THE EFFECT OF METHAMPHETAMINE ON ASTROCYTES WITH IMPLICATIONS FOR FELINE IMMUNODEFICIENCY VIRUS AND CXCR4

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By

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*****

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ABSTRACT

There is a growing population of human immunodeficiency virus-1 (HIV-1) infected drug abusers and epidemiological evidence suggests that the psychostimulant drug methamphetamine (METH) is a risk factor for HIV dementia. However, the mechanism by which this occurs is not well defined. The feline immunodeficiency virus (FIV) is a lentivirus that causes a natural immunodeficiency syndrome in cats including encephalitis and neurological symptoms resembling HIV dementia in humans. Astrocytes maintain homeostasis in the brain and may participate in the FIV-induced neurotoxicity and/or act as a reservoir for FIV infection. Our findings show that METH (1 µM) had short lived proliferative effects on cell growth of G355-5 cells, a feline astrocyte cell line, with a peak at 24 h. During this time period, METH promoted cell-mediated FIV infection of astrocytes. These results indicate that METH may increase FIV infection by one or both of the following mechanisms: cell proliferation and/or expression of lentiviral entry receptors. Recent studies show that both OX40 and CXCR4 are important for FIV entry into cells. FIV infected peripheral blood mononuclear cells (PBMC) are needed to mediate FIV infection of G355-5 cells. However, FIV-infected PBMC in the presence of METH did not increase cell proliferation or RNA expression of OX40 or CXCR4 expression until 24 h. G355-5 cells exposed to METH however, increased astrocytic CXCR4 RNA expression at early time points (3, 6 and 12 h). The presence of METH also increased cell surface CXCR4 receptor expression at 12 and 24 h. The increase in RNA expression, in addition to the increase in total CXCR4 protein strongly suggests CXCR4 protein synthesis. These results indicate that an increase in proliferation and upregulation of CXCR4 receptors on G355-5 cells may contribute to METH-induced susceptibility of astrocytes to FIV infection.
These results lead us to further study the role of METH on the modulation of CXCR4 receptors in primary rat astrocytes. This animal model more closely mimics the human disease. In this group of studies, we confirmed that METH increased expression CXCR4 RNA (3 and 6 h) and surface protein expression (12 and 24 h). Furthermore, studies with pharmacological inhibitors indicated that the METH modulation of CXCR4 expression on astrocytes was occurring through reactive oxygen species (ROS) and nuclear factor-kappa B (NFkB). Taken together, these results suggest that METH may alter astrocytes to increase the brain’s vulnerability to disease.
DEDICATION

Dedicated to my parents, family and my husband.
ACKNOWLEDGMENTS

I would like to acknowledge many people for helping and supporting me during my doctoral work. First, I am grateful to my parents, Dr. and Mrs. Hien Tran, Dr. and Mrs. Wu-Hsiung Tseng and my sister, Dr. Justina Tseng for all of the support and love they have given me. Most of all I would like to thank my husband, Dr. Ray Tseng whose support gave me the strength and courage to stand up for myself. I feel very lucky to have such a supportive husband. I would like to thank the Neff Laboratory including Dr. Maria Hadjiconstantinou, Dr. Norton Neff and Dr. Anne-Marie Duchemin for their guidance and advice early in my graduate studies. I am grateful to both Trina Wemlinger and Debra Moore for technical support and friendship. I also would like to acknowledge the Whitacre laboratory, Dr. Fei Song, Aaron Kithcart, Ingrid Gienapp, Todd Shawler, Jessica Williams and Kristin Smith for welcoming me into their scientific family. I wish to thank my good friend Erin Fink for her friendship through difficult times and Erik and Kelly Hill for letting me stay with them for many months. During these short months I had a lot of help many people from many laboratories. In addition to Dr. Whitacre’s lab, I would especially like to thank both Dr. Mike Farrow and Jacki Mays in Dr. Sheridan’s lab for their real-time PCR expertise and Dr. Richa Tripathi in Dr. Popovich’s and Dr. McTigue’s lab for the immunofluorescent images. I am extremely grateful to my
graduate committee (Dr. Mathes, Dr. Sanders and Dr. Sheridan) who has been instrumental in my development as a scientist. I would like to thank Dr. Lawrence Mathes for his guidance and insights as a co-advisor throughout my graduate studies, particularly in virology and immunology, and Dr. Virginia Sanders, whose scientific and personal encouragement has gotten me through one of the most difficult experiences of my life. I am very grateful to Dr. John Sheridan for his time, wise counsel and guidance. This dissertation would not be possible without Dr. Caroline Whitacre. I have complete admiration for Dr. Whitacre and her sense of doing what is right in a difficult situation. She has shown me a side of science that is worth fighting for and has set professional standards for me that I hope, over my scientific career, that I will able to live up to.
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<td>α</td>
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<td>AMPA</td>
<td>Alpha-Amino-3-Hydroxy-5-Methyl-4-Isoxazole Propionic Acid</td>
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<td>AIDS</td>
<td>Acquired Immunodeficiency Syndrome</td>
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<td>AMPH</td>
<td>amphetamine</td>
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<tr>
<td>bcl-2</td>
<td>B cell Lymphoma 2</td>
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<td>bax</td>
<td>bcl-2-associated X protein</td>
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<tr>
<td>β</td>
<td>beta</td>
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<tr>
<td>BBB</td>
<td>blood brain barrier</td>
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<tr>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>calcium</td>
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<td>CCR5</td>
<td>CC motif chemokine receptor-5</td>
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<td>CNS</td>
<td>central nervous system</td>
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<td>CXCR4</td>
<td>C-X-C motif chemokine receptor-4</td>
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<tr>
<td>CD4</td>
<td>cluster of differentiation 4</td>
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<tr>
<td>COCA</td>
<td>cocaine</td>
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<td>ConA</td>
<td>concanavalin A</td>
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<tr>
<td>CuZn-SOD</td>
<td>Cooper, Zinc- superoxide dismutase</td>
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<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<td>DA</td>
<td>dopamine</td>
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<td>DAT</td>
<td>dopamine transporter</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>DFF</td>
<td>DNA Fragment Factor 45</td>
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<td>FIV</td>
<td>feline immunodeficiency virus</td>
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<td>FACS</td>
<td>fluorescence-activated cell sorting</td>
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<td>GPCRs</td>
<td>G protein-coupled receptors</td>
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<td>γ</td>
<td>gamma</td>
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<tr>
<td>GABA</td>
<td>γ-aminobutyric acid</td>
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<td>GFAP</td>
<td>glial fibrillary acidic protein</td>
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<td>GLU</td>
<td>glutamate</td>
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<tr>
<td>gp</td>
<td>glycoprotein</td>
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<td>HIV-1</td>
<td>human immunodeficiency virus-1</td>
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<td>HIVD</td>
<td>HIV-1 Associated Dementia</td>
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<tr>
<td>IFN</td>
<td>interferon</td>
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<td>IL</td>
<td>interleukin</td>
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<td>lactate dehydrogenase</td>
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<td>lipopolysaccharide</td>
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<td>MIP-1</td>
<td>Macrophage inflammatory protein 1</td>
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<td>mRNA</td>
<td>messenger RNA</td>
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<td>METH</td>
<td>methamphetamine</td>
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<td>MDMA</td>
<td>3,4-methylenedioxymethamphetamine</td>
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<td>MAO</td>
<td>monoamine oxidase</td>
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<td>MCP-1</td>
<td>Monocyte chemotactic protein-1</td>
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<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>nNOS</td>
<td>neural nitric oxide synthase</td>
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<tr>
<td>NO</td>
<td>nitric oxide</td>
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<tr>
<td>NE</td>
<td>norepinephrine</td>
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<tr>
<td>NERT</td>
<td>norepinephrine transporter</td>
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<td>NFκB</td>
<td>nuclear factor-kappa B</td>
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<td>PCR</td>
<td>polymerase chain reaction</td>
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<td>ROS</td>
<td>reactive oxygen species</td>
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<tr>
<td>RANTES</td>
<td>regulated on activation normal T Cell expressed and secreted</td>
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<tr>
<td>RT</td>
<td>reverse transcriptase</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
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<tr>
<td>5-HT</td>
<td>serotonin</td>
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<td>SERT</td>
<td>serotonin transporter</td>
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<tr>
<td>SDF-1</td>
<td>stromal cell-derived factor-1</td>
</tr>
<tr>
<td>SI</td>
<td>syncytium inducer</td>
</tr>
<tr>
<td>T cells</td>
<td>T-lymphocytes</td>
</tr>
<tr>
<td>NED</td>
<td>N-1-naphthylethylenediamine dihydrochloride</td>
</tr>
<tr>
<td>NBQX</td>
<td>1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzo[f]quinoxaline-7-sulfonamide</td>
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<td>TNF-α</td>
<td>tumor necrosis factor</td>
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CHAPTER 1
INTRODUCTION

