Significant loss of white matter microstructural integrity in the insula, endopiriform nucleus, and part of the striatum was observed in the 7 day Tat-induced mice compared to uninduced GT-tg mice, suggesting a progressive effect of Tat protein on white matter, with increasing exposure to Tat resulting in more severe damage. Western blot analysis was run in parallel to confirm Tat protein expression in whole brain as well as specific brain areas. Additionally, limited analysis of histology staining used to detect activated microglia and cell death was performed to correlate in vitro evidence of the toxicity of Tat to in vivo demonstrations of neurodegeneration in our animal model.

In conclusion, this thesis addressed an under-investigated topic, identifying the contribution of HIV-1 Tat protein to neurodegeneration and neuropsychological impairments associated with NeuroAIDS, correlating cognitive and neuropsychological dysfunction with Tat-induced abnormalities in brain gray and white matter. Notably, this research expands on current literature evidence of Tat-induced dysfunction in the dopamine system with possible behavioral consequences of this dysfunction, such as the potentiation of psychostimulant effects. Evaluating the consequences of Tat activity in the brain may improve our understanding of the neurological underpinnings of NeuroAIDS and the neurodegeneration associated with HIV-1 infection. Moreover, understanding the effects of Tat and utilizing this model could one day facilitate the identification and development of preventative therapies or adjunctive countermeasures for the treatment of the neurodegeneration and behavioral dysfunction that persists in the HIV-1-infected population even with the use of current antiretroviral drugs.
ACKNOWLEDGEMENTS

Foremost, I wish to give my deepest thanks to my advisor, Dr. Jay P. McLaughlin. As the most important mentor in my career thus far, he has helped mold me to become a confident and rigorous scientist. Without his mentorship, I would have never believed that I have what it takes to be a scientist. He has not only helped me to believe that I can succeed, but through his leadership, he is responsible for providing me with the skills and confidence to bring this to fruition. From the bottom of my heart, thank you for everything.

Gratitude is also extended to the members of my dissertation committee, Drs. Martin Block, Bill Carlezon, Marcelo Febo, Marc Kaufman, and Rich Melloni, for their valuable input throughout the development of this dissertation. An especially heartfelt “thank you” needs to be extended to Dr. Marc Kaufman who welcomed me into his lab and provided me with integral mentorship during this last year. Thank you for use of your laboratory facilities and especially for your guidance, both academically and emotionally. I would also like to thank a member of Dr. Kaufman’s laboratory, Michael Liu, for his technical advice and assistance with the Neuroimaging project.

I want to thank Dr. Rachel Galli, my undergraduate mentor, who is a wonderful and inspiring teacher. I also want to thank Dr. Barbara Shukitt-Hale. I am so grateful that we have developed a friendship over the years and I appreciate your continued mentorship deeply. My gratitude is also extended to the Psychology Department at Northeastern University. The outstanding faculty and graduate students have created a supportive and stimulating environment for education. In particular, gratitude is extended to Drs. Rhea Eskew and Joanne Miller for taking an interest in my success as a graduate student.

I have been fortunate to work with an amazing group of people over the past 5 years. I acknowledge a fellow graduate student and a wonderful scientist, Susan Rasakham, for her
friendship and camaraderie during our time of overlap in the lab. Gratitude is also extended to all of the outstanding undergraduates who have passed through the lab over the years. The undergraduate who deserves my utmost gratitude is Elizabeth Sypek. Serving as my research assistant this last year, she assisted me during a critical time in my research and without her many of my ideas would not have come to fruition. Thank you for being such a dedicated assistant and for being my friend (and for the delicious “savory angels”).

Thank you to my friends, particularly Shelley McLeod and Derek Fisher, for being ears for my complaining and sources of support when the going got rough. I want to convey my love and appreciation for my colleague and friend, “Beck” Grayhem, who took this journey with me. I could not have succeeded in this journey without you. I would also like to thank Mike Marcoux for sticking with me these past few years, especially given the stress that can accompany the final moments of a dissertation. I appreciate your unconditional love and understanding (and that you bought me an adorable kitten).

Most importantly, I would like to thank my “parents”, my mom, Teresa Moran and my grandma, Natalie Moran for their never-ending love and unwavering support. Words cannot express how much your love has meant to me over the years. You have proven time and time again that a child does not need money or a traditional family structure to thrive; your unselfish and unconditional love were integral to the development of the confidence and ambition that was necessary to achieve my dreams. I love you.

Finally, I dedicate this work to two people that I have lost this year. My friend, Steve Foxx, inspires me to pursue in research that can benefit people suffering from terrible disease. I also dedicate this work to James A. Joseph, an amazing scientist and an inspiring mentor. Thank you for giving me the opportunity and means to engage in research. The impact you have on the scientific community will be a legacy for generations to come and I hope to be able to honor that legacy in my future career. You both will be missed.
# TABLE OF CONTENTS

Abstract 2

Acknowledgements 7

Table of Contents 9

List of Abbreviations 12

List of Figures 16

Introduction 19

Chapter 1. Literature Review
  i. Human Immunodeficiency Virus 24
  ii. Neuropathology Associated with HIV Infection 26
  iii. Neurological Implications of HIV Infection 32
    a. HIV-associated Dementia 32
    b. Neuropsychiatric Complications 34
    c. Limitations of Highly Active Antiretroviral Therapies 36
  iv. HIV-1 Viral Proteins 38
    a. HIV-1 Tat Protein 39
    b. Tat Produced Oxidative Stress and Dysfunction of the Blood Brain Barrier 40
    c. Tat Increases Cellular Calcium Concentrations and Stimulates N-methyl-D-aspartic Acid Receptors 41
    d. Activation of Cytokines and Chemokines by Tat Protein 43
    e. Tat Activates Multiple Intracellular Signal Transduction Pathways 44
    f. The Effects of Tat on the Dopaminergic System and Synergism with Drugs of Abuse 45
    g. HIV Clade-Specific Differences in Tat and the Development Of HIV-associated Dementia 47
  v. Animal Models of HIV-1 Central Nervous System Pathology for Behavioral Research 48
    a. Non-human Primates and Felines 48
    b. Rodents 49
    c. Tat Animal Models: The GT-tg Bigenic Mouse 51
  vi. Significance 53
  vii. Chapter References 55

Chapter 2. Study 1: Expression of HIV-Tat protein is associated with spatial learning and memory deficits and a long lasting impairment in novel object recognition learning and memory
  i. Introduction 73
  ii. Materials and Methods 77
Chapter 3. Study 2: Expression of HIV-Tat protein is associated with increases in anxiety and behavioral disinhibition that are dependent on the extent of Tat protein exposure

i. Introduction 117
ii. Materials and Methods 120
iii. Results 126
iv. Discussion 135
v. Chapter References 140

Chapter 4. Study 3: HIV-1 Tat protein expression in mouse brain potentiates the behavioral psychostimulant effects of cocaine

i. Introduction 144
ii. Materials and Methods 148
iii. Results 152
iv. Discussion 166
v. Chapter References 170

Chapter 5. Study 4: The brains of mice expressing HIV-Tat protein exhibit reductions in gray matter density, loss of white matter microstructural integrity, cell death, and microglia activation

i. Introduction 173
ii. Materials and Methods 176
iii. Results 183
iv. Discussion 197
v. Chapter References 202

Chapter 6. Summary and Discussion

i. Effects of Tat on Learning and Memory 206
ii. Modulation of Anxiety and Behavioral Disinhibition by Tat 213
iii. The Interaction of Tat with the Rewarding Effects of Cocaine and Reinstatement of Drug-seeking Behavior 216
   a. Connection between Behavioral Disinhibition and Drug Abuse 220
iv. The Utility of the GT-tg Bigenic Mouse Model 221
v. Neuroanatomical Effects of Tat Protein and Possible Consequences 223
   a. Effects of Tat on Gray Matter Density 223
   b. Tat-induced Changes in White Matter Microstructure 224
   c. Histological Abnormalities 226
   d. Summary of Neuroanatomical Findings 228
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-CSRTT</td>
<td>5-choice serial reaction time task</td>
</tr>
<tr>
<td>ADHD</td>
<td>Attention deficit hyperactivity disorder</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquire immune deficiency syndrome</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>ASR</td>
<td>Acoustic startle reflex</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood brain barrier</td>
</tr>
<tr>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>Calcium</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CPP</td>
<td>Conditioned place preference</td>
</tr>
<tr>
<td>CREB</td>
<td>Cyclic adenosine monophosphate response element binding protein</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td>CXCR4</td>
<td>Chemokine receptor 4</td>
</tr>
<tr>
<td>D&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Dopamine receptor subtype 2</td>
</tr>
<tr>
<td>DA</td>
<td>Dopamine</td>
</tr>
<tr>
<td>DAT</td>
<td>Dopamine transporter</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>Dox</td>
<td>Doxycycline</td>
</tr>
<tr>
<td>DTI</td>
<td>Diffusion tensor imaging</td>
</tr>
<tr>
<td>EPM</td>
<td>Elevated plus maze</td>
</tr>
<tr>
<td>ERK 1/2</td>
<td>Extracellular signal-regulated kinase 1 and 2</td>
</tr>
<tr>
<td>ETL</td>
<td>Echo train length</td>
</tr>
<tr>
<td>FA</td>
<td>Fractional anisotropy</td>
</tr>
<tr>
<td>FIV</td>
<td>Feline immunodeficiency syndrome</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>FOV</td>
<td>Field of view</td>
</tr>
<tr>
<td>FPS</td>
<td>Fear potentiated startle</td>
</tr>
<tr>
<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
</tr>
<tr>
<td>gp120</td>
<td>HIV structural envelop glycoprotein 120</td>
</tr>
<tr>
<td>HAART</td>
<td>Highly active antiretroviral therapies</td>
</tr>
<tr>
<td>HAD</td>
<td>HIV-associated dementia</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>HIVE</td>
<td>HIV encephalitis</td>
</tr>
<tr>
<td>HSD</td>
<td>Honestly significant difference</td>
</tr>
<tr>
<td>Ib1a</td>
<td>Ionized calcium binding adaptor molecule 1</td>
</tr>
<tr>
<td>i.c.v.</td>
<td>Intracerebroventricular</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>i.p.</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>ITI</td>
<td>Inter-trial interval</td>
</tr>
<tr>
<td>LD</td>
<td>Light-dark, as in light-dark testing or apparatus</td>
</tr>
<tr>
<td>LSD</td>
<td>Least significant difference, as in Fisher's LSD</td>
</tr>
<tr>
<td>LTP</td>
<td>Long-term potentiation</td>
</tr>
<tr>
<td>MAIDS</td>
<td>Murine acquired immune deficiency syndrome</td>
</tr>
<tr>
<td>MAP</td>
<td>Mitogen activate protein</td>
</tr>
<tr>
<td>MND</td>
<td>Mild neurological disorder</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>MWM</td>
<td>Morris water maze</td>
</tr>
<tr>
<td>NAc</td>
<td>Nucleus accumbens</td>
</tr>
<tr>
<td>NFκB</td>
<td>Nuclear factor kappa B</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
</tr>
<tr>
<td>---------</td>
<td>------------</td>
</tr>
<tr>
<td>NIH</td>
<td>National Institutes of Health</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartic acid</td>
</tr>
<tr>
<td>NNRTI</td>
<td>Non-nucleoside reverse transcriptase inhibitors</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NOR</td>
<td>Novel object recognition</td>
</tr>
<tr>
<td>NP</td>
<td>Neuropsychiatric</td>
</tr>
<tr>
<td>NRTI</td>
<td>Nucleoside reverse transcriptase inhibitors</td>
</tr>
<tr>
<td>n.s.</td>
<td>Not significant</td>
</tr>
<tr>
<td>OF</td>
<td>Open field</td>
</tr>
<tr>
<td>OS</td>
<td>Oxidative stress</td>
</tr>
<tr>
<td>PD</td>
<td>Personality disorder</td>
</tr>
<tr>
<td>PI</td>
<td>Protease inhibitors</td>
</tr>
<tr>
<td>RBE4</td>
<td>Rat brain endothelial cell line-4</td>
</tr>
<tr>
<td>REGWF</td>
<td>Ryan-Einot-Gabriel-Welsch multiple F</td>
</tr>
<tr>
<td>RI</td>
<td>Recognition index</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>s.c.</td>
<td>Subcutaneous</td>
</tr>
<tr>
<td>SCID</td>
<td>Severe combined immunodeficient</td>
</tr>
<tr>
<td>S.E.M</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SIV</td>
<td>Simian immunodeficiency virus</td>
</tr>
<tr>
<td>T</td>
<td>Tesla, a measure of magnetic field strength used in MRI studies</td>
</tr>
<tr>
<td>Tat</td>
<td>HIV transactivator of transcription, as in Tat protein</td>
</tr>
<tr>
<td>tat</td>
<td>The gene that codes for Tat protein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>TBS-T</td>
<td>Tris-buffered saline-Tween</td>
</tr>
<tr>
<td>TE</td>
<td>Echo time</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor-alpha</td>
</tr>
<tr>
<td>TR</td>
<td>Repetition time</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling</td>
</tr>
<tr>
<td>UNAIDS</td>
<td>Joint United Nations Programme on HIV/AIDS</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>VBM</td>
<td>Voxel based morphometry</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

Chapter 1

Figure 1.1: Tat protein expression in GT-tg bigenic mouse is limited to brain 54

Chapter 2

Figure 2.1: Schematic of the Barnes maze and novel object recognition assay 82
Figure 2.2: Saline- and Dox-treated C57Bl/6J control mice display greater latencies and made more errors in the acquisition trials of the Barnes maze than the uninduced GT-tg mice 87
Figure 2.3: Tat-induced mice demonstrated impaired acquisition and inefficient search strategies in the Barnes maze compared to uninduced GT-tg mice 91
Figure 2.4: Mice induced to express Tat were not successful in finding the escape location during a probe trial 94
Figure 2.5: Tat-induced mice required more trials to learn a new escape location during a reversal learning task in the Barnes maze 96
Figure 2.6: Optimization of the conditions for the novel object recognition assay 98
Figure 2.7: GT-tg mice induced to express Tat demonstrated a Dox dose- and duration of induction-dependent deficit in phase 3 novel object recognition 103
Figure 2.8: GT-tg mice induced to express Tat protein with the
maximum Dox-induction regimen demonstrated lasting suppression of performance in phase 3 novel object recognition

Chapter 3

Figure 3.1: Schematic of the open field arena, light-dark box, and elevated plus maze

Figure 3.2: GT-tg mice induced to express Tat demonstrated Dox dose- and duration of induction-dependent anxiety-like behavior in the open field test

Figure 3.3: Tat protein expression produced anxiety-like behavior in the light-dark box that was dependent on the duration of Tat induction

Figure 3.4: GT-tg mice induced to express Tat demonstrated a Dox dose- and duration of induction-dependent behavioral disinhibition in the elevated plus maze

Chapter 4

Figure 4.1: Expression of HIV-1-Tat produced sensitization of cocaine-induced locomotor activity

Figure 4.2: HIV-1 Tat expression potentiated cocaine-CPP

Figure 4.3: Induction of Tat potentiated the effect of cocaine place conditioning in previously place-conditioned mice

Figure 4.4: The duration of the HIV-1 Tat protein-mediated potentiation of cocaine-CPP was consistent with the unpotentiated
response of uninduced mice 163

Figure 4.5: Cocaine-CPP was reinstated by induction of Tat protein 164

Chapter 5

Figure 5.1: Administration of Dox induced expression of HIV-1 Tat protein in GT-tg mouse brain 185

Figure 5.2: The 5 day Tat-induced mice showed potential brain region-specific reductions in gray matter density 188

Figure 5.3: The brains of the 7 day Tat-induced GT-tg mice showed significant loss of white matter microstructural integrity 190

Figure 5.4: The brains of mice induced to express Tat showed more cell degeneration and death in the perirhinal cortex compared to control mice 193

Figure 5.5: Mice induced to express Tat showed changes in microglia morphology indicative of inflammation in the prefrontal cortex 195

Chapter 6

Figure 6.1: Summary of relevant findings 207
INTRODUCTION

NeuroAIDS is the syndrome consisting of neuropsychological manifestations and central nervous system pathology resulting from human immunodeficiency virus (HIV-1) infection (Everall et al., 1993). NeuroAIDS is characterized by progressive loss of cognitive and motor function (Price et al., 1988; Glass et al., 1993), ranging from mild to severe, and can ultimately progress to a debilitating condition referred to as HIV-associated dementia. NeuroAIDS is also linked to other psychological disturbances that include changes in mood (i.e., depression, anxiety, apathy, and irritability), “personality” (i.e., aberrant motor behavior, disinhibition, and agitation) (Peters et al., 2008), and drug abuse (Nath et al., 2002; Joint United Nations Programme on HIV/AIDS and World Health Organization, 2005). In fact, a greater prevalence of mood, anxiety, and substance use disorders is observed in HIV-1 positive patients relative to the general population (Rabkin et al., 1997). This is likely due to both the direct neurobiological effects of the virus on frontostriatal function as well as indirect effects of the psychosocial stigma associated with HIV-infection. The cognitive dysfunction and changes in behavior associated with HIV-infection are difficult to disentangle due to overlapping symptoms, highlighting the importance of understanding the brain areas affected and specific mechanism(s) underlying these symptoms. Research suggests that the cognitive and neuropsychological dysfunction may have a common etiological and biological basis (Castellon et al., 2000), although research directly testing this is limited.

In seeking a biological understanding of NeuroAIDS, it is important to note that HIV-1 is not known to directly infect neurons. Instead, the predominant cell type in the brain that is infected with HIV-1 is the monocyte/macrophage (including microglia) (Gabuzda et al., 1986; Wiley et al. 1986; Gendelman et al., 1994), along with a smaller but significant number of brain astrocytes (Nath et al., 1995; McCarthy et al., 1998). It is theorized that the release of a number
of toxic viral proteins from HIV-infected cells is a key mechanism by which HIV-1 indirectly and adversely affects neurons (Jones et al., 1998). These proteins have been implicated to varying degrees in the pathogenesis of HIV-1 infection (Frankel and Young, 1998), but most studies examining viral protein mediation of HIV-neuropathology have concentrated on two gene products of HIV-1: the structural envelop glycoprotein, gp120 and the transactivator of transcription regulatory protein, Tat. This thesis focuses on HIV-Tat protein, which has been implicated through numerous studies in the pathogenesis of HIV-1 neurological complications and consequently, in the progression of NeuroAIDS (Frankel and Young, 1998; Romani et al., 2010; for full review, see Chapter 1). Tat is released in the extracellular environment from unruptured, HIV-infected lymphoid cells and microglia, producing a host of effects on neurons (Tardieu et al., 1992; Ensoli et al., 1993; Chang et al., 1997). In vitro studies have demonstrated Tat to be excitotoxic as well as pro-inflammatory, and also associated with increased oxidative stress and apoptosis (Price et al., 1988; Power et al., 1998; Aksenov et al., 2001). Moreover, Tat has been associated with alteration of intracellular calcium homeostasis and stimulation of the cytokines and chemokines (Hayman et al., 1993; New et al., 1998; Haughey et al., 1999). Direct injections of Tat into rat brain results in multiple histopathological and behavioral manifestations that model aspects of HIV-associated dementia (Rappaport et al., 1999; Bruce-Keller et al., 2003). Furthermore, a mutation in the tat gene has been directly associated with neurological insult, as demonstrated by HIV-1 clade-specific human neuron toxicity that correlates with specific mutations within the first exon of tat (Mishra et al., 2008; Rao et al., 2008). HIV-Tat may also be at least partly responsible for the dysfunction of the dopaminergic system implicated in NeuroAIDS-related behavioral dysfunction (Maragos et al., 2002). Very recently, Tat has been reported to influence dopamine recycling and uptake kinetics (Ferris et al., 2009; Zhu et al., 2009) and cause rapid dysfunction of the dopamine transporter (Wallace et al., 2006). However, evidence that Tat has significant effects on broad systemic brain function
or pathogenesis *in vivo*, or that it specifically mediates HIV- and NeuroAIDS-induced behavioral disorders is undetermined.

In summary, HIV-1 infection in the brain is associated with cognitive dysfunction and aberrant behavior. The underlying etiology of the NeuroAIDS is likely complex and almost certainly involves a number of pathogenic factors associated with HIV-1 infection. However, the previous research demonstrating the neurotoxic and neuromodulatory effects of Tat suggests the potential for a significant contribution to the symptoms of NeuroAIDS. Determining the behavioral consequences of HIV-Tat protein activity in brain is crucial to understanding the complex role of Tat in NeuroAIDS. The present thesis attempts to understand the role of a potential pathogenic factor, HIV-1 Tat, in the development of cognitive (i.e., learning and memory) and neuropsychological (i.e., anxiety and behavioral disinhibition) dysfunction along with Tat-induced abnormalities in brain gray and white matter. Moreover, this research also attempts to link the current evidence that Tat causes dysfunction in the dopamine system with possible behavioral implications of this dysfunction, such as increased reward from psychostimulant drugs.

These studies employed the GT-tg bigenic mouse (Kim et al., 2003) to examine the contribution of Tat to behavioral alterations associated with NeuroAIDS. The GT-tg mouse possesses an inducible and brain-selective *tat* gene that codes for Tat. As reviewed in Chapter 1, these mice are uniquely suited for this study, as the expression of Tat protein is under the control of the researcher through the administration of Doxycycline. The dosage and number of days mice are administered Doxycycline may be varied in order to control the amount and the length of time of Tat expression. The central hypothesis tested in this thesis is that HIV-Tat protein, when expressed in brain, mediates behavioral dysfunction and neurodegeneration in an exposure-dependent manner. The central hypothesis was tested with the specific aims outline below, corresponding to specific chapters of this thesis:
Chapter 2: To test the hypothesis that the activity of Tat in brain is sufficient to impair learning and memory processes. These studies utilized two learning and memory assays to identify the types of deficits associated with expression of Tat protein. The Barnes maze was used to assess spatial learning and memory, while the novel object recognition task was used to assess visual recognition memory. As literature pertaining to the assay protocols is diverse, initial studies were performed using a parent strain of mice, C57Bl/6J, to determine the optimal parameters for each task to detect intact learning and memory. Then, the responses of GT-tg mice induced to express the viral protein Tat and tested in either the novel object recognition task or Barnes maze were compared to the responses of uninduced GT-tg littermates. Deficits noted in the novel object recognition task in mice that were tested 48 h after the completion of Tat induction were further evaluated in separate groups of mice one week and one month after the completion of induction to determine if deficits were persistent.

Chapter 3: To test the hypothesis that exposure to Tat protein increases behavioral signs of anxiety. Uninduced and Tat-induced mice were initially tested in the open field test for anxiety 48 h and one week after the completion of Tat induction to determine if any increases in anxiety-like behavior were transient or persistent. Increases in anxiety-like behavior were apparent in mice induced to express Tat compared to controls, but mice administered the maximum induction regimen (Doxycycline, 100 mg/kg, 7 days) were noted to have a more complex behavioral profile. These data were confirmed in the light-dark box. Mice induced to express Tat using various Doxycycline induction regimens were also tested in the elevated plus maze. Tat-induced mice demonstrated behavioral changes in the elevated plus maze that were suggestive of behavioral disinhibition (not anxiety), a behavior commonly associated with humans suffering from dementia. Together, this battery of tests was used to characterize the
behavioral profile of the transgenic mice induced to express varying amounts of Tat for different durations.

Chapter 4: To test the hypothesis that Tat protein can potentiate the psychostimulant and rewarding effects of the reinforcing drug cocaine. First, the locomotor sensitizing effect of acute cocaine exposure was evaluated in Tat-induced mice. Subsequently, the ability of Tat protein expression to potentiate the rewarding effect of cocaine in a Doxycycline dose- and duration of induction-dependent manner was evaluated in the conditioned place preference assay. These experiments further investigated if induction of Tat protein could potentiate reward in cocaine non-naïve subjects. Furthermore, the duration of the potentiated reward state was determined as was the ability of Tat protein to reinstate cocaine-seeking behavior after extinction.

Chapter 5: To test the hypotheses that Tat protein expression in the central nervous system is associated with decreases in gray matter density, white matter microstructural changes, and histological abnormalities in brain areas known to be affected in NeuroAIDS patients. In the study, brains were excised from Tat-induced, uninduced, and C57Bl/6J mice for ex vivo anatomical histology-grade magnetic resonance imaging using a 9.4 Tesla Varian magnet. Anatomical images were analyzed using voxel based morphometry to determine reductions in gray matter volume. As changes in volume of particular brain regions may be small, we also used diffusion tensor imaging to examine fractional anisotropy of white matter microstructure. Western blot analysis was run in parallel to confirm Tat protein expression in whole brain as well as specific brain areas. Additionally, limited analyses of histology staining used to detect activated microglia and cell death were performed.

Chapter 6 provides a summary and discussion of the data presented in this thesis.
CHAPTER 1

LITERATURE REVIEW

I. HUMAN IMMUNODEFICIENCY VIRUS

Human immunodeficiency virus (HIV) is the etiological agent of acquired immune deficiency syndrome (AIDS). According to the Joint United Nations Programme on HIV/AIDS (UNAIDS) and the World Health Organization (WHO), as of 2009 there were over 33 million adults and children living with HIV-infection, and by 2005 AIDS had caused over 20 million deaths worldwide (McArthur et al., 2005; UNAIDS/WHO, 2009). HIV belongs to a group of virulent retroviruses called lentiviruses. The retrovirus designation stems from the actions of reverse transcriptase that transcribes the ribonucleic acid (RNA) genome back into deoxyribonucleic acid (DNA) prior to incorporation into the host cell chromosome (Fauci, 1988). A retrovirus subtype, the lentivirus is named after the Latin word *lentus*, meaning slow, and is so named because of the gradual course of disease progression associated with lentiviral infection (see Fauci, 1988; Brew, 2001). Infection with the HIV lentivirus results in a slow and insidious deterioration of the immune system. The lentivirus causes immune deficiency in their hosts in addition to progressive wasting disorders, neurodegeneration, and death (Haase, 1986). One feature that distinguishes lentiviruses from other retroviruses is the complexity of their viral genomes. While most retroviruses that are capable of replication contain only three genes (*env, gag* and *pol*) (Varmus, 1988), HIV also contains the complex regulatory genes *tat, rev, nef*, and auxiliary genes *vif, vpr and vpu* (Greene, 1991). It is believed that the actions of these additional genes contribute to the profound virulence that differentiates HIV from many other retroviruses (Frankel and Young, 1998).
There are two main forms of HIV: HIV-1 and HIV-2. HIV-2 has greater similarity to simian immunodeficiency virus (SIV) and is restricted to a small West African population (Clavel, 1987; de Cock et al., 1993). The blanket term “HIV” typically refers to HIV-1, and it should be noted that in this thesis the term HIV is referring to HIV-1. Variants of HIV-1 are known to exist, and are further divided into three groups: major, new, and outlier. Within the major-group there are at least ten clades (i.e., subtypes): A, B, C, D, E, F, G, H, I, J, and K. Clade B is dominant in the U.S., Europe, Southeast Asia, and South America, whereas clades E and C are dominant in Asia, and A, C, and D are dominant in Africa (Hu et al., 1996; see Coffin et al., 1997 for review). Importantly, the classification of HIV strains into subtypes is a complex issue and it should be emphasized that definitions and categorization schemes are changing with our evolving knowledge of the complexity and diversity of HIV.

The CNS is one of many compartments in the human body. HIV enters the CNS via two mechanisms: it can cross the blood brain barrier (BBB) in infected lymphocytes and monocytes or can enter through the cerebrospinal fluid (CSF) compartment. Two types of infection can occur in the CNS: productive infection and restricted/latent infection. In productive infection, the infected host cell supports viral replication and transmission, and the infection is cytopathic (i.e., results in degeneration and death of the host cell; see Petito, 2004 for review). The main sources of productive infection in the CNS are monocytes-derived macrophages and a distinct class of macrophage called microglia (Gabuzda et al., 1986; Wiley et al., 1986; Gendelman et al., 1994). In addition to productive infection, the CNS provides a reservoir or “sanctuary” for the virus in which there is restricted infection in a large number of undividing cells (Ho et al., 1985; Levy et al., 1985; Resnick et al., 1985). Astrocytes are the targets of restricted HIV infection in the CNS (Kleinschmidt et al., 1994). After a brief phase of productive infection, astrocytes enter a latent phase (Nath et al., 1995; Gorry et al., 1998), where HIV can exist for an indefinite period. Although the proportion of astrocyte infection can be limited compared to productive
infection in macrophages, 70% of the brain is comprised of astrocytes, suggesting that even limited infection could affect a significant proportion of brain cells (see Lawrence and Major et al., 2002). Research suggests that this latent reservoir for HIV infection in astrocytes may be crucial to the neuropathology that persists with the treatment of HIV with highly active antiretroviral therapies (HAART). In this latent stage, HIV has the potential to elude these drugs (Nath, 1999; but see below for more detail). During restricted infection, HIV infectivity of cells is noncytopathic and nonreplicative, but HIV is thought to continue the production and possibly the over-expression of the viral regulatory proteins for release into the extracellular environment (Power et al., 1998; Brack-Werner, 1999; Johnston et al., 2001). The production of these viral proteins is important because it is generally believed that HIV is not directly responsible for the pathogenesis of brain dysfunction associated with AIDS (UNAIDS/WHO, 2004), as the virus does not infect neurons nor does the dysfunction consistently correlate with the viral burden in the brain (Everall et al., 1993). As such, it is postulated that HIV induces brain pathology through indirect means. There is accumulating evidence that the viral proteins encoded by HIV directly or indirectly mediate the cell damage and neuropathology associated with HIV infection, discussed further in Part IV.

II. NEUROPATHOLOGY ASSOCIATED WITH HIV INFECTION

It has been reported that as many as 80% of AIDS patients have neurologic abnormalities at the time of autopsy (Bendok et al., 1997). Autopsies of the HIV brain have found microglial nodules, which are small clusters of mononuclear cells including macrophages and microglia. These microglia nodulae are associated with a number of inflammatory disorders of the CNS, including viral infections (Gabuzda et al., 1986). Additionally, multinucleated giant cells, which are multiple HIV-infected macrophages fused together, have been noted in the subcortical grey matter (Navia et al., 1986). During active HIV infection, the
basal ganglia, a group of subcortical brain structures involved in motor control and learning and memory, exhibits one of the highest viral loads and greatest number of HIV-infected macrophages and multinucleated giant cells in brain, resulting in the eventual loss of dopaminergic neurons in the substantia nigra (Navia et al., 1986, Koutsilieri et al., 2001). The combined neuropathology of activated microglia, infiltrating macrophages, HIV-infected multinucleated giant cells, and pronounced astrocytosis is an indicator of chronic neuroinflammation termed HIV-encephalitis (HIVE) (Levy et al., 1985; Anthony and Bell, 2008).

Neuropathology may be found in AIDS patients without significant behavioral dysfunction, but it is not surprising that more severe neuropathology is detected in patients with HIV-associated dementia (HAD) (behavioral symptoms discussed in more detail in Part III). Viral loads in the hippocampus, globus pallidus, and frontal cortex correlate well with the clinical symptoms of HAD (Price et al., 1988; Glass et al., 1993; Fujimura et al., 1997; Wiley et al., 1998). Pathology of the HAD brain includes diffuse white matter pallor involving central and periventricular white matter, most often associated with astrocytic reaction; this is also apparent in non-demented AIDS patients (Navia et al., 1986; Koenig et al., 1986). Under post-mortem examination for histopathological abnormalities, the brains of subjects with HAD are found to have decreased synaptic density, neuronal loss, astrocytosis, microglial nodules, and signs associated with breakdown of the BBB (Everall et al., 1993; Thompson et al., 2001). Multinucleated giant cells are also evident, and most apparent in the basal ganglia and thalamus of HAD patients (Sharer et al., 1985; Everall et al., 1993).

Notable atrophy of brain is apparent in HIV infection and HAD. The cortical pathology is characterized by loss of a large number of neurons in the orbital frontal cortex, an area involved in sensory integration of decision making and hedonic processing (Ketzler et al., 1990; Gray et al., 1991; Kringelbach, 2005). Research has also found a 20% decrease in cortical width in the temporal and parietal lobes (Wiley et al., 1991). Morphometric studies have found evidence that
there is a 50-90% loss of interneurons and pyramidal neurons of the hippocampus, a key structure in long-term memory formation and spatial navigation (Masliah et al., 1992b; Spargo et al., 1993; Fox et al., 1997), as well as a 40% decrease in tissue density in the frontal and temporal lobes (Ketzler et al., 1990; Masliah et al., 1992a). Interestingly, Seilhean and colleagues (1995) did not find significant cortical or subcortical atrophy in a cohort with cognitive dysfunction, but did find widespread gliosis of the cortex and white matter, suggesting that neuronal loss does not have to be present for cognitive impairment and may instead be due to neuronal dysfunction or changes in neurotransmitters.

Imaging studies are commonly used to exclude opportunistic CNS infection in cases of possible dementia, but also to identify characteristic changes that occur in HAD and quantify atrophy and white matter changes in vivo. Magnetic resonance imaging (MRI) of the brains of AIDS patients has revealed enlargement of cerebrospinal fluid spaces, loss of white matter, and white matter signal abnormalities, such as deep white matter lesions (Broderick et al., 1993; Filippi et al., 2001; Archibald et al., 2004; McArthur et al., 2005). MRI has been used to examine brain atrophy in HIV-seropositive patients, with reduced volumes of the hippocampus, and to a lesser extent, the cerebral cortex consistently identified (Tornatore et al., 1994; Archibald et al., 2004). MR images have also shown significant atrophy in the basal ganglia, particularly the caudate nucleus, as well as basal ganglia calcification in pediatric AIDS (Brew, 2001). Of interest, diffusion tensor imaging (DTI) is a type of MRI that is sensitive to white matter microstructural integrity. DTI has demonstrated abnormalities in white matter tracts in HIV-seropositive patients, even in patients without identifiable white matter abnormalities in MR images (Filippi et al., 2001). Consistent with this, diffusion abnormalities were identified in patients infected with HIV in the splenium of the corpus callosum that connects the hemispheres of the brain, and the magnitude of alteration was correlated with the severity of dementia (Wu et al., 2006). These data are consistent with previous studies suggesting that white matter
abnormalities may be among the most important neuropathologic lesions in HIV-infection and an early hallmark of HAD (Barker et al., 1995; Marcus et al., 1998). Research suggests that even the mild cognitive impairment observed early in the disease process may arise from affected white matter (Wu et al., 2006). However, despite this, the etiology of white matter abnormalities and their contribution to behavioral dysfunction remains to be extensively evaluated.

It should be noted that other biological responses to HIV infection may contribute to neuropathology. Considerable interest has focused on the HIV-induced increase in chemokines (Vargas et al., 2005). Also, elevated cytokine messenger RNAs (mRNAs) and protein products have been identified in the brains of those dying with AIDS (Tyor et al., 1992, 1993; Wesselingh et al., 1993). Chemokines and cytokines are immune factors excreted by cells to mediate the inflammatory immune response of cells to events such as injury, allergens, and foreign microorganisms by attracting leukocytes, monocytes, and neutrophils to the area of damage (Murdoch and Finn, 2000). Interestingly, chemokine receptors have been identified in macrophages/microglia, astrocytes, and neurons in brain and chemokines demonstrate highly diverse mechanisms of action and cellular targets in the CNS (Lavi et al., 1998). Chemokines have been implicated in the pathogenesis of HIV infection as they are used as co-receptors for HIV entry into cells (Catani et al., 2003). The possible involvement of chemokines in HIV and HAD is not limited to virus entry, as it has been suggested that chemokine receptor signaling may modulate neuronal apoptosis and neurotoxicity (Klein et. al, 1999). In neurons, activation of the CXCR4 and CCR5 receptors by chemokines affects neuronal calcium signaling (Meucci et al., 1998). Another chemokine receptor, CX3CR1, is expressed on cell surfaces of microglia, neurons, and macrophages and it is suggested that it may be involved in pro-inflammatory responses and inflammatory brain diseases (Meucci et al., 2000). Moreover, HIV-infected patients diagnosed with neurological disorders have higher CSF levels of fractalkine, the ligand
activating CX3CR1, as compared to HIV-infected patients presenting without neurological disorder (Tong et al., 2000). Likewise, in SIV-infected primates, release of proinflammatory cytokines has been demonstrated to impact cell functioning and induce neuropathology similar to that seen in HAD (Zink et al., 1998). By virtue of their inflammatory role, cytokines may play a role in the development of HAD and HIVE (Valcour and Paul, 2006). These data suggest that cytokines and chemokine receptors may also play a causal role in the neurotoxicity and neuronal dysfunction associated with HAD.

Oxidative stress (OS) is also thought to be a significant component underlying the neuropathology seen in HIV infection and HAD. Research suggests that OS may contribute to the normal aging process (Harman, 1992) and to the progression of neurodegenerative diseases (Nunomura et al., 2006). Oxidation occurs as a normal byproduct of metabolism, but the body is protected against extensive damage from free radicals by endogenous antioxidant defense systems. However, when free radicals are produced in excess of the endogenous defense mechanisms, such as in neurodegenerative disorders like HAD, the imbalance can lead to a toxic environment with detrimental effects on neurons (Markesbery and Carney, 1999). OS appears to play a critical role in the progression of HAD (Mollace et al., 2001). Indices of OS are consistently detected in brain tissue and CSF of those with HAD. Patients with HAD have shown increases in nitric oxide (NO) synthase in astrocytes and microglia and superoxide dismutase activity in microglia, both markers for OS, when compared to HIV-infected patients without dementia (Adamson et al., 1996; Vincent et al., 1999).