I. INTRODUCTION

Acquired Immunodeficiency Syndrome (AIDS) was first reported in the United States in 1981 and is now considered a worldwide pandemic. AIDS follows infection with the human immunodeficiency virus (HIV). HIV progressively destroys the body’s immune system by preferentially infecting CD4 bearing T-lymphocytes (T cells), causing the body to be vulnerable to infection and various cancers. The incidence of HIV infection is 40,000 cases each year in the United States. This rate has not substantially decreased in recent years. As of 2006, an estimated 37.2 million adults worldwide are living with HIV/AIDS and worldwide estimates indicated that 2.9 million people have died from AIDS despite the development of antiretroviral therapy that reduces AIDS-related deaths (Harris and Bolus, 2008 and Holtgrave, 2007).

II. HIV ASSOCIATED DEMENTIA (HIVD)

HIV Dementia (HIVD) is also called AIDS dementia complex, HIV encephalopathy or NeuroAIDS. HIVD affects 30% of individuals in the late stages of AIDS and is a significant cause of disability (McArthur et al, 1993; Price et al., 1988, Kaul et al., 2001, Gonzalez-Scarano and Martin-Garcia, 2005). The clinical symptoms of HIVD include impaired short-term memory, concentration deficit, and psychomotor slowing that progressively lead to dementia (Wesselingh and Thompson, 2001). The pathological changes occur as a part of HIV infection of the central nervous system
(CNS), also known as HIV encephalitis. HIV encephalopathy is characterized by formation of multinucleated giant cells, diffuse myelin pallor, perivascular inflammation, reactive astrocitosis, macrophage/microglial activation and proliferation, atrophy and astrocyte and neuronal loss (Gorry et al., 2003; Wesselingh and Thompson, 2001). Current therapies for HIV, including highly active antiretroviral therapy (HAART), do not provide complete protection from HIVD or the reversal of the disease. Also, because of the availability of antiretroviral drugs, HIV infected individuals are living longer and the prevalence of dementia is increasing. There is no current therapy for HIVD and understanding of the disease may provide potential therapies (Kaul et al., 2001).

The pathogenesis of HIVD involves HIV entry into the brain early after systemic infection (Gonzalez-Scarano and Martin-Garcia, 2005). The mechanism(s) of how HIV-1 invades the CNS is largely unknown. There are two prevailing views: 1) HIV enters the CNS by transcytosis or by direct infection of endothelial cells or 2) the “Trojan horse hypothesis” by which HIV acts as a passenger in peripheral blood cells that traffic through the blood brain barrier (BBB) into the CNS (Gonzalez-Scarano and Martin-Garcia, 2005).

Irrespective of the mechanism of initial entry, after HIV-1 invades the CNS, it is still unclear how the virus causes neuronal injury and astrocyte dysfunction (Kaul et al., 2001). HIVD rarely occurs before the onset of advanced HIV disease, and some have hypothesized that there may be a threshold number of infected and activated immune cells in the CNS to cause neuronal injury due to immune and BBB dysfunction. The CNS disease may persist over time because the CNS may not be exposed to significant quantities of antiretrovirals. HIV does not directly infect neurons (due to lack of CD4, the principal receptor for HIV entry). It is hypothesized that neuronal cell may be caused by several possible mechanisms (Gabuzda et al., 1998; Kaul et al., 2001). It is possible that toxic viral envelope or structural proteins (gp120, Tat, gp41, Nef) act directly or perhaps via chemokine receptors on neurons to cause their death (Levy, 1998; Kaul et al., 2001). Alternatively, viral proteins, infection by HIV-1 or immune activation may cause CNS resident macrophages or/and microglia, to release excitotoxins (glutamate, quinolinic acid and its metabolites), lipid mediators (arachidonic acid, its metabolites, and
platelet-activating factor), free radicals (nitric oxide and superoxide), cytokines (TNFα, IL-1α and β, IL-6, TGF-β), or chemokines that may subsequently induce toxicity in neurons (Kaul et al., 2001; Wesselingh and Thompson, 2001; Lawrence et al., 2002; Jones and Power, 2006).

III. FIV AS ANIMAL MODEL FOR HIV:

Feline immunodeficiency virus (FIV) infection occurs naturally in cats and induces a disease similar to acquired immunodeficiency syndrome (AIDS). The feline disease has the same characteristic of progressive depletion of CD4+ T lymphocytes. FIV can be transmitted via mucosal exposure, blood transfer, as well as pre- and post-natal routes (Elder et al., 2008). FIV and simian immunodeficiency virus are the only two animal models, in which immunodeficiency and neurological impairment characteristic are both observed, similar to HIV-1.

FIV infection has three distinct clinical phases, each of which is characterized by specific clinical or pathological signs:

1. **Acute phase** - flu-like signs, increasing viral loads, febrile episodes, weight loss, lymphadenopathy

2. **asymptomatic period** - relatively strong antiviral immune responses, low viral titers, a gradual decline in CD4+ cells and minimal clinical signs

3. **clinically active disease** - with overt immunodeficiency and neurological signs defined by immunologic decline, exacerbation of plasma viral load and clinical signs of immunodeficiency with opportunistic infections (Elder et al., 2008, Nesbit et al., 2002, Podell et al., 2000).

The genomic structure of FIV is somewhat similar to HIV, but FIV lacks Vpr, Vpu and Nef, as “accessory” genes, that are present in the HIV genome (Elder et al., 2008). However, both are retroviruses and contain reverse transcriptase which transcribes viral RNA (ribonucleic acid) to viral DNA (deoxyribonucleic acid) in the host’s cell cytoplasm and they undergo a similar viral life cycle. The viral DNA is
inserted into the host’s DNA in the host cell’s nucleus where is may be quiescent or replicate actively. The viral DNA is transcribed into messenger RNA (mRNA), which then leaves the nucleus to enter the cytoplasm where it is translated into polyproteins. The viral protease then cleaves the polyprotein into proteins to assemble into a virion. The virion is formed at the plasma membrane of host cells, where the virion fuses with the host membrane and new viral buds form at the surface (Levy, 1998; Harris and Bolus, 2008).

The mechanism of FIV viral entry and infection of target cells closely parallels that of HIV, with respect to the interactions of the viral envelope with binding and entry receptors. FIV is tropic for T cells, macrophages and CNS cells (Elder et al., 2008). FIV utilizes the chemokine receptor CXCR4, similar to HIV-1, to enter target cells (Patrick et al., 2002; Podell et al., 2000). Studies have shown that FIV infection of certain cells may be solely mediated by CXCR4 if the expression is sufficiently high (deParseval and Elder, 2001; Joshi et al., 2005). The primary receptor for FIV entry was recently revealed to be CD134, also known as OX40 (Shimojima et al., 2004). OX40 is a member of the tumor necrosis factor (TNF) receptor/nerve growth factor superfamily. Primary isolates of FIV use OX40 and CXCR4 as a coreceptor for infection. Researchers hypothesize that primary binding of OX40 causes conformational changes in FIV surface glycoproteins that facilitate high affinity binding to CXCR4. Subsequent fusion events involve cell membrane and transmembrane proteins to facilitate viral entry (Elder et al, 2008). Studies show that FIV targets activated CD4 T cells that express upregulation of OX40 (deParseval et al., 2004). As the disease progresses, FIV may lose its dependence on the interaction with OX40 and more efficiently interact with CXCR4 (Willett et al, 2006).

Similar to HIV, FIV may cause active encephalopathy. In the brain, FIV infects microglia, macrophages and astrocytes and causes neuronal loss in the cortex (Zenger et al., 1997). FIV infection is rapid and persistent in the CNS, providing an excellent animal model for the neuropathogenesis of lentiviruses. FIV enters the brain early after infection and this is likely to occur via the blood-brain and blood cerebrospinal fluid barriers. As with HIV, the primary method of viral entry into the CNS is via infected lymphocytes
and monocytes/macrophages through blood-brain and blood-choroid plexus connections (Fletcher et al., 2008). FIV may also infect the CNS via cell-free virus or direct infection of endothelial cells and astrocytes of the BBB. Recent studies show that astrocytes may increase transmigration of immune cells (Hudson et al., 2005; Fletcher et al., 2008).