As previously discussed, the basal ganglia exhibits one of the highest viral loads and greatest number of HIV-infected macrophages and multinucleated giant cells. These infected cells have been demonstrated to cause a loss of dopamine (DA) neurons in the substantia nigra (Navia et al., 1986, Koutsilieri et al., 2001). This is significant, as basal ganglia dysfunction has been implicated in the development of clinical signs common to dopaminergic disorders like
Parkinson’s disease seen in HIV infection (Epstein et al., 1986; Post et al., 1988; Chrysikopoulos et al., 1990; Nath, 1999). HIV patients also have a heightened sensitivity to DA selective drugs and to psychostimulants that act on DA neurons in the basal ganglia (Nath et al., 2000). For example, neuroleptic exposure can exacerbate previously developed Parkinsonian symptoms in AIDS patients (Mirsattari et al., 1998). It has been reported that up to 78% of HIV patients treated with DA agonists (i.e., neuroleptics) develop extrapyramidal symptoms (Hriso et al., 1991). Not surprisingly, post mortem analysis has indicated that AIDS patients show decreased levels of DA and homovanillic acid, a metabolite of DA, in the caudate nucleus attributed to damage or loss of nigrostriatal neurons and striatal dysfunction (Sarder et al., 1996). Notably, AIDS patients also show depletion of DA levels in the CSF and research suggests that HAART does not improve these levels in the CSF of patients with HAD (Berger et al., 1994) or alleviate the symptoms associated with DA deficiency (Mirsattari et al. 1998). Importantly, HAART does not appear to completely alleviate the clinical symptoms associated with DA deficiency either (Mirsattari et al., 1998). In addition to DA deficiency, clinical research shows that patients with HIV have a 13-20% reduction in dopamine transporter (DAT) density in the basal ganglia compared to HIV-negative controls (Wang et al., 2004). Moreover, a reduction in DAT, possibly coupled with a loss of DAT function, was also noted in AIDS patients with HAD compared to AIDS patients without neurological symptoms (Berger et al., 1994). Furthermore, research has documented that neurons show a loss of function of DAT after exposure to HIV accessory proteins, suggesting a role for these proteins in the dysfunction of the dopaminergic system associated with HAD (Maragós et al., 2002). Clearly, there is a multitude of neuropathogenic changes associated with HIV infection, but the mechanisms behind these changes are not fully understood. There is accumulating evidence that the toxic viral proteins released by HIV-infected cells play a pivotal role in the development of NeuroAIDS-related pathology, highlighting the importance of further investigation into the effects of viral proteins.
like Tat. The role of HIV-Tat in the development of neuropathology and dysfunction in relation to NeuroAIDS is discussed in Part IV.

III. NEUROLOGICAL IMPLICATIONS OF HIV INFECTION

A. HIV-ASSOCIATED DEMENTIA

Not long after the recognition of AIDS in 1981, reports began to appear of an unusually high incidence of encephalitis in HIV-infected patients (i.e., HIVE) (Snider et al., 1983; Levy et al., 1985). The common clinical symptoms, many similar to those found in dementia, were collectively termed NeuroAIDS. The cognitive impairments have been categorized into two classes to differentiate between mild and more severe impairment: mild neurological disorder (MND) (also referred to as mild cognitive and motor disorder) and HAD (also referred to as AIDS dementia complex; Navia et al., 1986; UNAIDS/WHO, 2007).

HAD is the more severe of the two disorders and is associated with greater impairment of executive function (Bartlett and Gallant, 2005). HAD typically occurs with advanced immune suppression, but can also occur independently of other symptoms of HIV-1 infection (Navia and Price, 1987). Clinically, HAD is characterized by distinct changes in cognition (e.g., learning, memory, attention, and executive function), motor coordination (e.g., psychomotor slowing and hypertonia), and behavior (e.g., apathy, irritability, emotional lability, and affective blunting) (Heaton et al., 1994). HAD is characteristic of a subcortical dementia, with prominent basal ganglia dysfunction (Berger and Nath, 1997). It is important to note that subcortical and cortical dementias (e.g. Alzheimer’s disease) are not dichotomous disorders as previously believed (Savage, 1997). There is substantial overlap in the brain areas involved, functional changes observed, and the types of cognitive impairments, and it is therefore not surprising that cortical changes are apparent in HAD as well (Freedman et al., 1994; Moore et al., 2006). Although not
as severe as HAD, MND is also characterized by mild but significant cognitive dysfunction, pronounced motor impairment and is associated with decreased adherence to treatment regimens, greater levels of unemployment, and HIVE (McArthur et al., 2005).

The relevance of HAD is underscored by reports showing that HIV is the leading cause of dementia in people younger than 60 years old (Janssen et al., 1992; McArthur et al., 1993). In the pre-HAART era, the annual incidence of HAD was reported as 7%, with a cumulative lifetime risk of 5-20% (McArthur, 1987; Pakker et al., 1999). The Dana cohort (prospective study of cognitive impairment in HIV-infected patients) suggests that cognitive impairment was as prevalent as 23.2% (Sacktor et al., 2002). An Australian study found that the incidence of AIDS-defining illnesses have decreased after the advent of HAART, including HAD, but that HAD incidence has decreased less than other AIDS-defining illnesses (Dore et al., 1999). There is continued development of HAD despite measured improvement in immune function in treated patients (Dore et al., 1999). Furthermore, although HAD incidence has fallen with the advent of HAART, the cumulative prevalence may have risen with improved survival (see McArthur et al., 2003 for review). Jevtovic and colleagues (2009) found that a group of patients on stable HAART regimens demonstrated a 13.5% prevalence of HAD and 28.1% prevalence of MND. Sacktor and colleagues (2002) found a 15% prevalence of HAD and a 20% prevalence of MND in symptomatic HIV in a homosexual male subpopulation on HAART. Indeed, Masliah et al. (2000) found that in the era of HAART, after the lungs, the CNS is the most commonly involved organ in AIDS autopsy cases. Additionally, rates of HIVE identified at autopsy after the advent of HAART are similar to the incidence in the pre-HARRT era (Masliah et al., 2000; Anthony and Bell, 2008). There is also evidence that in the era of HAART, the inflammation associated with HIV infection may have shifted from a subcortical pathology to a cortical pattern (Brew, 2004) and from a rapidly progressing condition to a more subtle neurodegenerative process (Xu and Ikezu, 2009). Furthermore, in the pre-HAART era, the basal ganglia was the primary sight of
strong infiltration with HIV, but now infiltration is often identified in the hippocampus, and adjacent parts of the entorhinal cortex and temporal cortices (see Anthony and Bell, 2008 for review).

Recent evidence suggests that the incidence of HAD as an AIDS-defining illness may be on the rise, as HAART-mediated increases in life expectancies for those infected with HIV results in many more older individuals living with HIV as a chronic illness (Saksena and Smit, 2005). Research has shown an increased risk of developing HAD as an older individual after adjusting for important differences such as difference in age, race, substance abuse, HAART status, viral load, and depression (Valcour and Paul, 2006). Accordingly, HAD is becoming a significant factor for death due to AIDS (Ellis et al., 1997; Liner et al., 2008). Notably, there is an increased incidence of brain lesions in AIDS patients with long-term survival over age-matched uninfected subjects (Maehlen et al., 1995). Furthermore, cognitive impairment may be more profound in HIV infected older individuals compared to uninfected populations (Valcour et al., 2004; Saksena and Smit, 2005), and there may be more susceptibility in HIV patients to develop Alzheimer’s disease comorbidity (see Xu and Ikezu, 2009 for review). This increased prevalence suggests a synergism of the neuropathology associated with HIV-infection and the normal and abnormal declines associated with aging, such as cognitive slowing and dementia, respectively (Valcour and Paul, 2006). With increased survival, but persistence of cognitive decline, it becomes increasingly important to understand the neurological underpinnings of NeuroAIDS-related dysfunction, particularly to guide the development of preventative therapies or adjunctive countermeasures.

B. NEUROPSYCHIATRIC COMPLICATIONS

HIV-infected patients frequently demonstrate neuropsychiatric (NP) dysfunction; patients demonstrate elevated rates of depression and anxiety over both the general population and
populations of patients suffering from terminal diseases such as cancer (Cielsa and Roberts, 2001; Price et al., 2005). Unfortunately, few good estimates based on diagnostic data are available. Recent evidence from clinical studies suggest that the prevalence of clinical depression diagnosis in the HIV-infected population may range from 33% (Evans et al., 2005) to as much as 60% (Scharko, 2006), with anxiety disorders diagnosed in approximately 20-25% of those infected with HIV (American Psychiatric Association, 2000; Pence et al., 2006; Scharko, 2006). Furthermore, NP disturbances in HAD are common and present as agitation, behavioral disinhibition, aberrant motor behaviors, and irritability, which progresses with the severity of the disease (Srikanth et al., 2005). Additionally, personality disorders (PD) are common in HIV, although the estimates are greatly varied, ranging from 18-61% (Perkins et al., 1993; Johnson et al., 1995). In particular, the rate of PD is significantly higher than in substance abusing cohorts compared to non-abusing cohorts (Ladd and Petry, 2003), which is greater than the rates of PD in HIV-negative individuals, which ranges from 1-20% (Reich et al., 1989; Zimmerman and Coryell, 1990). Along those lines, it is notable that substance abuse is prevalent in the HIV-infected population. In fact, psychostimulant users comprise a significant segment of the population of HIV-infected patients (Nath et al., 2002; UNAIDS/WHO, 2005), and HIV-infected drug users present with a more marked neurological progression of HAD (UNAIDS/WHO, 2007).

While NP dysfunction and substance abuse in HIV could be psychogenic in origin, stemming from the psychosocial burdens of being infected with HIV, diverse evidence is emerging to suggest a physiological component. Recent clinical imaging studies have demonstrated functional abnormalities in dorsal frontal cortex of HIV-infected patients, an area of the brain implicated in mood disorders (Ernst et al., 2002; Chang et al., 2003). Also, research has correlated significant volumetric decreases in the nucleus accumbens (NAc) of HIV-positive patients displaying elevated behavioral apathy (Paul et al., 2005). As noted previously, the DA
system may be affected in HIV-infected patients; these findings are significant, as dopaminergic pathways in the midbrain are well known to mediate the rewarding effects of abused drugs; having excess dopaminergic tone in mesolimbic “reward circuits” that project through the ventromedial striatum is strongly linked to the reinforcing effects of drugs and drug addiction (Di Chiara et al., 2004; Kalivas and Volkow, 2005). Notably, a recent post mortem study of brains from patients with HIVe demonstrated abnormalities in striatal dopaminergic markers, specifically an increase in the presynaptic DAT where cocaine is known to bind, and significant decreases in the number of inhibitory type 2 dopamine receptors (D$_2$; Gelman et al., 2006). The authors suggest that HIVe may down-regulate striatal D$_2$ receptor expression as it progressively damages striatum, thereby resulting in a net stimulation of dopaminergic circuits. However, other studies regarding the number of transporters and function of DAT do not corroborate this. For example, a case study of a HAD patient found bilateral reduction of DAT in the posterior striatum (Sporer et al., 2005). Moreover, Wang et al. (2004) showed using positron emission tomography that viral load negatively correlated with levels of DAT in the putamen and the striatum, without concomitant decrease in D$_2$ receptors. Although conflicting, these studies suggest that changes in DAT number and function may play a pivotal role in the molecular profile of NeuroAIDS, although behavioral demonstrations of these effects remain poorly studied. Furthermore, research has demonstrated that Tat protein may contribute to the dysfunction of dopaminergic signaling associated with HIV-infection and HAD (Maragos et al., 2002), and this will be further discussed in Part IV.

C. LIMITATIONS OF HIGHLY ACTIVE ANTIRETROVIRAL THERAPIES

The use of HAART has helped many infected with HIV live longer and relatively healthier lives in comparison to drug regimens used to treat patients in the “pre-HAART” era (Antiretroviral Therapy Cohort Collaboration, 2008). HAART therapies are administered as
combination therapies involving three classes of drugs: protease inhibitors (PI), nucleoside reverse transcriptase inhibitors (NRTI), and non-nucleoside reverse transcriptase inhibitor (NNRTI). Combination antiretroviral therapies can often prevent or delay the progression of AIDS and may even decrease the severity of NeuroAIDS-related disorders (Kaul, 2009). However, a number of reports suggest that HAART cannot prevent the eventual progression of neurological disorder (Tardieu, 1999), and fails to provide complete protection from the development of NeuroAIDS (McArthur et al., 2005; Brew et al., 2009; Boisse et al., 2008).

It is not surprising then, that the neuropathology associated with NeuroAIDS also persists in the HAART era. The capacity for HIV to cause disease in the CNS may remain, as the CNS acts as a compartment or reservoir for the virus even during maximal treatment with HAART (Jevtovic et al., 2009). This may be due to poor penetration of HAART through the BBB (Doughtery et al., 2002). The BBB has a unique anatomical structure that protects the CNS against intrusive chemicals, also making it difficult for therapeutic interventions to penetrate and efficacy the CNS. For example, PIs have little to no BBB penetration ability because they are highly protein bound (Lin et al., 1996; Brew, 2001). The NRTIs are known to have variable penetration. For example, Zidovudine (commonly known as AZT) has shown moderate efficacy at reversing and preventing some neurological changes associated with HIV-infection (Brouwers et al., 1997; Gisslen et al., 1997), but is only effective when used at doses which are hematologically intolerable (Sidtis et al., 1993; Brew, 2001). NNRTIs may also have the potential to cross the BBB effectively as determined by in vitro endothelial cell models of the BBB, but similar to NRTIs, this is not true for all of the NNRTIs (Glynn and Yazdanian, 1998).

The lack of success of HAART in completely alleviating neurological decline has significant implications for adherence to treatment. Forgetfulness, which can be exacerbated by HAD, is the most common reason indicated by patients for non-adherence (Amassari et al., 2002, 2004). Depression and anxiety further complicate adherence (Starace et al., 2002;
Ammassari et al., 2004; see Vervoort et al., 2007 for review), and as noted above, continued substance abuse, can exacerbate the progression of HAD, counteracting the benefit of HAART (Witteveen and van Ameijdan, 2002; Powell-Cope et al., 2003). Given that incomplete adherence or noncompliance to proper medication regimens can rapidly lead to the emergence of drug resistance (Condra et al., 1996), complications of HAD present a significant challenge in the successful treatment of HIV-infected patients. Moreover, as an adherence rate of up to 95% of scheduled medication is necessary to prevent the development of drug resistant mutant strains (Low-Beer et al., 2000; Bangsberg et al. 2003), even mild forgetfulness resulting in partial adherence (as seen with MND) may favor the formation of genetically distinct viral variants in the CNS compared to the blood and periphery (Smit et al., 2004). These issues stress the increasing importance of developing not only adjunctive therapies to directly deal with the issue of continued damage in the CNS, but developing new insights into the underlying causes of the neurobiological mechanisms producing HAD and the symptoms of NeuroAIDS.

IV. HIV-1 VIRAL PROTEINS

As previously discussed, HIV encodes several accessory and regulatory proteins in addition to the prototypical retroviral genes encoded by primate lentiviruses. These proteins have been implicated to varying degrees in the pathogenesis of HIV-1 infection (Frankel and Young, 1998). A majority of studies examining viral protein mediation of HIV-neuropathology have concentrated on gp120 and Tat. It should be noted that although this thesis focuses on the effects of Tat protein, gp120 has also been implicated in the neuropathogenesis of HIV in vivo and in vitro. For example, gp120 has been demonstrated to be toxic to cultured DA neurons (Bennett et al., 1995) and stimulate apoptotic effects in rodent cortical and hippocampal neurons and retinal ganglion (see Corasaniti et al., 2003 for review). Furthermore, intracerebroventricular (i.c.v.) injection of gp120 into rat has been associated with learning and memory impairment
(Hill et al., 1993). However, this research focuses on Tat protein, as a mutation in the \textit{tat} gene has been directly implicated in neurological insult (Mishra et al., 2008; Rao et al., 2008). Also, Tat may be at least partly responsible for the dysfunction of the dopaminergic system implicated in NeuroAIDS-related behavioral dysfunction (Maragos et al., 2002; Ferris et al., 2009; Zhu et al., 2009; Wallace et al., 2006). For example, there is some evidence to suggest that Tat may increase locomotor responses to cocaine (Harrod et al., 2008), which is of interest in the present study, but the data is limited. Furthermore, based on a multitude of evidence that Tat is involved in NeuroAIDS neuropathology (see below for details), this research aims to determine if Tat alone is sufficient to induce behavioral dysfunction and identifiable changes in brain.

A. \textbf{HIV-1 TAT PROTEIN}

The \textit{tat} gene within the HIV-1 genome encodes the 86-101 amino acid viral regulatory protein Tat (Hauber et al., 1987), which initiates the transcription of HIV-1 RNA (Wei et al., 1998). In fact, efficient viral replication of HIV is not viable without Tat (Dayton et al., 1986; Fisher et al., 1986). Notably, evidence suggests that Tat uses other means to contribute significantly to the neuropathology associated with HIV-infection and NeuroAIDS. Tat is actively secreted by HIV-infected cells into the extracellular environment (Tyor et al., 1995; Ozdener, 2005) where it can be directly taken up by nearby uninfected cells, including neurons (Thomas et al., 1994). Tat can also indirectly interact with cells, including microglia, astrocytes, as well as neurons and endothelial cells (Mayne et al., 2000; Bruce-Keller et al., 2001). For example, Tat has been shown to interact with cell surface receptors, including integrin receptors (Vogel et al., 1993), members of the vascular endothelial growth factor receptor family (Albini et al., 1996), the chemokine receptors (Albini et al., 1998), which may interfere with or activate signaling cascades (Noonan & Albini, 2000) or induce inflammatory responses like Tumor necrosis factor-alpha (TNF-\(\alpha\)) production (Mayne et al., 2000). \textit{In vitro} studies have shown Tat to be excitotoxic,
pro-inflammatory, and associated with increased OS and apoptosis (Price et al., 1988; Power et al., 1998; Aksenov et al., 2001). Tat has also been associated with alteration of intracellular calcium (Ca$^{2+}$) homeostasis and stimulation of the cytokines and chemokines (Hayman et al., 1993; New et al., 1998; Haughey et al., 1999). Furthermore, Tat has been demonstrated to induce gliosis and the infiltration of macrophages (Hayman et al., 1993). Importantly, even transient exposure to Tat may be sufficient to trigger a cascade of events leading to neuronal degeneration. This is referred to as the “hit and run” phenomenon and it suggests that viral proteins may induce progressive damage without needing to be continuously present (Nath et al., 1999; Nath et al., 2002).

B. TAT PRODUCES OXIDATIVE STRESS (OS) AND DYSFUNCTION OF THE BLOOD BRAIN BARRIER (BBB)

Reactive oxygen species (ROS) are chemicals naturally generated in cells as byproducts of electron transport in mitochondria and redox enzyme reactions in the cytoplasm. While carefully controlled by the homeostatic functions of the body under normal conditions, elevated cellular levels of ROS resulting in periods of OS are thought to be major contributing agents to chronic neurodegenerative disease (Toda et al., 2007). In vivo and in vitro research has shown that HIV proteins are released from cells infected with HIV and may directly trigger OS (Kruman et al., 1998; Askenov et al., 2001). The OS evident in HIV has been attributed to the direct interactions of HIV proteins with neuronal cells as well as the result of chronic inflammation of CNS tissue in reaction to virotoxins (Nath et al., 2002). Significant evidence suggests Tat is involved in the initiation and/or intensification of OS (Kruman et al., 1998). Moreover, Tat-induced OS results in neurotoxicity and functional deficits in surviving cells, notably pyramidal hippocampal and midbrain dopaminergic neurons (Aksenov et al., 2001, 2003; Aksenova et al., 2005, 2006). For example, in vitro studies conducted by Aksenova and
colleagues (2005), demonstrated a Tat-induced increase in intracellular ROS followed by an increase in protein oxidation (i.e., indices of OS) in cultured midbrain neurons.

ROS not only has deleterious effects on the homeostasis of CNS tissues, but can also modify the permeability of the BBB (Mollace et al., 2001). The microvascular endothelial cells that comprise the BBB are rich in polyunsaturated fatty acids, making it susceptible to lipid peroxidation occurring during OS (Avison et al., 2004). The subsequent weakening of the BBB is of great importance, as it provides the means by which HIV and infected immune cells enter the brain from the circulatory system, accelerating the pace of infection and illness (Banks, et al., 2001). A disruption of the BBB is more often seen in AIDS patients with HAD than in those AIDS patients without HAD or in HIV-negative controls (Toborek, et al., 2003). Price and colleagues (2005) examined the effects of Tat on the BBB using the immortalized rat brain endothelial cell line-4 (RBE4) derived from capillaries. Results showed that Tat significantly decreased levels of intracellular glutathione, glutathione peroxidase activity, and glutathione reductase activity, all of which are endogenous antioxidant agents and enzymes protecting the BBB from oxidative damage. Tat also increased levels of malondialdehyde and a significantly decreased ratio of glutathione/oxidized glutathione in the RBE4 cells, which are indices of an OS-rich environment. The neurotoxic environment resulting from OS-induced damage of the endothelial cells of the BBB is thought to lead to an increase in HIV infection and deleterious effects in the CNS, that ultimately suggest that Tat is an important factor underlying the neuropathology associated with HAD.

C. TAT INCREASES CELLULAR CALCIUM CONCENTRATIONS AND STIMULATES N-METHYL-D-ASPARTIC ACID RECEPTORS

Ca$^{2+}$ is one of the most important second messengers within the cell. It regulates a variety of cell functions including gene expression, cell proliferation, and cell death (Contreras et
In vitro studies have demonstrated that Tat is toxic to fetal neurons through a Ca\(^{2+}\)-dependent mechanism (Nath et al., 2002). Moreover, Tat has been found to dysregulate Ca\(^{2+}\) within the cells it infects. After release from infected cells, Tat can directly depolarize neurons, increasing intracellular Ca\(^{2+}\) concentrations (Bonavia et al., 2001). A number of studies have shown that astrocytes exposed to Tat protein permit the passage of extracellular Ca\(^{2+}\) into the cell while additionally triggering the internal release of Ca\(^{2+}\) from inositol 1,4,5-triphosphate-regulated pools, causing further Ca\(^{2+}\) dysregulation and cellular dysfunction (Lipton, 1994; Nath and Geiger, 1998; Haughey et al., 1999; Mayne et al., 2000). The Tat-induced surge in intraneuronal Ca\(^{2+}\) increases mitochondrial Ca\(^{2+}\) uptake, the generation of ROS and activation of caspases, leading to eventual apoptosis (see Romani et al., 2010 for review).

Tat has also been shown to activate N-methyl-D-aspartic acid (NMDA) receptors (Haughey et al., 2001; Chandra et al., 2005), possibly increasing excitatory amino acid receptor-mediated Ca\(^{2+}\) flux (Haughey et al., 2001) and increasing the release of the neurotransmitter glutamate (Haughey et al., 1999). Glutamate signaling further increases levels of intracellular Ca\(^{2+}\), which may contribute to excitotoxicity (Haughey et al., 1999). Surprisingly, it has been demonstrated that Tat protein additionally complexes with zinc to produce the disinhibition of NMDA receptors, potentiating glutamate signaling even further (Chandra et al., 2005). This is intriguing because the zinc cation alone blocks NMDA-receptor ion channels, protecting cells from excess glutamate signaling. These data suggest that Tat may be able to negate the actions of neuroprotective molecules, like zinc, exacerbating neurotoxicity. The role of Tat in the dysregulation of Ca\(^{2+}\) homeostasis in various cell populations and the activation of NMDA receptors further demonstrates how Tat may contribute significantly to HIV pathology.
D. **ACTIVATION OF CYTOKINES AND CHEMOKINES BY TAT PROTEIN**

Tat exhibits chemokine activity through molecular mimicry (Albini et al., 1998; Peruzzi et al., 2005). Chemokines are low molecular weight cytokines that recruit inflammatory cells to sites of cellular damage (Bornemann et al. 1997; Rollins et al., 1997). It is widely accepted that the activation of microglia and astrocytes by viral proteins results in the secretion of chemokines, cytokines and other potentially neurotoxic factors; Tat is noted as a strong inducer of chemokines in microglia and astrocytes (McManus et al., 2000), particularly CCL2, CCL4, and CXCL8 (D’Aversa et al., 2005). Consistent with this observation, various chemokines are elevated in HIV-infected patients (Schmidtmayerova et al., 1996), and the chemokine mimicry produced by Tat is correlated with HAD (Ranga et al., 2004).

In human microglia, application of Tat protein also increases the production of the cytokines interleukin (IL)-1β, IL-6 and TNF-α (Nath et al., 1999; Bruce-Keller et al., 2001) and increases IL-6 levels in human astrocytes (Nath et al., 1999), thereby further contributing to neurotoxicity and neurodegeneration (Gupta et al., 2008). However, it should be noted that the effects of chemokines and cytokines are highly dependent upon their environment. For instance, in neurons, CCL2 has neuroprotective properties, shown by its ability to decrease Tat-induced cell death (Eugenin et al., 2007). This is in direct contrast to findings of Bruce-Keller et al. (2001), where Tat-induced CCL2 increases were neurotoxic in microglia. Together these studies suggest different effects of CCL2 and the other cytokines across cell types, but nonetheless demonstrate another mechanism by which Tat may induce neurotoxicity.

Once activated by Tat, microglia also upregulate cell surface proteins such as CD40; this action is often a response to changes in the cell’s environment, and serve to increase the inflammatory response. This normal function becomes problematic as chronic upregulation of CD40 receptors further increases cytokine production. These cytokines trigger further upregulation of CD40 receptors, thus initiating a positive feedback loop (D’Aversa et al., 2005).
Cytokines and nuclear factor kappa B (NFκB) have been observed to act in a similar fashion in monocytes and astrocytes (Nath et al., 1999), where NFκB signaling was necessary for the increased expression of IL1-β, IL-6, and TNF-α. These cytokines then function to increase NFκB activation, again creating a positive feedback loop. Unchecked, these two positive feedback loops present a significant neurotoxic threat and further demonstrate how an initial Tat-induced activation of microglia may trigger a prolonged neurotoxic response in the absence of Tat. Supporting this, research has demonstrated that although the protein was no longer detected six hours after a single injection of recombinant Tat into the rat brain, pathological changes and apoptosis were observed for several more days (Haughey et al., 1999). It therefore seems that Tat protein may induce long lasting changes in neuropathology and in neurological activity, even after it is no longer present in the brain.

**E. TAT ACTIVATES MULTIPLE INTRACELLULAR SIGNAL TRANSDUCTION PATHWAYS**

Extracellular Tat protein influences a number of intracellular signal transduction pathways, resulting from transactivation of either the long terminal repeat (promoter region for viral gene expression), or cellular genes following direct cellular Tat uptake (Nabel et al., 1994; Ganju et al., 1998; Ranga et al., 2004). Although literature on Tat signaling is not extensive, Tat exposure alone does appear to specifically modulate a number of intracellular pathways that can influence cell survival (Hauser et al., 2005). For example, Tat interacts with cell surface receptors to induce the activation of mitogen activated protein (MAP) kinase, including c-Jun N-terminal kinase, p38, and extracellular signal-regulated MAP kinase (ERK 1/2, or p44/42), and Ca\(^{2+}\) signaling pathways (Borgatti et al., 1997; Badou et al., 2000; Bennasser et al., 2000; Manna and Aggarwal, 2000). Extracellular Tat stimulation of growth factor receptors has been demonstrated to transiently activate specific isoforms of the protein kinase C pathway (Borgatti
et al., 1998; Sano et al., 2005). Activation of protein kinase C by Tat has been linked to nuclear translocation of NFkB (Conant et al., 1996). Furthermore, extracellular Tat translocates to the nucleus where it is known to activate various transcription factors including cyclic adenosine monophosphate response element binding protein (CREB) (Gibellini et al., 1998). In fact, Tat has been shown to bidirectionally modulate CREB (Zauli et al., 2001). Zauli and colleagues (2001) found that exposure of PC12 cells to extracellular Tat resulted in an initial rapid induction of CREB phosphorylation, but then a subsequent Tat-induced prolonged down-regulation of CREB expression. These changes in CREB phosphorylation and expression temporally correlated with PC12 cell viability, with early protection from apoptosis (24 h), followed by increased apoptosis (72–96 h). These results suggest that Tat can modulate signaling cascades that can result in diminished cell survival.

The involvement of the MAP kinase pathway, particularly ERK 1/2 (Hauser et al., 2005), is also notable for this thesis, as activation of MAP kinase is associated with changes in mood (Einat et al., 2003) and drug reward and abuse (Olive and Messing, 2004; Self, 2004) in animals, and regulates the function and trafficking of the DAT (Morón et al., 2003). This regulation of MAP kinase may provide Tat with an influence over the activity of dopaminergic systems mediating neuropsychiatric disorders (Nutt, 2006), which are additionally observed in animal neurotoxicity studies (Bennett and Hart, 1995; Everall et al., 1993) and human imaging studies (Paul et al., 2005) to be damaged by HIV-infection. As such, activation of MAP kinase may be a mechanism by which Tat mediates the neuropathology, neuronal dysfunction and the behaviors associated with NeuroAIDS.

F. THE EFFECTS OF TAT ON THE DOPAMINERGIC SYSTEM AND SYNERGISM WITH DRUGS OF ABUSE
Research has demonstrated a loss of DAT functioning in neurons exposed to Tat, suggesting that Tat may contribute to the dysfunction of dopaminergic signaling associated with HAD (Maragos et al., 2002). Consistent with this, no-net flux microdialysis studies demonstrated that intra-accumbal administration of Tat_{1-86} significantly reduced local DAT efficiency with little change in DA release, an effect accentuated by addition of cocaine (Ferris et al., 2009). The Tat-induced loss of DAT function was confirmed *in vitro* with radioligand binding and uptake studies following Tat incubation with rat synaptosomes, in a dose-dependent and reversible manner (Zhu et al., 2009). Loss of DAT function has significant implications for the response to drugs of abuse that act on the dopaminergic systems in the brain. Although behavioral studies are limited, intra-accumbal microinjections of Tat to rats significantly increased both acute and sensitized locomotor responses to cocaine (Harrod et al., 2008). Together, these results suggest Tat inhibition of DAT might initially increase synaptic levels of DA, enhancing the rewarding effects of addictive drugs.

The interaction of psychostimulant abuse and Tat may also accelerate the progression of HAD (Nath, et al., 2002). HIV-infected psychostimulant users present with more marked neurological impairment than HIV non-drug users, and it is theorized that this may be due to a synergy of abused drugs with HIV proteins like Tat to potentiate the neurotoxic effects of either insult alone (Nath et al., 2002). It has been suggested that DA neurons and pathways are most vulnerable to the effects of the synergistic neurotoxicity (Nath et al., 2000). Commonly abused psychostimulants, such as cocaine and methamphetamine are reinforcing due to their ability to activate dopaminergic pathways (Kalivas and Volkow, 2005), but this effect is neurotoxic over time (Cass et al., 2003). These drugs could potentially confer an increased risk of developing HAD or exacerbate preexisting HAD symptoms by inducing basal ganglia dysfunction in an already compromised dopaminergic system (Mirsattari et al., 1998). Additionally, it has been reported that lentiviral infection of the brain in the presence of the psychostimulants may result
in enhanced astrocyte viral replication, producing a more rapid and increased brain viral load (Gavrilin et al., 2002).

Of course, the occurrence of oxidative stress may also accelerate symptoms of dementia caused by psychostimulants. Tat preferentially increases neurotoxicity among DA neurons, possibly due to loss of mitochondria and the increased production of ROS (Askenov, et al., 2001), much as cocaine can decrease mitochondrial respiration and increase production of ROS (Boess, et al., 2000). Thus, with a common mitochondrial target, exposure to both cocaine and Tat likely trigger similar mitochondrial dysfunction and apoptosis, and suggests another possible mechanism by which viral proteins produce neurotoxicity. However, more research is necessary to characterize the synergy of psychostimulants with HIV proteins and their production of increased toxicity, as well as to elucidate the behavioral implications of this expected synergy in the context of the response to drugs of abuse.

G. **HIV CLADE-SPECIFIC DIFFERENCES IN TAT AND THE DEVELOPMENT OF HAD**

Direct injections of Tat into rat brain result in multiple histopathological and behavioral manifestations that model aspects of HAD (Bansal et al., 2000; Bruce-Keller et al., 2003). Evidence is accumulating that a mutation in the Tat gene may be directly associated with the severity of this neurological insult. This observation stems from recent reports that HIV clade-specific differences in HAD prevalence are specifically linked to mutations within the first exon of tat (i.e., the neurotoxic epitope) (Ranga et al., 2004). Analysis of Tat sequences indicates a natural variation within the cysteine-rich domain of HIV-Tat that conveys differing chemotactic functions between the clades, with the Tat sequence derived from clade C being less efficient at recruiting monocyte immune cells (Ranga et al., 2004). Furthermore, Tat from clade B, but not C, up-regulates the surface expression of chemokine receptor 4 (CXCR4) on resting CD4+ T
cells, rendering a larger population of these cells more susceptible to HIV-1 infection by viral strains that rely on CXCR4 for cell entry (Campbell et al., 2010).

More relevant to the present thesis, infection with the HIV-1 clade B that is prevalent in North America and Europe is associated with higher rates of cognitive dysfunction than clade C, which is most prevalent in India (Satischandra et al., 2000). Recently, it was demonstrated in vitro that Tat derived from clade C was significantly less neurotoxic to human neurons than Tat derived from clade B (Mishra et al., 2007), phenotypes attributed to differing abilities to induce mitochondrial dysfunction and OS. These studies not only highlight the role of Tat in neurodegeneration, but also in the development of neurological complications associated with HIV infection. However, animal behavior studies are needed to directly test this theory in vivo.

V. ANIMAL MODELS OF HIV-1 CNS PATHOLOGY FOR BEHAVIORAL RESEARCH

A. NON-HUMAN PRIMATES AND FELINES

A variety of animal models have been developed to examine aspects of HIV (or HIV-like) infection in the CNS. Many of these models are limited, as HIV infection is species-specific to humans and a limited number of primates. A close alternative to HIV infection is SIV. Similar to HIV in genomic organization and sequence (Desrosiers, 1990), SIV infection of macaques results in an AIDS-like disease (Letvin et al., 1985) that follows an accelerated but similar pattern of human disease progression (Reimann et al., 1994). Rhesus macaques are well suited to behavioral studies, and like HIV-infected humans, develop cognitive and motor deficits during the progression of the disease (Murray et al., 1992; Rausch et al., 1999). Despite these strengths, the SIV system is not an ideal system for investigating the role of HIV-Tat in neurological dysfunction, as the very breadth of physiological responses due to SIV-infection negates their utility in studying the specific effects of Tat protein on the brain and behavior.
Furthermore, SIV-infected primates are expensive to maintain, difficult to work with from a technical perspective, and it is difficult to obtain a sufficient number of subjects to achieve statistical power. Alternatively, the feline immunodeficiency virus (FIV) also reasonably approximates HIV infection (Bennett and Hart, 1995), and results in a number of similar neurological and behavioral deficits (Phillips et al., 1994). For example, the FIV model demonstrates motor slowing and abnormal reflexes, similar to humans, and delayed auditory and visual evoked potentials, suggesting altered neurological parameters (Phillips et al., 1994). Also, potential interactions between methamphetamines and FIV-infected felines have been reported, both on a neurotoxic and behavioral level (Phillips et al., 2000). However, it is difficult to produce a full spectrum of immunodeficiency-associated problems in FIV-infected animals, and a long latency between infection and clinical disease significantly reduces the value of this model (Nesbit and Schwartz, 2002).

B. RODENTS

Rodents are extensively utilized and established models for conducting a variety of behavioral assays. Because rodents cannot be infected with HIV, researchers have employed numerous strategies to develop non-infectious small animal models of HIV neuropathogenesis. For example, in vivo rat models have demonstrated the neurotoxic effects of an administered HIV-derived peptide (Barks et al., 1993), or HIV-accessory proteins such as gp120 or Tat (Bansal et al., 2000). In addition to several transgenic mouse models (Vogel, 1988), a transgenic rat has been developed that carries a gag-pol deleted HIV-1 provirus and constitutently expresses seven of the nine HIV-1 genes (including tat; Reid et al., 2001), with multiple immune alterations and brain pathologies similar to human infection (Reid et al., 2001). It has been demonstrated that these transgenic rats show impaired spatial learning when tested in an adapted Morris water maze (MWM) (Vigorito et al., 2007). However, although the HIV-1
transgenic rat developed deficits in spatial learning and memory performance in a MWM task, over time they suffer a significant incidence of hind-limb paralysis and are rendered blind by congenital cataracts (Reid et al., 2001; Vigorito et al., 2007), limiting their value in behavioral assays.

Mice, which are easily studied in behavioral assays, have also been manipulated to model HIV-like disease. Inoculation of C57Bl/6 mice with the LP-BM5 murine leukemia virus causes murine acquired immunodeficiency syndrome (MAIDS; Hartley et al., 1989), which has a number of similarities to HIV infection, making it a potentially useful model for the study of behavioral disorder induced by an HIV-like infection (Hartley et al., 1989; Klinman and Morse, 1989). Mice with MAIDS show losses of cognition and memory, demonstrated by spatial reference deficits in the MWM (Sei et al., 1992) and increased errors and response times in a two-choice serial reaction task reflective of sustained attention performance deficits (Lee et al., 2002). HIV is also modeled with a strain of severe combined immunodeficient (SCID) mice, which contain a genetic defect rendering their B and T cells immunologically incompetent. As such, SCID mice are susceptible to a variety of xenographs of HIV-infected human tissues (Mosier et al., 1991; Persidsky et al., 1996). Chronic injection of HIV-positive monocytes into SCID mice produced progressive deficits in cognitive function and motor slowing, suggesting a potential model of HIV-dementia (Persidsky et al., 1996; Avgeropoulos et al., 1998). HIV-infected SCID mice also demonstrate neuropathology similar to that seen in humans with HIV encephalitis, suggesting a broader demonstration of HIV-like symptoms than seen in FIV (Limoges et al., 2000). Cocaine has been demonstrated to enhance HIV-infection in this model (Roth et al., 2005), suggesting an interaction of psychostimulants and the progression of grafted HIV infections. Finally, transgenic animals containing a humanized immune system (such as the RAG-hu mouse model) are susceptible to HIV infection and may prove to be valuable models in the study of NeuroAIDS related behaviors (Berges et al., 2006). However, the very breadth of
the physiological responses to infection precludes the use of these mouse and animal models in the present thesis. This proposal requires the use of an animal model to specifically determine whether Tat alone is sufficient to cause neurological dysfunctions of the CNS in the absence of HIV infection, thereby providing direct evidence to link Tat protein to the neurodegeneration, cognitive dysfunction, and behavioral disorders observed in NeuroAIDS patients. As such, a more specific animal model focusing on the production of Tat alone is required.

C. **TAT ANIMAL MODELS: THE GT-TG BIGENIC MOUSE**

Previous research with Tat protein has commonly used direct stereotaxic administration of recombinant Tat into murine brain (Jones et al., 1998; Rappaport et al., 1999; Cass et al., 2003). Although, stereotaxic administration of Tat protein is an option, it is difficult and time consuming to perform, and could potentially confound the behavioral results with the stress and physical trauma of the procedure. Transgenic “knock in” approaches to expressing HIV proteins in mice have also been developed (Leonard et al., 1988; Corallini et al., 1993; Santoro et al., 1994; Wu et al., 2002). While useful in the study of the immune response to HIV proteins, neurotoxicity, and tumorgenesis, these approaches have important developmental and locomotor complications. As Tat expression occurs constitutently throughout development in all or most of the tissues, these mice demonstrate significant developmental, phenotypic and eventual locomotor complications that limit their use for behavioral experiments.

He and colleagues developed the GT-tg bigenic mouse model (Kim et al., 2003), which provides an opportunity to test the effects of HIV-Tat on the brain and behavior. These mice have a Tat “knock in” gene found only in brain astrocytes containing glial fibrillary acidic protein (GFAP), resulting in brain-specific Tat protein expression. Moreover, Tat expression is achieved using an inducible system controlled through a tet-on promotor strategy, which becomes
transcriptionally active only when Doxycycline (Dox), a tetracycline derivative, is introduced into the body.

Notably, as described, Tat gene expression in the GT-tg bigenic mouse model relies on a GFAP promoter, limiting Tat protein production to astrocytes. There are limitations to this model as brain macrophages and microglial cells constitute the major cell populations that are productively infected in vivo with HIV (Tardieu et al., 1992; Ensoli et al., 1993). However, there are also strengths associated with this particular model system. Astrocytes represent a minor but consistently infected population of HIV-infected cells in human infection of the CNS. This HIV-infected cell type has been closely linked with the pathogenesis of HIV-associated neurological disease (Gorry et al., 2003; Nath, 2010). Astrocytes may be an important reservoir for HIV within the brain and release toxic viral products like Tat (Nath et al., 2002). Tat protein has been detected in HIV-infected astrocytes, producing trimming of neurites, mitochondrial dysfunction, and cell death in neurons (Chauhan et al., 2003; Pocernich et al., 2004). Furthermore, there are no microglia/macrophage specific promoters commercially available and the GFAP promoter-driven gene expression in astrocytes has been successfully used to characterize CNS functions of a number of genes, including gp120, and ApoE4 (Campbell et al., 1998). Additionally, the GFAP promoter in this model has been demonstrated to effectively targeted Tat expression in astrocytes, and in a Dox-dependent manner both in vivo and in vitro (Kim et al., 2003).

When GT-tg bigenic mice were administered Dox, a dose and time-dependent induction of Tat mRNA and protein was observed specifically in the brain (Kim et al., 2003, Figure 1.1). Tat-induced GT-tg mice show symptoms over subsequent months similar to that observed in HIV transgenic studies and HIV-infected humans, including failure to thrive, apoptosis, astrocitosis, neurodegeneration of cerebellum and cortex, degeneration of dendrites, inflammation, seizures, and premature death (Kim et al., 2003). These results help confirm the
validity of the GT-tg mouse model in Tat-related neuropathologies without the logistical or
developmental complications of previous approaches. As such, the model provides an
opportunity to test the effects of HIV-Tat on the brain and behavior to determine whether Tat is
sufficient to induce dysfunction.

**SIGNIFICANCE**

This thesis addresses an under-investigated topic, identifying the contribution of HIV-1
Tat protein to neurodegeneration and neuropsychological impairments associated with
NeuroAIDS. There are limited behavioral studies examining the effects of Tat, and this thesis
will be the first to do so using a transgenic animal with brain-selective and controllable Tat
protein expression. A novel technique, *ex vivo* mouse magnetic resonance imaging, is also
employed in the research, adding a neuroanatomical correlate to the behavioral examination of
Tat protein effects. Evaluating the consequences of Tat activity in the brain will simultaneously
improve our understanding of the neurological underpinnings of NeuroAIDS and the
neurodegeneration associated with HIV-1 infection. This is particularly important given the
prolonged survival of HIV-infected populations that is now possible as a result of improved
treatment regimens; although the improved antiretroviral therapies afford an increased life span
for HIV-1 infected patients, evidence suggests these medications are minimally effective at
treating HIV-1 in the brain. This increase in life span may result in unforeseen increases in
mental disorders that significantly impair both the quality of life and efficacy of treatment. As
such, understanding a neurobiological mechanisms underlying dysfunction is important for both
our understanding of this disease and to accelerate the development of potential
neuroprotective agents useful in the treatment of NeuroAIDS.
Figure 1.1. **Tat protein expression in GT-tg bigenic mouse is limited to brain.** Tat expression in the brain of the GT-tg bigenic mice. (a) **Dox-dependent Tat expression.** GT-tg bigenic or wild-type mice were fed with Dox (6 mg/ml/7d)-containing water and the brains were harvested. Tat expression was analyzed by reverse transcription-polymerase chain reaction (RT-PCR) of the isolated total RNA using Tat-specific and glyceraldehyde-3-phosphate dehydrogenase primers (as controls). (b) **Tat expression is Dox dose-dependent.** GT-tg bigenic mice were fed drinking water containing Dox at the concentrations as indicated, and Tat expression was analyzed by RT-PCR. (c) **Exclusive Tat expression in the brain.** RT-PCR amplification products in all tissues except brain were determined to be nonspecific, because no hybridization signals were detected on the Southern blot using a Tat-specific probe. (Figure was taken from Kim et al., 2003)
REFERENCES


transcription factor in both Jurkat lymphoblastoid T cells and primary peripheral blood mononuclear cells. *J Immunol.* **160:** 3891-3898.


Navia BA and Price RW. (1987) The acquired immunodeficiency syndrome dementia complex as the presenting or sole manifestation of human immunodeficiency virus infection. *Arch Neurol.* **44:** 65-69.


CHAPTER 2

STUDY 1: EXPRESSION OF HIV-TAT PROTEIN IS ASSOCIATED WITH SPATIAL LEARNING AND MEMORY DEFICITS AND A LONG LASTING IMPAIRMENT IN NOVEL OBJECT RECOGNITION LEARNING AND MEMORY

Mild to moderate cognitive deficits are common among HIV-infected patients, and can worsen to a debilitating dementia despite HAART (Navia et al., 1986; Tardieu, 1999; UNAIDS/WHO, 2007). Even with the use of HAART, it is theorized that there may be a restricted infection of brain astrocytes by HIV-1, where the level of production is limited to that of the regulatory proteins like Tat (Power et al., 1998; Brack-Werner, 1999; Johnston et al., 2001). The continued activity of HIV-1 and/or the viral proteins in the brain may be responsible for the persistence of HIV-related neuropathology and subsequent cognitive and psychomotor slowing (Frankel and Young, 1998).

Evidence suggests that the hippocampus, an area critical to certain types of memory, is damaged in patients with NeuroAIDS (Lawrence and Major, 2002). AIDS patients show more marked morphological changes in the hippocampus (see Mollace et al., 2001 for review) that may be responsible for memory deficits seen in HIV-infected individuals (Navia et al., 1987, 1986). These results are consistent with the limited available data collected from animal models of HIV-infection. HIV-infected SCID mice and mice with MAIDS display learning deficits in the Morris water maze (MWM) (Sei et al., 1992; Zink et al., 2002; Griffin et al., 2004), which has been used extensively to examine learned performance on a hippocampal-dependent task (Morris et al., 1982; Brandeis et al., 1989; see D’Hooge and De Deyn, 2001 for review). LP-BM5-inoculated mice display several deficits in the MWM, without the finding of detectable neuropathological alterations (Sei et al., 1992). Mice with MAIDS show losses of cognition and
memory, demonstrated by spatial reference deficits in the MWM (Sei et al., 1992). Moreover, rats administered daily gp120 (i.c.v.) show impaired acquisition in the MWM (Glowa et al., 1992), but further studies demonstrated that a certain type of impairment noted (i.e., probe trial deficit) was only evident in aged mice, suggesting a progression of disease state in mice administered gp120 (D’Hooge et al., 1999). FIV- and HIV-derived gp120 injected into brain has also been shown to impair performance in the Barnes maze, another type of spatial maze (Sanchez-Alavez et al., 2000). The HIV-1 transgenic rat (Reid et al., 2001, 2004) demonstrated deficits in acquisition of the specific location of the escape platform in the MWM, but intact memory for the general location of the escape platform (Vigorito et al., 2007). However, these results are difficult to interpret, as the HIV-1 transgenic rat presents with congenital cataracts. Therefore these rats cannot use the typical visual cues necessary to navigate the maze, requiring the authors to develop a modified version of the maze dependent on olfactory and tactile cues. The modification of the maze does not negate the findings of a learning impairment, but must be interpreted with caution as this modified maze has not yet been used in other studies.

As noted, various animal models of HIV-1 have displayed specific deficits in learning in memory, particularly in mazes dependent on the intact functioning of the hippocampus. Fewer studies have specifically looked at how HIV-Tat can affect learning and memory, but it has been suggested that Tat may play a crucial role in the neurotoxicity and cognitive impairment evident in NeuroAIDS (Rappaport et al., 1999). Research suggests that HIV-Tat protein may create a potentially toxic extracellular environment while additionally inducing direct damage to cells (Aksenov et al., 2001; Aksenov et al., 2003). Of interest, recent reports indicate that HIV-1 clade-specific differences in HAD prevalence are specifically linked to mutations within the neurotoxic epitope of the tat gene between clades of HIV-1 (Ranga et al., 2004). The mutation in the tat gene has been directly associated with the toxicity of HIV-Tat protein on human
neurons (Mishra et al., 2008; Rao et al., 2008). In vitro research suggests that Tat exposure can result in CA1 hippocampal and entorhinal cell dysfunction, as incubation of hippocampal-entorhinal cortex slices and CA1 only hippocampal slices with Tat$_{1-86}$, resulted in suppression of long-term potentiation (LTP) (Behnisch et al., 2004; Li et al., 2004, respectively). This is relevant, as LTP is thought to be the neuronal basis for hippocampal-dependent learning (see Sarvey et al., 1989; Teyler, 1987-1988 for reviews). Consistent with this, the Tat-induced suppression of LTP was correlated with deficits in eight-arm radial arm maze spatial learning and memory in rat, as indicated by increased number of errors committed after i.c.v. administration of recombinant Tat protein (Li et al., 2004).

Behavioral studies examining the contribution of Tat to learning and memory deficits are limited. Therefore, we used the GT-tg mouse model to assess the effects of brain-specific Tat expression on learning and memory performance. We tested the hypothesis that the activity of Tat in brain is sufficient to impair learning and memory processes. Mice were tested in the Barnes maze, which is a dry-land hippocampal-dependent spatial learning and memory task (Barnes et al., 1990: Bach et al., 1995). This experiment was performed to confirm previously demonstrated spatial deficits in mice administered a single i.c.v. injection of Tat (Li et al., 2004), which could help to validate the GT-tg bigenic mouse model as a tool to study the effects of Tat protein on cognition. The Barnes maze was chosen over the well-used MWM because water-based mazes were initially developed for rats (Morris et al., 1982), but may not be as ecologically valid for mice, as mice are more likely to burrow than swim (D’Hooge and De Deyn, 2001; Wishaw and Tomie, 1996). (Indeed, it has been determined that if optimal performance is desired on a spatial task in mice, it is more likely to be obtained on a dry-land maze as opposed to a swimming task, as mice are not as good swimmers as are rats, and easily develop hypothermia in water (Bach et al., 1995; Wishaw and Tomie, 1996)). We then elaborated on the types of learning and memory impaired by Tat expression by employing the novel object
recognition (NOR) assay (Genoux et al., 2002; Save et al., 1992; Carey et al., 2009). As a simple test of learning and memory, NOR is dependent on the ability of the animals to differentiate between a novel object and those objects that have previously been encountered. This is not a spatial ability task and therefore tests a different type of learning and memory process (i.e., object recognition) than the Barnes or MWM. Furthermore, the brain regions that underlie performance on the NOR task are different than spatial mazes. Research suggests that NOR performance is not based on hippocampal function, but may be largely mediated by the entorhinal and other activity of the perirhinal cortex in the medial temporal lobe (Mumby and Pinel, 1994; Murray and Richmond, 2001). This area does receive input from the hippocampus, but Ainge et al. (2006) demonstrated that lesions in the hippocampus do not disrupt object recognition memory. Given the imaging work reviewed later in this thesis (see Chapter 5), we used NOR to determine if there are different types of learning and memory deficits attributable to Tat, and if those deficits may be mediated by damage to different brain regions.
MATERIALS AND METHODS

Animals and housing

Adult male GT-tg bigenic (bred in a colony at Northeastern University; see below) and C57Bl/6J wild-type (Jackson Labs, Bar Harbor, ME) mice, 8-14 weeks of age, were used in all experiments. Mice were housed and cared for in the Northeastern University animal facility in accordance with the 1996 National Institutes of Health Guide for the Care and Use of Laboratory Animals as approved by the Institutional Animal Care and Use Committee. The creation and development of the GT-tg bigenic mouse and genotype confirmation of the inducible and brain-targeted HIV-1 Tat protein were described in detail by Kim et al., 2003. The GT-tg bigenic mice are engineered to express the Tat86 gene upon Dox-mediated activation of a brain-specific promoter. A colony of GT-tg bigenic mice, previously back-crossed seven generations onto the C57Bl/6 line, was established at our facilities using two breeding pairs of bigenic mice to provide animals for this study. The use of the C57Bl/6J line is expected to allow a direct comparison to determine if there are differences in behavior displayed in uninduced mice compared to the parent strain of mouse.

Chemicals

Doxycycline hyclate (Dox; see below), obtained from Sigma-Aldrich (St. Louis, MO), was dissolved in 0.9% saline prior to injection.

Induction of brain-targeted Tat with Doxycycline treatment
To express Tat86 protein, GT-tg bigenic mice were administered Dox via intraperitoneal (i.p.) injection with a single daily dose (25, 50, or 100 mg/kg, dissolved in 0.9% saline in a volume of 0.3 ml/30 mg body weight) for 1, 3, 5, or 7 days as indicated. Note that for the majority of induction experiments, the maximum amount of Dox was administered at a dose of 100 mg/kg, i.p., once daily for 7 days on the basis of previously demonstrated efficacy of Tat induction (JJ He, personal communication (dose) and Zou et al., 2007 (duration of administration)). C57Bl/6J mice were administered Dox following the same protocol to control for any possible effects of Dox-treatment on behavior. Vehicle (0.9% saline) treatment of GT-tg mice was included as a direct control for the comparison of behavior to the Tat-induced GT-tg mice. The vehicle-treated GT-tg mice are referred to as “uninduced” throughout the thesis.

Barnes Maze

The Barnes Maze is a cognitive task that assesses spatial learning and memory and is based on the natural tendency of mice to explore and escape through holes (Bach et al., 1995; Dawood et al., 2004). The mice are required to use fixed and distinct spatial cues located in the testing room to navigate to an escape in the maze. The Barnes Maze used for testing here was a brightly lit 105 cm circular surface with 40 holes, 5 cm in diameter, which are evenly spaced around the perimeter (Figure 2.1a). An escape chamber (15 X 8 X 3 cm) was located beneath one of the holes and remained fixed throughout the acquisition period. Mice were habituated by allowing them to freely explore the maze for 1 min with no escape. After this trial, mice were placed directly into the escape box (not yet attached to the escape hole), for 30 s to familiarize them to the box.

After habituation, mice were trained to locate an escape tunnel (i.e., acquisition trials). Each trial started by placing a mouse in a cylinder located in the center of the maze. The mice
were placed in the central cylinder so that they were randomly facing a different direction when
the trial initiated. Recording of the trial began when the cylinder was removed. Upon removal of
the cylinder, a static radio noise was played as additional motivation to escape the maze (i.e., in
addition to the bright light) (Sarkisyan and Hedlund, 2009). During training, the mice were
allowed to explore for 3 min; if the escape was not found in this time the mice were guided to
the tunnel and allowed to enter the hole. When the escape was found, the radio static noise was
terminated and the mice remained in the escape box for 15 s. In between every trial the maze
was wiped with 70% ethanol to control for odor cues.

The acquisition period consisted of two trials/day for four days with a 15-min inter-trial
interval (ITI). During these acquisition trials, latency to escape the maze and number of errors
made prior to escaping were recorded. A mouse was considered to have escaped when the last
foot of the mouse left the maze surface. Errors were deemed as nose and head deflection into a
hole that did not lead to the escape box. Reference memory errors were recorded when a
mouse made a nose and head deflection into a hole in the maze that was not the escape hole,
whereas working memory errors were recorded when a mouse made a nose and head
deflection into an incorrect hole that the mice had already visited during that same trial.
Furthermore, the type of search strategy employed by the mice on each trial was recorded as
serial (searching the holes in a clockwise or counterclockwise order), random (hole searches
separated by crossing through the center of the maze or unorganized search), or direct search
(moving directly to escape hole or within the two adjacent holes before visiting the escape)
(Barnes, 1979).

At the end of day 4, a probe trial was performed. During this trial, the escape location
was removed and mice were given 90 s to explore the maze. The variables recorded during this
trial were success in finding the former escape location and time spent in the target quadrant
(i.e., quadrant where the escape was located). This selective search and spatial bias for the
target quadrant constitutes evidence for spatial memory. **Reversal learning** was tested on day 5 by moving the escape tunnel 135 degrees from the previous location and conducting four trials. Latency to escape at the new location was recorded.

On the last day of testing the mice were given a single trial to locate a visible escape tunnel in a novel location (made visible with a contrasting object placed behind the escape). This assessed if there were any vision problems that may afflict the mice. Notably, all mice in the study successful found the visually-cued escape location in the time allotted (3 min), so none were excluded from the study on the basis of visual disturbance.

**Novel object recognition (NOR) assay**

Learning and memory performance was examined using the NOR assay (Save et al., 1992; Genoux et al., 2002; Carey et al., 2009). The NOR task for rats and mice is based on the spontaneous exploration of their environment, in which normal animals will spend more time exploring a novel object than a familiar one (Ennaceur and Delacour, 1988; Frick and Gresack, 2003). As the assay does not require motivation such as food or water deprivation, this assay also minimizes external, physical stressors (Ennaceur and Delacour, 1988). The paradigm was carried out over three phases as describe in Carey et al. (2009); the first two were training phases and the third phase was the testing phase. The NOR is a test for both learning and memory, as the final output of the assay is affected by both. During each trial, mice freely explored the environment before being returned to their home cages. In phase 1, object A and B (a pair of dice) were centered across from each other, 2 cm away from the walls of a rectangular cage (16 X 24 X 12 cm) (Figure 2.1b). In phase 2, object B was moved 1 cm from the edge of the cage whereas object A remained fixed. In phase 3, after completion of the training phases, object B (the die) was replaced with a marble, the novel object. Phase 2 served as a control, to
demonstrate that subsequent performance in phase 3 was based on novel object recognition, rather than simple changes in object location. In contrast, phase 3 testing assesses both learning and memory, as mice will recognize and reject previously encountered objects to spend more time with the novel object. For each phase, the time that the mice spent attending to each object was recorded with stopwatches. Attending to the object was defined as the duration of time the mouse spent in physical contact with the object using any body part other than the tail, or whenever it was within 0.5 cm of the object, facing it, and engaged in active exploration (e.g., sniffing or manipulating). Data are presented as a percent recognition index (RI) for object B: RI = (time attending to object B/time attending to object A+B)*100.

Initial pilot experiments were performed in untreated-C57Bl/6J control mice to determine the optimal trial and ITI duration (Carey et al., 2009, but see Results). Also, a control experiment was performed to confirm that the increased time spent attending to object B in phase 3 resulted from the novelty of, rather than the preference for, a particular object. Mice were tested following the described procedure except that the familiar objects were marbles and the novel object in phase 3 was a die. No differences in performance with either novel object in phase 3 was detected ($F_{1,33} = 0.316, p = 0.578$, one-way analysis of variance (ANOVA)), and there was no significant interaction of stimuli X phase ($F_{2,100} = 0.96, p = 0.39$, two-way ANOVA), establishing novel object interchangeability. However, use of marble and dice as novel objects was randomized across the study.

**Statistical analyses**

**General:** Data from all experiments were analyzed using SPSS 14 or 16 statistical package for the social sciences (Chicago, Illinois, USA) and are presented as mean ± standard error of the mean (S.E.M.), unless noted otherwise. Significance was set at $p \leq 0.05$. 
a.

Object A

Phase 1
2 Die
10 min

Object B

Phase 2
Object B moved to corner
10 min

Phase 3
Object B replaced with marble
10 min

\[
\% \text{ Recognition Index} = \left( \frac{\text{Time attending to object B}}{\text{Time attending to object A + B}} \right) \times 100
\]
Figure 2.1. Schematic of the Barnes maze and NOR assay. (a) The Barnes Maze consisted of a 105 cm white circular surface with 40 holes, 5 cm in diameter, which were evenly spaced around the perimeter. An escape chamber (15 X 8 X 3 cm) was affixed beneath any hole as necessary with Velcro. (b) Images represent testing cages as they were arranged in each phase of testing. Objects A and B were identical in phases 1 and 2, although object B was moved to a different position during phase 2 testing. In phase 3, object B was replaced with a novel object. In all phases, the percentage of time spent attending to object B was calculated as a percentage RI by the formula shown. The use of dice and marbles as novel objects was randomized. Each phase lasted 10 min followed by a 10-min ITI (but see also Fig 2.6 for justification of these variables).
Barnes maze: Latency was analyzed with a two-way repeated measures ANOVA with day (acquisition, day 1-4) or trial (reversal, trial 1-4) as the within subjects repeated measure and group as the between subjects variable. The number of errors was analyzed with one-way ANOVA. Tukey Honestly Significant Difference (HSD) post hoc test was used to further elucidate all significant ANOVA effects. Search strategies were analyzed with Mann-Whitney U and are presented as proportions and 95% confidence intervals. Probe trial success was analyzed with Fisher’s exact test (alternate to Chi-square for smaller samples sizes) and also presented as proportions and 95% confidence intervals. Time in the target quadrant during the probe trial was analyzed with a one-sample t-test comparing the time spent in the quadrant to chance (22.5 s).

Novel object recognition: Optimization of the NOR assay was analyzed using two-way ANOVA, with phase as a repeated measure, to examine the effects of phase or ITI duration on NOR. Fisher’s Least Significant Difference (LSD) was used to further examine between group effects, while paired-samples t-tests were used to make within group comparisons between recognition indices in phase 1 and phase 3. For all other NOR data, an omnibus repeated measures ANOVA was run with phase as the within subjects repeated measure, and time of testing, Dox-dose and duration of administration as the between subjects variables. Interactions and main effects were further analyzed with one-way ANOVA and/or Tukey HSD post hoc testing.
RESULTS

RESULTS OF BARNES MAZE TESTING:

Saline- and Dox-treated C57Bl/6J mice performed differently in the Barnes maze than uninduced GT-tg mice

C57Bl/6J mice were treated with either vehicle (0.9% saline, i.p.) or Dox (100 mg/kg, i.p) for 7 days and subsequently tested in the Barnes maze. An additional set of uninduced GT-tg mice (0.9% saline, i.p., 7 days) were tested in parallel. A repeated measures ANOVA indicated an overall difference between groups ($F_{2,90} = 4.55, p < 0.05$) in latency to escape the maze during acquisition trials (Figure 2.2a). Further analyses indicated that there were no differences in latencies to escape the maze on any of the four days of acquisition testing between saline- and Dox-treated C57Bl/6J mice ($p = 0.96, p = 0.84, p = 0.16, p = 0.84$, days 1-4, respectively, Tukey HSD). However, uninduced GT-tg bigenic mice showed a shorter latency to find the escape location compared to saline- and Dox-treated mice on days 1 ($p = 0.06$ (trend) and $p < 0.01$, respectively) and 2 ($p < 0.05$ from both C57Bl6/J groups).

Results also indicated a significant difference between groups in the mean number of errors committed prior to escaping the maze over the four acquisition days (excluding the first trial because the mice have not had an encounter with the escape tunnel location at this point) ($F_{2,370} = 19.32, p < 0.001$, one-way ANOVA, Figure 2.2b). The saline- and Dox-treated C57Bl/6J mice did not differ in the number of errors committed ($p = 0.51$). However, uninduced GT-tg mice committed fewer errors over the course of the acquisition trials ($5.7 \pm 0.4$) compared to saline- ($9.9 \pm 0.8, p < 0.001$, Tukey HSD) and Dox-treated ($9.0 \pm 0.5, p < 0.001$) C57Bl/6J mice.

Although latency only differed on days 1 and 2 in uninduced GT-tg mice versus C57Bl/6J mice, the mice showed a difference learning pattern. It should be noted, however, that Dox-
treatment itself had no effects on performance, as C57Bl/6J mice treated with Dox did not show any difference in latency or number of errors committed compared to mice of the same strain treated with saline. These data suggest that a direct comparison of the GT-tg mice to the C57Bl/6J mice may not be appropriate for this particular behavioral task, as there may be subtle strain differences in the transgenic C57Bl/6-back-crossed mice and the C57Bl/6J mice purchased from Jackson Laboratories in spatial acquisition performance.

Tat-induced mice demonstrated impaired acquisition in the Barnes maze compared to uninduced GT-tg littermates

GT-tg bigenic mice were induced to express Tat protein by administering Dox (100 mg/kg, i.p.) daily for either 5 or 7 days prior to being tested in the Barnes maze. For the sake of clarity, these mice will be referred to as 5 day Tat-induced and 7 day Tat-induced mice, respectively. Performance was compared to uninduced (0.9% saline, i.p., 7 days) GT-tg littermates. A two-way repeated measures ANOVA indicated an overall difference between groups in their latencies to escape the maze ($F_{2,89} = 14.99, p < 0.001$). The average latencies over the four acquisition days of the 5 day Tat-induced mice ($148 \pm 8$ s) and the 7 day Tat-induced mice ($133 \pm 6$ s), although not different from each other ($p = 0.32$, Tukey HSD), were significantly longer than the average latency of their uninduced littermates ($100 \pm 6$ s, $p < 0.001$). Further analysis of the individual acquisition days (Figure 2.3a), indicated that there were no differences in escape latencies on any of the four acquisition days between the 5 day and 7 day Tat-induced mice ($p = 0.53, p = 0.22, p = 0.99, p = 0.65$, days 1-4, respectively). However, the 5 day Tat-induced mice demonstrated longer latencies to escape from the maze compared to uninduced GT-tg mice on days 1, 2, and 4 ($p < 0.05$), but not on day 3 ($p = 0.20$).
a. 
Latency to escape (s) 

- uninduced GT-tg bigenic mice 
- C57Bl/6J + Saline 
- C57Bl/6J + Dox 

Day 
1 2 3 4 

b. 
Number of errors 

C57Bl/6J + Saline 
C57Bl/6J + Dox 
GT-tg mice uninduced 

*
Figure 2.2. Saline- and Dox-treated C57Bl/6J control mice displayed greater latencies and made more errors in the acquisition trials of the Barnes maze than the uninduced GT-tg mice. (a) There were no differences in latencies to escape the maze on any of the four days of acquisition testing between saline- (white squares) or Dox-treated (gray squares) C57Bl/6J mice. However, uninduced GT-tg mice (white circles) showed a shorter latency to find the escape location compared to saline- and Dox-treated mice on days 1 and 2, suggesting a different pattern of learning. (n = 9-19; ^ = p = 0.06, trend from saline-treated C57Bl/6J mice, * = different from Dox-treated C57Bl/6J mice, † = different from saline-treated C57Bl/6J mice, p < 0.05, repeated measures ANOVA followed by Tukey HSD) (b) C57Bl/6J mice treated with either saline- or Dox- made a similar amount of errors during the acquisition trials. However, uninduced GT-tg mice committed fewer errors during the acquisition trials compared to saline- and Dox-treated C57Bl/6J mice. (n = 9-19; * = different from all other groups, p < 0.001, one-way ANOVA followed by Tukey HSD)
The 7 day Tat-induced mice demonstrated longer latencies to escape from the maze compared to uninduced mice on days 1 and 4 ($p < 0.01$), but not days 2 and 3 ($p = 0.54$, $p = 0.09$, respectively).

There was also an overall difference in the total number of errors made over acquisition testing (excluding trial 1) between groups ($F_{2,362} = 11.03$, $p < 0.001$, one-way ANOVA, Figure 2.3b). Post hoc analyses indicated that the 7 day Tat-induced mice made significantly more errors (8.3 ± 0.5) than the uninduced mice (5.6 ± 0.4, $p < 0.001$). However, while the 5 day Tat-induced mice did not commit significantly more errors (6.6 ± 0.6) than the uninduced mice ($p = 0.21$), the number or errors committed was also not different from the 7 day Tat-induced mice ($p = 0.09$).

To further evaluate errors, total errors were divided into two types of errors, reference memory and working memory errors (Figure 2.3b). A significant overall difference was found between groups in the number of reference memory errors made ($F_{2,360} = 12.11$, $p < 0.001$, one-way ANOVA). Post hoc analyses indicated that the 7 day Tat-induced mice made significantly more reference memory errors (7.1 ± 0.4) on average than the uninduced mice (4.8 ± 0.3, $p < 0.001$, Tukey HSD). However, the 5 day Tat-induced mice did not commit more errors (5.7 ± 0.5) than the uninduced mice ($p = 0.23$) and committed significantly fewer errors than the 7 day Tat-induced mice ($p < 0.05$). Although, all mice committed fewer working memory errors overall than reference memory errors, there was difference between groups in the number of working memory errors made ($F_{2,361} = 6.70$, $p = 0.001$). The 7 day Tat-induced mice committed significantly more working memory errors on average (1.1 ± 0.1) than the uninduced mice (0.5 ± 0.1, $p < 0.01$, Tukey HSD). The 5 day Tat-induced mice did not commit significantly more working memory errors (0.9 ± 0.2) than the 7 day Tat-induced mice ($p = 0.82$), but did show a trend toward committing more working memory errors than uninduced mice ($p = 0.06$).
The search strategy the mice employed during each trial on days 2-4 was also examined (Figure 2.3c). The mice could use three difference strategies to escape the maze: random, serial, and direct. The 5 day and 7 day-Tat induced mice demonstrated different patterns of search strategy usage compared to the uninduced mice who favored the direct search strategy (vs 5-day Tat induced mice, \( U = 2230, p < 0.01 \); vs 7-day Tat-induced mice, \( U = 5171.5, p < 0.05 \), Mann-Whitney U). The 5 and 7 day Tat-induced mice did not show differences in their pattern of search strategy used (\( U = 2583, p = 0.20 \)), with both groups favoring the random strategy.

**Mice induced to express Tat demonstrated deficits in probe trial performance**

On day 4, after the acquisition trials, a single probe trial was performed. During this probe trial, the escape chamber was removed from the maze and mice were given 90 s to locate the former escape hole. We analyzed the amount of time mice spent in the quadrant in which the escape box was formerly located (i.e., target quadrant) and whether mice found this escape hole (Figure 2.4). Uninduced GT-tg mice (0.9% saline, i.p., 7 days) spent 36.5 ± 5.2 s in the target quadrant, which was significantly more time than chance would predict (22.5 s) (\( p < 0.05 \), one-sample t-test). However the 5 day Tat-induced mice (100 mg/kg Dox, i.p., 5 days) and the 7 day Tat-induced mice (100 mg/kg Dox, i.p., 7 days) did not spend more time in the target quadrant than chance (33.8 ± 11.3 s, \( p = 0.35 \) and 34.6 ± 6.9 s, \( p = 0.10 \), respectively). Moreover, the uninduced mice were significantly more successful at finding the escape hole during the probe trial, with 74% of the mice finding the escape, compared to the 5 day Tat-induced mice (13%, \( p < 0.01 \), Fisher’s exact test, Figure 2.4). The 7 day Tat-induced mice displayed a trend toward being less successful at finding the escape than the uninduced mice (41%, \( p = 0.09 \)). Notably, the 7 day Tat-induced mice but did not find the escape at a higher frequency than the 5 day Tat-induced mice (\( p = 0.21 \)).
C.

Percent Search Strategy

* * *

7 d saline 5 d Dox 7 d Dox
(uninduced) 100 mg/kg, day

Pretreatment
GT-tg bigenic mice
Figure 2.3. Tat-induced mice demonstrated impaired acquisition and inefficient search strategies in the Barnes maze compared to the uninduced GT-tg mice. (a) The escape latencies of 5 day Tat-induced mice (100 mg/kg Dox, i.p; gray squares) or 7 day Tat-induced mice (100 mg/kg, i.p.; black diamonds) did not differ on any of the acquisition days. However, the 5 day Tat-induced mice demonstrated longer escape latencies compared to uninduced GT-tg mice (0.9% saline, i.p., 7 days) (white circles) on days 1, 2, and 4, while The 7 day Tat-induced mice demonstrated longer latencies compared to uninduced mice on days 1 and 4. (n = 9-18; n.s. = not significant, * = different from 5 day Tat-induced mice, † = different from 7 day Tat-induced mice, p < 0.05, repeated measures ANOVA followed by Tukey HSD) (b) The 7 day Tat-induced mice (dark gray patterned bars) committed more total (left bars), reference memory (middle bars), and working memory errors (right bars) than the uninduced mice (white bars). The 5 day Tat-induced mice (gray bars) did not commit more total errors than the uninduced or the 7 day Tat-induced mice, but committed less reference memory errors than the 7 day Tat-induced mice. Also, the 5 day Tat-induced mice showed a trend toward committing more working memory errors than the uninduced mice. (n= 8-16; ^ = trend from uninduced mice, p = 0.06, * = different from uninduced mice, † = different from 5 day Tat-induced mice, p < 0.05, one-way ANOVA followed by Tukey HSD) (c) The 5 day (middle bars) and the 7 day (right bars) Tat-induced mice demonstrated different patterns of search strategy usage compared to the uninduced mice (left bars), who favored the more efficient direct strategy. The 5 day Tat-induced mice and 7 day Tat-induced mice did not show differences in the overall pattern of search strategy utilized, both groups favoring the random strategy. (data plotted as proportion of trials where that strategy was employed, with 95% confidence intervals) (n = 9-18; * = different overall pattern of search strategy used compared to uninduced mice, p < 0.05, Mann-Whitney U)
Figure 2.4. Mice induced to express Tat were not successful in finding the escape location during a probe trial. Upon completion of the acquisition trials, a single probe trial was performed during which the escape was removed from the maze and mice were given 90 s to locate the former escape hole. The uninduced GT-tg mice (0.9% saline, i.p., 7 days) succeeded in finding the previous escape location more often than the GT-tg mice administered Dox (100 mg/kg, i.p.) for 5 days to express Tat protein. The GT-tg mice induced to express Tat protein by administering Dox (100 mg/kg, i.p.) for 7 days displayed a trend toward being less successful at finding the escape than the uninduced mice and did not perform better than the 5 day Tat-induced mice. (Data are plotted as percentages with 95% confidence intervals based on each individual proportion.) (n = 8-19; * = different from uninduced mice, p < 0.05; ^ = p = 0.09 from uninduced mice, Fisher’s exact test)
Tat-induced mice took longer to learn a new escape location during a reversal learning task in the Barnes maze

On day 5, mice were given four trials to learn the location of a new escape hole (Figure 2.5). A two-way repeated measures ANOVA indicated an overall difference in escape latency between groups ($F_{2,42} = 4.86, p < 0.05$). There were no differences in the average latencies over the four trials displayed by 5 day Tat-induced mice (148 ± 8 s) and the 7 day Tat-induced mice (128 ± 7 s, $p = 0.19$, Tukey HSD). However, these latencies were both significantly longer than those of their uninduced GT-tg littermates (102 ± 7 s, vs 5 day Tat-induced mice $p < 0.001$, vs 7 day Tat-induced mice $p < 0.05$). Further analysis of the individual trials (Figure 2.5) indicated that the escape latencies demonstrated by the 5 day and 7 day Tat-induced mice were not different on any of the four trials ($p = 0.85$, $p = 0.62$, $p = 0.37$, $p = 0.80$, trials 1-4, respectively). However, although the 5 day Tat-induced mice demonstrated longer latencies to escape from the maze compared to uninduced mice on trials 2 and 3 ($p < 0.01$ and $p < 0.05$), they showed only a subtle trend on trial 1 ($p = 0.11$) and no difference on trial 4 ($p = 0.81$). Furthermore, the 7 day Tat-induced mice demonstrated increased latencies to escape from the maze compared to uninduced mice on trials 1 and 2 (both $p < 0.05$), but not trials 3 and 4 ($p = 0.30$ and $p = 0.99$).
Figure 2.5. Tat-induced mice required more trials to learn a new escape location during a reversal learning task in the Barnes maze. On day 5, mice were given four trials to find a new escape location. There were no differences in latencies to escape the maze on any of the 4 trials between the GT-tg mice induced to express Tat by administered Dox (100 mg/kg, i.p.) for 5 days (gray squares) or for 7 days (black diamonds). However, the 5 day Tat-induced mice demonstrated longer escape latencies compared to uninduced GT-tg mice (0.9% saline, i.p., 7 days) (white circles) on trials 2 and 3, but showed only a subtle trend on trial 1 and no difference on trial 4. The 7 day Tat-induced mice demonstrated longer latencies to escape from the maze compared to uninduced mice on trials 1 and 2 only. (n = 9-19; ^ = trend from 5 day Tat-induced mice, * = different from 5 day Tat-induced mice, † = different from 7 day Tat-induced mice, p < 0.05, repeated measures ANOVA followed by Tukey HSD)
RESULTS OF NOVEL OBJECT RECOGNITION TESTING:

Optimization of phase and inter-trial interval in novel object recognition task

To optimize the assessment of object recognition, NOR was conducted with 1-, 5-, 10-, or 20-min phases with a fixed 10-min ITI on C57Bl/6J mice (Fig. 2.6a). Results indicated a significant main effect of phase duration ($F_{3,216} = 8.6, p < 0.001$, two-way ANOVA). Mice tested with 1-min phases displayed significantly less object recognition over three phases compared to mice tested using 5-, 10-, and 20-min phases ($p < 0.01$ each, Fisher’s LSD). Mice tested using 5-, 10-, and 20-min phases spent significantly more time interacting with the novel object in phase 3 as compared to time attending to object B in phase 1 ($p < 0.05$; paired-samples t-test), whereas mice tested with 1-min phases did not ($p = 0.06$, n.s.). As a peak RI for the novel object was demonstrated after testing with 10-min phases (71.7 ± 3.4%), this phase duration was used for the remainder of the study.