FIV and HIV induce similar neuropathological changes, including gliosis, microglial activation, perivascular mononuclear cell infiltration, multinucleated giant cells, neuronal damage and loss. Neurological manifestations include delayed auditory and visual evoked potential changes and sleep pattern alterations (Phillips et al., 1994, Elder et al., 2008). The behavioral and neurophysiological impairment of the infected cat, as in HIV, is not directly linked to neuronal infectivity. Cats infected at a younger age present more severe neurological signs than cats infected in life (Podell et al., 2004). The neurological manifestations persist throughout the disease, however, and in later stages of the disease, the CD4 T cells decline and viral load increases causing increasing susceptibility to opportunistic infection. In summary, FIV represents an excellent animal model for HIV; since the viruses share common structural and biochemical properties, the clinical signs are similar, FIV is a reproducible disease model, it is a naturally model, there is relatively low cost of cats, availability of specific pathogen free (SPF) cats and the nervous and immune systems of cats are well characterized. Substantial research focuses on FIV to further develop both drug and vaccine treatments for HIV, especially since FIV provides the opportunity to test interventions in vivo in felines (Elder et al., 2008). Therefore, for many reasons, FIV is a good model to study the temporal course of the pathogenesis of HIVD (Podell et al., 2000).

IV. PSYCHOSTIMULANTS: METHAMPHETAMINE

Psychostimulants refer to a group of highly addictive compounds including cocaine (COCA), amphetamine (AMPH) and substituted amphetamines, such as METH, 3,4 methylenedioxymethamphetamine (MDMA, ecstasy) and methylphenidate (Ritalin). Psychostimulants have immediate effects such as an increased in energy, cardiovascular stimulation, euphoria, suppression of fatigue and decreased appetite (Nestler, 2001, Rothman et al., 2003, Barr et al., 2006). Prolonged use of psychostimulants can cause
disordered thought processes, including severe psychotic episodes. Psychostimulants have strong addictive and reinforcing properties and are subsequently commonly abused. The addictive use consists of intermittent binges and escalating intake leading to significant morbidity and mortality (Nestler, 2001). Despite the potential for abuse, therapeutic uses for psychostimulants have been identified and include use as a local anesthetic and treatment for attention deficit disorder with hyperactivity and narcolepsy (Rothman et al., 2003).

Psychostimulants interact with monoamines (dopamine (DA), norephinephrine (NE) and serotonin (5-HT)) neurons in the central nervous system. Cocaine (COCA) and amphetamines have similar overall effect of increasing the monoaminergic neurotransmission (Rothman et al., 2003). However, the mechanism utilized to increase neurotransmission is different. Amphetamines are considered substrate-type releasers because they enter through monoaminergic transporters of cells and then displace both vesicular and intracellular monoamines whereas COCA directly blocks the monoamine transporters (Davidson et al., 2001).

A. METHAMPHETAMINE

METH is one of the most commonly abused psychostimulant drugs. Other names that METH is commonly known as are speed, go, crystal meth, ICE, and poor man’s cocaine (Yu et al., 2002). There has been a recent surge in popularity of the drug especially in the past two decades, leading to the belief that METH abuse is a growing epidemic (Cadet et al., 2003, Barr et al., 2006). The 2004 National Survey on Drug Abuse and Health estimates that 1.4 million people use METH in the United States (SAMHSA). Recently, it has been estimated that as many as 600,000 people in the US use the drug on a weekly basis (Roehr B, 2005). The US National Association of Counties reported that METH usage was responsible for more emergency department visits than any other drug (Cadet et al., 2003). In addition, recreational METH users often use METH in combination with other drugs. The growing METH use is not limited to the United States. The World Health Organization, in 1996, estimated that over 35 million people worldwide regularly used METH, second only to cannabis and surpassing the
number of cocaine and heroin users (Rawson et al., 2002; Thompson et al., 2004, Barr et al., 2006).

METH is a synthetic compound, derived from a one-step reduction process of ephedrine or pseudoephedrine, ingredients that are commonly found in cold and asthma medications (Ellinwood, 1998, Barr et al., 2006). METH can be administered in a variety of ways: inhaled, vaporized, orally ingested, intravenously injected or administered as a rectal suppository. The intoxicating effects of METH may last up to 12-16 h (Barr et al., 2006). METH is highly lipid soluble and therefore can rapidly transverse the BBB to directly affect the CNS. The acute effects of METH, similar to other psychostimulants, include increased energy and alertness, decreased appetite, feelings of power and invulnerability, tachycardia, hypertension, myocardial ischemia, cerebrovascular accidents and hyperthermia. These effects can escalate until users develop severe insomnia, aggression, and anxiety (acute negative side effects) as well as more chronic effect such as hallucination, paranoia and schizophrenia (Hart, 2001, Barr et al., 2006).

Acute methamphetamine intoxication is treated with supportive care and second-generation atypical antipsychotics such as, aripiprazole, risperidone and quetiapine. Another current therapy focuses on agonist-like replacement similar to methadone for morphine addiction. Similar to antipsychotics, drugs that increase dopamine levels may be effective in treating METH addiction. These drugs include selegline (monoamine oxidase, MAO inhibitor), buproprion or GBR12909 (DA reuptake inhibitor) and lobeline (inhibits uptake of DA into synaptic vesicles). A new promising therapy is immunopharmacotherapy, which is based on the generation or administration of antibodies that are capable of binding to METH before it can reach the brain (Barr et al., 2006). Long-term consequences of chronic METH use is associated with physical problems such as, weight loss/malnutrition, depression, dental caries, heart failure, infection of the skin and impaired cognitive performance. METH withdrawal/termination is also associated problems with depression, anergia, agitation and insomnia. Symptoms can last from weeks to months, with relapses to methamphetamine use common (Colfax and Guzman, 2006, Barr et al., 2006).
METH, like other amphetamines, causes an overall increase in the levels of monoamines, NE, 5HT, and principally DA (Barr et al., 2006). A majority of the METH literature focuses on DA. METH has pharmacological effects as a potent releaser of monoamines through many different molecular processes. METH increases levels of cytosolic monoamines in between cells by several means including release of monoamines from synaptic vesicles (Brown et al., 2001), inhibition of MAO activity (Mantle et al., 1976) and increase in tyrosine hydroxylase (DA-synthesizing enzyme) activity and expression (Mandell et al., 1970, Hotchkiss et al., 1980). Additionally, METH increases monoamine levels by affecting monoamine transporters by reversing transport (Khoshbouei et al., 2003) and blocking the activity and expression of monoamine transporters (Schmitz et al., 2001, Sauders et al, 2000, Barr et al., 2006). The high levels of DA are thought to be responsible for the acute physiological and psychological effects of METH as well as its reinforcing properties (Davidson et al., 2001, Fleckenstein et al., 2000, Riddle et al., 2006, Barr et al., 2006). The long-term consequences of increased DA levels include reduction in dopamine transporter (DAT) activity and the degeneration of dopaminergic nerve terminals (Fleckenstein et al., 2000; Riddle et al., 2006 and Colfax and Guzman, 2006).