To establish the optimal ITI for novel object recognition, the duration of the interval between each 10-min phase was examined using 1-, 10-, or 20-min delays (Fig. 2.6b). A significant main effect of ITI duration was demonstrated ($F_{2,144} = 3.74, p < 0.05$, two-way ANOVA), but no significant interaction between phase and ITI was found ($F_{4,144} = 0.394, p = 0.81$). Significant differences in performance were demonstrated between mice tested with a 1-min and 10- ($p < 0.01$, Fisher’s LSD) or 20-min ITI ($p < 0.05$), but not between the mice tested with a 10- and 20-min ITI ($p = 0.48$). Further analysis found significant increases in the RI values in phase 3 over phase 1 when the ITI lasted 10 min ($p < 0.001$; paired-samples t-test) or 20 min ($p < 0.05$), but not 1 min ($p = 0.20$, n.s.). As the peak response was demonstrated using a 10-min delay between phases, this ITI was used throughout the remainder of the study.
Figure 2.6. **Optimization of conditions for the novel object recognition assay.** Data plotted as % RI ± S.E.M. for each phase. (a) **Effect of phase duration.** Vehicle-treated C57Bl/6J mice were used in the three phases of the NOR assay. Time in each phase was limited to 1 (black bars), 5 (gray bars), 10 (white bars) or 20 (striped bars) min. Mice demonstrated a significant increase in time spent attending to the novel object in phase 3 as compared to the time spent attending to the equivalent object in phase 1, but a peak phase 3 response was demonstrated with a phase duration of 10 min. (n = 15-23 mice/bar; * = significantly different from phase 1 response, paired-samples t-tests, \( p < 0.05 \)) (b) **Effect of inter-trial interval.** The duration of the interval between each 10-min trial was varied from 1 (black bar), 10 (white bar) or 20 (grey bar) min. Mice tested with a 10- or 20- min ITI demonstrated significant increases in recognition index for the novel object in phase 3 as compared to matching phase 1 responses, with a peak response demonstrated in experiments utilizing a 10-min ITI. (n = 7-28 mice/bar; * = significantly different from phase 1 response, paired-samples t-tests, \( p < 0.05 \))
Mice expressing Tat protein demonstrated an overall impairment in novel object recognition

For this project, GT-tg mice were induced to express Tat with various doses of Dox (25, 50, or 100 mg/kg, i.p.) for 7 days or were induced for different durations (1, 3, 5, 7 days) with 100 mg/kg Dox. Mice were then tested in the 3-phase NOR task (Figure 2.1b). Separate groups of mice were tested at either 48 h or one week after the completion of induction. Also, a group of mice induced with the maximal dose of Dox for the maximum duration (100 mg/kg, i.p., 7 days) was also tested one month after the completion of induction. These induced mice were compared to Dox- treated C57Bl/6J mice (100 mg/kg, i.p., 7 days) and uninduced GT-tg mice (0.9% saline, i.p., 7 days).

For initial analysis an omnibus repeated measures ANOVA was employed, which included all the aforementioned groups and conditions to determine the main effects and interactions before subsequent, more detailed analyses. Results indicated a main effect of phase of task ($F_{2,706} = 11.78$, $p < 0.001$), Dox dose ($F_{2,353} = 3.71$, $p < 0.05$), and induction duration ($F_{3,353} = 4.16$, $p < 0.01$). Also noted were an interaction of phase X duration ($F_{46,706} = 5.78$, $p < 0.001$) and phase X dose ($F_{4,706} = 4.42$, $p < 0.01$). No main effect of time ($F_{2,353} = 0.86$, $p = 0.42$) or phase X time interaction ($F_{64,706} = 0.71$, $p = 0.60$) was revealed, suggesting performance across time remained consistent (i.e., did not recover). Further analysis of these main effects and interactions using one-way ANOVA indicated that there were no overall differences in performance in phase 1 ($F_{14,353} = 1.22$, $p = 0.26$) or phase 2 ($F_{14,353} = 1.23$, $p = 0.25$) performance between groups. There was however, an overall difference between groups in phase 3 performance ($F_{14,353} = 6.05$, $p < 0.001$).
GT-tg mice induced to express Tat demonstrated a duration-of-induction-dependent deficit in phase 3 novel object recognition

To evaluate the main effect of duration of Dox of administration, post hoc analyses were conducted on data from GT-tg mice administered the maximum dose of Dox (100 mg/kg, i.p.) for 1, 3, 5, and 7 days (Figure 2.7a). These groups were tested in NOR 48 h and one week after the completion of induction. These groups were compared to uninduced GT-tg mice (0.9% saline, i.p., 7 days) and C57Bl/6J mice pretreated with Dox (100 mg/kg, i.p., 7 days). Data were analyzed for two comparisons of interest: differences in phase 3 RIs between groups and/or differences within a group between phase 1 and phase 3 RIs. Recall that a significant increase in time spent on object B in phase 3 compared to phase 1 suggests intact learning and memory performance. The 7 day Tat-induced mice showed a difference in phase 3 RI when compared to C57Bl/6J mice and uninduced littermates (48 h, \( p < 0.01 \), and one week, \( p < 0.05 \), Tukey HSD). This pattern of impairment was also apparent in the 5 day Tat-induced mice. The 5 day Tat-induced mice demonstrated differences in phase 3 RIs compared to C57Bl/6J mice and uninduced littermates at 48 h (\( p < 0.001 \)) and one week (\( p < 0.05 \)). Accordingly the 5 day and 7 day Tat-induced mice did not show an increase in phase 3 NOR performance when compared to phase 1 at either 48 h or one week (\( p = 0.99 \)). The 3 day Tat-induced mice demonstrated a partial impairment of performance, as their phase 3 performance was not significantly different from the C57Bl/6J and uninduced mice when tested 48 h after the completion of induction (\( p = 0.24 \) and \( p = 0.39 \), respectively) or in the group tested one week after the completion of induction (\( p = 0.27 \) and \( p = 0.44 \), respectively). However, the 3 day Tat-induced mice did not show an increase in phase 3 NOR performance when compared to phase 1 at either 48 h or one week (\( p = 0.99 \)), suggesting NOR was to some extent impaired. There was no impairment in the 1 day Tat-induced mice. The 1 day Tat-induced mice did not perform differently in phase 3 than C57Bl/6J or uninduced mice in groups tested 48 h (\( p = 0.99 \)) or one week (\( p = 0.99 \)) after the
completion of induction and showed significant increases in phase 3 RI compared to phase 1 ($p = 0.05$, both time points tested).

**GT-tg mice induced to express Tat demonstrated a Dox dose-dependent deficit in phase 3 novel object recognition**

To evaluate the main effect of Dox dose-dependent changes in NOR, post hoc analyses were conducted on data from GT-tg mice administered 25, 50, or 100 mg/kg Dox dose of Dox for 7 days (Figure 2.7b). These groups were tested in NOR 48 h and one week after the completion of induction and were compared to uninduced GT-tg mice (0.9% saline, i.p., 7 days) and C57Bl/6J mice pretreated with Dox (100 mg/kg, i.p., 7 days). As describe in the previous section, the mice induced for 7 days with 100 mg/kg Dox were impaired in phase 3 NOR. Mice induced with 50 mg/kg Dox for 7 days also demonstrated impairment in phase 3 NOR. These mice showed no increase in phase 3 RIs compared to phase 1 in groups tested 48h ($p = 0.99$, Tukey HSD) and one week ($p = 0.99$) after the completion of induction. Interestingly, although phase 3 NOR was significantly different from C57Bl/6J and uninduced littermates in the group tested 48 h after the completion of induction ($p < 0.01$), there was a curious mild improvement in the group tested at one week after the completion of induction as they did not differ from controls (vs C57Bl/6J, $p = 0.14$, and vs uninduced, $p = 0.25$). Mice induced with 25 mg/kg Dox for 7 days showed an increase in phase 3 RIs compared to phase 1 when tested 48 h after the completion of induction ($p < 0.01$), but there was no increase from phase 1 to phase 3 in the group tested one week after induction ($p = 0.99$). However, phase 3 performance in the mice administered 25 mg/kg Dox for 7 days did not differ from C57Bl/6J or uninduced mice in the groups tested 48 h ($p = 0.99$) or one week after induction ($p = 0.68$ and $p = 0.85$, respectively).
a. % Recognition Index - Phase 3

- C57Bl/6J + Dox
- Uninduced
- 100 mg/kg Dox each day

1d, 3d, 5d, 7d

- * tested 48 h post induction
- ** tested 1wk post induction

b. % Recognition Index - Phase 3

- C57Bl/6J + Dox
- Uninduced
- Dox injected for 7 days (mg/kg): 25, 50, 100

- * tested 48 h post induction
- ** tested 1wk post induction

†
Figure 2.7. GT-tg mice induced to express Tat demonstrated a Dox dose- and duration of induction-dependent deficit in phase 3 novel object recognition. GT-tg mice were administered the maximum dose of Dox (100 mg/kg, i.p.) for 1, 3, 5, and 7 days (a, gray bars) or 25, 50, or 100 mg/kg Dox for 7 days (b, gray bars). These groups were tested 48 h (solid bars) or one week (thatched bars) after the completion of induction and were compared to uninduced GT-tg mice (0.9% saline, i.p., 7 days, black bars) and Dox-treated C57Bl/6J mice (100 mg/kg, i.p., 7 days, white bars). (a) The 5 and 7 day Tat-induced mice showed differences in phase 3 RI when compared to C57Bl/6J mice and uninduced littermates. Accordingly, the 5 and 7 day Tat-induced mice did not show an increase in phase 3 RIs compared to phase 1 when tested at either 48 h or one week. Phase 3 performance was not significantly different between the 3 day Tat-induced mice and control mice. However, the 3 day Tat-induced mice did not show an increase in phase 3 NOR performance when compared to phase 1. There was no impairment in the 1 day Tat-induced mice. (b) As described above, the mice induced for 7 days with 100 mg/kg Dox were impaired in phase 3 NOR. Furthermore, mice induced with 50 mg/kg Dox for 7 days demonstrated no increase in phase 3 RIs compared to phase 1 in groups tested 48 h and one week after the completion of induction. Mice induced with 25 mg/kg Dox for 7 days showed an increase in phase 3 RIs compared to phase 1 when tested 48 h after the completion of induction, but there was no increase from phase 1 to phase 3 in the group tested one week after induction. (n = 17-53 mice/bar; * = different from matching phase 1 response, † = different from phase 3 response of C57Bl/6J and uninduced mice, p ≤ 0.05, Tukey HSD)
Mice induced to express Tat protein with the maximum Dox-induction regimen demonstrated lasting suppression of phase 3 novel object recognition

We then further examined the effects of maximal Tat-induction (Figure 2.8). Mice that were induced to express Tat with 100 mg/kg Dox for 7 days and either tested within 48 h, one week, or one month after the completion of induction. These groups were compared to uninduced GT-tg mice (0.9% saline, i.p., 7 days) and C57Bl/6J mice pretreated with Dox (100 mg/kg, i.p., 7 days). Regardless of testing within 48 h, one week, or one month after induction, Tat-induced mice spent less time on the novel object in phase 3 than C57Bl/6J mice ($p < 0.05$, Tukey HSD) or their uninduced littermates ($p < 0.05$). There was no difference in phase 3 NOR performance between C57Bl/6J and uninduced mice ($p = 0.99$). Furthermore, while both the uninduced and C57Bl/6J mice spent significantly more time on the novel object in phase 3 compared to phase 1 (both $p < 0.01$, Tukey HSD), Tat-induced mice when tested either within 48 h, one week, or one month after induction did not show increased RIs in phase 3 compared to matching group performance in phase 1 ($p = 0.99$), suggesting a deficit in novel object recognition.
GT-tg mice induced to express Tat protein with the maximum Dox-induction regimen demonstrated lasting suppression of performance in phase 3 novel object recognition. Mice that were induced to express Tat with 100 mg/kg Dox (i.p.) for 7 days and either tested within 48 h (light gray thatched bars), one week (dark gray thatched bars), or one month (black bars) after the completion of induction. These groups were compared to uninduced GT-tg mice (0.9% saline, i.p., 7 days, white thatched bars) and C57Bl/6J mice pretreated with Dox (100 mg/kg, i.p., 7 days, white solid bars). NOR of the C57Bl/6J and uninduced mice in phase 3 was not different and both groups spent significantly more time on the novel object in phase 3 compared to phase 1. Tat-induced mice groups tested at the various time points spent less time on the novel object in phase 3 than controls. Also, all three groups of Tat-induced mice showed no increase in phase 3 RIs from phase 1, suggesting a lasting NOR deficit. (n = 17-53 mice/bar; * = significant increase from phase 1 response, † = different from phase 3 response of C57Bl/6J and uninduced mice, p < 0.05, Tukey HSD)
DISCUSSION

In the present study we sought to determine if brain-specific expression of Tat protein was linked to different types of learning and memory impairment. As the data concerning behavioral studies examining the contribution of Tat to learning and memory deficits are limited, we used the GT-tg mouse model to test the hypothesis that the activity of Tat in brain is sufficient to impair learning and memory processes. Supporting our hypothesis, we demonstrated that Tat-induced mice were impaired in spatial acquisition, probe trial memory performance, and reversal learning in the Barnes maze. Furthermore, we found that Tat-induced mice show long-lasting novel object recognition learning and memory deficits.

Mice were tested in the Barnes maze, a hippocampal-dependent, spatial learning and memory task (Barnes, 1979; Barnes et al., 1990; Bach et al., 1995). A previous study found that a single i.c.v. administration of Tat protein was sufficient to impair spatial learning and memory in an eight-arm radial maze (Li et al., 2004). In this study rats injected with Tat were tested for five days (one trial a day) and entry into an already visited arm (i.e., re-entry) of the eight possible arms was recorded. The researchers found that the Tat-injected rats made more errors than controls on days 3 and 4 of testing, but on day 5 this difference was smaller. The present study found similar types of deficits, but extended the results by using a task that allowed for examination of more variables. We found that GT-tg mice induced to express Tat by administering Dox (100 mg/kg, i.p.) for 5 or 7 days demonstrated longer latencies to find an escape hole compared to their uninduced GT-tg littermates (0.9% saline, i.p., 7 days). Furthermore, the 7 day Tat-induced mice committed more total, reference, and working memory errors than uninduced mice, with the 5 day Tat-induced mice showing a less robust increase in the number of errors committed. The working memory errors recorded here corroborate the results of the Li et al. (2004) study, as the type of errors the Tat-injected mice made in that study
were based on a similar behavior. Moreover, the 5 and 7 day Tat-induced mice used less efficient search strategies when exploring the maze during acquisition training than the strategies used by the uninduced mice. The Tat-induced mice favored the inefficient random search strategy over more efficient direct and serial search strategies. This is in contrast to the uninduced mice, who favored the efficient direct search strategy. Use of the direct search strategy suggests knowledge of the escape location, whereas the random search strategy (or lack of a strategy) is less efficient and suggests that the animals may have little knowledge of the escape hole location (Barnes, 1979).

Studies have shown that tetracycline and its analogs can impair memory when administered chronically and in high doses (Dunning and During, 2003). These results suggest that Dox treatment itself may have directly impaired memory performance in the present study. However, this alternative is unlikely, given that the tet-on promoter strategy used in the GT-tg mouse model minimizes the length of exposure to Dox compared to the tet-off strategy where animals are maintained on Dox for many months. Moreover, to further control for this effect, C57Bl/6J mice were treated with the highest dose of Dox used (100 mg/kg, i.p., 7 days) and performance in the Barnes maze was compared to saline-treated C57Bl/6J mice (0.9% saline, i.p., 7 days). Importantly, the saline- and Dox-treated C57Bl/6J mice did not perform differently from each other in any part of this study, confirming that Dox administration itself did not have an effect on spatial learning and memory under the conditions used here.

It should be noted the Tat-induced GT-tg mice were not compared to C57Bl/6J mice in the Barnes maze. It was demonstrated that the C57Bl/6J mice showed significant learning differences during acquisition training when compared to the uninduced GT-tg mice, therefore precluding direct comparison in Barnes maze experiments. The C57Bl/6J mice used as controls here are direct ancestors of mice born at a breeding colony from Jackson Laboratories, whereas the GT-tg mice were created and back-crossed in the laboratory of Dr. Johnny J He. Although
the GT-tg mice have been back-crossed seven generations onto the C57Bl/6 line, it is possible that there is some divergence in phenotype from the C57Bl/6J strain, resulting in subtle differences in spatial acquisition due to the ancestry of the mice. Importantly, although, this effect highlights the importance of making direct comparisons between the uninduced GT-tg mouse and the parent strain of mice throughout the remainder of the studies, it does not negate the findings that the Tat-induced mice demonstrated acquisition deficits compared to uninduced littermates.

In addition to deficits in acquisition in the Barnes maze, the Tat-induced mice also demonstrated deficits in a probe trial memory performance. At the end of day 4, a probe trial was performed during which the escape location was removed and mice were given 90 s to explore the maze. The variables recorded during this trial were success in finding the former escape location and time spent in the target quadrant (i.e., quadrant where the escape was located). The amount of time spent in the target quadrant by both the 5 day and 7 day Tat-induced mice was not greater than that predicted by chance (chance performance = 90 s / 4 quadrants = 22.5 s in each quadrant), whereas their uninduced littermates spent more time in the target quadrant than chance would predict. Furthermore, fewer of the 5 day Tat-induced mice were successful in finding the former escape hole location compared to uninduced mice. Also, fewer of the 7 day Tat-induced mice found the escape hole compared to controls, but this was only a statistical trend. The selective search evidenced by finding the escape hole and spatial bias for the target quadrant constitutes evidence for spatial memory, which is clearly impaired in the Tat-induced mice.

Tat-induced mice were also impaired in reversal learning in the Barnes maze. On the fifth day of testing, mice were given four trials to find and learn the location of a new escape hole. The perseveration often observed in patients suffering from progressive neurodegenerative diseases suggests a lack of cognitive flexibility (Traykov et al., 2002) which
may be modeled with deficits in reversal learning in animal models. The 5 day and 7 day Tat-induced mice showed deficits in reversal learning, as demonstrated by the greater latencies to find the new escape hole location compared to their uninduced littermates. However, by trial 4, Tat-induced mice did not perform differently than uninduced mice. This suggests that although the Tat-induced mice re-learned the new location less efficiently, they are not completely impaired in the ability to re-learn and display only moderate cognitive inflexibility.

It is interesting, and somewhat counterintuitive, that the 5 day Tat-induced mice in some cases performed worse than the 7 day Tat-induced mice. It should be noted that this is not the case for results concerning the number of errors committed, where the 7 day Tat-induced mice performed more poorly than the 5 day Tat-induced mice. However, it is the case that 5 day Tat-induced mice performed more poorly in the probe trial. Moreover, although never reaching significance, there are differences apparent in the acquisition and reversal learning latencies, suggesting the 5 day Tat-induced mice have slightly increased latencies over the 7 day Tat-induced mice. This could potentially be attributable to different levels of motivation anxiety in the Barnes maze (Patil et al., 2009). The maze is based on the natural tendency of mice to explore and escape through holes (Bach et al., 1995; Dawood et al., 2004), with the bright light and static radio noise providing additional external motivations to escape the surface of the maze (Sarkisyan and Hedlund, 2009). It could be that the 7 day Tat-induced mice, although displaying a similar impairment in learning and memory as the 5 day Tat-induced mice, were more motivated to escape the maze, possibly due to increase anxiety levels. The anxiogenic properties of Tat protein are evaluated in more detail in Chapter 3 (Study 2), and will be discussed further in the context of how it could affect performance in the Barnes maze in Chapter 6. However, it should be noted that the NOR task involves no additional outside motivation, minimizing external and physical stressors (Ennaceur and Delacour, 1988), and therefore would not be significantly affected by anxiety like the Barnes maze.
To extend the previous results, we used the NOR assay to determine if there are different types of learning and memory deficits attributable to Tat that may be mediated by damage to different brain regions. NOR is a simple test of learning and memory that is dependent on the ability of the animals to differentiate between a novel object and those objects that have previously been encountered and is not based on spatial ability (Genoux et al., 2002; Save et al., 1992; Carey et al., 2009). It should first be noted that there was no difference in performance between the C57Bl/6J mice and uninduced mice in this task. This allowed for statistical comparison of the C57Bl/6J mice to the GT-tg bigenic mouse strain, and suggests that possible performance differences of mouse strain may be limited to the Barnes maze. NOR results indicate that mice induced to express Tat with Dox (100mg/kg, i.p.) for 7 days show deficits in learning and memory performance versus Dox-treated C57Bl/6J mice (100 mg/kg, i.p., 7 days) and uninduced GT-tg mice (0.9% saline, i.p., 7 days) as measured in the test phase of the novel object recognition assay. These deficits were not transient, as performance was significantly impaired up to one month after Tat-induction. Furthermore, NOR impairment was dependent on the dose and duration of Dox exposure. Interestingly, a very low dose of Dox (25 mg/kg) did not result in significant impairment of test phase novel object recognition in immediate testing (i.e., within 48 h of the completion of induction), but did result in impairment in a separate group of mice so treated and tested one week after induction. This suggests that there could be progressive deficits attributable to the effects of Tat over time. It has been demonstrated that viral proteins like Tat may induce progressive damage without needing to be continuously present (Nath et al., 1999) and could possibly account for this finding. We also found that mice administered 100 mg/kg Dox (i.e., the maximum dose used in this study) for only 5 days demonstrated significant impairment in NOR performance for up to one week, with a partial deficit seen in the mice treated for only 3 days to induce Tat expression. However, there was no significant impairment in mice treated with Dox for only 1 day. Together, these data
suggest that the deficits in learning and memory were related to the amount of Tat produced and the duration of exposure to Tat.

In summary, here we have shown four types of learning and memory impairment correlated with brain-specific Tat-protein expression: (1) spatial acquisition learning, (2) spatial memory in a probe trial, (3) spatial reversal learning, and (4) enduring novel object recognition impairment. This study is one of very few to show that Tat expression may be sufficient to mediate deficits in spatial learning and memory. It is also the first study to show that Tat expression is correlated with decrements in non-spatial ability, the less complex form of learning and memory of novel object recognition. This study verifies that, like gp120, Tat protein is a possible mediator of the cognitive deficits seen in HIV-infected individuals and may be responsible for some of the behavioral pathology seen in HAD. As Tat is known to have numerous toxic and dysfunctional effects on the cellular environment, it is not surprising that its activity may result in behavioral deficits. However, more research is needed to study the specific brain regions affected by the activity of Tat and other NeuroAIDS-related behaviors that may be modulated by HIV-Tat. These topics will be addressed in the subsequent studies in this thesis.
REFERENCES


CHAPTER 3

STUDY 2: EXPRESSION OF HIV-TAT PROTEIN IS ASSOCIATED WITH INCREASES IN ANXIETY AND BEHAVIORAL DISINHIBITION THAT ARE DEPENDENT ON THE EXTENT OF TAT PROTEIN EXPOSURE

HIV-infected patients frequently demonstrate neuropsychiatric (NP) dysfunction, with elevated rates of depression and anxiety over both the general population and populations of patients suffering from terminal diseases such as cancer (Cielsa and Roberts, 2001; Price et al., 2005). Recent evidence from clinical studies suggests that the prevalence of clinical depression diagnosis in the HIV-infected population may range from 33% (Evans et al., 2005) to as much as 60% (Scharko, 2006). Moreover, anxiety disorders are diagnosed in approximately 20-25% of those infected with HIV (American Psychiatric Association, 2000; Pence et al., 2006; Scharko, 2006), with a recent study of South African patients living with HIV reporting that more than a fifth demonstrated elevated symptoms of anxiety (Kagee and Martin, 2010). Furthermore, it is also notable that NP disturbances in HAD are common and present as agitation, behavioral disinhibition, aberrant motor behaviors, and irritability, which progresses with the severity of the disease to significantly complicate patient treatment (Srikanth et al., 2005).

Neuropsychiatric dysfunction in NeuroAIDS could be psychogenic in origin, stemming from the psychosocial burdens of being infected with HIV. However, evidence is emerging to suggest a number of physiological alterations may contribute, at least in part, to the development of NP disorders in NeuroAIDS. Previous clinical imaging studies have demonstrated abnormalities in dorsal frontal cortex (Ernst et al., 2002; Chang et al., 2003), amygdala, hippocampus (Castelo et al., 2006), and NAc (Paul et al., 2005) of HIV-infected patients. These areas of the brain have been implicated in the development of depression and
anxiety and have shown to be subject to Tat toxicity (Aksenova et al., 2005; Bansal et al., 2000). Basal ganglia calcification is also common in pediatric AIDS (Brew, 2001), which is relevant as Oliveira et al. (2004) correlated basal ganglia calcification with anxiety and other personality and behavioral changes. Furthermore, as discussed in Chapter 1, the DA system is affected in HIV-infected patients. HIV may down-regulate striatal D$_2$ receptor expression as it progressively damages the striatum (Gelman et al., 2006). This is significant, as it has been demonstrated that low D$_2$ receptor binding is associated with the potentiation of social anxiety symptoms (Tiihonen et al., 1997; Scheneier et al., 2000), suggesting that DA system dysfunction may physiologically mediate anxiety in HIV patients. Notably, it has been suggested that there may also be a link between high levels of anxiety and OS (Bouayed et al., 2009). For example, researchers found that mice with vitamin E deficiency had increases in brain OS markers that were correlated with anxiogenic behavior (Desrumaux et al., 2005). Although, the specific connection between OS and anxiety has not been extensively evaluated, OS alters gene expression and protein conformation, affects cell signaling, and alters neurotransmission and neuronal functioning (LeBel and Bondy, 1991; Valko et al., 2007). The correlation between OS and anxiety is important given the significant evidence implicating Tat in the initiation and/or intensification of OS (Kruman et al., 1998). Finally, in human microglia, application of Tat protein increases the production of the cytokines IL-1$\beta$, IL-6 and TNF-$\alpha$ (Nath et al., 1999; Bruce-Keller et al., 2001) and increases IL-6 levels in human astrocytes (Nath et al., 1999). Proinflammatory cytokines are elevated in anxiety disorders, with a recent study reporting that 87% of sampled anxiety patients displayed six or more detectable levels of proinflammatory cytokines and chemokines in their blood, compared to the 25% incidence in controls (Hoge et al., 2009). These elevated proinflammatory agents included IL-1$\beta$, IL-6 and TNF-$\alpha$, all known to be produced in response to Tat (Nath et al., 1999; Bruce-Keller et al., 2001). Although an explicit cause and effect relationship has not been established between the discussed biological
changes and increases in anxiety, these studies together suggest possible biological mediators that are known elements in the disease process of NeuroAIDS. Moreover, it appears plausible that Tat protein activity may mediate or contribute to the increased risk of developing anxiety in HIV-infection.

We tested the hypothesis that exposure to Tat protein increases behavioral signs of anxiety. Dox-treated C57Bl/6J, uninduced GT-tg, and Tat-induced GT-tg mice were tested in the open field (OF), light-dark (LD) box, and elevated plus maze (EPM) tests. This battery of tests was used to behaviorally characterize the effect of varying amounts of Tat expressed for different durations on anxiety-like behaviors. Interestingly, mice displayed Tat-dependent anxiety-like behaviors in OF and LD box testing, but behavioral disinhibition in EPM testing. These data suggest a more complex behavioral profile than simple anxiety. These studies are the first to demonstrate the complex relationship between Tat and behavioral dysfunction that could potentially mimic the progression of the NeuroAIDS disease process.
MATERIALS AND METHODS

Animals and housing

Adult male GT-tg bigenic (Kim et al., 2003) and C57Bl/6J wildtype (Jackson Labs, Bar Harbor, ME) mice were used in all experiments as described in Chapter 2.

Chemicals and induction of brain-targeted Tat with Doxycycline treatment

Dox, obtained from Sigma-Aldrich (St. Louis, MO), and was dissolved in 0.9% saline prior to injection. The induction of Tat protein expression was performed as described in Chapter 2.

General behavioral methods

Mice were tested between 24 and 48 h after the completion of induction, unless noted otherwise. All behavior was monitored and digitally encoded for automated analysis by Noldus Ethovision Pro 3 (Noldus Information Technology, Wageningen, the Netherlands). Testing equipment was cleaned with 70% ethanol between trials.

Open field (OF)

The OF test is used to study behavioral responses in mice that are placed in a novel and bright arena (Simon et al., 1994; Prut and Belzung, 2003). Mice tend to avoid brightly illuminated areas, and in this assay tend to avoid the center of the arena and remain near the walls. This decreases gradually during the first minutes of exploration in normal mice, but remains elevated
in “anxious” animals (Choleris et al., 2001; Ramos et al., 2008). In this study, the mice were placed in the center of the apparatus, which was a square white Plexiglas box (46 x 46 x 30 cm) (Figure 3.1a), and allowed to explore the whole field for 15 min. Time spent in the center (i.e., open area; 25 X 25 cm) was measured.

**Light-dark box (LD)**

The LD test is also based on the innate aversion of rodents to brightly illuminated areas, as well as the spontaneous exploratory behavior of rodents in response to the mild stressors of novel environment and light (Crawley and Goodwin et al., 1980; Onavi and Martin, 1989; Belzung and Griebel, 2001). The test apparatus used here consisted of a small dark compartment (39 X 20 X 13 cm) and a large illuminated aversive compartment (40 X 39 X 13 cm) (Figure 3.1b). The two compartments were separated by a partition with an opening (6 X 5 cm). The animals were placed in the center of the lit compartment and left to explore the space for a 10-min period. The time spent in the dark compartment was evaluated.

**Elevated plus maze (EPM)**

The EPM is a widely used and validated anxiety paradigm that is based on the unconditioned response (natural aversion) of rodents to height and open spaces (i.e., a potentially dangerous environment) (Pellow et al., 1985; Lister, 1987). High anxiety states are directly related to the degree to which the rodent avoids the open arms of the maze. Mice were tested in an apparatus built by Med Associates, Inc. (St. Albans, VT) and following methods similar to the one described in Lister (1987). The mice were placed in the center (6 X 6 cm) of an elevated (100 cm) 4-arm maze with 2 arms open and 2 enclosed arms (35 X 6 cm each) (Figure 3.1c). The
closed arms had walls on all sides that are 20 cm high. The open arms had a 2 mm lips on the edges to prevent mice from falling off, which could confound the results. Each mouse was placed in the central square, facing an open arm, and allowed to explore for a single 5-min trial, which is a trial length that has been established to produce a reliable response to the maze environment without confounds of hyperactivity in a novel environment or prolonged familiarity (Lister, 1987). The time each mouse spent in the open arms was recorded. All testing was performed during the light phase of the light–dark cycle as established previously (Smith et al., 1998; Vetter et al., 2002).

Data Analysis of Behavioral Experiments

**General:** All data were analyzed using MedCalc software (Mariakerke, Belgium). All data are presented as mean ± S.E.M. Significance was set at $p \leq 0.05$.

**Open field:** Time spent in the center was analyzed using the Kruskal-Wallis H-test, with post hoc pairwise comparisons performed according to Conover (1999). This non-parametric test was chosen as the data did not satisfy all the assumptions underlying a parametric ANOVA. These data were found to violate the homogeneity of variance assumption ($p < 0.01$, Levene's statistic) and did not follow a Gaussian distribution ($p < 0.001$, D'Agostino-Pearson test of normality, $p < 0.001$, skewness).

**Light-dark box:** Time in the dark box was analyzed with a one-way ANOVA (data did not violate the variance or normality assumption). Student-Newman-Keuls post hoc tests were chosen because only 3 comparisons were being made.

**Elevated plus maze:** Time spent in the open arms was analyzed using the Kruskal-Wallis H-test, with post hoc pairwise comparisons performed according to Conover (1999). This non-parametric test was chosen as the data were found to violate the homogeneity of variance.
assumption (Levene’s statistic, \( p < 0.01 \)), and therefore did not satisfy all the assumption underlying a parametric ANOVA. A Pearson’s R statistic was used to determine if a significant correlation existed between the OF and EPM data.
Figure 3.1. **Schematic of the open field arena, light-dark box, and elevated plus maze.**

(a) The OF apparatus was a square white Plexiglas box. Time spent in the center (represented by the gray area) was measured during a 15-min trial. Spending less time in the center and more time in the periphery is considered an anxiety-like behavior. (b) The LD box used here consisted of a small dark compartment (black) and a large illuminated compartment (white). The two compartments were separated by a partition with an opening (gray, 6 X 5 cm) through which the animal could pass from one compartment to the other. The animals were placed in the center of the lit compartment and allowed to explore for 10-min testing period. Increased time spent in the dark compartment compared to controls indicates anxiety-like behavior. (c) For testing in the EPM mice were placed in the center (6 X 6 cm) of an elevated (100 cm) 4-arm maze with 2 arms open and 2 enclosed arms (35 X 6 cm each) for 5 min. The closed arms have black Plexiglas walls on all sides that are 20 cm high, the open arms have a 2 mm lips on the edges to prevent mice from falling off. Less time spent in the open arms compared to controls is indicative of anxiety behavior.

(EPM image from: http://www.jefferson.edu/neurosurgery/research/images/EPM.jpg)
RESULTS

Tat protein expression produced dose- and duration of induction-dependent increases in anxiety-like behavior in the open field

GT-tg mice were induced to express Tat with various doses of Dox (25, 50, or 100 mg/kg, i.p.) for 7 days and were then tested in the OF test (Figure 3.2a). These induced mice were compared to saline-treated C57Bl/6J mice (0.9% saline, i.p., 7 days), Dox-treated C57Bl/6J mice (100 mg/kg, i.p., 7 days), and uninduced GT-tg mice (0.9% saline, i.p., 7 days). Results indicated an overall difference in time spent in the center of the open field (H = 22.57, p < 0.001, Kruskal-Wallis), indicative of differences in anxiety-like behavior. Post hoc analyses indicated that there were no differences between saline-treated C57Bl/6J, Dox-treated C57Bl/6J, and uninduced GT-tg mice (78 ± 8.7 s, 84 ± 5.8 s, and 79 ± 10.7 s, respectively, p > 0.05, Kruskal-Wallis post hoc test). GT-tg mice induced with 25 mg/kg Dox for 7 days did not spend any more or less time in the center of the open field (68 ± 7.0 s) than saline-treated C57Bl/6J, Dox-treated C57Bl/6J, and uninduced GT-tg mice (p > 0.05). However GT-tg mice induced to express Tat with 50 and 100 mg/kg Dox (i.e., under conditions expressing more Tat protein) spent significantly less time in the center of the OF (45 ± 8.4 s and 44 ± 5.9 s, respectively) compared to saline-treated C57Bl/6J, Dox-treated C57Bl/6J, or uninduced GT-tg mice (p ≤ 0.05), suggesting an increase in anxiety-like behavior.

Next, the effect of Tat expression duration on OF anxiety behavior was examined (Figure 3.2b). GT-tg mice were induced to express Tat for different durations (0, 1, 3, 5, or 7 days) with 100 mg/kg Dox (i.p.) and tested within 48 h of the completion of induction in the OF. Also, a separate set of mice were induced for 7 days with 100 mg/kg and were tested one week after the completion of induction. Results indicated an effect of induction duration on anxiety behavior (H = 14.53, p < 0.05, Kruskal-Wallis). GT-tg mice induced with Dox (100 mg/kg, i.p.)
for 1 day did not differ in time spent in the center (59 ± 7.0 s) when compared to uninduced GT-tg mice (i.e., 0 days Dox induction) \( (p > 0.05, \text{Kruskal-Wallis post hoc test}) \). However, mice that were induced for 3 or 5 days spent less time in the center of the OF (5.1 s and 40 ± 8.9 s) than uninduced GT-tg littermates \( (p \leq 0.05) \). As discussed above, the 7 day Tat-induced mice (i.e., maximally induced mice) demonstrated increased anxiety behavior when tested within 48 h of the completion of induction and compared to uninduced GT-tg littermates. However, when a separate group of 7 day Tat-induced mice was tested one week after induction, the time animals spent in the center returned to control values \( (73 ± 8.6 \, \text{s}, p > 0.05 \, \text{compared to uninduced and} \, p \leq 0.05 \, \text{compared to matched treatment group tested at 48 h}) \). Overall, these data suggest a Dox dose- and duration of induction-dependent increase in anxiety-like behavior in the OF, dependent on the time of testing.

**Tat protein expression produced an increase in anxiety-like behavior in the light-dark box dependent on the duration of Tat induction**

Anxiety-like behavior was also assessed in the LD box in a small group of animals to confirm data collected in the OF test. GT-tg mice were induced to express Tat for different durations (3, 5, 7 days) with 100 mg/kg Dox and the time spent in the dark compartment was compared to that of uninduced GT-tg mice (0.9% saline, i.p., 7 days). It should be noted that Tat-induced GT-tg mice were not compared to the C57Bl/6J mouse strain in this test, as an initial analysis of saline-treated C57Bl6/J, Dox-treated C57Bl/6J, and uninduced GT-tg mice indicated a significant difference in performance in the LD box \( (F_{2,31} = 8.22, \, p < 0.01, \text{one-way ANOVA}) \). Although saline-treated C57Bl6/J and Dox-treated C57Bl/6J mice did not differ from each other in the time spent in the dark compartment \( (325 ± 31 \, \text{s and} \, 354 ± 29 \, \text{s, respectively}) \), they spent considerably more time in the dark box than uninduced GT-tg mice \( (228 ± 16 \, \text{s,} \, p \leq \)
a. Time in Center (s) for different groups:

- C57-Saline
- C57-Dox
- Uninduced GT-tg
- GT-tg mice injected with Dox for 7 days (mg/kg)

b. Time in Center (s) for different days after induction:

- Uninduced
- Number of days administered 100 mg/kg Dox

- tested 48h post induction
- tested 1wk post induction

Significance indicated by * and †.
Figure 3.2. GT-tg mice induced to express Tat demonstrated Dox dose- and duration of induction-dependent anxiety-like behavior in the open field test. (a) GT-tg mice were administered the 25, 50, or 100 mg/kg Dox for 7 days (gray bars) and the amount of time spent in the center of the arena during a 10-min trial was compared to saline-treated C57Bl/6J (white bar), Dox-treated C57Bl/6J white hatched bar), and uninduced GT-tg mice (black bar). Notably, the saline-treated C57Bl/6J, Dox-treated C57Bl/6J mice, and uninduced GT-tg mice did not demonstrate any significant differences in time spent in the center of the arena. GT-tg mice induced with 25 mg/kg Dox for 7 days did not spend any more or less time in the center of the open field than any of the three groups of control mice. In contrast, the mice induced to express Tat with 50 or 100 mg/kg Dox for 7 days did spend significantly less time in the center compared to the three control groups. (n = 20-24; * = different from saline-treated C57Bl/6J, Dox-treated C57Bl/6J, and uninduced GT-tg mice, $p \leq 0.05$, Kruskal-Wallis H-test).

(b) The effect of the duration of Tat expression on OF anxiety behavior was also examined. GT-tg mice were induced with the maximum dose of Dox (100 mg/kg, i.p.) for 1, 3, 5, or 7 days (gray bars) and compared to uninduced GT-tg littermates (black bar). The 1 day Tat-induced mice did not display differences in anxiety behavior compared to control mice. However, the 3 and 5 Tat-induced mice spent significantly less time in the center than uninduced GT-tg mice. The 7 day Tat-induced mice spent less time in the center compared to uninduced littermates when tested at 48 h (rightmost solid gray bar), but not one week after induction (rightmost hatched gray bar). (n = 14-24; * = different from uninduced GT-tg mice, † = different from 48 h matched-treatment group, $p \leq 0.05$, Kruskal-Wallis H-test).
suggesting differences in baseline responses in this assay between the two strains of mice. When the behavior of uninduced and Tat-induced GT-tg mice only was analyzed, results indicated a significant difference overall in time spent in the dark compartment (\( F_{3,43} = 3.60, p < 0.05 \), one-way ANOVA, Figure 3.3). Subsequent analyses indicated that the 3 and 5 day Tat-induced mice demonstrated elevated anxiety-like behavior, spending significantly more time in the dark compartment (308 ± 35 s and 297 ± 14 s, respectively), compared to their uninduced littermates (228 ± 16 s, \( p \leq 0.05 \), Student-Newman-Keuls). However, it should be noted that the mice induced for 7 days to express Tat did not spend more time in the dark compartment as compared to uninduced littermates (248 ± 18 s, \( p \geq 0.05 \)).

**Tat protein expression produced a behavioral disinhibition response in elevated plus maze testing that was dependent on the dose and duration of Dox induction**

GT-tg mice were induced to express Tat with various doses of Dox (25, 50, or 100 mg/kg, i.p.) for 7 days (Figure 3.4a) and were then tested in the EPM. These induced mice were compared to Dox-treated C57Bl/6J mice (100 mg/kg, i.p., 7 days) and uninduced GT-tg mice (0.9% saline, i.p., 7 days). Results indicated an overall difference in time spent in the open arms of the EPM (\( H = 13.79, p < 0.01 \), Kruskal-Wallis). Post hoc analysis indicated that Dox-treated C57Bl/6J mice and uninduced GT-tg mice did not differ in the time spent in the open arms of the EPM (99 ± 7.5 s and 110 ± 11.0 s, respectively, \( p > 0.05 \), Kruskal-Wallis post hoc). GT-tg mice induced with 25 mg/kg Dox for 7 days spent more time in the open arms (139 ± 11.2 s) compared to Dox-treated C57Bl/6J mice (\( p \leq 0.05 \)), but not uninduced GT-tg mice (\( p > 0.05 \)). In contrast, the mice induced to express Tat with 50 mg/kg Dox for 7 days spent more time in the open arms (144 ± 10.4 s) compared to both the Dox-treated C57Bl/6J mice and uninduced GT-tg mice (\( p \leq 0.05 \)). Interestingly, mice induced with 100 mg/kg Dox for 7 days spent significantly
Figure 3.3.  

Tat protein expression produced anxiety-like behavior in the light-dark box that was dependent on the duration of Tat induction. GT-tg mice were induced to express Tat for different durations (3, 5, 7 days) with 100 mg/kg Dox and the time spent in the dark compartment was compared to uninduced GT-tg mice (0.9% saline, i.p., 7 days). The 3 and 5 day Tat-induced mice (gray bars) demonstrated significantly elevated anxiety, spending significantly more time in the dark compartment compared to their uninduced littermates (white bar). In contrast, the 7 day Tat-induced mice (black bar) did not spend more time in the dark compartment compared to uninduced littermates. (n = 9-14; * = different from uninduced GT-tg mice, p ≤ 0.05, one-way ANOVA, followed by Student-Newman-Keuls)
more time in the open arms (124 ± 6.5 s) compared to Dox-treated C57Bl/6J mice (p ≤ 0.05),
but not their uninduced GT-tg littermates (p > 0.05).

Next, the effect of the duration of Tat expression on EPM behavior was examined. GT-tg mice were induced to express Tat for different durations (0, 1, 3, 5, or 7 days) with 100 mg/kg Dox (i.p.) (Figure 3.4b). Results indicated an effect of induction duration on behavior (H = 9.67, p ≤ 0.05, Kruskal-Wallis). GT-tg mice induced with Dox (100 mg/kg, i.p.) for 1 day spent significantly more time in the open arms (143 ± 10.8 s) compared to uninduced GT-tg mice (p ≤ 0.05). The 3 day and 5 day Tat-induced mice also spent more time in the open arms (144 ± 16.6 s and 152 ± 8.5 s, respectively) than uninduced GT-tg littermates (p ≤ 0.05). As noted, the maximum Dox induction regimen (100 mg/kg, 7 days, i.p.) did not produced a significant increase in time spent in the open arms compared uninduced GT-tg mice. Overall, these data suggest a potential Dox dose- and duration of induction-dependent decrease in anxiety-like behavior in the EPM. However, these results also indicate an increased willingness of the Tat-induced animal to explore an otherwise aversive environment, an example of behavioral disinhibition. Notably, anxiety-like behavior in the open field assay was significantly negatively correlated with the demonstration of behavioral disinhibition behavior in the EPM (R = -0.79, p < 0.05, Pearson’s correlation), with increasing anxiety in OF being associated with increasing behavioral disinhibition in EPM.
Figure 3.4. GT-tg mice induced to express Tat demonstrated a Dox dose- and duration of induction-dependent behavioral disinhibition in the elevated plus maze. (a) GT-tg mice were administered the 25, 50, or 100 mg/kg Dox for 7 days and the amount of time spent in the open arms of the EPM during a 5-min trial was compared to Dox-treated C57Bl/6J (100 mg/kg, i.p., 7 days, white bar), and uninduced GT-tg mice (0.9% saline, i.p., 7 days, white hatched bar). Notably, the Dox-treated C57Bl/6J mice and uninduced GT-tg mice did not demonstrate any significant differences in time spent in open arms of the EPM. GT-tg mice induced with 25 mg/kg (light gray) or 100 mg/kg (black) Dox for 7 days spent more time in the open arms than Dox-treated C57Bl/6J mice, but not uninduced littermates. However, the GT-tg mice induced to express Tat with 50 mg/kg for 7 days spent significantly more time in the open arms compared to both control groups. (b) The effect of the duration of Tat expression on EPM behavior was also examined. GT-tg mice were induced with the maximum dose of Dox (100 mg/kg, i.p.) for 1, 3, 5, (gray bars) or 7 days (black bar) and compared to uninduced GT-tg littermates (white hatched bar). The 1, 3, and 5 day Tat-induced mice spent significant more time in the open arms compared to uninduced GT-tg littermates. However, the 7 day Tat-induced mice did not. (n = 18-24; † = different from Dox-treated C57Bl/6J, * = different from uninduced GT-tg mice, p ≤ 0.05, Kruskal-Wallis H-test)
DISCUSSION

As behavioral studies examining the contribution of Tat to NP dysfunction are almost non-existent, we used the GT-tg mouse model to test the hypothesis that Tat protein expression in brain results in increased anxiety-like behavior. Supporting our hypothesis, we demonstrated that Tat-induced mice demonstrated Dox-induction dose- and duration-dependent increases in anxiety-like behavior in the OF, and Dox duration-dependent increases in anxiety-like behavior in the LD box. However, we found that the maximally Tat-induced mice (Dox 100 mg/kg for 7 days) did not display profound increases in anxiety-like behavior in LD box testing or when tested one week after induction in the OF. Even more interesting, mice induced to express Tat demonstrated a Dox dose- and duration-dependent increase in behavioral disinhibition, not anxiety-like behavior, in the EPM. The U-shaped pattern of the data was similar to that seen in LD box testing, although the specific behavior observed was different. While seemingly contradictory, upon review, these data together are consistent with the progression of different elements of the NP dysfunction observed over the full duration of a NeuroAIDS disease state.

Behavioral disinhibition is thought to be a manifestation of impulsivity observed in a variety of NP disorders such as attention deficit hyperactivity disorder (ADHD), drug abuse, mania, and PD (Evenden, 1999; Moeller et al., 2001). Behavioral disinhibition is also evident in dementia (Zamboni et al., 2008; Raczka et al., 2010) and after brain injury (Rao et al., 2007; Ciurli et al., 2010). It has been demonstrated that low D₂ mRNA and decreased D₂ receptor binding may lead to behavioral disinhibition in rats (Flagel et al., 2010). This is notable, given the progressive HIVE-induced down-regulation of striatal D₂ receptor expression (Gelman et al., 2006).

Interestingly, data from OF and LD box testing support the role of Tat protein in the development of anxiety-like behavior, but the EPM data suggest a more complex behavioral
profile that extends beyond simple anxiety. There are two main questions that arise when examining this somewhat perplexing data. First, why do the maximally Tat-induced mice demonstrate no profound changes in behavior in LD box and EPM, and variable behavior in OF? Secondly, why do Tat-induced mice in general demonstrate increased anxiety behavior in OF and LD box, but behavioral disinhibition in EPM? The latter question can be broken down into two parts: the evaluation of the possibility that these two behaviors can exist simultaneously and a discussion of the differences between the two assays. These questions will be discussed in turn.

Why do the maximally Tat-induced mice demonstrate no profound changes in behavior in LD box and EPM testing, and variable behavior in OF testing?

Although, more tests and evaluations are needed to elucidate this issue, these data clearly show a different behavioral profile with increased Tat production and exposure. The U-shaped nature of the data acquired in LD box and EPM testing and the modulating behavior observed in the OF, may suggest a possible progression of behavioral deficits. The shape of the data in LD box and EPM appears to mimic the Yerkes Dodson law (Yerkes and Dodson, 1908). This law dictates that performance increases with physiological or mental arousal, but only up to a point. When levels of arousal become too high, performance decreases. The process is often illustrated graphically as a curvilinear, inverted U-shaped curve (Yerkes and Dodson, 1908). Although this relationship is often discussed in relation to how arousal affects cognition, it would apply to any behavioral state where the response follows this curvilinear shape, such as behaviors measured in the EPM (Crawley, 1985). Accordingly, the behavioral state could be related to the extent of damage induced by HIV-Tat protein. If true, the dysfunction in the maximally Tat-induced mice may exceed the basic level of function necessary for task performance. Therefore, it becomes difficult to interpret if these maximally Tat-induced animals
suffer from anxiety or behavioral disinhibition, although OF data suggest that anxiety is evident in these mice at least acutely. As anxiety reaches a maximal peak, it may suppress overall performance in the LD box and EPM. Alternatively, it is possible that the progression of both neurological dysfunction and toxicity in relevant brain structures following increased exposure to Tat protein may underlie these findings. Presumably, damage to structures mediating cognition such as the medial prefrontal cortex may produce both behavioral disinhibition (as judgment is impaired; see Ciurli et al., 2010; Raczka et al., 2010) and alterations in anxiety-like behaviors (as inhibitory feedback on neurocircuits mediating anxiety-like behavior is itself reduced; see Marowsky et al. 2005; Bortolato et al., 2009). Detailed examination of Tat-protein effects over time and magnitude of exposure on neuroanatomical integrity of individual brain regions would be required to examine this possibility, and will be discussed in Study 4 (Chapter 5).

Why do Tat-induced mice in general demonstrate increased anxiety behavior in OF and LD box, but behavioral disinhibition in EPM?

It is possible to display both behavioral disinhibition and anxiety, although when taken at face value they appear as opposite behaviors in the assays used in this study. There are instances of co-morbidity between ADHD impulsivity and anxiety (Schatz and Rostain, 2006). Furthermore, the appearance of behavioral disinhibition in high anxiety situations has been documented in patients suffering from social anxiety disorders (Kashdan et al., 2008). Further studies need to be performed to determine if Tat protein expression results in behavioral disinhibition independent of anxiety. This may be achieved by using a task, such as the 5-choice serial reaction time task (5-CSRTT). This test is used extensively to examine impulsivity (Diergaard et al., 2007; Pattijen et al., 2009). For example, rats with damage to the medial prefrontal cortex, particularly the anterior cingulate and infralimbic cortex, have shown behavioral disinhibition in the 5-CSRTT (Muir et al., 1996).
Also, other types of anxiety-like behaviors need to be evaluated in Tat-induced mice. It is important to determine if demonstrations of anxiety in one assay cross-generalize to other models of anxiety to exclude artifacts and false positives. Testing this dictates the use of multiple assays of anxiety-like behavior to confirm the mediating effects of Tat. All three assays used here in this study are based on unconditioned anxiety and use avoidable stimuli, but anxiety tests that examine conditioned anxiety or use unavoidable stimuli could help to confirm the data and provide insight into the types of anxiety behaviors modulated by Tat. Furthermore, use of different tests could help to elucidate possible brain regions affected by Tat, as conditioned versus unconditioned anxiety is thought to be modulated by different areas of the brain (see Fanselow and Ponnusamy, 2008). Fear-potentiated startle (FPS) (Fendt and Fanselow, 1999) is a well validated conditioned anxiety test that is different from the assays used in the current study, as anxiety to a conditioned stimulus is evaluated. The acoustic startle reflex (ASR) test (Ralph and Caine, 2005) is different from the OF, LD, and EPM tests because it involves unavoidable anxiety-provoking stimuli. By assessing FPS and ASR, we could determine the effects of Tat protein expression on anxiety elicited by conditioned and/or unavoidable stimuli.

The results of this study, using three unconditioned anxiety tests, suggest that it may be possible that anxiety tests based on unconditioned and avoidable stimuli can detect different behavioral states. Even anxiety tests based on the same ethological design may examine different facets of anxiety. Physiological and neurobiological features of a group of assays believed to test the same behavior are to some extent overlapping, but also partially independent (Ramos, 2008). There are studies where two similar anxiety tests result in different results (File, 2001), specifically one study which reported no change in OF behavior, but increased open arm time in EPM in mice with reduced neural plasticity (Ognibene et al., 2006). Furthermore, Hodgson et al. (2008) found increased time spent in the open arms during morphine withdrawal
(not due to changes in locomotion), even though it is thought that withdrawal precipitates an anxious state. Therefore it may be possible that EPM behavior may not be exclusively driven by anxiety, but other behaviors as well, and this could be the case in the current study.

Summary

These studies are the first to demonstrate the complex relationship between Tat and NP dysfunction associated with anxiety disorders. In summary, here we have shown that Tat-induced mice demonstrate increases in anxiety-like behavior and behavioral disinhibition, dependent on the extent of Tat induction and time of testing. However, there is much work to be done in order to delineate the exact nature of the anxiety-like behavior, as well as if anxiety and behavioral disinhibition exist as co-morbid conditions. With the use of more specific tests for impulsivity and other types of anxiety, these issues may be elucidated. Furthermore, more research is needed to study the implications of these behavioral changes. For example, evidence suggests impulsivity may be causally involved in drug abuse vulnerability (Perry et al., 2005). Therefore, a greater understanding of the possible impulsivity displayed by Tat-induced mice may have further implications for drug abuse and addiction (see Chapters 4 and 6 for further discussion).
REFERENCES


CHAPTER 4

STUDY 3: HIV-1 TAT PROTEIN EXPRESSION IN MOUSE BRAIN POTENTIATES THE BEHAVIORAL PSYCHOSTIMULANT EFFECTS OF COCAINE

Research implicates the dopaminergic systems known to mediate drug reward (Di Chiara et al., 2004; Kalivas and Volkow, 2005) in the pathogenesis of neurological complications associated with HIV-1 infection (see Berger and Arendt, 2000 for review; Cass et al., 2003). However, there are limited behavioral demonstrations examining the effects that HIV-1 or its associated proteins have on the reinforcing and rewarding properties of abused drugs. This is an important avenue of investigation, as psychostimulants are highly abused in both general and HIV-infected populations (Gorman, 1998). In fact, psychostimulant abusers comprise one of the fastest growing populations of HIV-infected patients (Nath et al., 2002; UNAIDS/WHO, 2005).

A post mortem analysis of brains from patients with HIVE demonstrated abnormalities in striatal dopaminergic markers, specifically significant increases in the presynaptic DAT where cocaine is known to bind, and significant decreases in the number of inhibitory D_2 receptors (Gelman et al., 2006). The authors suggest that HIVE may down-regulate striatal D_2 receptor expression as it progressively damages striatum, thereby resulting in a net stimulation of dopaminergic circuits. However, it must be noted that other studies do not corroborate this. For example, a case study of a patient suffering from HAD showed bilateral reduction of DAT in the posterior striatum (Sporer et al., 2005). Moreover, Wang et al. (2004), used positron emission tomography to demonstrate that viral load negatively correlated with levels of DAT in the putamen and the striatum, and without a concomitant decrease in D_2 receptors. Although conflicting in details, these studies together suggest that changes in DAT number and
dopaminergic function correlate with the progression of HIV infection, and may play a pivotal role in the molecular profile of NeuroAIDS. However, the behavioral consequences of these biological changes remain under studied, especially in regards to the rewarding response of abused drugs.

A growing set of evidence demonstrates that Tat protein contributes to the dysfunction of dopaminergic signaling associated with HIV-infection and HAD (Maragos et al., 2002). Tat-induced OS has been associated with neurotoxicity and loss of function (Price et al., 2005) in midbrain dopaminergic neurons (Aksenov et al., 2001, 2003; Aksenova et al., 2006). Research also suggests that the toxic viral products released from HIV-1 infected cells are responsible for neuronal damage (Kolson and Pomerantz, 1996; Nath and Geiger, 1998) in the DA-rich basal ganglia (Kure et al, 1990), a collection of brain structures implicated in drug reward. Furthermore, acute exposure to Tat can lead to rapid dysfunction of the DAT by decreasing uptake (Wallace et al., 2006) and binding (Aksenova et al., 2006). Consistent with this, no-net flux microdialysis studies demonstrated that intra-NAc administration of Tat_{1-86} significantly reduced local DAT efficiency with little change in DA release, an effect accentuated by addition of cocaine (Ferris et al., 2009). In vitro radioligand binding and uptake studies confirmed the Tat-induced loss of DAT function in a dose-dependent and reversible manner following Tat incubation with rat synaptosomes (Zhu et al., 2009). This is of interest, as the loss of DAT function has significant implications for the response to drugs of abuse that act on the dopaminergic systems in the brain. Behavioral studies testing this are limited, but microinjection of Tat into the NAc of rats has been shown to significantly increase acute locomotor responses to cocaine (Harrod et al., 2008). Together, these results suggest that Tat-mediated inhibition of DAT might initially increase synaptic levels of DA, possibly enhancing the rewarding effects of psychostimulant drugs.
Research regarding the effects of HIV-1 or associated proteins on the reinforcing and rewarding properties of abused drugs is important because psychostimulants are highly abused in HIV-infected populations (Gorman, 1998) and because evidence suggests that the interaction of psychostimulant abuse and Tat that might accelerate the progression of HAD (Nath, et al., 2002), complicating patient care. Clinically, HIV-infected psychostimulant users present with more marked neurological impairment than HIV non-drug users. It is theorized that this impairment may be due to a synergism of abused drugs with HIV proteins like Tat, resulting in greater neurotoxic effects than conferred by either insult alone (Nath et al., 2002). Furthermore, it has been suggested that DA neurons and pathways are most vulnerable to the effects of the synergistic neurotoxicity (Nath et al., 2000). Commonly abused psychostimulants are reinforcing due to their ability to activate dopaminergic pathways (Kalivas and Volkow, 2005), but this effect is neurotoxic over time (Cass et al., 2003). These drugs could potentially confer an increased risk of developing HAD or exacerbate pre-existing HAD symptoms by inducing dysfunction in an already compromised dopaminergic system (Mirsattari et al., 1998). However, more research is necessary to characterize the synergy of psychostimulants with proteins expressed by HIV and their production of increased toxicity, as well as to elucidate the behavioral implications of this expected synergy in the context of the response to drugs of abuse.

The contribution of Tat to dopaminergic system dysfunction suggests a biological connection between the progression of neurological disease and the motivation for drugs of abuse, but determinations of the behavioral significance are lacking. We hypothesized that expression of HIV-1 Tat protein can potentiate the psychostimulant and rewarding effects of the reinforcing drug cocaine. Using the GT-tg mouse, with Dox-inducible and brain-selective Tat expression, we first evaluated the locomotor sensitizing effects of acute cocaine exposure. Next, we tested the effects of various doses of Dox and durations of exposure to Tat on cocaine reward in the conditioned place preference (CPP) assay. Using this assay, we also determined
the effects of Tat expression on a previously established place preference. Finally, we examined the duration of the potentiated preference in Tat-induced mice, and determined if Tat protein induction was sufficient to reinstate cocaine-seeking behavior in mice demonstrating extinction of an established cocaine-conditioned place preference response.
MATERIALS AND METHODS

Animals and housing

Adult male GT-tg bigenic (Kim et al., 2003) and C57Bl/6J wildtype (Jackson Labs, Bar Harbor, ME) mice were used in all experiments as described in Chapter 2 (Study 1).

Chemicals

Chemicals were obtained from Sigma-Aldrich (St. Louis, MO), unless otherwise noted. Cocaine (10 mg/kg, subcutaneous (s.c.)) and Dox were dissolved in 0.9% saline prior to injection. A dose of 10 mg/kg cocaine was selected for this study as it has been shown previously to produce a reliable CPP response in C57Bl/6 mice (Brabant et al., 2005).

Induction of brain-targeted Tat with Doxycycline treatment

This was performed as described in Chapter 2 (Study 1).

Locomotor activity

Distance traveled (m) was recorded as described in Carey et al. (2009) using the Noldus EthoVision Pro 3 tracking software (Noldus Information Technology, Wageningen, The Netherlands). The testing cages were translucent Plexiglas mouse home cages (16 X 24 X 12 cm) without bedding. After 30 min of habituation to the testing cages, mice were administered saline (0.9%, s.c.) and immediately returned to the testing cages to establish baseline
locomotion. Mice were then administered cocaine (10 mg/kg, s.c.) and activity was again measured. All recorded trials were 90 min.

**Conditioned place preference**

Mice were conditioned using the previously established biased cocaine-CPP paradigm with the apparatus and automated measurement system (San Diego Instruments, San Diego, CA) described in Carey et al. (2007). The biased place-conditioning protocol produces a sensitive indicator of conditioned drug reward that is consistent across studies (Shimosato and Ohkuma, 2000; Szumlinski et al., 2002, McLaughlin et al., 2003 and 2006), equivalent to alternative methods (e.g., counterbalanced design; see Bardo et al., 1995 for review; Sperling et al., 2010). Moreover, the biased conditioned place preference design has the advantage of controlling for the individual animal’s bias for in the apparatus, and allows for more efficient use of the animals available. It has also been demonstrated as an effective protocol for the study of extinction and reinstatement (Szumlinski et al., 2002; Carey et al., 2007; Aldrich et al., 2009; see also next page). The apparatus was a compartmentalized box divided into two equal-sized outer chambers (25 × 25 × 25 cm) with distinct cues, each joined to a small central section (8.5 × 25 × 25 cm) accessed through a single doorway (3 cm high). The entire unit was fitted with infrared beams, the breaking of which allows an automated measure of the time animals spend in each chamber. The chambers differed in wall striping (vertical vs. horizontal alternating black and white lines, 1.5 cm in width) and floor texture (lightly mottled vs. smooth). In these studies, GT-tg mice were treated with vehicle (0.9% saline), 100 mg/kg Dox for 1, 3, 5 or 7 days, or 25 or 50 mg/kg Dox for 7 days. Note that this treatment was administered before the start of conditioning in most experiments. However, one set of mice received Dox (100 mg/kg, 7 days) following completion of conditioning to test for the ability of Tat protein to alter established place
preference responses (see Figure 4.3 for more details). An initial pre-conditioning preference was determined by measuring the amount of time the individual mice spent in each chamber during a 30-min testing period. After administration of cocaine, mice were immediately confined for 30 min in the initially non-preferred chamber. Conditioning with assay vehicle (0.9% saline, 0.3 ml/30 g body weight, s.c.) followed 4 h later in a similar manner, but paired to the initially preferred chamber. To determine the direct effects of Tat protein on the rewarding effects of cocaine, this cocaine-saline conditioning “cycle” was repeated for two cycles, one cycle per day. Mice were tested for preference 24 h after the completion of conditioning by allowing the mice free access to the apparatus and measuring the time they spent in each chamber over a 30-min testing period.

Duration, extinction, and reinstatement of cocaine-seeking behavior

Mice were place-conditioned for four cycles (rather than two cycles as described above), as this paradigm has been demonstrated to be optimal to produce a long lasting CPP (i.e., up to approximately 3 weeks) using this dose of cocaine (Brabant et al., 2005). The place preference response to cocaine was made extinct similar to the methods described previously (Carey et al., 2007). First, the duration of the potentiation of cocaine CPP associated with the expression of Tat protein was examined. Uninduced GT-tg mice (0.9% saline, 7 days) and maximally induced mice (100 mg/kg Dox, 7 days) were conditioned with cocaine once a day for four days (Brabant et al., 2005; Carey et al., 2007). Place-conditioned mice were subsequently tested twice weekly by placing animals into the CPP apparatus with free access to all compartments for 30 min until they demonstrated extinction, which was defined as the return of the preference response to values statistically similar to initial responses (Brabant et al., 2005; Carey et al., 2007). In an additional experiment, a set of initially untreated GT-tg mice were place conditioned using the 4-
cycle paradigm and extinct as described above. Subsequent to extinction, these mice were
induced to express Tat protein with 100 mg/kg Dox for 7 days. 24 h after the completion of
induction, mice were given a preference test by placing animals into the CPP apparatus with
free access to all compartments for 30 min. This experiment tested if Tat protein induction alone
was sufficient to reinstate drug seeking behavior, without the need for a drug prime, retraining
session, or external stressor.

**Data analysis of behavioral experiments**

Data were analyzed by ANOVA (SPSS 16.0, Chicago, IL). Locomotor and CPP data were
analyzed using ANOVA and Ryan-Einot-Gabriel-Welsch (REGWF) multiple range test based on
the F-statistic (unless otherwise noted) to further examine the significant main effects or
interactions. CPP data are plotted as the difference in time spent in the eventual cocaine- and
saline-paired compartments, such that a negative value is generated to represent initial bias,
and a positive value reflects a conditioned preference for the cocaine-paired side. All data are
presented as mean ± S.E.M., with significance set at $p \leq 0.05$. 
RESULTS

HIV-1 Tat potentiated cocaine-induced locomotor activity

We first tested the neurobehavioral effect of brain-specific HIV-1 Tat protein expression on psychostimulant activity by examining cocaine-induced locomotor activity. GT-tg mice were induced to express Tat with Dox (100 mg/kg, i.p.) for 7 days and were subsequently tested for baseline locomotion (0.9% saline, s.c.) and cocaine-induced locomotion (10 mg/kg, s.c.). The locomotor responses of the induced mice were then compared to uninduced GT-tg mice (0.9% saline, i.p., 7 days) and Dox-treated C57Bl/6J mice (100 mg/kg, i.p., 7 days; Figure 4.1). A two-way ANOVA indicated an interaction of mouse (Dox-treated C57Bl/6J, uninduced, Tat-induced) X locomotion trial (baseline, cocaine) ($F_{2,215} = 5.73$, $p = 0.004$). Examining the simple main effects of this interaction, we found that there were no significant differences in baseline saline-induced locomotion between Dox-treated C57Bl/6J, uninduced, and Tat-induced GT-tg mice (85 ± 4.2 m, 91 ± 3.9 m, 80 ± 3.6 m, respectively, $p = 0.12$, REGWF, Figure 4.1). In contrast, there was a significant difference in cocaine-induced locomotion. Uninduced mice did not demonstrate significant differences in the locomotor response to cocaine compared to Dox-treated C57Bl/6J control mice (93 ± 5.4 m vs. 95 ± 7.0 m, respectively, $p = 0.87$). However, induction of Tat in GT-tg mice was associated with a significant potentiation of the locomotor effects of cocaine (120 ± 10.9 m; Figure 4.1) when compared to C57Bl/6J control mice or uninduced GT-tg mice ($p \leq 0.05$, REGWF). Furthermore, this increase in cocaine-mediated locomotion demonstrated by the Tat-induced mice was significantly greater than the magnitude of baseline locomotor activity demonstrated by the same mice ($p \leq 0.05$, REGWF).

Notably, vehicle- and Dox-treated C57Bl/6J mice discounted any potential locomotor effects of Dox on the non-transgenic control mice. The administration of Dox (100 mg/kg/d, 7 d) did not significantly alter baseline locomotion (vehicle-pretreated; 80 ± 12.1 m vs. Dox-
pretreated; 85 ± 4.2 m, $F_{1,42} = 0.19, p = 0.67$), nor did Dox pretreatment affect cocaine-mediated (10 mg/kg, s.c.) locomotor responses (vehicle-pretreated, 95 ± 9.3 m vs. Dox-pretreated 95 ± 7.0 m, $F_{1,40} = 0.00, p = 0.99$).

**HIV-1 Tat potentiated cocaine-conditioned place preference**

To initially test the hypothesis that HIV-1 Tat protein expression would potentiate the rewarding effect of psychostimulants, C57Bl/6J and GT-tg mice were treated with Dox (100 mg/kg) or vehicle (0.9% saline) once daily for 7 days. Following treatment, all mice were place-conditioned with cocaine (10 mg/kg, s.c.) as described in the methods. Mice were tested for final preference 24 h after the completion of conditioning.

A two-way ANOVA determined that there was a significant interaction of mouse X conditioning trial (pre- vs. post-conditioning) ($F_{3,146} = 9.16, p = 0.001$). Further analysis of this interaction effect indicated that prior to place conditioning, there were no significant differences between the initial place preference responses of Tat-induced (-224 ± 36 s) or uninduced (-193 ± 29 s) GT-tg mice, and those of vehicle-treated (-195 ± 26 s) or Dox-treated C57Bl/6J mice (-232 ± 54 s) ($p = 0.99$, REGWF, Figure 4.2a). Following place conditioning with cocaine, all groups of mice demonstrated significant cocaine-CPP, as indicated by a significant increase in the time mice spent in the cocaine-paired chamber compared to pre-conditioning baseline responses ($p \leq 0.05$). There were no significant differences between the post-conditioning preferences of vehicle- or Dox-pretreated C57Bl/6J mice (127 ± 54 and 198 ± 60 s, respectively) or uninduced GT-tg mice (91 ± 53 s) ($p > 0.05$, n.s.). However, mice expressing Tat protein showed a potentiation of cocaine-CPP (593 ± 108 s) that was significantly greater than post-conditioning responses of the vehicle- and Dox-pretreated C57Bl/6J mice and uninduced mice ($p \leq 0.05$; Figure 4.2a).
Figure 4.1. Expression of HIV-1-Tat produced sensitization of cocaine-induced locomotor activity. C57Bl/6J or GT-tg mice were pretreated for 7 days with vehicle (0.9% saline, solid bars) or Dox (100 mg/kg, striped bars). Baseline locomotor activity (white bars) did not differ significantly between groups. However, cocaine-mediated (10 mg/kg, s.c., gray bars) locomotor activity was significantly increased in mice expressing Tat protein (striped gray bar, far right) compared to the activity of uninduced littermates (solid gray bar, center) and Dox-treated C57Bl/6J mice (first striped gray bar). (n = 28-49 mice/group; * = significant difference from matching baseline; † = significant difference from cocaine-mediated locomotion of other groups, p ≤ 0.05, REGWF following two-way ANOVA)
Control experiments were performed replacing the cocaine in conditioning trials with saline alone, to verify the need for cocaine in place preference conditioning. Tat-induced and uninduced GT-tg mice, as well as C57Bl/6J mice were conditioned with saline in both chambers. There were no significant increases in time spent in the non-preferred chamber compared to matching pre-conditioning preference when mice were conditioned with saline (data as pre- vs. post-CPP; C57Bl/6J, -183 ± 42 vs. -136 ± 62 s, p ≤ 0.35; uninduced, -182 ± 56 vs. -41 ± 70 s, p = 0.23; Tat-induced, -228 ± 76 vs. -93 ± 151, p = 0.3, n.s.; Bonferroni-corrected t-tests comparing pre- vs. post-conditioning preferences).

**HIV-1 Tat potentiated cocaine-CPP in an induction dose- and duration-dependent manner**

Additional GT-tg mice were administered one of two different doses of Dox (25 or 50 mg/kg, i.p.) for 7 days or a 100 mg/kg dose for one of three different durations (1, 3, or 5 days). The magnitude of the potentiation of cocaine-CPP in GT-tg mice was dependent on the dose (Figure 4.2b) and duration of Dox administration (Figure 4.2c). This was demonstrated by the significant main effects of both Dox dose ($F_{2,182} = 8.18, p = 0.001$) and duration of administration ($F_{3,182} = 6.53, p = 0.001$) as well as significant interactions of Dox dose X conditioning trial ($F_{2,182} = 8.64, p = 0.001$) and duration of Dox administration X conditioning trial ($F_{3,182} = 6.42, p = 0.001$) revealed by three-way ANOVA. Post-hoc analyses were performed to explore these main and interaction effects. As previously described, the maximally Tat-induced mice (100 mg/kg Dox, 7 days, i.p.) demonstrated a significant potentiation of cocaine-CPP. The cocaine-CPP responses of mice administered 25 and 50 mg/kg Dox (142 ± 52 s and 311 ± 44 s, respectively) were not significantly greater than uninduced GT-tg mice (91 ± 53 s) ($p = 0.21$, REGWF, Figure 4.2b), although there is an apparent elevation in the cocaine-CPP preference of mice induced for 7 days with 50 mg/kg Dox. Compared to uninduced mice, there was a significant potentiation of cocaine-CPP in the mice administered 100 mg/kg Dox for 5 (394 ± 62
s, $p \leq 0.05$, REGWF), but not 1 or 3 days ($170 \pm 118$ s and $105 \pm 71$ s, respectively, $p = 0.21$) (Figure 4.2c). There was no difference between the post-conditioning preference for cocaine in the mice administered 100 mg/kg Dox for either 5 or 7 days ($p = 0.14$).