DAT plays a major role in METH-induced dopamine release and toxicity. Investigators hypothesize that there are high concentrations of DAT in terminal regions of the nigrostriatal dopamine pathway (i.e. the striatum) which is quite susceptible to METH-induced neurotoxic effects are most vulnerable (Eisch et al., 1992). Animal studies of genetically engineered mice without the DAT (Fumagallo et al., 1998) and mice treated with DAT blockers (bupropion, Marek et al., 1990) show decreased vulnerability to the neurotoxic effects of METH. Depletion of DA using pharmacological agents (such as reserpine or α-methyl p-tyrosine) decreases the neurotoxic effects of METH administration (Lew et al., 1997). These results in mice have similar to findings in humans. Long-term users of METH have 50-60% reduction in dopamine levels in the striatum (nucleus accumbens, caudate, putamen) as measured in chronic users of METH (Wilson et al., 1996). Genetic mutation of the DAT gene
(3’VNTR, variable number tandem repeat) has been linked to long-term psychosis after METH termination (Barr et al., 2006; Ujike et al., 2003).

Neurotoxic effects of METH on 5HT and NE is less studied. While METH seems to affect both DA and 5HT function, and another substituted amphetamine, MDMA, seems to be more specific to the 5HT terminal markers (Quinton et al., 2006). There is a decrease in overall levels of 5HT and serotonin transporters (SERT, Hanson et al., 2004; Itzhak et al., 2004), however, the effects are much more diffuse than DA to different regions of the brain (prefrontal cortex, hippocampus, striatum, Brunswick et al., 1992). Yu and Liao (2000) reported serotonergic neurotoxicity after METH treatment and administration of a SERT blockers showed neuroprotection (Davidson et al., 2001). Interestingly, METH has the greatest capacity to stimulate NE of all the monoamines however, it is the least studied (Rothman et al., 2001). There have also been reports showing a decrease in NE transporter (NET) binding (Brunswick et al., 1992). METH facilitates NE release from adrenergic nerve terminals and inhibits its reuptake which gives users a feeling a euphoria (Yu et al., 2002).

METH causes neural damage, but the precise mechanism is unclear. Initially, it was believed that METH-induced toxicity was limited to monoaminergic terminals. Recent in vitro studies suggest that neurons undergo apoptosis (Cadet et al. 1997 and Stumm et al. 1999). In the following paragraph, I will briefly outline many pathways that have been implicated in METH-induced toxicity including oxidative stress, excitotoxicity and hyperthermia.

METH causes an increase in DA in the extracellular space (via reverse transport of DAT). Excess amounts of DA in the extracellular space results in free radical formation either by enzymatic oxidation or auto-oxidation. Along with quinone and semiquinone formation, there is production of superoxide radicals and hydrogen peroxide (also called reactive oxygen species, ROS). The administration of antioxidants or free radical scavengers such as vitamin E, N-acetylcysteine and ascorbic acid (Wagner et al., 1986, Fukami et al., 2004, Quinton et al. 2006) and the transgenic overexpression of the Cu,Zn- superoxide dismutase (CuZn-SOD) gene, each attenuate METH-induced toxicity (Cadet et al., 1994). Another source of oxidative stress has been proposed to be
mitochondrial disruption causing the inhibition of the electron transport chain (Davidson et al., 2001). These findings establish ROS, specifically oxygen-based free radicals, as part of the toxic action of METH.

Neuronal cell death is often associated with excitotoxic damage. It has been documented that METH increases extracellular concentrations of glutamate in the striatum and the hippocampus, secondary to DA release. Glutamate (GLU) antagonists (including N-methyl-D-aspartate (NMDA), AMPA and metabotropic glutamate antagonists) attenuate the METH-induced toxicity (Quinton et al., 2006, Kita et al., 2003). GLU induced toxicity is hypothesized to be linked to the production of nitric oxide in some models (Cadet et al., 2003). Some suggest that glutamate activates the NMDA receptor allowing calcium (Ca\(^{2+}\)) into the cell. Once inside the cell, Ca\(^{2+}\) binds to calmodulin activating nNOS (neural nitric oxide synthase) to produce NO and peroxynitrite (Davidson et al., 2001 and Cadet et al., 2003). nNOS inhibition by either pharmacological treatment (7-nitroindazole) or targeted gene disruption (Davidson et al., 2001 and Itzhak et al., 1996) is neuroprotective under conditions of METH treatment (Kawasaki et al., 2006, Kita et al., 2003).

Changes in temperature can influence METH toxicity (Albers and Sonsalla, 1995; Cadet et al., 2003). Drugs that prevent hyperthermia and even cool ambient temperatures have been shown to abate the toxic effects of METH (Miller and O’Callaghan, 2003, Davidson et al., 2001). All of the pathways described above, including oxidative stress, excitotoxicity and hyperthermia, could lead to neuronal apoptosis during METH-induced neural damage. It has been established that components of the biochemical apoptotic cascade are activated after METH treatment, such as caspase 3 and 9, proapoptotic B cell lymphoma (bcl-2) family genes, bcl-2-associated x protein (bax) expression and DNA Fragmentation Factor 45 (DFF45, Cadet et al., 1997, 2003). Interestingly, p53 knockout mice have been shown to have an attenuated response to METH in terms of neurotoxicity. P53 is an apoptosis promoting factor and a tumor suppressor gene. It is regulated by the transcription factor, nuclear factor \(\kappa\) B, (NFkB), which is activated by ROS (Asanuma et al., 1998, 2002, Hirata and Cadet, 1997). These are all pathways that
METH has been observed to activate and interestingly, by these pathways are also activated during HIV infection.