**Induction of HIV-Tat potentiated the effect of cocaine place conditioning in previously place-conditioned mice**

Two groups of untreated GT-tg mice were place conditioned with cocaine for two days to establish cocaine-CPP. Following this, one group was induced to express Tat (100 mg/kg Dox i.p. daily, 7 days), while the other group was administered vehicle for 7 days (i.e., remained uninduced). All mice were subsequently trained with a single additional cycle of cocaine place conditioning and re-tested for place preference. Results indicated a main effect of conditioning trial (i.e., pre-CPP, post-CPP, post-retraining; $F_{2,120} = 16.81$, $p = 0.001$, two-way ANOVA) as well as a main effect of induction treatment (Dox or vehicle, $F_{1,120} = 5.00$, $p = 0.03$). Post-hoc testing of these main effects indicated that the two groups of untreated GT-tg mice place conditioned with cocaine daily for two days (represented as squares in Figure 4.3a) demonstrated equivalent post-conditioning preference for the cocaine-paired chamber ($73 \pm 80$ and $140 \pm 63$ s; $p = 0.06$, n.s., REGWF, Figure 4.3b). When mice were subsequently administered Dox to induce Tat protein expression or received vehicle as a control, and then exposed to an additional cocaine-saline place conditioning cycle (see Figure 4.3a for schematic), the vehicle-treated (uninduced) GT-tg mice did not demonstrate a difference from their prior preference for the cocaine-paired chamber ($73 \pm 80$ vs. $106 \pm 73$ s, $p \leq 0.06$, Figure 4.3b). In contrast, Tat-induced GT-tg mice displayed a post-induction cocaine-CPP that was significantly greater than their prior preference response ($140 \pm 63$ vs. $438 \pm 125$ s, $p \leq 0.05$, Figure 4.3b, right-most bar). This preference was also significantly greater than the response of the mice than remained uninduced ($p \leq 0.05$).
Figure 4.2. HIV-1 Tat expression potentiated cocaine-CPP. (a) Post-conditioning preferences were potentiated in Tat-induced mice. C57Bl/6J mice (white bars left of dashed line) pretreated with Dox (100mg/kg, striped white bar) displayed a cocaine-CPP response that was similar to that of vehicle-pretreated C57Bl/6J mice (0.9% saline, solid white bar), demonstrating that there was no effect of Dox pretreatment itself on CPP. Post-conditioning responses of the C57Bl/6J and uninduced mice (solid gray bar right of dashed line) did not differ significantly from each other. In contrast, Tat-induced mice (100 mg/kg Dox, 7 days, striped gray bar) showed a significantly increased cocaine-CPP compared to C57Bl/6J and uninduced mice. (n = 12-25 mice/group; * = significant difference from all other post-conditioning responses, $p \leq 0.05$, REGWF following two-way ANOVA) (b) Tat-induced potentiation of cocaine-CPP was Dox dose-dependent. GT-tg mice induced with 25 mg/kg Dox for 7 days (striped light gray bar) did not demonstrate a significantly potentiated place preference for the cocaine-paired chamber compared to uninduced GT-tg mice (solid white bar). Mice administered 50 mg/kg Dox for 7 days (striped gray bar) showed an elevated cocaine-CPP. The response was not different from uninduced mice, but was notably also not different from mice induced with the maximum Dox dose (100 mg/kg, 7 days, striped dark gray bar). (n = 11-25 mice/group; * = significant difference from uninduced GT-tg mice, $\alpha = $ different from maximally Tat-induced mice, $p \leq 0.05$, REGWF following three-way ANOVA) (c) Potentiation of cocaine-CPP was dependent on the duration of Tat induction. GT-tg bigenic mice induced with 100 mg/kg Dox for 1 or 3 days (first two gray bars to the right of dotted line) did not demonstrate a significant difference in cocaine-place preference as compared to uninduced mice (solid white bar). However, mice administered 100 mg/kg Dox for 5 and 7 days (last two gray bars to the right of dotted line) showed a significant potentiation of cocaine-CPP. (n = 10-25 mice/group; * = significant difference from uninduced GT-tg mice, $p \leq 0.05$, REGWF following three-way ANOVA)
a. 

Dox/Veh

Day: 1 2 3 4 5 6 7 8 9 10 11 12

b. 

Difference in time spent in cocaine-paired chamber (s)

<table>
<thead>
<tr>
<th></th>
<th>Post-CPP preference</th>
<th>Post-trx preference</th>
</tr>
</thead>
<tbody>
<tr>
<td>- - - - - - - - -</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- - - - - - - - -</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- - - - - - - - -</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- - - - - - - - -</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- - - - - - - - -</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ Dox</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

N.S. *
Figure 4.3. Induction of Tat potentiated the effect of cocaine place conditioning in previously place-conditioned mice. (a) Schematic of experimental design. Untreated GT-tg mice were tested for place preference (triangles) the days before and after place-conditioning with cocaine and saline for two days (squares). Mice were then either administered Dox to induce Tat expression or vehicle (as a control) daily for 7 days and given an additional cycle of conditioning (day 12) and re-tested for place preference (day 13). (b) Result of Dox or vehicle treatment on CPP after a previously established place-conditioning response. Both groups of initially untreated GT-tg mice demonstrated equivalent post-conditioning cocaine preference (solid bars). Mice that were administered 0.9% saline for 7 days (i.e., uninduced) and were given an additional cycle of conditioning demonstrated no change in cocaine-CPP (striped white bar). However, Tat-induced mice displayed a post-induction cocaine-CPP (striped gray bar) that was significantly potentiated from both previous matching group preference (solid gray bar) and the preference of the mice that were uninduced (solid white bar). (n = 17-25 mice/group; * = significant difference from uninduced GT-tg mice and matching post-conditioning preference, $p \leq 0.05$; n.s. = no significant difference between groups, REGWF following two-way ANOVA)
Duration of cocaine-CPP in uninduced and Tat-induced GT-tg mice

The duration of cocaine-CPP (10 mg/kg, s.c.) was characterized in uninduced (0.9% saline, 7 days, i.p.) and maximally Tat-induced GT-tg mice (100 mg/kg Dox, 7 days, i.p.) to determine if the potentiated preference demonstrated by Tat-induced mice was a persistent or transient effect. Both uninduced and Tat-induced GT-tg bigenic mice were place-conditioned with four “cocaine-saline cycles” (see Figure 4.4a schematic, squares). Mice were tested for preference 24 h after the completion of conditioning (Figure 4.4a, triangle) and twice weekly thereafter (Figure 4.4a, circles) until there was no longer a significant preference for the cocaine-paired chamber (i.e., a demonstration of extinction). A repeated measures ANOVA indicated a significant within subjects main effect of test session (F_{3,72} = 23.43, p = 0.001) and a significant interaction of test session X Tat expression (i.e., uninduced vs. induced) was also revealed (F_{3,72} = 9.29, p = 0.01). Examining these significant effects further and consistent with the initial results, the mice expressing Tat protein demonstrated a significant potentiation of cocaine-CPP compared to the post-conditioning preference of uninduced littermates (p ≤ 0.05, REGWF, Figure 4.4b). Tat-induced mice maintained this potentiated cocaine-CPP response two weeks after conditioning (vs. initial post-CPP response p = 0.90, n.s.). However, three weeks after the completion of place conditioning, cocaine-CPP was not statistically different from pre-conditioning responses in both the Tat-induced and uninduced mice (p = 0.10).

Reinstatement of cocaine-CPP by Tat induction

An additional group of mice was used to determine if Tat protein induction alone was sufficient to reinstate drug seeking behavior, without the need for a drug prime or additional retraining cycle of conditioning. A set of untreated GT-tg mice were place conditioned using the 4-cycle paradigm and extinct as described above. Subsequent to extinction, these mice were induced to express Tat protein with 100 mg/kg Dox for 7 days and given a preference test (see
A repeated measures ANOVA indicated a significant main effect of test session ($F_{5,105} = 10.59$, $p = 0.01$). Post hoc analysis revealed that untreated GT-tg mice demonstrated a significant preference for cocaine after conditioning (Figure 4.5b, $130 \pm 49$ s) compared to pre-conditioning preference ($-178 \pm 31$ s, $p \leq 0.05$, REGWF). Consistent with the previous data, this preference lasted up to two weeks, but was no longer apparent after three weeks of extinction training ($-129 s \pm 52$ s, $p = 0.82$ compared to pre-conditioning preference). Subsequent to extinction, GT-tg mice were induced to express Tat with 100 mg/kg Dox for 7 days and were given a single preference test 24 h after induction completion. Tat induction reinstated drug seeking behavior in the GT-tg mice, as post-induction preference ($186 \pm 54$ s) was significantly different from pre-conditioning preference ($p \leq 0.05$) and no different that the post-conditioning preference ($p = 0.43$) demonstrated prior to extinction.
Figure 4.4. The duration of the HIV-1 Tat protein-mediated potentiation of cocaine-CPP was consistent with the unpotentiated response of uninduced mice. (a) Schematic of the experiment. In the first week, mice were preference tested (triangles) the days preceding and following place-conditioning with cocaine (10 mg/kg, s.c.) and saline daily for 4 days (squares). Mice were then tested for place preference twice weekly during weeks 2, 3 and 4 (circles). (b) Tat-induced mice (black bars) showed a significantly potentiated cocaine-CPP compared to matching pre-conditioning preference and the cocaine-CPP of the uninduced (gray bars). This potentiation was maintained for up to two weeks after conditioning. Extinction of place preference was demonstrated by uninduced and Tat-induced mice three weeks after the completion of conditioning. (n = 12-14 mice/group; * = significant difference from response of uninduced mice in same test session, \( p \leq 0.05 \), REGWF following repeated measures ANOVA)
Figure 4.5.  Cocaine-CPP was reinstated by induction of Tat protein. (a) Schematic of the experiment. In the first week, mice were preference tested (triangles) the days preceding and following place-conditioning with cocaine (10 mg/kg, s.c.) and saline daily for 4 days (squares). Mice were then tested for place preference twice weekly during weeks 2, 3 and 4 (circles). After demonstration of extinction mice were induced to express Tat protein (100 mg/kg Dox, 7 days, i.p.) and were given an additional preference test (last triangle) (b) Untreated GT-tg mice demonstrated a significant preference for cocaine after conditioning compared to pre-conditioning preference. Consistent with the previous data, this preference lasted up to two weeks and was no longer apparent after three weeks of extinction training. Subsequent to extinction and induction of Tat protein, the now induced GT-tg mice were given a single preference test. Tat induction reinstated drug seeking behavior in the GT-tg mice, as post-induction preference was significantly different from pre-conditioning preference, but no different that the post-conditioning preference demonstrated prior to extinction. (n = 22 mice; * = significant difference from pre-CPP response, $p \leq 0.05$, REGWF following repeated measures ANOVA)
DISCUSSION

These experiments utilized a bigenic mouse model capable of brain-specific expression of HIV-1 Tat protein to examine if Tat would have neurobehavioral consequences on the psychostimulant effects of cocaine. We tested the hypothesis that expression of HIV-1 Tat protein can potentiate the psychostimulant and rewarding effects of the reinforcing drug *cocaine*. Once expressed, HIV-1 Tat produced sensitization to the locomotor effects of cocaine, potentiated cocaine-CPP in a Dox dose- and duration of induction-dependent manner, and induced reinstatement of an extinct cocaine-CPP response. These results demonstrate that the activity of Tat was sufficient to influence the motivation for drugs of abuse and may suggest a possible biological connection between HIV infection and reward systems.

A number of studies using HIV-animals models have suggested an interaction between psychostimulants and the progression of grafted HIV infections. For example, the SCID mouse model has demonstrated enhancement of HIV-infection in the presence of cocaine (Mosier et al., 1991). More directly of interest to the present work, a recent study showed methamphetamine-induced rearing and stereotypical head movement was increased in HIV-1 transgenic rats, suggestive of enhanced drug sensitization in these animals (Liu et al., 2009). However, it is notable that while these models offering clear strengths, the breadth of physiological response to HIV infection or persistent HIV-protein expression precludes our use of these models, as we sought to determine whether the neurological dysfunction induced by HIV-Tat alone is sufficient to increase drug seeking behavior.

Recent reports have demonstrated that Tat modulates the activity of the dopaminergic system in brain (Aksenov et al., 2001; 2006; Ferris et al., 2009; Zhu et al., 2009), suggesting that HIV-1 Tat might be sufficient to influence the effects of, and motivation for drugs of abuse. Accordingly, we first tested the neurobehavioral effect of brain-specific Tat on the locomotor
response to a single administration of cocaine. The Dox-treated GT-tg mice demonstrated a significant Tat-mediated increase in locomotion from saline-baseline locomotion and when compared to C57Bl/6J control or uninduced GT-tg mice. This sensitization to the locomotor effects of cocaine was not due to Dox treatment or hyperactivity, as there were no significant differences in baseline locomotion across groups. These results further discounted possible subtle effects of mouse strain on locomotor activity, as uninduced GT-tg mice did not demonstrate significant differences in cocaine locomotor responses compared to Dox-treated C57Bl/6J control mice. The effects here are in agreement with research demonstrating that direct microinjection of Tat into the NAc of rats resulted in increased locomotion in response to a single intravenous injection of cocaine (Harrod et al., 2008). This is significant given that the NAc is a major reservoir in the brain for HIV (Wiley et al., 1998) and an area integral to the addictive effects of psychostimulants (Kalivas and Volkow, 2005). Interestingly, Harrod et al. (2008) found that the locomotor sensitization response that occurs in animals after repeated exposure to cocaine was itself suppressed in the rats administered Tat into the NAc. The effects of repeated exposure to cocaine were not tested in this study, but future studies using the GT-tg mouse to investigate the effects of chronic psychostimulant exposure on locomotor responses and reward would be of value.

Mice expressing Tat showed a greatly potentiated preference for the cocaine-paired chamber compared to C57Bl/6J or uninduced mice, suggesting that brain expression of Tat protein enhanced the rewarding effects of cocaine. The magnitude of this effect was dependent on the dose of Dox administered and the duration of Tat induction, with increasing Dox doses and durations of exposure associated with greater potentiation. We also confirmed that the potentiated preference was not a transient event, and lasted as long as a “normal” cocaine preference; the potentiated cocaine preference demonstrated by the Tat-induced mice lasted at least two weeks and up to three weeks, following a similar time course as the preference
demonstrated by the uninduced mice. Experiments further demonstrated the effects of Tat on
an established reward state, with the induction of Tat protein significantly potentiating the effect
of an additional exposure to place conditioning over both prior preference and the response of
mice that were uninduced. Together, these experiments suggest that induction of Tat protein
can potentiate reward in subjects with no previous experience with cocaine (Figure 4.2) and in
drug non-naïve subjects (Figure 4.3).

Finally, we demonstrated that after extinction of cocaine preference in untreated GT-tg
mice, induction of Tat protein directly resulted in reinstatement of cocaine-seeking behavior.
Previous research has demonstrated that various types of stressors can potentiate the
rewarding properties of drugs of abuse (Piazza et al., 1990) and that stress is a major
contributor to the reinstatement of drug-seeking behavior in abstinent subjects (Shaham et al.,
2000), but this marks the first demonstration that these behaviors may be due to endogenous
Tat protein expression in brain. Future experiments using C57Bl/6J mice in the same paradigm
used here would be of value to confirm that Dox itself has no effect on the reinstatement of
cocaine-seeking behavior. Notably, in the other experiments discussed in this thesis, Dox does
not appear to have significant effects on behavior. Moreover, a recent experiment in our
laboratory using a counterbalanced CPP assay found no reinstatement of cocaine-seeking
behavior in C57Bl/6J mice after Dox administration (unpublished data). Although these data
cannot be directly compared to the Tat-induced reinstatement data presented in the current
study because a different experimental design was used (i.e., counterbalanced versus biased
design), we hypothesize that administration of Dox in C57Bl/6J mice would not result in
reinstatement of cocaine-seeking behavior in our paradigm. Overall, these results are of
interest, as they could have implications for HIV-associated increases in psychostimulant use
and signal an increased likelihood of relapse in drug users who become infected with HIV,
although a direct determination of this would require further pre-clinical and clinical examinations.

In summary, the present data suggest that HIV-Tat protein is sufficient to potentiate the psychostimulant effects of cocaine, and support the utility of the Tat bigenic mouse as an in vivo model to investigate the behavioral modifications attributed to HIV-1 Tat in the context of the intact organism. Given that psychostimulant users comprise a significant segment of the population of HIV-infected patients (UNAIDS/WHO, 2005), and HIV-infected drug users present with a more marked neurological progression of HIV dementia (UNAIDS/WHO, 2005), studies examining the synergism of drug abuse and neurotoxic substances released during HIV infection, such as viral proteins like Tat (Aksenov et al., 2001; 2006) may offer new insights into the behavioral changes correlated with the progression of HIV-1 infection.
REFERENCES


Price TO, Ercal N, Nakaoke R, Banks WA. (2005) HIV-1 viral proteins gp120 and Tat induce oxidative stress in brain endothelial cells. *Brain Res.* **1045**: 57-63.


CHAPTER 5

STUDY 4: THE BRAINS OF MICE EXPRESSING HIV-TAT PROTEIN EXHIBIT REDUCTIONS IN GRAY MATTER DENSITY, LOSS OF WHITE MATTER MICROSTRUCTURAL INTEGRITY, CELL DEATH, AND MICROGLIAL ACTIVATION

Imaging studies are commonly used to identify and quantify characteristic changes that occur in the HIV-infected brain in vivo. Atrophy and white matter lesions are the most common MR findings in the brains of those patients with HAD (Chrysikopoulos et al., 1990; Wu et al., 2006). MRI examination of HIV-seropositive patients has consistently identified reduced volumes of the hippocampus and, to a lesser extent, the cerebral cortex (Tornatore et al., 1994; Archibald et al., 2004). Moreover, white matter abnormalities, such as deep white matter lesions, are a common finding in MR images of AIDS patients’ brains (Broderick et al., 1993; Filippi et al., 2001). Diffusion tensor imaging (DTI), a type of MRI that is sensitive to the microstructural integrity of scanned tissue, has revealed abnormalities in white matter tracts in HIV-seropositive patients, even in patients without identifiable white matter abnormalities in standard MR imaging (Filippi et al., 2001). Consistent with this, diffusion abnormalities were identified in patients infected with HIV in the splenium of the corpus callosum and the magnitude of alteration was correlated with the severity of dementia (Wu et al., 2006).

Research suggests that HIV-Tat protein may be an important factor in the viral pathogenesis leading to the development and progression of HIV-associated neurotoxicity. As discussed in Chapter 1, a number of studies have shown that Tat may mediate cell death through a variety of mechanisms (Kruman et al., 1998; Jones et al., 1998; Corasaniti et al., 2003; Manna and Aggarwal, 2000; Eugenin et al., 2007). Moreover, in vitro research suggests that Tat may be indirectly involved in the development of white matter abnormalities in HIV-
infected patients by compromising BBB integrity through increased OS (Price et al., 2005) and apoptosis (Kim et al., 2003). Tat has also been shown to activate microglia \textit{in vitro} (Bruce-Keller et al., 2001), an event correlated though histology studies of post-mortem tissue with the presence of white matter abnormalities detected in MR images of cognitively impaired vascular dementia patients (Black et al., 2009). However, few data are available detailing the neurotoxic effect of Tat \textit{in vivo}, especially in those brain regions thought to be crucial for learning, memory, and mood. Histological examination using hematoxyloin and Eosin straining demonstrated progressive atrophy of cortex (Kim et al., 2003) and hippocampus (Zou et al., 2007) in GT-tg mice administered increasing doses of Dox. The increases in terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)-positive neurons in the same regions also suggest the incidence of cell death may be attributable to Tat (Zou et al., 2007). However, no quantitative imaging data is available to document regional volumetric changes of the brain in the intact organism following Tat expression.

Using ultra-high magnetic field (9.4 Tesla) \textit{ex vivo} micro-imaging and histology, we examined alterations in brains of mice with brain-selective and inducible Tat-protein expression. Specifically, we tested the hypotheses that \textbf{Tat protein expression in the CNS would be associated with decreases in gray matter density, microstructural changes in white matter, and histological abnormalities in brain areas known to be affected in NeuroAIDS patients}. First, we confirmed the expression of Tat protein in brain by performing Western blots with tissue taken from both whole brain and specific brain regions. Then, brains were excised from Tat-induced GT-tg (100 mg/kg Dox, i.p., for 5 or 7 days), uninduced GT-tg (0.9% saline, 7 days), and Dox-treated C57BI/6J mice (100 mg/kg Dox, 7 days) for \textit{ex vivo} anatomical MRI using a 9.4 T Varian magnet. Anatomical images were analyzed using voxel based morphometry (VBM) to determine gray matter density. Also, diffusion tensor imaging was used to examine fractional anisotropy...
(FA) of white matter microstructure. Finally, a preliminary analysis of histology staining was performed to detect activated microglia and cell death.
MATERIALS AND METHODS

Animals and housing

Adult male GT-tg bigenic (Kim et al., 2003; described in Chapter 2) and C57Bl/6J wildtype (Jackson Labs, Bar Harbor, ME) mice were used in all experiments.

Chemicals and Induction of brain-targeted Tat with Doxycycline treatment

Dox, obtained from Sigma-Aldrich (St. Louis, MO), was dissolved in 0.9% saline prior to injection. The induction of Tat protein expression was performed as described in Chapter 2.

Western blot

Whole brains were isolated from GT-tg mice pretreated with vehicle (0.9% saline, 7 days) or Dox (50 or 100 mg/kg, i.p. for 7 days), the cerebellum removed and remaining tissue homogenized for use in Western blot analysis. Additionally, C57Bl/6J mice lacking the Tat transgene were pretreated with Dox (100 mg/kg, i.p. for 7 days) as a control. For region-specific analysis, additional groups of mice pretreated with vehicle (0.9% saline, 7 days) or Dox (100 mg/kg, i.p. for 7 days) were sacrificed and regions of interest (hippocampus, prefrontal cortex, cerebellum, anterior cortex, striatum, midbrain, and brain stem) pooled by treatment conditions after dissection guided by established C57Bl/6J mouse brain maps (URL: http://www.mbl.org/atlas/atlas.php). All membrane protein was harvested by centrifugation and solubilized, with protein (90 μg/lane) resolved in duplicate by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis as described (McLaughlin et al., 2004). Membranes were
incubated overnight at 4°C in 3%BSA/Tris-buffered saline-Tween (TBS-T) containing primary antibodies for either β-actin (0.2 μg/ml, Cell Signaling Technologies, Ipswich, MA) or Tat protein (1.6 μg/ml, ab43014, Abcam, Cambridge, MA) before washing, incubation with a horseradish peroxidase-conjugated goat antirabbit IgG (diluted 1:5000 in 1%BSA/TBS-T), and visualization of labeled protein with enhanced chemiluminescence (Cell Signaling Technologies, Ipswich, MA) detection on Kodak X-OMAT film. Western blots were performed in triplicate, and β-actin signal intensity bands and bands corresponding to Tat protein weights in GT-tg mice were quantified. β-actin antibody labeling was measured at the expected weight of 45 kDa, whereas Tat protein labeling was quantified from bands found at both the predicted weight of Tat protein, 14 kDa (Pugliese et al., 2005) and 19 kDa, which has been suggested to be the observed weight of expressed Tat protein in the GT-tg mouse model (J.J. He, personal communication).

Magnetic Resonance Imaging

**General methods:** Harvested mouse brains for *ex vivo* micro-imaging were perfused, fixed, and processed according to Cyr et al. (2005). Imaging studies were conducted on Varian 9.4 T (horizontal bore) scanner in the Translational Imaging Laboratory at McLean Hospital, using the micro-imaging gradient/shim insert set and a Varian ¹H (400 MHz) surface coil with an inner diameter of 25 mm. The micro-imaging insert had outer/inner diameters of 115/60 mm, a gradient strength of 1000 mT/m, and a 150 μsec rise time.

**Ex vivo micro-imaging:** Imaging was performed on extracted fixed mouse brains using a protocol similar to that described by Cyr et al. (2005). Mice (n = 8) from each treatment group were anesthetized with isoflurane and perfused transcardially with 20 ml of 0.9% saline (37°C), followed by a chilled (4°C) solution of 10% buffered formalin and ProHance (Gadoteridol, Bracco Diagnostics, Inc., NJ), in a volume ratio of 20:1. Extracted brains were stored in solution
at 4°C and scanned within 72 h of perfusion with the following scan parameters: Repetition Time (TR) = 3033 ms, Echo Train Length (ETL) = 8, matrix size = 512 x 512, Field of View (FOV) = 20 x 20 mm, Effective Echo Time (TE) = 25.9 ms, averages = 64, slice number = 28, slice thickness = 0.5 mm, 0 gap, scan time = 3.33 hours.

**Diffusion tensor imaging:** *Ex vivo* high resolution diffusion imaging was performed using a fast-spin echo-multi-slice DTI protocol subsequent to structural imaging, utilizing previously established methods (Boretius et al., 2009; Lehmann et al., 2010). Note that DTI scans were performed on the same set of prepared brains as were used for structural imaging both to maximize use of animals and to provide within-subject correlates of structural changes in grey and white matter. DTI data were acquired volumetrically using the following parameters: TR = 4000 ms, ETL = 8, matrix size = 256 x 256, FOV = 20 x 20mm, Effective TE = 27 ms, averages = 8, diffusion gradients were applied at 6 noncollinear directions, diffusion gradient amp = 18G/cm, duration = 5 ms, separation = 11.5 ms, giving a equivalent b-value of 1140 mm2/s, scan time = 1.75 h.

**Histology**

GT-tg mice administered vehicle (0.9% saline, 7 days) or Dox (100 mg/kg i.p. for 5 or 7 days) and Dox-treated C57Bl/6J mice (100 mg/kg, 7 days) were euthanized with isoflurane and transcardially perfused with 4% paraformaldehyde. Brains were removed from skulls 24 h later and placed in phosphate buffered saline. Sectioning and histology was performed by Neuroscience Associates (Knoxville, TN). Brains were sectioned to 35 µM using MultiBrain® technology. Histology for degeneration was performed using deOlmos’ amino cupric silver stain. Amino-cupric silver identifies degeneration in neuronal cell bodies, dendrites, axons and terminals and is visualized as a black stain (Switzer, 2000) Microglia were identified using
ionized calcium-binding adapter molecule 1 (Iba1) antibody (Schluesener et al., 1998) and visualized with DAB as chromagen. Iba1 is a protein specifically expressed in microglia and upregulated during the activation of these cells (Schluesener et al., 1998). It is used to identify and examine the morphology of microglia. Brain sections were photographed with Openlab under 20X magnification using a Zeiss axioskop 2 microscope.

Data Analysis

**Western blot:** β-actin signal intensity bands (45 kDa) and bands corresponding to Tat protein weights in GT-tg mice (14 and 19 kDa) were quantified with ImageJ 1.62 software (National Institutes of Health (NIH)). These values were plotted as percent of C57Bl/6J control labeling.

**VBM:** Structural image analyses were performed in FSL v.4.1 (FMRIB Software Library), similar to Sawiak et al. (2009). Brains were first extracted from background and then a study-specific template was constructed. For template construction, the brains of the uninduced GT-tg and Dox-treated C57Bl/6J mice were co-registered to a selected control brain and then averaged to create a study-specific template for each control group as per Sawiak (2009). The brains of mice from the Tat-induced GT-tg group (100 mg/kg Dox, i.p., 5 days) were aligned to the study-specific templates using a non-linear approach. Signal intensities from each aligned image were normalized to the mean image pixel value to minimize scan-to-scan variance. Registered images for each treatment group were spatially smoothed using a 0.05 mm Gaussian kernel. Lastly, the permutation-based nonparametric inference processing algorithm was executed in FSL using the “randomize” command, resulting in creation of nonparametric brain maps documenting structural difference distributions. The nonparametric brain maps were compared using MRICro analysis software (freeware, Rorden and Brett, 2000), and significant
differences identified with cluster corrected t-tests with a threshold set at \( p \leq 0.05 \). The C57Bl/6J mouse brain atlas from the Mouse Brain Library (URL: http://www.mbl.org/atlas/atlas.php) was used as a reference to identify the brain regions where significant differences were observed. Also, a non-parametric regional analysis was also performed comparing all groups using a composite region of interest (ROI) determined in four standardized brain slices where bilateral differences existed between the 5 day Tat-induced mice and the Dox-treated C57Bl6/J. The mean gray matter density in this ROI was calculated for each treatment group. Kruskal-Wallis non-parametric H-test with post hoc testing according to Conover (1999) was used to test for significant differences between groups (MedCalc software, Mariakerke, Belgium).

**DTI:** The diffusion tensor calculation was performed using the TrackVis DTI software (http://trackvis.org, Martinos center for Biomedical Imaging, Massachusetts General Hospital). The linear least square fitting method was used for diffusion tensor calculation. FA maps were generated from the calculated diffusion tensor map. The FA maps were compared using MRIcro analysis software (freeware, Rorden and Brett, 2000), and significant differences identified with cluster corrected t-tests with a threshold set at \( p \leq 0.05 \). The C57Bl/6J mouse brain atlas from the Mouse Brain Library (URL: http://www.mbl.org/atlas/atlas.php) was used as a reference to identify the brain regions where significant differences were observed.

**Histology:**

**General:** All histology data were analyzed with the nonparametric Kruskal-Wallis H-test and post hoc comparisons performed according to Conover (1999) using MedCalc software (Mariakerke, Belgium). Significance was set at \( p \leq 0.05 \).

**Amino Cupric Silver:** The left hemisphere of the perirhinal cortex was analyzed in three consecutive sections per brain (i.e., 105 \( \mu \)M), with \( n = 3 \) brains per group (for a total of 9 brain sections per treatment group). The left hemisphere of the perirhinal cortex was chosen because in Study 1 (Chapter 2) we found deficits in novel object recognition that may be attributed to
changes in this brain region; a recent clinical imaging study indicated that, although both hemispheres play a role in object memory, object encoding is partially lateralized to the left hemisphere of the perirhinal cortex (Bellgowan et al., 2009). We hypothesized that the brains of Tat-induced GT-tg mice would show increased staining with amino cupric silver compared to control mice. Images were imported into ImageJ (NIH) and converted to red-blue-green stacks. A threshold was determined on a randomly selected brain section to select for only stained areas and was then standardized for all images. Percent area above threshold (stained) was measured. A nonparametric test was selected for this analysis because the data were found to violate the homogeneity of variance assumption \( (p < 0.001, \text{Levene’s statistic}) \) and did not follow a Gaussian distribution \( (p < 0.001, \text{D’Agostino-Pearson test of normality}) \). All data are presented as mean optical density of staining ± S.E.M.

*Iba1*: The prefrontal cortex was examined by sampling three consecutive bilateral sections of the both the orbital frontal and cingulate cortex (210 µM in total) per brain, with \( n = 2-5 \) brains per group (for a total of 6-15 brain sections per treatment group). These brain regions were chosen as they are areas that have been implicated in behavioral disinhibition (Muir et al., 1996; Passetti et al., 2002, and see Chapter 6 for further discussion). We hypothesized that the microglia of Tat-induced mice would show increases in signs of microglia activation/reactivity (see Figure 5.5a for specific details) and increased intensity of labeling with Iba1 as visualized by a darker brown stain compared to the microglia of control mice. Microglia were rated by two independent raters that were blind to the treatment groups. The rating scale ranged from 1 to 4 and was based on microglia morphology and phenotypes described in (Ladeby et al., 2005) and were as follows: 1: Resting-state microglia with ramified and long processes and a round cell body; 2: Early activated microglia showing shortening of the processes, with minimal increases in immunoreactivity (i.e., darkened staining) in the cell body; 3: Activated/reactive microglia displaying shortening of the distal and thickening of the proximal processes and increased
immunoreactivity in the cell body; 4: Reactive microglia show a progression from the previous phenotype, but the cell body is irregular and indistinct from the processes. (See also Figure 5.5a for representative images of this rating scale.) Ratings were analyzed with a nonparametric test as data did not follow a Gaussian distribution ($p < 0.01$, D’Agostino-Pearson test of normality). All data are presented as mean rating ± S.E.M.
RESULTS

HIV-1 Tat protein is expressed in GT-tg mouse brain

HIV-1 Tat expression in this model has been previously monitored and confirmed to be brain-specific by RT-PCR of Tat mRNA (Kim et al., 2003), but this is a proxy for Tat protein expression and not a direct measurement. To attempt a more direct demonstration of Dox-induced Tat expression, we performed Western blots on whole homogenized brains isolated from GT-tg mice pretreated with vehicle (0.9% saline, i.e., uninduced) or Dox (50 or 100 mg/kg, i.p. for 7 days). C57Bl/6J mice were also pretreated with Dox (100 mg/kg, 7 days) as a control for the effects of Dox treatment. As expected, incubation with a β-actin antibody labeled a single band of similar intensity among all samples, suggesting equivalent amounts of protein in each sample (Figure 5.1a, top panel). In contrast, at both the predicted weight of Tat protein (14 kDa, Figure 5.1a, lower arrow in lower panel and Figure 5.1b, white bars) and the suggested observed weight (19 kDa, Figure 5.1a, upper arrow in lower panel and Figure 5.1b, black bars), the antibody demonstrated a difference in labeling intensity that correlated with the presence and dose of Dox administered. Notably, although the Tat antibody demonstrated a modest degree of non-selective binding by labeling proteins of similar kDa in samples of C57Bl6/J mouse brain in these ranges of protein weight, the magnitude of labeling was similar to that of uninduced GT-tg mice (Figure 5.1b, left pair of bars). In contrast, a greater amount of adjusted Tat antibody labeling was demonstrated in the GT-tg mice induced to express Tat with Dox (Figure 5.1b). Although the small sample size and non-selective labeling made statistical analysis unfeasible, the data suggest an apparent Dox dose-dependent increase in Tat-antibody labeling consistent with the increased expression of Tat protein. Importantly, increases in Tat-labeling were observed in all brain regions dissected from GT-tg mice treated with 100 mg/kg Dox for 7 days (Figure 5.1c, lower panel) as compared to uninduced GT-tg mice (Figure 5.1c,
middle panel) or C57Bl/6J mice treated 7 days with 100 mg/kg Dox as a control (Figure 5.1c, upper panel). No significant differences in β-actin antibody labeling were observed.

**Voxel based morphometry of ex vivo MR images suggest a possible brain region-specific reduction in gray matter density in 5 day Tat-induced GT-tg mice**

Ex vivo structural MR images were acquired from Dox-treated C57Bl/6J mice (100 mg/kg, 7 days), uninduced GT-tg mice (0.9% saline, 7 days), and Tat induced GT-tg mice treated with Dox (100 mg/kg/day) for 5 or 7 days. Images were analyzed using VBM whole brain analysis methods described above. Analysis revealed no significant differences between any brain region of the uninduced GT-tg and Dox-treated C57Bl/6J mice. In contrast, significant reductions were found in gray matter density in the amygdala, amygdala-hippocampal area, piriform, perirhinal, and entorhinal cortices in the 5 day Tat-induced mice compared to Dox-treated C57Bl/6J mice (corrected $p \leq 0.05$, two-tailed t-test, Figure 5.2a). However, there were no significant gray matter density differences between 5 day Tat-induced and uninduced GT-tg mice. Although, if the threshold for significance was relaxed to $p < 0.10$, differences were revealed between the 5 day Tat-induced and the uninduced GT-tg mice in the right-hemisphere in the amygdala and piriform cortex. Interestingly, there were also no significant differences in gray matter between the 7 day Tat-induced mice and either control group, possibly due to increased variability in measured volumes in the 7-day Tat induced animals (see below and Figure 5.2b).

A post-hoc analysis using non-parametric statistics was also performed. Regions of interest (ROIs) were determined from four standardized brain sections where bilateral differences were identified using VBM between the 5 day Tat-induced and the Dox-treated C57Bl6/J mice (see Figure 5.2a, brain sections 6-9). An analysis of gray matter density was
a. GT-tg mouse

<table>
<thead>
<tr>
<th></th>
<th>C57Bl/6J</th>
<th>+ Dox (50)</th>
<th>+ Dox (100)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+ Saline</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>GT-tg bigenic mouse</th>
<th>+ Dox (50)</th>
<th>+ Dox (100)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (saline)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>GT-tg bigenic mouse</th>
<th>+ Dox (50)</th>
<th>+ Dox (100)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (saline)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Arbitrary Units (mean + SEM)

<table>
<thead>
<tr>
<th>Dose Dox (mg/kg, i.p.)</th>
<th>B-Actin (Mean ± SEM)</th>
<th>Tat 14 kD (Mean ± SEM)</th>
<th>Tat 19 kD (Mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57Bl/6J mouse 100</td>
<td>8041 ± 2714</td>
<td>711 ± 264</td>
<td>1039 ± 609</td>
</tr>
<tr>
<td>GT-tg bigenic mouse 0</td>
<td>9303 ± 2836</td>
<td>883 ± 338</td>
<td>652 ± 69</td>
</tr>
<tr>
<td>GT-tg bigenic mouse 50</td>
<td>7666 ± 2276</td>
<td>2018 ± 607</td>
<td>1756 ± 637</td>
</tr>
<tr>
<td>GT-tg bigenic mouse 100</td>
<td>7422 ± 2756</td>
<td>2645 ± 1309</td>
<td>1824 ± 691</td>
</tr>
</tbody>
</table>

b. % Control Labeling

C57Bl/6J mouse

<table>
<thead>
<tr>
<th>Lane</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>19 kDa</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15 kDa</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

+ Dox (100 mg/kg, 7d)

GT-tg mouse

<table>
<thead>
<tr>
<th>Lane</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>19 kDa</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15 kDa</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

+ Saline (7d)

+ Dox (100 mg/kg, 7d)
Figure 5.1. Administration of Dox induced expression of HIV-1 Tat protein in GT-tg mouse brain. (a) Western blots were performed with solubilized mouse brain protein from whole homogenized brains isolated from GT-tg bigenic mice pretreated with vehicle (0.9% saline) or Dox (50 or 100 mg/kg, i.p. for 7 days). Additionally, C57Bl/6J mice lacking the Tat transgene were treated with Dox (100 mg/kg, i.p. for 7 days) as a control. Samples were incubated with primary antibodies for either β-actin (upper panel) or Tat protein (lower panel). The β-actin antibody labeled a single band corresponding to the weight of the β-actin protein of similar band intensity across all samples (upper panel). In contrast, the Tat antibody labeled a number of proteins non-selectively (lower panel), but demonstrated a difference in labeling intensity that corresponded to the presence and dose of Dox administered. (b) In this summary graph of the quantified whole brain Western blots, all band intensities are plotted as percent C57Bl/6J labeling. Dox treatment resulted in a dose-dependent increase in Tat-antibody labeling in GT-tg mice. The differences in labeling intensity corresponded to the presence and dose of Dox administered at both the predicted weight of Tat protein (14 kDa, white bars), and at 19 kDa (black bars), which has been suggested to be the observed weight of expressed Tat protein in the GT-tg mouse model. (c) Increases in Tat-labeling were observed in all brain regions dissected from GT-tg mice treated with 100 mg/kg Dox for 7 days (lower panel) as compared to uninduced GT-tg mice (middle panel) and C57Bl/6J mice treated with Dox (100 mg/kg, i.p. for 7 days) as a control (upper panel). Lanes: 1. weight markers, 2. hippocampus, 3. prefrontal cortex, 4. cerebellum 5. anterior cortex, 6. striatum, 7. thalamus, 8. brain stem.
performed in this composite ROI across all groups. Kruskal-Wallis non-parametric testing found an overall significant difference between groups (H = 12.46, \( p = 0.006 \)). Notably, the ROIs of the Dox-treated C57Bl/6J and uninduced GT-tg mice were not different. Post hoc testing further demonstrated a significant reduction in gray matter density in the ROIs of the 5 day and 7 day Tat-induced compared to the Dox-treated C57Bl/6J mice (\( p \leq 0.05 \), Kruskal-Wallis post hoc, Figure 5.2b). Furthermore, the brains of the 5 day Tat induced mice also demonstrated significantly reduced gray matter density in these ROIs compared to uninduced GT-tg mice (\( p \leq 0.05 \)).

*Ex vivo* DTI revealed a significant loss of white matter microstructural integrity in the brains of Tat-induced GT-tg mice

FA maps were created from DTI scans acquired from in Dox-treated C57Bl/6J mice (100 mg/kg, 7 days), uninduced GT-tg mice (0.9% saline, 7 days), and Tat induced GT-tg mice treated with Dox (100 mg/kg/day) for 5 or 7 days (i.e., using the same brains as above). The analysis of the FA maps revealed significant decreases in white matter microstructural integrity in the insula, endopiriform nucleus, and part of the striatum in the 7 day Tat-induced compared to uninduced GT-tg mice (corrected \( p \leq 0.05 \), two-tailed t-test, Figure 5.3), but not compared to Dox-treated C57Bl/6J mice. It should be noted, however, that there were no significant differences between any brain region of the uninduced GT-tg and Dox-treated C57Bl/6J mice. Also, there were no differences between 5 day Tat-induced and either control group.
Figure 5.2. The 5 day Tat-induced mice showed potential brain region-specific reductions in gray matter density. (a) VBM analysis. The brains of the 5 day Tat-induced mice show significant loss of gray matter compared to the brains of Dox-treated C57Bl/6J mice in the amygdala (1), amygdala-hippocampal area (2), piriform (3), perirhinal (4), and entorhinal (5) cortices. Areas of significant loss of gray matter in the 5 day Tat-induced mice are shown as a colorimetric image overlaid on a composite brain created with the 32 total brains imaged. (+0.74 mm to -4.20 mm Bregma; n = 8/group; areas in color area are $p \leq 0.05$, corrected t-tests)

(b) Composite region of interest (ROI) analysis. The ROI was determined from four brain slices where bilateral differences existed between the 5 day Tat-induced mice and the Dox-treated C57Bl6/J (see Figure 5.2a, brain sections 6-9). The mean gray matter density in these composite ROIs was calculated in each group. There was a significant reduction in gray matter density in the ROIs of the 5 day and 7 day Tat-induced compared to the Dox-treated C57Bl/6J mice. The brains of the 5 day Tat induced mice also demonstrated significantly reduced gray matter density in these ROIs compared to uninduced GT-tg mice. Notably, the ROIs of the Dox-treated C57Bl/6J and uninduced GT-tg mice were not different. (* = different from Dox-treated C57Bl/6J mice, † = different from uninduced GT-tg mice $p \leq 0.05$, Kruskal-Wallis H-test with post hoc testing)
Figure 5.3. The brains of the 7 day Tat-induced GT-tg mice showed significant loss of white matter microstructural integrity. The analysis of the FA maps created from images acquired with DTI revealed significant loss of white matter microstructural integrity in the insula (1), endopiriform nucleus (2), and part of the striatum (3) in the 7 day Tat-induced compared to uninduced GT-tg mice. Areas of significant loss of white matter microstructural integrity in the 7 day Tat-induced mice compared to uninduced GT-tg mice are shown as a colorimetric image overlaid on a composite brain created with the 32 total brains imaged with DTI. (+2.22 mm to -4.20 mm Bregma, n = 8/group, areas in color area = $p \leq 0.05$, corrected t-tests)
Tat-induced mice demonstrated increased cell death in the perirhinal cortex and changes in microglia morphology in the prefrontal cortex

Mice induced to express Tat (100mg/kg Dox, i.p., 7 or 5 days) showed increased signs of cell degeneration and death in the left hemisphere of the perirhinal cortex compared to control mice, as revealed by amino cupric silver staining (H = 21.97, p = 0.001, Kruskal-Wallis H-test, see Figure 5.4a for representative images). The perirhinal cortices from 5 day and 7 day Tat-induced mice demonstrated significantly greater optical density (3.5 ± 1.2 and 4.4 ± 1.1) compared to Dox-treated C57Bl/6J mice (100 mg/kg Dox, i.p., 7 days) (1.0 ± 0.1, p ≤ 0.05, Kruskal-Wallis post hoc test, Figure 5.4b). The increased optical density was caused by increased labeling with amino cupric silver, which identifies cell death and degeneration. Moreover, the perirhinal cortex from 7 day Tat-induced mice showed a significantly greater optical density than the uninduced GT-tg mice (0.9% saline, 7 days) (1.2 ± 0.2, p ≤ 0.05), while the 5 day Tat-induced mice showed a trend from the uninduced GT-tg mice (p = 0.06). Notably, there was no difference in optical density in perirhinal cortices of the Dox-treated C57Bl/6J and uninduced GT-tg mice (p > 0.05). These data corroborate the volumetric losses noted in the VBM MR image analysis in the same region.

The prefrontal cortex was analyzed for changes in microglia morphology. Slides were rated by two independent raters on a scale of 1 through 4, with a rating of 1 indicating normal resting microglia and rating of 4 suggesting a predominance of reactive microglia (see Figure 5.5a for examples of cell morphology). The microglia ratings of the prefrontal cortex showed a significant difference between groups (H = 13.74, p = 0.0018, Kruskal-Wallis H-test, Figure 5.5b). Post hoc testing revealed that the 7 day Tat-induced mice showed higher ratings for reactive microglia (3.5 ± 0.2) than Dox-treated C57Bl/6J (2.4 ± 0.2) and uninduced GT-tg mice (2.7 ± 0.3) (p ≤ 0.05, Kruskal-Wallis post hoc test). Interestingly, the 5 day Tat-induced mice demonstrated a contrasting result, with a slightly lower rating (2.0 ± 0.2) than the other groups.
tested, but the rating was not significantly different from Dox-treated C57Bl/6J or uninduced GT-tg mice ($p > 0.05$). Notably, there was no difference in average rating of microglia morphology between the Dox-treated C57Bl/6J and uninduced GT-tg mice ($p > 0.05$). These data suggest a progressive development of inflammation in the brains of Tat-induced mice.
a. 

Control

Tat-induced

b. 

Optical Density

C57BL/6J + Dox

days of Dox (100 mg/kg) GT-tg mice

0 5 7

*^ * *^
Figure 5.4. The brains of mice induced to express Tat showed more cell degeneration and death in the perirhinal cortex compared to control mice. (a) Representative images selected from a Dox-treated C57Bl/6J (left) and a Tat-induced mouse (right) illustrate the black staining indicating cellular debris due to cell death and degeneration of cell bodies, axons, and dendrites. (b) There was no difference in staining in the perirhinal cortex of the Dox-treated C57Bl/6J (white solid bar) and uninduced GT-tg (white hatched bar) mice. The brains of 5 day (gray bar) and 7 day (black bar) Tat-induced mice demonstrated greater amount of staining in the perirhinal cortex compared to the brains of Dox-treated C57Bl/6J. Also, the perirhinal cortex from 7 day Tat-induced mice showed a greater amount of staining than the perirhinal cortex of the uninduced GT-tg mice (white hatched bar). (\(^*\) = different from Dox-treated C57Bl/6J mice, \(\dagger\) = different from uninduced GT-tg mice \(p \leq 0.05\), \(^\wedge\) = trend from uninduced GT-tg mice, \(p = 0.06\), Kruskal-Wallis H-test)
a. Resting Early Activated Activated/Reactive Reactive

b. 

C57Bl/6J + Dox

days of Dox (100 mg/kg) GT-tg mice

Morphology Rating
Figure 5.5. Mice induced to express Tat showed changes in microglia morphology indicative of inflammation in the prefrontal cortex. (a) Microglia phenotypes: Resting microglia show long, ramified processes and round cell bodies; Early Activated microglia show shortening of the processes, with minimal increases in cell body immunoreactivity (i.e., darkened staining); Activated/Reactive microglia display shortening of the distal and thickening of the proximal processes and increased cell body immunoreactivity; Reactive microglia show a progression from the previous phenotype, but the cell bodies are now irregular and indistinct from the processes. (b) There was no difference in microglia morphology in the prefrontal cortices of Dox-treated C57Bl/6J (white solid bar) and uninduced GT-tg (white hatched bar) mice. The mean ratings in the prefrontal cortex of the 7 day Tat-induced mice (black bar) were higher and suggestive of more microglia activation and reactivity compared to both the prefrontal cortices of Dox-treated C57Bl/6J and uninduced GT-tg mice. In contrast, the 5 day Tat-induced mice (gray bar) did not show any differences in microglia morphology compared to control mice. (* = different from Dox-treated C57Bl/6J and uninduced GT-tg mice, † = different from 5 day Tat-induced mice p ≤ 0.05, Kruskal-Wallis H-test)
DISCUSSION

In the present study, we tested the hypotheses that Tat protein expression in the CNS would be associated with decreases in gray matter density, white matter microstructural changes, and histological abnormalities in brain areas known to be affected in NeuroAIDS patients. Specifically, we examined gray matter density and white matter microstructural integrity using ex vivo micro-imaging. Furthermore, we performed preliminary histological examinations of cell death and microglia morphology in mouse brains expressing Tat. Although imaging studies are commonly used to identify characteristic changes that occur in the HIV-infected brain and quantify atrophy and white matter changes, there are no quantitative imaging studies investigating the contribution of Tat protein specifically to regional brain alterations.

We first confirmed Tat protein expression in brain by performing whole brain and brain region-specific Western blots. Although the small sample size and non-selective labeling made statistical analysis unfeasible, the data suggest a Dox dose-dependent increase in Tat-antibody labeling consistent with the increased expression of Tat protein. Importantly, increases in Tat-labeling were observed in all brain regions dissected from GT-tg mice treated with 100 mg/kg Dox for 7 days (i.e., hippocampus, prefrontal cortex, cerebellum, anterior cortex, striatum, thalamus, and brain stem). The non-selective labeling observed in the Dox-treated C57Bl/6J and saline-treated GT-tg bigenic mice also hindered analysis, but may be attributed to the poor quality of antibody used to detect Tat protein. It has been suggested that the commercially available Tat antibodies demonstrate limited selectivity for native Tat protein in Western blot analysis (J.J. He, K. Hauser, and R.M. Booze, personal communications), reducing the value of using traditional Western blotting procedures to measure Tat protein. Despite this limitation, this experiment confirmed Tat protein expression in the GT-tg mouse brain in response to Dox administration, corroborating previous research confirming the expression and distribution of
induced Tat protein in GT-tg mouse brain (Kim et al., 2003; Zou et al., 2007). Kim and
colleagues (2003) demonstrated that GT-tg mice that were treated with Dox-containing (6
mg/ml) drinking water for 7 consecutive days exhibited Tat mRNA expression in brain from both
heterozygous and homozygous GT-tg bigenic mice. Importantly, while “leaky expression” of a
minute amount of Tat mRNA transcripts occurred in the absence of Dox (i.e., uninduced mice),
the overwhelming majority of brain Tat expression in GT-tg mice was induced and regulated by
Dox administration, consistent with other uses of tetracycline-mediated transgene expression
models (Chen et al., 1998; Mansuy et al., 1998; Kistner et al., 1996). Furthermore, the RT-PCR
results showed that Tat expression occurred only in brain, but not in eye, heart, kidney, liver,
lung, spleen, and thymus (Kim et al., 2003). Moreover, an enzyme-linked immunosorbent assay
analysis detected Tat expressed in isolated brain homogenates and in supernatants of primary
astrocyte cultures prepared from Dox-treated GT-tg mouse brain tissue, demonstrating Tat
expression in astrocytes (Kim et al., 2003). In situ hybridization detected higher Tat mRNA
levels in cerebellum, cerebellar cortex, hippocampus, brain stem, tectum, and white matter
tracts in the brains of GT-tg mice treated with Dox, many fewer Tat mRNA transcripts in brains
of uninduced GT-tg mice, and no Tat mRNA transcripts in wild-type mouse brains (Kim et al.,
2003). Together, these data verify that Tat protein is expressed after administration of Dox
throughout and solely in the GT-tg mouse brain.

Using MRI and VBM analysis techniques and supporting the hypothesis that Tat protein
expression in the CNS would be associated with decreases in gray matter density, reductions in
gray matter density in the amygdala, amygdala-hippocampal area, piriform, perirhinal, and
entorhinal cortices were found in the brains of 5 day Tat-induced mice compared to Dox-treated
C57Bl/6J mice. These areas have been implicated in various types of learning and memory and
may play a role in anxiety (discussed in greater detail in Chapter 6, but see Murray and
Richmond, 2001; Cannistraro and Rauch, 2003; Aggleton et al., 2010; Gavrilovici et al., 2010;
Interestingly, the whole brain VBM analysis did not detect any differences in gray matter between 5 day Tat-induced and uninduced GT-tg mice or between 7 day Tat-induced mice and either control. However, we then performed a post hoc ROI analysis to test for between-group effects in the specific regions exhibiting gray matter density differences in the whole brain analysis of 5 day Tat-induced mice. With this analysis, additional differences were observed between the 5 day Tat-induced and uninduced GT-tg mice as well as a differences between the 7 day Tat-induced mice and Dox-treated C57Bl/6J mice. There are number of experimental issues that could have independently or collectively contributed to the detection of some group differences with an ROI analysis that we could not detect with a whole brain analysis. First, strain differences could account for the difference detected in the whole brain analysis between the 5 day Tat-induced and Dox-treated C57Bl/6J mice. However, this is unlikely to have contributed to the data significantly given that in both the whole brain VBM analysis and ROI analysis, there were no noted differences between the uninduced GT-tg mice or the Dox-treated C57Bl/6J mice. Secondly, group differences in variance could obscure effects in the whole brain analysis. Figure 5.2b suggests that the data variance in the 7 day Tat-induce mice was greater than in the other groups, which could have contributed to the inability to detect a difference in the 7 day Tat-induced mice in the VBM analysis. Furthermore, there may not have been enough statistical power in the sample to detect effects that have a small effect size (e.g., when variances are large). This is supported by evidence that when the threshold for significance was relaxed to \( p < 0.10 \), differences were revealed between the 5 day Tat-induced and the uninduced GT-tg mice in brain structures that were significantly different between the 5 day Tat-induced and Dox-treated C57Bl/6J mice. As with traditional statistical tests, the power to detect differences between groups is typically affected by the sample size. Increasing sample sizes in future studies could reveal smaller effect size differences using the VBM technique. Lastly, our cross-sectional study design has the disadvantage of possible
cohort effects. This issue could be mitigated by conducting an *in vivo* longitudinal MRI study to examine the progressive changes in the brains of the same Tat-induced mice over days of induction and several weeks subsequent to induction. The disadvantages of *in vivo* imaging are that scan time must be shortened, thereby reducing image resolution while increasing the occurrence of respiratory motion artifacts that further reduce image quality. Clearly, a number of issues could have obscured possible difference between some of the Tat-induced mouse groups and controls. Further studies that compensate for the issues described above could help to resolve these issues.

Also supporting our hypothesis, we found significant loss of white matter microstructural integrity in the insula, endopiriform nucleus, and part of the striatum in the 7 day Tat-induced compared to uninduced GT-tg mice. These areas have been implicated in emotional processing (Critchley et al., 2005), anxiety (Paulus and Stein, 2006), drug reward and craving (Di Chiara et al., 2004; Kalivas and Volkow, 2005; Vorel et al., 2007), planning of movement (Penney and Young, 1986; Doyon et al., 1997), and epilepsy (Kowianski et al., 2004). These areas and their functions will be further discussed in Chapter 6. Interestingly, we found no differences between the 5 day Tat-induced mice and either control group. This could be due to a number of issues as described above. Moreover, these results could be attributed to a progressive effect of Tat protein on white matter, with increasing exposure to Tat resulting in more severe damage to this cell type.

In terms of histological findings, the perirhinal cortex was examined using a histological marker for cell death and degeneration. The brains of 5 and 7 day Tat-induced mice demonstrated a greater amount of staining in the perirhinal cortex compared to the brains of Dox-treated C57Bl/6J. Compared to uninduced GT-tg mice, only the 7 day Tat-induced mice showed significantly more staining, but the 5 day Tat-induce mice displayed a strong trend. These data suggest that cell death and degeneration may underlie the loss of gray matter
density detected by MRI in the same area. We also examined the prefrontal cortex, specifically the orbitofrontal and cingulate cortex, for microglia activation, as Tat has been shown to activate microglia in vitro (Bruce-Keller et al., 2001). The phenotype of microglia in the brains of the 7 day Tat-induced mice suggested more microglia activation and reactivity compared to both the prefrontal cortices of Dox-treated C57Bl/6J and uninduced GT-tg mice. In contrast, the 5 day Tat-induced mice did not show any differences in microglia morphology compared to control mice. This suggests that an increased duration of Tat exposure may correlate linearly with the magnitude of microglia pathology. Although MRI did not detect differences in these areas of the prefrontal cortex, inflammation and immune cell abnormalities may be a predecessor to gross anatomical changes and may itself have an impact on brain function and behavior.

Research suggests that HIV-Tat protein may be an important factor in the viral pathogenesis leading to the development and progression of HIV-associated neurotoxicity. However, few data are available detailing the neurotoxic effect of Tat in vivo. The use of structural MRI, DTI, and histology facilitated a neuropathological examination of alterations in the brains of Tat-induced mice. These results suggest that Tat may play a role in the development of CNS pathology and neurodegeneration associated with HIV infection and provides new insights into the biological basis for the behavioral syndrome of NeuroAIDS.
REFERENCES


The goal of this dissertation was to investigate the role of HIV-Tat protein in mediating behavioral dysfunction and neurodegeneration associated with NeuroAIDS. Specifically, this project determined the ability of Tat to mediate cognitive dysfunction, anxiety, behavioral disinhibition, and drug-seeking behavior. Furthermore, changes in gross anatomical brain structure as well as microstructure were also evaluated (see Figure 6.1 for a summary table of results and use as a reference throughout the discussion). These studies employed the GT-tg bigenic mouse (Kim et al., 2003), which is unique for its inducible and brain-selective tat gene, to test the central hypothesis that HIV-Tat protein, when expressed in brain, mediates behavioral dysfunction and neurodegeneration in an exposure-dependent manner.

I. EFFECTS OF TAT ON LEARNING AND MEMORY

Research suggests that HIV-Tat protein may create a potentially toxic environment (Aksenov et al., 2001) and may also directly induce damage to cells (Aksenov et al., 2003). Consequently, it has been suggested that Tat may play a crucial role in the neurotoxicity and cognitive impairment evident in NeuroAIDS (Rappaport et al., 1999). HIV-related cognitive deficits, which range from mild cognitive and motor dysfunction to global dementia, are thought to be in part the result of Tat expression in brain (Frankel and Young, 1998).

In Study 1, expression of Tat protein was linked to different types of learning and memory impairment. Tat-induced GT-tg mice demonstrated impaired acquisition of the Barnes maze, which is a spatial maze dependent on intact hippocampal function (Barnes et al., 1990;
### Figure 6.1. Summary of relevant findings

This table displays a summary of the behavioral alterations associated with Tat protein expression and the brain areas that may mediate these specific behavioral changes. Furthermore, neuropathological changes in gray matter density, white matter microstructural integrity, and histological markers in the implicated brain regions are also summarized. (↓ = decreases, ↑ = increases, GM = gray matter, WM = white matter)

<table>
<thead>
<tr>
<th>Behavioral alterations</th>
<th>Brain areas implicated in behavioral change</th>
<th>Brain-related changes in these regions</th>
</tr>
</thead>
<tbody>
<tr>
<td>↓ NOR</td>
<td>Perirhinal cortex</td>
<td>↓ GM density &amp; ↑ cell death</td>
</tr>
<tr>
<td>↓ NOR</td>
<td>Entorhinal cortex</td>
<td>↓ GM density</td>
</tr>
<tr>
<td>↑ anxiety &amp; ↑ disinhibition</td>
<td>Amygdala</td>
<td>↓ GM density</td>
</tr>
<tr>
<td>↑ anxiety &amp; ↑ drug craving</td>
<td>Insula</td>
<td>↓ WM microstructural integrity</td>
</tr>
<tr>
<td>↑ drug reward &amp; ↑ disinhibition</td>
<td>Striatum</td>
<td>↓ WM microstructural integrity</td>
</tr>
<tr>
<td>↑ disinhibition</td>
<td>Prefrontal cortex</td>
<td>↑ microglia reactivity</td>
</tr>
<tr>
<td>↓ spatial learning &amp; memory</td>
<td>Hippocampus</td>
<td>not determined</td>
</tr>
</tbody>
</table>
Bach et al., 1995). The GT-tg mice induced to express Tat demonstrated longer latencies to find an escape hole compared to their uninduced GT-tg littermates, suggesting impaired spatial learning. Moreover, Tat-induced GT-tg mice committed more total, reference, and working memory errors and favored a less efficient search strategy during acquisition than uninduced mice. Tat-induced mice also demonstrated poor probe trial performance, suggesting impaired spatial memory. Furthermore, reversal learning was impaired in Tat-induced mice, as the mice learned a new escape location slower than uninduced littermates.

Notably, strain differences in learning pattern were observed between the C57Bl/6J mice (both saline and Dox pretreated groups) and uninduced GT-tg mice. Although this was addressed in detail in the discussion of Study 1, it highlights the need for further investigation into the key phenotypic differences between the C57Bl/6J and GT-tg mice that may result in performance differences during some tasks. Furthermore, the use of multiple spatial mazes may provide further insight into the spatial learning and memory performance differences between the C57Bl/6J and GT-tg mice, and may also elaborate on the types of learning and memory deficits demonstrated by the Tat-induced GT-tg mice. In this study, the Barnes maze was chosen over the well-used MWM because the water-based maze may not be as ecologically valid for mice, as they are more likely to burrow than swim (D’Hooge and De Deyn, 2001; Wishaw and Tomie, 1996) and because mice are not as good swimmers as are rats, and easily develop hypothermia in water (Bach et al., 1995; Wishaw and Tomie, 1996). It has also been demonstrated that there are mouse strain- and task-dependent differences between water- and land-based spatial mazes (Patil et al., 2009). Therefore, a more fair evaluation of spatial memory may require the use of both water and land mazes. As various animal models of HIV-1 have displayed specific deficits in learning in memory in water-based spatial mazes, specifically the MWM (Sei et al., 1992; Zink et al., 2002; Griffin et al., 2004; Vigorito et al., 2007), we would hypothesize that the Tat-induced mice would continue to show spatial learning and memory.
impairment when tested in the MWM. Importantly, although, this effect highlights the importance of further characterizing spatial learning and memory deficits in Tat-induced mice, it does not negate the findings that the Tat-induced mice demonstrated acquisition deficits compared to uninduced GT-tg littermates.

There is a possibility that Tat-induced locomotor deficits could affect the latency of the mouse to escape the Barnes maze, confounding the results. An increased latency to escape the maze is considered an index of learning and memory ability; mice with intact learning and memory escape the maze more quickly. Notably, a deficit in locomotion could also increase the latency to escape the maze. However, in Study 3 we measured baseline locomotor activity in 7 day Tat-induced mice and verified that there were no significant locomotor deficits confounding the data. Although the locomotor testing was performed in a separate group of Tat-induced mice, the search strategy employed to escape the maze and the number or errors made during each trial was also suggestive of an impairment in the Tat-induced mice that were tested in the Barnes maze. It is notable that these measures would not be expected to be greatly affected by locomotor deficits. Overall, based on this combined evidence, we do not believe that locomotor impairment may account for the performance deficits in the Barnes maze by Tat-induced mice.

It is interesting, and somewhat counterintuitive, that the 5 day Tat-induced mice in some cases performed worse than the 7 day Tat-induced mice (e.g., probe trial) in the Barnes maze. This could potentially be attributed to different levels of motivational anxiety (Patil et al., 2009). Possibly supporting this, in Study 2, the 5 and 7 day Tat-induced mice showed exceedingly different anxiety and behavioral disinhibition profiles, particularly in the LD box and EPM (see further discussion in next section). However, overall Barnes maze performance was similarly impaired in the two Tat-induced groups, even when minor differences existed. This may be more a result of a cohort effect than the effects of anxiety. Nonetheless, an alternative explanation for the results of this study is that anxiety was responsible for the changes in
learning and memory behavior. For example, glucocorticoids released during anxious states, like corticosterone, have been reported to both enhance (Der-Avakian et al., 2005) and impair learning and memory performance (de Quervain et al., 1998), although this is further complicated by findings demonstrating that glucocorticoids are not always necessary (Wood et al., 2001) or sufficient (Beylin and Shors, 2003) to produce learning and memory deficits. Thus, the involvement of glucocorticoids and/or anxiety cannot be discounted entirely with the present results. However, performance in the Barnes maze has been demonstrated to be less susceptible to the effects of anxiety and corticosterone. Supporting this, a previous study demonstrated that anxiety measures were not significantly correlated with overall performance in the Barnes maze, and Barnes maze learning was not significantly associated with corticosterone levels (Harrison et al., 2009). On the other hand, performance deficits in the MWM were demonstrated to be correlated with corticosterone levels (Harrison et al., 2009), suggesting that spatial learning and memory may in part be affected by anxiety and stress and anxiety-mediating hormones. However, the Barnes maze may be more resistant to these effects. Therefore, it is unlikely that anxiety significantly affected Barnes maze performance in any systematic manner. However, to further clarify this issue, it would be beneficial to perform future experiments testing the same mice in behavioral models of anxiety prior to Barnes maze testing, directly seeking a correlation in a manner similar to the experiment described above. Also, a mediation analysis of Barnes maze performance, with anxiety status as a mediator, would also help to elucidate this issue further.

Tat-induced mice demonstrated deficiencies in novel object recognition (NOR), a behavioral model for a different type of learning and memory performance than the Barnes maze. Tat-induced mice displayed long-lasting (i.e., up to one month) NOR deficits. Furthermore, NOR impairment was dependent on the dose and duration of Dox exposure, suggesting that the amount and duration of exposure to Tat progressively mediated deficits.
interest, the brain regions that underlie performance on the NOR task are thought to be different than those regulating performance in spatial mazes. Research suggests that NOR performance may be largely mediated by the entorhinal and perirhinal cortices in the medial temporal lobe (Mumby and Pinel, 1994; Murray and Richmond, 2001; Murray et al., 2007; Aggleton et al., 2010). These results suggest that areas that subserve distinct types of learning and memory may be damaged by Tat protein expression. In fact, using MRI and the VBM analysis technique discussed in Study 4, reductions in gray matter density in the perirhinal and entorhinal cortices were found in the brains of 5 day Tat-induced mice. Furthermore, when the perirhinal cortices of 5 and 7 day Tat-induced mice were evaluated using a histological marker for cell death and degeneration, a greater amount of staining was observed than in the brains of control mice. Together, these results support that Tat does indeed damage areas underlying NOR performance.

It should be noted that anxiety and behavioral disinhibition could also affect NOR performance. It has been suggested that disinhibited animals seek novelty (Johansson and Hansen, 2001), while anxious animals avoid novelty (Weiss et al., 2000; note however that this is debated in the literature; see Li et al., 2010). However, Krskova et al. (2009) demonstrated that rats with an anxious phenotype showed increased anxiety in the EPM and, although the rats showed slightly less overall object exploration, they demonstrated intact NOR performance. This suggests that the NOR task may still be sensitive to learning and memory performance even when anxiety is a factor, as the anxious rats spent more time on the novel, rather than familiar object. Moreover, direct evidence from our study suggests that anxiety was not mimicking a learning and memory deficit. The 7 day Tat-induced mice showed anxiety-like behavior in the OF when tested 48 h after induction, but normal OF behavior when tested one week after induction. In NOR, the 7 day Tat-induced mice showed a deficit in NOR performance 48 h and one week after induction, showing a stable deficit in NOR. If NOR performance was
modulated by anxiety and not an actual learning and memory deficit, NOR should not have been impaired when tested one week after induction. Moreover, behaviorally disinhibited animals tend to demonstrate increased novelty seeking (Johansson and Hansen, 2001). This suggests that if behavioral disinhibition were driving the NOR results, that the Tat-induced mice would actually spend more time on the novel object than control mice. This was not the case in our study. Therefore, overall, we do not believe that anxiety or behavioral disinhibition were modulating the NOR deficit displayed in the Tat-induced mice. Although these effects cannot be entirely discounted, our data suggest a Tat induction-dependent persistent deficit in NOR attributable to learning and memory impairment.

In human studies, HIV infiltration is often identified in the hippocampus, and adjacent parts of the entorhinal cortex and temporal cortices (see Anthony and Bell, 2008 for review). Although we did not observe significant damage directly to the hippocampus by Tat protein, the deficit in spatial learning and memory in the Barnes maze is consistent with data collected from other animal models of HIV-infection showing impaired spatial ability in MWM performance (Sei et al., 1992; Zink et al., 2002; Griffin et al., 2004; Vigorito et al., 2007) and eight arm radial arm maze (Li et al., 2004). Furthermore, in vitro research suggests that Tat exposure can result in CA1 hippocampal and entorhinal cell dysfunction by suppressing LTP (Behnisch et al., 2004; Li et al., 2004), the neuronal basis for hippocampal-dependent learning (see Sarvey et al., 1989; Teyler, 1987-1988 for reviews). This suggests that cellular dysfunction, as opposed to gray matter density loss, may mediate the Barnes maze impairment observed in this study. Supporting this, Seilhean and colleagues (1995) did not find significant cortical or subcortical atrophy in a cohort of HIV-infected patients with cognitive dysfunction, suggesting that neuronal loss does not have to be present for cognitive impairment and may instead be due to neuronal dysfunction or changes in neurotransmitters.
The results of the Barnes maze and NOR tests, along with imaging results, provide evidence that Tat alone may be sufficient to induce behavioral dysfunction and brain damage. This study verifies that Tat protein may be a possible mediator of the cognitive deficits seen in HIV-infected individuals and may be responsible for some of the behavioral pathology seen in HAD. It is also one of the first studies to show that Tat expression is correlated with non-spatial learning and memory declines. It should be noted that previous research has demonstrated that expression of another HIV accessory protein, gp120, also impairs MWM (Glowa et al., 1992; D'Hooge et al., 1999) and Barnes maze performance (Sanchez-Alavez et al., 2000). It is notable that in vitro research suggests that Tat and gp120 can produce synergistic neurotoxic effects. Nath and colleagues (2000) demonstrated that subtoxic concentrations of Tat and gp120, when incubated together, resulted in neuronal cell death and prolonged increases in levels of intracellular Ca$^{2+}$. As such, future experiments examining the combined effects of gp120 and Tat on learning and memory performance would be of value in more accurately establishing the biological mechanisms mediating learning and memory deficits in patients suffering from NeuroAIDS.

II. MODULATION OF ANXIETY AND BEHAVIORAL DISINHIBITION BY TAT

Literature reports an increased incidence of anxiety disorders in HIV-infected patients (Scharko, 2006; Pence et al., 2006). Obviously, neuropsychiatric (NP) dysfunction could be a result of the psychosocial burdens of being infected with HIV. However, diverse evidence is emerging to suggest a number of possible physiological components that may, at least in part, mediate or increase the risk of developing NP disorders (see Chapter 3 for further discussion). Imaging studies have demonstrated abnormalities in areas of the brain implicated in the development of depression and anxiety, such as the dorsal frontal cortex (Ernst et al., 2002; Chang et al., 2003), amygdala, hippocampus (Castelo et al., 2006), and nucleus accumbens
(Paul et al., 2005) of HIV-infected patients. Furthermore, these areas have also shown to be subject to Tat toxicity (Aksenova et al., 2005; Bansal et al., 2000). Thus, it appears plausible that Tat protein activity may mediate or contribute to the increased risk of developing anxiety in HIV-infection.

The experiments in Study 2 were the first to demonstrate and evaluate the complex relationship between Tat and NP dysfunction. Tat-induced mice demonstrated Dox-induction dose- and duration-dependent increases in anxiety-like behavior in the OF, and duration-dependent increases in anxiety-like behavior in the LD box. As demonstrated by Western Blot analysis in Study 4, this Dox-dose dependency in GT-tg bigenic mice correlates with the expression of Tat protein in these animals. Curiously, the maximally Tat-induced mice did not display profound increases in anxiety-like behavior in LD box testing or when tested one week after induction in the OF. Even more interesting, mice induced to express Tat demonstrated a Dox dose- and duration-dependent increase in behavioral disinhibition, not anxiety, in the EPM. The U-shaped pattern of the data was similar to that seen in LD box testing, although the specific behavior observed was different. These studies overall suggest a Tat exposure-dependent modulation of two different, but possibly opposing behaviors: increases in anxiety and behavioral disinhibition. These behaviors may be dependent on the context in which they are tested (specifically in this case, the type of assay employed) and the extent of Tat protein exposure.

Dysfunction in the amygdala may be involved in pathological anxiety (see Cannistraro and Rauch, 2003 for review). The amygdala is a part of the limbic system that is involved in controlling fear and arousal (see Talarovicova et al., 2007 for review). This structure performs a primary role in the formation and storage of memories associated with emotional events; damage to the amygdala impairs the acquisition and expression of Pavlovian fear conditioning, a form of classical conditioning of emotional responses (Amunts et al., 2005). In Study 4, using
ex vivo MRI and VBM analysis techniques, we demonstrated that the brains of 5 day Tat-induced mice show reduced gray matter density in the amygdala. (A trend towards additional losses of grey matter density in the 7-day Tat-induced mice was also observed, although variability in this sample cohort precluded statistical significance.) Furthermore, we found significant loss of white matter microstructural integrity in the insula in the 7 day Tat-induced mice compared to uninduced GT-tg mice. The insula has also been implicated in emotional processing (Critchley et al., 2005) and anxiety (Paulus and Stein, 2006). Together, these data suggest that alterations and damage to the amygdala and insula may play a role in the anxiety behavior demonstrated by the Tat-induced mice in the OF and LD box.

Behavioral disinhibition is thought to be a manifestation of impulsivity observed in a variety of NP disorders such as ADHD, drug abuse, mania, and PD (Evenden, 1999; Moeller et al., 2001). Behavioral disinhibition is also evident in dementia (Zamboni et al., 2008; Raczka et al., 2010) and after brain injury (Rao et al., 2007; Ciurli et al., 2010). The behavioral disinhibition of EPM performance in Tat-induced mice is similar to results found in investigations using animal models of various types of dementia. Huntington’s disease mice have been shown to spend more time in the open arms of the EPM (File, 2001). Furthermore, Tg2576 Alzheimer’s disease (AD) transgenic mice developed disinhibited behavior in the EPM and, interestingly, demonstrated deficits in spatial object recognition (Ognibene et al., 2005), which is similar to the behavioral changes seen in the Tat-induced mice. The research investigating the biological underpinnings of behavioral disinhibition has suggested various mechanisms. It has been demonstrated that low D$_2$ mRNA and decreased D$_2$ receptor binding may lead to behavioral disinhibition in rats (Flagel et al., 2010). This is notable, given the progressive HIVE-induced down-regulation of striatal D$_2$ receptor expression (Gelman et al., 2006). Also, lesions to the cingulate have been shown to result in disinhibited behavior demonstrated by premature responding in the 5-CSRTT (Muir et al., 1996; Passetti et al., 2002). In Study 4, Tat-induced
mice were shown to have more microglia activation and reactivity in the cingulate cortex (and orbitofrontal cortex) compared to both the prefrontal cortices of Dox-treated C57Bl/6J and uninduced GT-tg mice. Although this is not a lesion per se, it suggests that the cingulate may be an area where Tat-induced pathology may have an effect on behavior, possibly behavioral disinhibition. Further studies report that the severity of disinhibition may be associated with gray matter loss in right mediotemporal structures (amygdala and hippocampus) (Zamboni et al., 2008). This is also relevant to our results discussed in Study 4, where Tat-induced mice demonstrated reductions in gray matter density in the amygdala and the amygdala-hippocampal area.

In summary, in Study 2 we have shown that Tat-induced mice demonstrate increases in anxiety-like behavior and behavioral disinhibition, dependent on the extent of Tat induction and time of testing. Admittedly, there is much work to be done in order to delineate the exact nature of the anxiety-like behavior, as well as if anxiety and behavioral disinhibition exist as co-morbid behaviors. With the use of more specific tests for impulsivity and other types of anxiety, these issues may be elucidated. Furthermore, histology and imaging results discussed in Study 4 indicate alterations in areas that may mediate anxiety and/or behavioral disinhibition and support a connection between the anatomical and behavioral changes noted in the Tat-induced mice. These studies provide evidence that NP dysfunction in HIV could be more than psychogenic in origin and suggest a physiological component.