**B. METHAMPHETAMINE AND THE IMMUNE RESPONSE**

Recent studies suggest that METH may have immunomodulatory activity particularly for the cellular response (Barr et al., 2006, Colfax and Guzman, 2006, Yu et al., 2003). Two studies by Yu et al., and In et al., report that *in vivo* administration of METH significantly reduced concanavalin A (ConA) -induced T-cell proliferation. Both studies found suppression of interleukin-2 (IL-2) production after METH treatment (Yu et al., 2002 and In et al., 2005). Both *in vitro* and *in vivo* administration of METH caused an increase in natural killer cell cytotoxicity (House et al., 1994, In et al., 2005, Saito et al., 2006). In et al., (2005) also reported reduced lipopolysaccharide (LPS)-induced B cell proliferation after METH treatment. However, Yu et al., (2002) did not find a difference after METH treatment B cell proliferation. Yu et al., (2002) further investigated the cytokine profile and found that interferon-γ (IFNγ) production was suppressed while TNFα was enhanced. Reduced macrophage numbers and activity including phagocytosis, cytotoxicity, nitric oxide (NO) production, TNFα, IL-1 and IL-6 were observed after METH administration (In et al., 2004). Iwase et al, 1996 found that high concentrations of METH *in vivo* caused apoptotic cell death in the thymus and spleen. In summary, METH has immunosuppressive affects on immune cells therefore influence intracellular communication in the immune system as well as the CNS.

Studies investigating the effect of other amphetamines have found results similar to those associated with METH. House et al. (1994) found that high concentrations of AMPH *in vitro* suppressed IL-2 secretion and LPS-induced B cell proliferation. *In vivo*, Kubera et al. (2002) found a decrease in ConA-induced T-cell proliferation and Freire-Garabal et al. (1991) found that chronic AMPH administration reduced peripheral T-cell populations *in vivo*. MDMA, another structurally related compound, has also been found to reduce lymphocyte proliferation in response to Con A and LPS as well as to enhance secretion of TNFα (Connor et al., 1998 and Connor et al., 2000). *In vitro*, MDMA has
also been found to increase natural killer cell cytotoxicity (House et al., 1995). Amphetamines, as a group of drugs, thus appear to cause immune dysfunction. Since these effects are somewhat varied, it is important for further studies to be conducted to better clarify the effects of each AMPH drugs on immune function.

V. ASTROCYTES

Astrocytes are the most abundant cell type in the CNS. Astrocytes maintain homeostasis in many ways including participation in formation of the blood-brain barrier, releasing growth factors (neurotrophic factors), regulating neurotransmitter release and regulating the immune response (Gee and Keller, 2005). Astrocytes have been shown to remove neurotransmitters such as DA, 5HT, NE, gamma-aminobutyric acid (GABA) and GLU from the extracellular space via their respective transporters (Inazu et al., 1999; 2001; Seifer and Steinhauser, 2001; Schools and Kimelberg, 2001).

Astrocytes play a crucial role in protecting the CNS from insult. For example, astrocytes remove excess excitatory amino acids (GLU), detoxifying the local environment. Astrocytes contribute to both the structural and functional integrity of the CNS and protect the brain (Gorry et al., 2003). A common hallmark of many neurodegenerative diseases is astrogliosis (Hatten et al., 1991, Dong and Benveniste, 2001), a process whereby astrocytes proliferate and undergo morphological changes such as the upregulation of glial fibrillary acidic protein (GFAP). GFAP is an intermediate filament present in mature astrocytes and is used as a marker for this cell type (Dong and Benveniste, 2001). Astrocytes have the ability to produce a wide variety of cytokines and chemokines that facilitate neuronal-glial interactions (Gorry et al., 2003). Thus, astrocytes play a pivotal role in determining the type and extent of CNS immune and inflammatory responses (Dong and Benveniste, 2001).

A common characteristic of HIV CNS infection is astrogliosis (Power et al., 1997, Jones and Power, 2006). Astrocytes are infected at low levels because they lack CD4, in addition to having intracellular inhibitors that block HIV-1 synthesis and post-translational modification. Astrocytes are infected in a CD4-independent manner by T-tropic strains of HIV-1. Infected astrocytes do not produce newly synthesized viral
proteins nor release infectious virus. The lentiviral infection of astrocytes makes it very difficult to eliminate cellular reservoir of HIV-1 in the brain (Wesselingh et al., 2001). In HIVD, a number of astrocyte functions are disrupted and it is not known how this occurs. Astrocytes normally act as a buffer, balancing neurotoxicity and neuroprotection (Levy, 1998; Lawrence et al., 2002), however, in HIVD there are excess levels of neurotoxins (Wesselingh and Thompson, 2001). Astrocyte function is not the only change in the microenvironment as productive infection of microglia occurs along with altered functionality of the BBB which might affects the homeostasis of neurons. Astrocytes are also important in relaying and amplifying neurotoxic signals from activated or HIV-infected microglia (Wesselingh and Thompson, 2001).

Thompson et al., (2001) conducted a clinical study which demonstrated a strong correlation between the numbers of HIV-1 DNA positive astrocytes and rapidly progressive dementia. Moreover, a correlation between the degree of astrocyte apoptosis and the severity and rate of HIVD progression was found (Thompson et al., 2001). Astrocytes are susceptible to Fas (CD95) induced apoptosis, which is mediated by the ccpp32/caspase-3 pathway. Exposure of astrocytes to a microenvironment of HIV-1 antigens or infected microglial cells or stimulation with cytokines such as, TNF-α, may substantially increase astrocyte susceptibility to cell death. Astrocytic cell death implies a loss of these cells’ and their capacity to control the CNS inflammatory response and detoxify excitatory neurotoxins (Gorry et al., 2003, Wesselingh and Thompson, 2001).

Evidence indicates that there is inflammation and an immune response to METH within the CNS (Thomas et al., 2003). Both types of glial cells, microglia and astrocytes are activated during METH-induced neurotoxicity (Thomas et al., 2004, Kawasaki et al., 2006). Microglia are thought to play a role in METH-neurotoxicity. Microglia are considered the resident immune cells of the CNS because of their ability to phagocytose and secrete factors such as ROS and cytokines (LaVoie et al., 2004 and Thomas et al., 2004). There are conflicting results as to whether microglial activation precedes METH exposure. However, microglia seems to participate throughout the process of neuronal damage (Asanuma et al., 2003, LaVoie et al., 2004, Thomas et al., 2005).
Many studies have demonstrated that METH induces astrogliosis in vivo and in vitro (Pu and Vorhees, 1993, Pu et al., 1994, Fukumara et al., 1998, Stadlin et al., 1998). Stadlin et al., 1998 treated cultured mouse astrocytes with high concentrations of METH, which resulted in activation and cell death. These studies also showed a difference in response depending on the site of origin of the astrocytes. Cortical astrocytes were more resistant to toxicity than striatal or mesencephalic astrocytes. Lau et al., 2000 using a similar experimental design reported an increase in ROS after METH treatment. This was confirmed in vivo when administration of radical scavengers prevented astrocyte activation (Kawasaki et al., 2006). In vivo, astrocyte activation persists, peaking at day 2 and lasting for at least 7 days after METH exposure after METH exposure (O’Callaghan and Miller, 1994). Even though numerous studies have shown that these glial cells are affected by METH, it is still unclear whether the activation of both glial cell types can be neuroprotective and aid in the recovery of function or contribute to neurotoxicity.

VI. CHEMOKINE AND CHEMOKINE RECEPTOR: SDF-1/CXCR4

Chemokines are a group of proteins that regulate leukocyte trafficking by interactions with specific receptors (Bajetto et al., 2002, Ruibal-Ares et al., 2004). The first chemokine was discovered in 1987 and now the chemokine family has grown to more than 50 separate molecules that interact with at least 20 receptors (Bajetto et al., 2002b). In addition to effecting chemotaxis of immune cells, chemokines have been implicated in other biological responses including enzyme secretion, cell adhesion, cytotoxicity, tumor cell growth, degranulation, inflammation, establishment of microenvironment, organogenesis and T cell activation (Bajetto et al., 1999; Bajetto et al., 2002). Chemokines are classified into groups by the number and spacing of conserved N-terminal cysteines. Chemokines that lack of cysteines are given the designation C, those with two cysteines adjacent (CC, β chemokines), those with cysteines separated by one amino acid residue (CXC, α chemokines) or three amino acid residues (CX3C) (Bajetto et al., 2001; Murphy et al., 2000; Rollins, 1997). Chemokines in the CNS are postulated to participate in neurogenesis, neuronal survival and neuromodulation (regulate neurotransmission and neuron-glia communication, Bonavia et al., 2003). More
importantly, brain chemokines participate in pathological processes such as multiple sclerosis, Alzheimer's disease, HIV-1 encephalitis, ischemia and brain tumors (Bajetto et al., 2002).

CXCR4 is expressed constitutively in the brain, heart, kidney, liver, lung and spleen (Nagasawa et al., 1996b). CXCR4 is the only identified receptor for stromal cell-derived factor 1 (SDF-1 also called CXCL12). The interaction between SDF-1 and CXCR4 is unique since most other chemokines bind to multiple receptors (i.e. CCR5). SDF-1 was originally defined as a pre-B cell stimulatory factor, but was later found to be a chemotactic factor for T cells, monocytes, dendritic cells and haematopoietic progenitor cells. There are three isoforms (\(\alpha\), \(\beta\), \(\gamma\)) of SDF-1 defined by differential splicing of a single gene. SDF-1\(\alpha\) is expressed intensely in astrocytes compared to SDF-1\(\beta\) (Ohtani et al., 1998). SDF-1 is highly conserved and there is only one amino acid difference between the human and mouse proteins (Nagasawa et al., 1994). Homozygous CXCR4 deficient mouse were embryonically lethal. SDF-1 deficient mice exhibit haematopoietic and cardiac defects and abnormal cerebellar development similar to CXCR4 deficient mice (Nagasawa et al., 1996b; Zou et al, 1998). CXCR4 and SDF-1 seem to specifically regulate neuronal migration in cerebellum and their deletion results in disruption of laminar architecture (Ma et al., 1998). Both SDF-1 and CXCR4 are expressed in the CNS specifically in neurons, astrocytes and microglial cells (Ohtani et al., 1998; Bajetto et al., 1999).

SDF-1 and its receptor CXCR4 are expressed in astrocytes under normal conditions in vivo and in vitro, but their regulation is not well characterized. Bajetto et al. (1999) demonstrated that CXCR4 and SDF-1 are expressed on rat cortical type-I astrocytes. SDF-1 stimulation of CXCR4 induced Ca\(^{2+}\) mobilization in astrocytes (Bajetto et al., 1999). Bajetto et al. (1999) proposed that CXCR4 can be downregulated by autocrine stimulation of SDF-1, as shown by a desensitized functional Ca\(^{2+}\) response. Bajetto et al., (2001) reported that SDF-1, behaved like a growth factor, in that it directly stimulated the proliferation of cultured astrocytes via Bordetella pertussis toxin-sensitive G-protein, phosphatidyl inositol-3 kinase and extracellular signal-regulated kinase activation. Furthermore, they hypothesized that CXCR4/SDF-1 may play a role in glial
proliferation, in an autocrine and paracrine regulated manner, in physiological and pathological conditions such as brain development, reactive gliosis and tumor formation (Bajetto et al., 2001). Bezzi et al., 2001 found that SDF-1 stimulated the release of glutamate from astrocytes and influenced synaptic activity independent of the presence of CXCR4 receptors on neurons. They proposed that SDF-1 intervenes in communication between glial and neurons, specifically glia-mediated regulation of synaptic transmission (Bezzi et al., 2001).