III. THE INTERACTION OF TAT WITH THE REWARDING EFFECTS OF COCAINE AND REINSTATEMENT OF DRUG-SEEKING BEHAVIOR

In Study 3, the ability of Tat to modulate the rewarding and sensitizing effects of cocaine was examined. Once expressed, Tat produced sensitization to the locomotor effects of acutely administered cocaine, as a single injection of cocaine was sufficient to produce more
locomotion, which is typically seen after repeated exposure to cocaine. Furthermore, mice expressing Tat showed a greatly potentiated preference for the cocaine-paired chamber compared to C57Bl/6J or uninduced mice in cocaine-CPP, suggesting that brain expression of Tat protein enhanced the rewarding effects of cocaine. The magnitude of this effect was dependent on the dose and duration of Dox administered and the corresponding Tat induction. We also confirmed that the potentiated preference was not a transient event, and lasted as long as a “normal/typical” cocaine preference. Experiments further demonstrated the effects of Tat on an established reward state, with the induction of Tat protein significantly potentiating the effect of an additional exposure to place conditioning over both prior preference and the response of mice that were uninduced. Together, these experiments suggest that induction of Tat protein can potentiate reward in subjects with no previous experience with cocaine and in drug non-naïve subjects. Finally, we demonstrated that after extinction of cocaine preference in untreated GT-tg mice, induction of Tat protein directly resulted in reinstatement of cocaine-seeking behavior in the mice. These studies suggest that HIV-Tat protein is sufficient to enhance the locomotor effects of cocaine, potentiate the psychostimulant effects of cocaine, and reinstate cocaine-seeking behavior. As results suggest that Tat will potentiate the conditioned place preference response to cocaine, future studies determining if this potentiation will cross-generalize to other reinforcing psychostimulant drugs like methamphetamine or to opiate drugs like morphine would be of value.

It is interesting that the reinstatement of cocaine-CPP demonstrated by the Tat-induced mice was not potentiated. However, there is a key difference between the initial demonstrations of Tat-induced potentiation of reward and the reinstatement experiment. Tat was induced prior to conditioning in the experiments demonstrating potentiation of cocaine reward, whereas Tat was induced after cocaine conditioning in the reinstatement experiment. It may be that the synergy between the effects of Tat and cocaine is necessary to elicit a potentiated response,
but further tests are required to evaluate this hypothesis. Notably, these data parallel studies investigating stress-induced reinstatement of cocaine-seeking behavior. Exposure of animals to external stressors is known to potentiate cocaine reward in CPP (McLaughlin et al., 2003; Kreibich et al., 2009), similar to the findings in this study. However, when similar stressors are used to elicit reinstatement of cocaine-seeking behavior, the reinstated preference is not always found to be potentiated (Lu et al., 2002; Carey et al., 2007).

In Study 4, we found significant loss of white matter microstructural integrity in the insula and part of the striatum in the 7 day Tat-induced mice compared to uninduced GT-tg mice. The insula has been implicated in drug craving (Blakeslee et al., 2007; Vorel et al., 2007). Furthermore, the striatum has also been linked to drug reward (Di Chiara et al., 2004; Kalivas and Volkow, 2005). The damage observed in these areas may provide evidence for identifying the regions mediating the abnormal response of Tat-induced mice to drugs of abuse. The striatum may be of particular importance, as striatal damage and dysfunction is common in HIV-infected patients, particularly those suffering from HAD (Wang et al., 2004; Sporer et al., 2005; Gelman et al., 2006) and has been shown to be damaged by Tat in other models (Aksenov et al., 2001; 2003). Notably, post mortem analysis of brains from patients with HIVe demonstrated abnormalities in striatal dopaminergic markers, specifically significant increases in the presynaptic DAT where cocaine is known to bind, and significant decreases in the number of inhibitory D2 receptors (Gelman et al., 2006).

Although the direct biological mechanisms underlying the Tat-mediated alterations of the psychostimulant effects of cocaine are not known, Tat has been demonstrated to have a host of detrimental effects on the CNS. Tat-induced OS has been associated with neurotoxicity and loss of function (Price et al., 2005) in midbrain dopaminergic neurons (Aksenov et al., 2001). Furthermore, acute exposure to Tat can lead to rapid dysfunction of the DAT by decreasing DAT uptake (Wallace et al., 2006) and binding (Aksenova et al., 2006). Moreover, Tat has been
shown to inhibit DA uptake into striatal synaptosomes in a time- and concentration-dependent manner (Zhu et al., 2009). All this may contribute to decreased DA recycling, subsequently increasing extracellular DA to potentiate the psychostimulant effects of cocaine (Ferris et al., 2009). Having excess dopaminergic tone in mesolimbic “reward circuits” that project through the ventromedial striatum is strongly linked to the reinforcing effects of drugs and drug addiction (Di Chiara et al., 2004; Kalivas and Volkow, 2005). Additionally, research suggests that extracellular Tat may modulate intracellular signaling, either directly or through interaction with cellular receptors (Gibellini et al., 1998; Noonan and Albini, 2000). Tat protein modulates a number of intracellular pathways in vitro (Gibellini et al., 1998), notably ERK1/2 MAP kinase (Rusnati et al., 2001). ERK1/2 MAP kinase activity has been implicated in the reinforcing properties of cocaine (Valjent et al., 2000) and the reinstatement of cocaine self-administration (Lu et al., 2005). As such, activation of MAP kinase might serve as a mechanism by which Tat biologically potentiates drug reward. However, the host of interactions between Tat and the brain highlight many avenues by which Tat may influence drug reward, which will require additional research. For example, studies examining if ERK 1/2 MAP kinase suppression could suppress the drug-seeking behavior demonstrated by the Tat-induced mice in CPP testing would help to evaluate the role of MAP kinase in the Tat-induced potentiation of cocaine reward. Moreover, in vivo microdialysis of synaptic DA levels in Tat-induced mice with and without cocaine exposure would assist in identifying the neurochemical changes that occur in Tat-induced mice during the potentiated behavioral response to cocaine. Finally, studies examining self-administration of cocaine would corroborate the evidence presented in these studies if there is concordance between the behaviors demonstrated by the Tat-induced in these two assays.

It is also important to note that the interaction of psychostimulant abuse and Tat may also accelerate the progression of HAD (Nath, et al., 2002). HIV-infected psychostimulant users present with more marked neurological impairment than HIV non-drug users, and it is theorized
that this may be due to a synergy of abused drugs with HIV proteins like Tat to potentiate the neurotoxic effects of either insult alone (Nath et al., 2002). It has been suggested that DA neurons and pathways are most vulnerable to the effects of the synergistic neurotoxicity (Nath et al., 2000). Commonly abused psychostimulants, such as cocaine and methamphetamine are reinforcing due to their ability to activate dopaminergic pathways (Kalivas and Volkow, 2005), but this effect is neurotoxic over time (Cass et al., 2003). These drugs could potentially confer an increased risk of developing HAD or exacerbate preexisting HAD symptoms by inducing dysfunction in an already compromised dopaminergic system (Mirsattari et al., 1998). Although the effects of the synergistic toxicity of Tat and psychostimulants on HAD-related symptoms were not directly examined here, more research is necessary to characterize this phenomenon. This is particularly important as psychostimulant users comprise a significant segment of the HIV-infected population (Nath et al., 2002; UNAIDS/WHO, 2005) and because the incidence of HAD as an AIDS-defining illness may be on the rise (Saksena and Smit, 2005).

A. CONNECTION BETWEEN BEHAVIORAL DISINHIBITION AND DRUG ABUSE

There may be a connection between alterations of the DA system, behavioral disinhibition, and drug-seeking behavior. Moreover, evidence suggests impulsivity may be causally involved in drug abuse vulnerability (Perry et al., 2005). Impulsivity, sensation-seeking, and risk-taking behavior are various manifestations of behavioral disinhibition (Dawe et al., 2004). It has been demonstrated that loss of ventral striatum neurons in rats is associated with increased home case alcohol consumption and disinhibited behavior in a punished drinking test, with increased punished responding and reduced behavioral suppression (Johansson and Hansen 2000). Moreover, studies examining behavioral disinhibition in rats study showed that rats selectively bred for their disinhibited tendencies had a greater tendency to attribute incentive salience to cues predictive of both food and cocaine (Flagel et al., 2009), poor
inhibitory control over behavior (Flagel et al., 2009), increased propensity for risk-taking behavior (Clinton et al., 2008), and drug self-administration (Davis et al., 2008). These behaviorally disinhibited rats were also shown to have a hypersensitive DA system (Flagel et al., 2009); specifically they have a higher proportion of high affinity D\textsubscript{2} receptors (Seeman et al., 2005) in the dorsal striatum. Although changes in D\textsubscript{2} receptor affinity has not been documented in HIV-infected patients or in response to Tat exposure, there are specific changes in the DA system (discussed previously) noted in HIV-patients (Gelman et al., 2006) and after Tat exposure (Aksenov et al., 2001; Aksenova et al., 2006; Wallace et al., 2006; Ferris et al., 2009; Zhu et al., 2009) that may potentially disinhibit behavior and increase the propensity for drug-seeking behavior. However, more work is needed to elucidate if there is a causal link between behavioral disinhibition and drug-seeking behavior. Alternately, there may not be a causal link between the two behaviors, but rather a similar underlying mechanism that produces both increased rewarding effect of cocaine and the disinhibition of behavior, such as an alteration of the DA system. This too requires extensive evaluation beyond the scope of the thesis, but evidence presented here evokes an interesting vein of novel research that has implications for the mental health of NeuroAIDS patients.

IV. THE UTILITY OF THE GT-TG BIGENIC MOUSE MODEL

Although there are various animal models of HIV-infection that are useful in HIV research, the GT-tg mouse provides a specific animal model for investigating the specific impact of Tat on neurological and behavioral functioning. Notably, tat gene expression in the GT-tg bigenic mouse model relies on a GFAP promoter, limiting Tat protein production to astrocytes. There are limitations to this model as brain macrophages and microglial cells constitute the major cell populations that are productively infected \textit{in vivo} with HIV (Tardieu et al., 1992; Ensoli et al., 1993). However, there are also strengths associated with this particular model system.
Astrocytes represent a minor but consistently infected population of HIV-infected cells in human infection of the CNS. This HIV-infected cell type has been closely linked with the pathogenesis of HIV-associated neurological disease (Gorry et al., 2003; Nath et al., 2010). Astrocytes may be an important reservoir for HIV within the brain and release toxic viral products like Tat (Nath et al., 2002). Tat protein has been detected in HIV-infected astrocytes, and its release into the extracellular environment resulting in trimming of neurites, mitochondrial dysfunction, and cell death in neurons (Chauhan et al., 2003; Pocernich et al., 2004). Furthermore, there are no microglia/macrophage specific promoters commercially available and the GFAP promoter-driven gene expression in astrocytes has been successfully used to characterize CNS functions of a number of genes, including HIV-1, gp120, and ApoE4 (Campbell et al., 1998). Also the GFAP promoter in this model has been demonstrated to effectively targeted Tat expression in astrocytes, and in a Dox-dependent manner both in vivo and in vitro (Kim et al., 2003). The GT-tg mouse model has previously been demonstrated to show symptoms over time similar to those observed in HIV-1 transgenic studies and HIV infected humans, including failure to thrive, apoptosis, astrocytosis, neurodegeneration of cerebellum and cortex, degeneration of dendrites, inflammation, seizures, and premature death (Kim et al., 2003). The results suggest validity of the GT-tg mouse as a model for HIV-related neuropathologies. Here we have taken the mouse model a step further and demonstrated the utility of using the GT-tg mouse model to study the effects of Tat on NeuroAIDS-related behavioral dysfunction. It should be noted that while not used for this purpose in the present thesis, this mouse model also presents broader opportunities for the study of possible NeuroAIDS therapeutics that specifically target the effects or actions Tat protein.
V. NEUROANATOMICAL EFFECTS OF TAT PROTEIN AND POSSIBLE CONSEQUENCES

A. EFFECTS OF TAT ON GRAY MATTER DENSITY

As discussed, in Study 4, reductions in gray matter density in the amygdala, amygdala-hippocampal area, piriform, perirhinal, and entorhinal cortices were found in the brains of 5 day Tat-induced mice compared to Dox-treated C57Bl/6J mice. The amygdala performs a primary role in the formation and storage of memories associated with emotional events (see Pape and Pare, 2010) and may notably also be involved in the development of certain types of anxiety (see Cannistraro and Rauch, 2003 for review). The perirhinal and entorhinal cortices also have strong links to learning and memory formation, particularly visual recognition memory (Murray and Richmond, 2001; Aggleton et al., 2010). The piriform cortex is most closely associated with identification and memory of odors (Gavrilovici et al., 2010). When an ROI analysis was performed to test for between-group effects in regions exhibiting gray matter density differences in the whole brain analysis of 5 day Tat-induced mice, additional differences were observed between the 5 day Tat-induced and uninduced GT-tg mice as well as a difference between the 7 day Tat-induced mice and Dox-treated C57Bl/6J mice were revealed.

As stated, the amygdala performs a primary role in the formation and storage of memories associated with emotional events (Amunts et al., 2005). Furthermore, research also suggests the amygdala is involved in anxiety (see Cannistraro and Rauch, 2003 for review) and as such, damage to the amygdala may underlie some of the behavioral alterations noted in this study. Moreover, the amygdala may play a role in the perception of social signals and the maintenance of social position (Amaral, 2003). Given the amygdalar damage in Tat-induced mice, it would be of interest to examine social interactions of Tat-induced mice. Specifically, examining the social behaviors of Tat-induced mice with novel and familiar mice may help
evaluate sociability, social approach, and social interaction (Mines et al., 2010). Furthermore, a resident-intruder paradigm (McLaughin et al., 2006) could evaluate submission and dominance behavior. These studies may help to identify other behaviors affected by Tat-induced damage to the amygdala. Findings that social interactions are affected by Tat protein may have broad implications for the neuropsychiatric health of HIV-infected patients, such as implications for social anxiety and/or aggressive behavior.

The perirhinal and entorhinal cortices mediate certain types of learning and memory. The perirhinal cortex is a cortical region in the medial temporal lobe that receives highly processed sensory information and is thought to be important for both visual perception and memory (Witter and Wouterland, 2002). Lesions to the perirhinal cortex in both monkeys and rats lead to the impairment of visual recognition memory, disrupting stimulus-stimulus associations and object-recognition abilities (Murray and Bussey, 1999; Aggleton et al., 2010). The perirhinal cortex is bordered medially and ventrally by the entorhinal cortex, which forms the main input to the hippocampus. The entorhinal cortex-hippocampal system is known to pay a key role in memory consolidation (Fyhn et al., 2004; Hargreaves et al., 2005). This is relevant to this thesis, as research suggests that the type of NOR performance impaired in the Tat-induced mice (Study 1) may be largely mediated by the entorhinal and perirhinal cortices in (Mumby and Pinel, 1994; Murray and Richmond, 2001; Murray et al., 2007; Aggleton et al., 2010). The imaging data noting a loss of gray matter density in these areas provide a possible and probable underlying neurological correlate to the NOR impairment of the Tat-induced mice.

B. TAT-INDUCED CHANGES IN WHITE MATTER MICROSTRUCTURE

Significant loss of white matter microstructural integrity in the insula, endopiriform nucleus, and part of the striatum was observed in the 7 day Tat-induced mice compared to uninduced GT-tg mice, suggesting a progressive effect of Tat protein on white matter, with
increasing exposure to Tat resulting in more severe damage. It should be noted that, although the specific etiology of white matter abnormalities is undetermined, there is pathologic evidence that the FA measured using DTI, which was decreased in the 7 day Tat induced mice, correlates directly with the amount of myelin in white matter (Schmierer et al., 2007) and also possibly correlates with axonal membrane integrity (Beaulieu, 2002). The loss of white matter microstructural integrity has implications for the development of cognitive impairment (Vernooij et al., 2009), but the possible wide range of behavioral implications remains to be extensively evaluated. Nonetheless, white matter abnormalities may be among the most important neuropathologic lesions in HIV-infection and an early hallmark of HAD (Barker et al., 1995; Marcus et al., 1998). Furthermore, research suggests that even the mild cognitive impairment observed early in the HIV-infection may arise from affected white matter (Wu et al., 2006).

As discussed previously in the context of this study, and to summarize, the insula has been implicated in emotional processing (Critchley et al., 2005), anxiety (Paulus and Stein, 2006) and drug craving (Blakeslee et al., 2007; Vorel et al., 2007). Activation in the insular cortex appears to be heightened in many of the anxiety disorders (Shin and Liberzon, 2010). The endopiriform nucleus is involved in numerous physiological and pathological processes and may play a role in epilepsy (Kowianski et al., 2004). The striatum is involved in planning of movement (Penney and Young, 1986; Doyon et al., 1997) and drug reward (Di Chiara et al., 2004; Kalivas and Volkow, 2005). Moreover, it is notable that striatal damage and dysfunction is common in HIV-infected patients, particularly those suffering from HAD (Wang et al., 2004; Sporer et al., 2005; Gelman et al., 2006), and has been shown to be damaged by Tat in other models (Aksenov et al., 2001; 2003).

Interestingly, the differences in the insula, endopiriform nucleus, and striatum revealed between 7 day Tat-induced and the uninduced GT-tg mice were mostly observed in the right-hemisphere. This could be due to issues of low statistical power and a small effect sizes as
describe in Study 4. However, specific damage to the right-hemisphere may also have behavioral implications. Patients with right insular infarction have been shown to display a significantly higher frequency of underactivity and tiredness than patients with either left or non-insula lesions (Manes et al., 1999). Furthermore, damage to the right insula in patients with stroke-induced unilateral neglect was associated with impaired spatial working memory performance (Malhotra et al., 2005). Moreover, studies in patients with closed head injuries, brain tumors, stroke lesions, and focal epilepsy have demonstrated a significant association between disinhibition syndromes and dysfunction of orbitofrontal and basotemporal cortices of the right hemisphere (see Starkstein and Robinson, 1997 for review). Finally, a study of cerebral blood flow found that manic patients had significantly lower perfusion of the right basotemporal cortex as compared to normal controls patients (Migliorelli et al., 1993). Together, these studies suggest that concentration of damage to the right hemisphere may have specific implications for behavior.

C. HISTOLOGICAL ABNORMALITIES

In terms of histological findings, the perirhinal cortex was examined using a histological marker for cell death and degeneration. The left hemisphere of the perirhinal cortex was chosen because in Study 1 we found deficits in NOR that may be attributed to changes in this brain region; a recent clinical imaging study indicated that, although both hemispheres play a role in object memory, object encoding is partially lateralized to the left hemisphere of the perirhinal cortex (Belligowan et al., 2009). Results indicated that the brains of 5 and 7 day Tat-induced mice demonstrated greater amount of cell death in the perirhinal cortex, suggesting that cell death and degeneration may underlie the loss of gray matter density detected by MRI in the same area. These data corroborate previous studies where Tat has been shown to induce cell death in vitro (Jones et al., 1998; Aksenov et al., 2001; Eugenin et al., 2007) and in vivo (Kim et
al., 2003). Admittedly, this study was limited, as only a single brain region was evaluated. Western blot analysis of isolated brain regions (Study 4) suggests widespread expression of Tat protein throughout the brain of Dox-treated GT-tg bigenic mice. A more in depth characterization is needed to identify other brain regions where Tat may induce cell death. Specifically, it would be of interest to examine the hippocampus to determine if cell death may be, at least in part, responsible for the spatial learning and memory impairment in the Barnes maze associated with Tat protein expression in GT-tg mouse brain. Notably, previous histology studies of the Tat-induced GT-tg mice found a significant increase in markers for cell death in the hippocampus (Zou et al., 2007).

We also examined the prefrontal cortex, specifically the orbitofrontal and a cingulate cortex, for microglia activation, as Tat has been shown to activate microglia in vitro (Bruce-Keller et al., 2001). These brain regions were chosen for analysis because they have been implicated in behavioral disinhibition (Muir et al., 1996; Passetti et al., 2002) and have been shown to be affected in HIV patients (Ketlzer et al., 1990). The phenotype of microglia in the brains of the 7 day Tat-induced mice suggested more microglia activation and reactivity compared to both the prefrontal cortices of Dox-treated C57Bl/6J and uninduced GT-tg mice. In contrast, the 5 day Tat-induced mice did not show any differences in microglia morphology compared to control mice. This suggests that with a greater duration of Tat exposure there may be more pathology associated with inflammation. Although MRI did not detect differences in these areas of the prefrontal cortex, inflammation and immune cell abnormalities may be a predecessor to gross anatomical changes and may itself have an impact on brain function and behavior. Accessory-protein induced microglial activation may have a causal role in neurological aspects of HIV infection given the cytotoxic processes implicated in the neuronal alterations associated with HAD. When activated, microglia upregulate a variety of receptors and other molecules involved in inflammation and phagocytosis. In the activated state, microglia also
produce large amounts of ROS, which leads to an environment of OS (Giulian, et al., 1995). Additionally, there are alterations of microglial phagocytosis in the HAD brain. Microglia have been shown to phagocytize neighboring healthy neurons in a process that is referred to as “bystander lysis”. Similarly to the disruption of the balance between oxidation and endogenous antioxidant defense systems in the HAD, this appears to be another homeostatic imbalance, given that microglia usually act to phagocytize apoptotic neurons, not healthy neurons (Tyor et al., 1995).

**D. SUMMARY OF NEUROANATOMICAL FINDINGS**

Research suggests that HIV-Tat protein may be an important factor in the viral pathogenesis leading to the development and progression of HIV-associated neurotoxicity. However, few data are available detailing the neurotoxic effects of Tat *in vivo*. The use of structural MRI, DTI, and histology facilitated a neuropathological examination of alterations in the brains of Tat-induced mice. Furthermore, these studies provide initial evidence of the neuropathology that may mediate the behavioral dysfunction displayed by the Tat-induced mice (see Table 6.1 for a summary of results). Studies using *in vivo* longitudinal MRI to examine the progressive changes in the brains of the same Tat-induced mice over days of induction and several weeks subsequent to induction would be valuable. Although further research is needed to elucidate the biological underpinnings of neurological dysfunction in Tat-induced mice, these results suggest that Tat may play a role in the development of CNS pathology and neurodegeneration associated with HIV infection and provides new insights into the biological basis for the behavioral syndrome of NeuroAIDS.
VI. ALTERNATIVE MEDIATORS OF TAT-INDUCED DYSFUNCTION

In this thesis, it has been demonstrated that Tat-induced mice show behavioral abnormalities that may be mediated by loss of gray matter, loss of integrity of white matter microstructure in the brain, activated microglia, or cell death. These results are suggestive of structural brain changes. However, the anatomical evidence is limited, as functional changes in the brain could also underlie or at least contribute to the behavioral changes associated with Tat protein expression. Furthermore, Tat-induced mice demonstrated a broad range of behavioral alterations that cannot be fully explained by the anatomical data presented here. Studies using blood oxygen level-dependent functional MRI to evaluate the activity in brains of intact Tat-induced animals may permit the correlation of altered functional changes to structural damage and behavioral impairment. Furthermore, given the pleiotropic effects of Tat demonstrated in vitro, there are a variety of cellular mechanisms through which Tat may mediate the behavioral dysfunction observed in this study. These mechanisms do not negate the effects of Tat on behavior and brain, but provide potential indirect mechanisms though which Tat may alter functioning. Also, it should be noted the effects of the possible biological mediators do not negate the MRI or DTI findings, but provide evidence for biological processes affected by Tat that may not be addressed specifically in this thesis. Furthermore, the potential mediators of Tat-induced dysfunction discussed below are not meant to be an exhaustive examination of the effects of Tat protein (Chapter 1 has a more extensive overview), but rather a pointed discussion of the components that may alter function at the cellular level and may at least in part modulate some of the behavioral dysfunction associated with the effects Tat protein.

A. CHEMOKINES

It has been suggested that chemokine receptor signaling may modulate neuronal apoptosis and neurotoxicity (Klein et. al, 1999). Moreover, HIV-infected patients diagnosed with
neurological disorders have higher CSF levels of fractalkine, the ligand activating CX3CR1, as compared to HIV-infected patients presenting without neurological disorder (Tong et al., 2000). Likewise, in SIV-infected primates, release of proinflammatory cytokines has been demonstrated to impact cell functioning (Zink et al., 1998). By virtue of their inflammatory role, cytokines may play a role in the development of HAD and HIVE (Valcour and Paul, 2006). The i.c.v. administration of the HIV protein gp120 has been demonstrated to induce the expression of the cytokine IL-1β mRNA in the hypothalamus and was associated with reduced food consumption and weight loss (Barak et al., 2002). More relevant to our findings, Tat is noted as a strong inducer of chemokines in microglia and astrocytes (McManus et al., 2000; D'Aversa et al., 2005). In human microglia, application of Tat protein increases the production of the cytokines IL-1β, IL-6 and TNF-α (Nath et al., 1999; Bruce-Keller et al., 2001) and increases IL-6 levels in human astrocytes (Nath et al., 1999), potentially resulting in neurotoxicity (Gupta et al., 2008). Moreover, once microglia are activated by Tat the cells further upregulate cell surface proteins such as CD40; chronic upregulation of CD40 receptors further increases cytokine production, which triggers further upregulation of CD40 receptors, thus initiating a positive feedback loop (D’Aversa et al., 2005). Cytokines and Nuclear Factor kappa B (NFκB) have been observed to act in a similar fashion in monocytes and astrocytes (Nath et al., 1999), where NFκB signaling was necessary for the increased expression of IL1-β, IL-6, and TNF-α. These cytokines then function to increase NFκB activation, again creating a positive feedback loop. Unchecked, these two positive feedback loops present a significant neurotoxic threat and demonstrate how an initial Tat-induced activation of microglia may trigger a prolonged neurotoxic response in the absence of Tat (Haughey et al., 1999).
B. OXIDATIVE STRESS

OS is also thought to be a significant component underlying the neuropathology seen in HIV infection and HAD. Research suggests that OS may contribute to the normal aging process (Harman, 1992) and to the progression of neurodegenerative diseases (Nunomura et al., 2006). OS appears to play a critical role in the progression of HAD (Mollace et al., 2001). Indices of OS are consistently detected in brain tissue and CSF of those with HAD. Patients with HAD have shown increases in NO synthase in astrocytes and microglia and SOD activity in microglia, both markers for OS, when compared to HIV-infected patients without dementia (Adamson et al., 1996; Vincent et al., 1999). The OS evident in HIV has been attributed to the direct interactions of HIV proteins with neuronal cells as well as the result of chronic inflammation of CNS tissue in reaction to virotoxins (Nath et al., 2002). Significant evidence suggests Tat is involved in the initiation and/or intensification of OS (Kruman et al., 1998). Moreover, Tat-induced OS results in neurotoxicity and functional deficits in surviving cells, notably pyramidal hippocampal and midbrain dopaminergic neurons (Aksenov et al., 2001, 2003; Aksenova et al., 2005, 2006).

C. AMYLOID-BETA

Very recent emerging evidence warns of potential convergent mechanisms underlying HIV- and amyloid-beta-mediated neurodegeneration. It is thought that the imbalance between the production and clearance of the amyloid-beta peptide results in amyloid plaque formation, which then results in subsequent inflammation, OS, and neuronal degeneration (Hardy and Selko, 2002). Formation of these plaques is thought to at least partially contribute to the development of AD (Hardy and Selko, 2002). HIV-1 Tat from Clade B has been shown to promote the secretion of toxic amyloid-beta in primary rat fetal hippocampal cell cultures (Aksenov et al., 2010). Furthermore, Giunta and colleagues (2009) used a hybrid transgenic mouse by crossing a Tat transgenic mouse with the AD PSAPP transgenic mouse. The
PSAPP/Tat transgenic mouse showed significantly more amyloid-beta deposition, neurodegeneration, neuronal apoptotic signaling, and phospho-tau than PSAPP mice, suggesting that HIV-1 Tat significantly synergizes with amyloid-beta effects or even promotes AD-like pathology in PSAPP/Tat mice. These studies suggest that Tat may be able to produce AD-like pathology and may exacerbate the pathology in patients developing AD pathology independently. It also stands to reason that Tat may also influence behavior through the mediation of AD-like pathology. Although this is not an all inclusive list of the possible biological mediators of Tat-induced dysfunction and these were not specifically tested here, they show the broad milieu Tat’s effects and illustrate the many avenues of research that need more investigation to fully assess the role of Tat protein in NeuroAIDS dysfunction.

VII. FRONTO-TEMPORAL DEMENTIA SYMPTOMS

Typically HAD is described as a subcortical dementia, with prominent basal ganglia dysfunction (Berger and Nath, 1997). However, in these studies we found more supporting evidence for medial temporal lobe and frontal cortex damage and behavioral dysfunction more comparable to that of fronto-temporal dysfunction. For example, the major clinical manifestations of fronto-temporal dysfunction include addictive behaviors and disinhibition (Caycedo et al., 2009). It is important to note that subcortical and cortical dementias (e.g. Alzheimer’s disease) are not dichotomous disorders as previously believed (Savage, 1997). There is substantial overlap in the brain areas involved, functional changes observed, and the types of cognitive impairments, and it is therefore not surprising that cortical changes are apparent in HAD as well (Freedman et al., 1994; Moore et al., 2006). There is also evidence that in the era of HAART, the inflammation associated with HIV infection may have shifted from a subcortical pathology to a cortical pattern (Brew, 2004) and from a rapidly progressing condition to a more subtle neurodegenerative process (Xu and Ikezu, 2009). Furthermore, in the
pre-HAART era, the basal ganglia were the primary sights of strong infiltration with HIV, but now infiltration is often identified in the hippocampus, and adjacent parts of the entorhinal cortex and temporal cortices (see Anthony and Bell, 2008 for review). It may be that the continued production of Tat in the HIV-infected brain may be responsible for the shift to a more cortical neurodegenerative process and this merits further research. This research may shed light on the origins of the divergent manifestations of NeuroAIDS in the pre-HAART versus HAART era of HIV infection.

VIII. TAT AS A THERAPEUTIC TARGET

Overall, expression of HIV-Tat in GT-tg mouse brain was associated with deficits in spatial and novel object learning and memory, increased anxiety and behavioral disinhibition, a potentiation of cocaine reward, decreased gray matter density, loss of white matter structural integrity, cell death, and increased microglia reactivity. This research may begin to improve our understanding of the detrimental consequences of HIV infection in the brain. NeuroAIDS is characterized by progressive loss of cognitive and motor function, (Price et al., 1988; Glass et al., 1993), and is also linked to changes in mood (Rabkin et al., 1997), “personality” (Peters et al., 2008), and drug abuse (Nath et al., 2002; UNAIDS/WHO, 2005). This underscores that the calamitous consequences of HIV-infection are not limited to bodily immune dysfunction and loss of life. HIV-infection has dire consequences for a patient’s quality of life, beyond the effects of a failing immune system and social stigma, but through the actions of the virus and its associated proteins on brain integrity and function. NeuroAIDS-related behavioral dysfunction has further implications for poor HAART adherence which, as discussed in Chapter 1, can result to drug-resistant strains (McArthur et al., 2005). This highlights the need for specific treatments designed to address NeuroAIDS neurological and behavioral dysfunction.
These studies provide evidence that implicates Tat in NeuroAIDS related behavioral changes and neuropathology, but also implicate Tat as a therapeutic target for the treatment of NeuroAIDS. Although HAART has dramatically increased the life span of HIV-infected patients by reducing the development of many HIV-associated complications, these drug therapies have proven to be less effective at reducing neuronal dysfunction and behavioral disorders (Masliah et al., 2000; Mollace et al., 2001). In fact, with increased life expectancy, some reports suggest the prevalence of cognitive and neurological impairment in HIV-infected patients is actually rising (Sacktor et al., 2002; McArthur et al., 2003). This may be due to the fact that the CNS provides a reservoir or “sanctuary” for the virus in which there is restricted infection in a large number of undividing cells, like astrocytes (Ho et al., 1985; Levy et al., 1985; Resnick et al., 1985; Kleinschmidt et al., 1994). After a brief phase of productive infection, astrocytes enter a latent phase (Nath et al., 1995; Gorry et al., 1998; Lawrence and Major et al., 2002), where HIV can exist for an indefinite period. Research suggests that this latent reservoir for HIV infection in astrocytes may be crucial to the neuropathology that persists with the treatment of HIV with HAART. In this latent stage, HIV has the potential to elude traditional HAART drugs (Nath, 1999). Furthermore, in this state, HIV is thought to continue the production and possibly the over-expression of the viral regulatory proteins for release into the extracellular environment (Power et al., 1998; Brack-Werner, 1999; Johnston et al., 2001). It is also notable that despite the HAART-induced inhibition of viral replication, the continued presence of at least some of these viral proteins have been observed in humans (Agbottah et al., 2006; Vigano et al., 2006) and animal models (Peng et al., 2010), and may play a role in the persistent cognitive deficits and slow continuing neurodegeneration (Vigano et al., 2006). As there are currently there are no therapeutics developed to specifically target these issues, and given its pleiotropic functions, Tat protein represents a promising target for therapeutic development. Furthermore, as HIV-1 Tat
protein is essential in the virus life cycle, the broader implications of targeting Tat may be the control of virus replication and the prevention of disease onset (Fanales-Belasio et al., 2002).

Moreover, there is an increased incidence of brain lesions in AIDS patients with long-term survival over age-matched uninfected subjects (Maehlen et al., 1995) and cognitive impairment may be more profound in HIV infected older individuals compared to uninfected populations (Valcour et al., 2004; Saksena and Smit, 2005). Furthermore, there may be more susceptibility in HIV patients to develop AD comorbidity (see Xu and Ikezu, 2009 for review). The possible increased prevalence of age-related dysfunction may be due to a synergism of the activity of viral proteins associated with HIV-infection and the normal and abnormal declines associated with senescence, such as cognitive slowing and dementia, respectively (Valcour and Paul, 2006). Given the increased life expectancy of HIV patients on HAART, future studies examining the effects of Tat protein in the aging brain would be of particular value. Specifically, the GT-tg mouse could be used to evaluate the effects of induction of Tat protein in aged mice, with the underlying hypothesis that behavioral dysfunction and neuropathology would be increased over than of younger Tat-induced mice.

Also, post-mortem examinations find a greater number of amyloid-beta plaques, a hallmark of AD, in the brains of older AIDS patients compared to younger AIDS patients (see Mollace et al., 2001 for review). This implies that, in the aging HIV-infected population, not only might there be a synergism of HIV-associated neurotoxicity and normal declines associated with aging that contributes to dysfunction and damage, but HAD symptoms and neuropathology may also be compounded by events that are responsible for other age-related neurodegenerative disorders. As discussed, HIV-Tat may play a role in the secretion of toxic amyloid-beta (Aksenov et al., 2010) and the promotion and exacerbation of AD-like pathology. This further suggests that HAD and other age-related dementing illnesses may have similar underlying etiologies, and therefore a better understanding of HAD could also lead to new insights into
other neurodegenerative disorders. However, it is necessary for researchers to also bear in mind that HAD is associated with viral infection, which is not true of most other dementing illnesses. However, the targeting of Tat protein with therapeutics in order to reduce AD-like pathology in AIDS patients may be an important avenue of investigation. Furthermore, it suggests that drugs used to allay and suppress symptoms and damage associated with AD may have some utility in treating NeuroAIDS-related dysfunction.

Also notable, infection with the HIV-1 clade B is associated with higher rates of cognitive dysfunction than clade C (Satishchandra et al., 2000; Rao et al., 2008). Recently, it was demonstrated in vitro that Tat derived from clade C was significantly less neurotoxic to human neurons than Tat derived from clade B (Mishra et al., 2008), phenotypes attributed to differing abilities to induce mitochondrial dysfunction and OS. The Tat expressed in the GT-tg mouse model is derived from a clade B sequence, providing supporting evidence for neurotoxicity of clade B Tat protein. Furthermore, these results suggest behavioral correlates that may be associated with the damage caused by clade B Tat and may underlie the higher rates of cognitive dysfunction in patients infected with HIV-1 clade B. The creation of a clade C Tat transgenic mouse, similar to the GT-tg mouse model, would be of interest for further studies investigating the differences in toxicity between the different clade-specific Tat proteins. Significantly less pathology and behavioral dysfunction in a clade C expressing transgenic mouse would support this in vitro and epidemiological evidence. Furthermore, it would highlight the importance of developing clade-specific adjunctive treatment regimes in the HIV-infected population.

IX. CONCLUSION

This thesis addressed an under-investigated topic, identifying the contribution of HIV-1 Tat protein to neurodegeneration and neuropsychological impairments associated with
NeuroAIDS. There are limited behavioral studies examining the effects of Tat, and this dissertation was one of the first to do so using a transgenic animal with brain-selective and controllable Tat protein expression. A novel technique, *ex vivo* mouse MRI, was also employed in the research, adding a neuroanatomical correlate to the behavioral examination of Tat protein effects. Evaluating the consequences of Tat activity in the brain may simultaneously improve our understanding of the neurological underpinnings of NeuroAIDS and the neurodegeneration associated with HIV-1 infection. Also, understanding the effects of Tat could help identify a target for development of preventative therapies or adjunctive countermeasures for the treatment of the neurodegeneration and behavioral dysfunction associated with HIV infection. This is particularly important given that antiretroviral drugs are less effective at allaying the effects of HIV in the brain. Furthermore, with increased survival, but persistence of cognitive decline, it becomes increasingly important to understand the potential mediators of NeuroAIDS-related dysfunction.
REFERENCES


Barnes CA, Markowska AL, Ingram DK, Kametani H, Spangler EL, Lemken VJ and Olton DS.


Johansson AK & Hansen S. (2001) Increased novelty seeking and decreased harm avoidance in rats showing Type 2-like behaviour following basal forebrain neuronal loss. Alcohol. 36: 520-524.


in the Barnes maze, the Multiple T-maze and in the Morris water maze. Behav Brain Res. 198: 58-68.