Chemokine receptors are seven transmembrane domain G-protein-coupled receptors (GPCR), which are expressed on various tissues such as hematopoietic cells, vascular endothelial cells, neurons and glia (Bajetto et al., 2001). CXCR4 and CCR5 are expressed in many different areas of the adult rat brain including the cortex, cerebellum, hippocampus, striatum and brainstem (Ohtani et al., 1998; Bajetto et al., 1999). In addition to their physiological roles in chemotaxis and development, the chemokine receptors, CCR5 and CXCR4 are also coreceptors for HIV. The \( \beta \) chemokine receptor CCR5 is expressed on macrophages and infection with HIV does not result in the formation of syncytia (initial stage of the disease). This observation is in contrast to HIV infection of T cells using CXCR4, which does cause syncytium formation (SF, late stages of the disease) (Ruibal-Ares et al., 2004). HIV infection is initiated by the fusion of the viral envelope with the cell membrane of the target cell. The fusion process requires binding of the membrane glycoprotein-120 (gp120) surface component of the viral envelope with CD4 and either CCR5 or CXCR4. The interaction with the coreceptor triggers a rearrangement of the transmembrane subunit of the envelope glycoprotein-41 (gp41) that leads to the fusion of the virus and the target cell membrane. HIV binding to the CD4- chemokine coreceptor complex is followed by HIV-membrane fusion and triggers biochemical signals that are important for the efficiency of infection. CCR5 is the receptor for CC chemokines including regulated on activation normal T cells expressed and secreted (RANTES), macrophage inflammatory protein (MIP)-1\( \alpha \) and MIP-1\( \beta \) and monocyte chemotactic protein (MCP)-1 and acts as an HIV-1 co-receptor. RANTES is the best studied member of this family of chemokines. In vitro activated astrocytes respond with increased production of RANTES to a variety of inflammatory stimuli,
including other chemokines, TNF-α, quinolinic acid, β-amyloid and HIV-1. Interestingly, RANTES can stimulate the production of other CC chemokines such as MCP-1, which has been shown to prevent apoptosis of neuron and astrocytes induced by NMDA or HIV-Tat. CXCR4 is also able to bind to gp120 (HIV-1 capsid glycoprotein) and may play a role in HIVD (Lazarini et al., 2000). This viral protein seems to mimic SDF-1 and induces the release of glutamate from astrocytes which can induce neuronal death (Bezzi et al., 2001). Chemokines may also contribute to HIVD by promoting the recruitment of monocytes and lymphocytes that facilitate HIV entry and spread within the brain (Bonavia et al., 2003).

VII. CONCLUSION: METHAMPHETAMINE AND HIV

METH use is a known risk factor for HIV infection (Page-Shafer et al., 1997, Buchbinder et al., 2005, Seage et al., 1992) and it has been hypothesized that METH lowers user’s inhibitions and encourages sexual activity. Recent data suggest that METH actually enhances HIV neurotoxicity (Rippeth et al., 2004). The relationship between METH use and acceleration of HIV-induced immune decline is not clearly defined. However, there is a growing body of evidence that METH’s harmful effects on the brain are exacerbated in HIV-infected individuals. There is an increased incidence of HIV encephalitis in drug users (Bell et al., 1998; Bouwman et al., 1988), suggesting that they are more sensitive to the neurological effects of HIV. Nath et al (2001) observed that METH and cocaine use accelerate HIVD even with antiretroviral therapy, and it was suggested that HIV and METH might act synergistically to cause neuronal death (Nath et al., 2001; Maragos et al., 2002). It has also been shown that Tat (HIV related viral protein) or HIV and METH synergistically deplete striatal dopamine levels and induce the loss of dopamine terminals (Maragos et al., 2002; Hauser et al., 2007). Furthermore, Turchan et al (2001) found that in human fetal neuronal culture, HIV-1 Tat plus METH caused neurodegeneration. In vivo, Chang et al., (2005) found declining neuronal function and increased glial activation in proportion to METH use in HIV patients.

Notably, the same toxic factors have been implicated in the HIV-1 neurotoxicity and neuronal death. However, the underlying mechanism of the interaction of HIV-1 and
METH that results in neural damage is not known. Furthermore, there is no currently available therapy for the growing population of neuroAIDS patients. It is therefore important to understand the synergistic neurotoxic effects of HIV-1 and METH on HIVD. Understanding these mechanisms will facilitate the development of new therapeutic strategies, and more importantly provide useful clues for early prevention.

METH may alter the astrocytic response, contributing to the synergistic effects of METH and HIV. Studies suggest that astrocytes can contribute to the pathogenesis of lentiviral encephalopathy (Fox and Phillips, 2002). The primary goal of this study was to utilize the feline model of HIVD to understand how METH may regulate infection of astrocytes either through proliferation or regulation of CXCR4. Thus I undertake the following:

1. To determine the effect of METH treatment on FIV infectivity of PBMC and astrocytes, using an astrocytic cell line (Chapter 2)

2. To determine the effects of various doses of METH on astrocyte function using an astrocyte cell line (Chapter 3)

3. To determine the effects of METH on primary astrocytes and the underlying mechanism (Chapter 4).
CHAPTER 2

METH AND CELL-ASSOCIATED FIV INFECTION OF ASTROCYTES

I. INTRODUCTION

METH is one of the most commonly abused drugs among HIV-infected individuals (National Institute on Drug Abuse, 2002, Carey et al., 2006). METH use has been shown to be a risk factor for HIV infection (Page-Shafer et al., 1997, Buchbinder, 2005 and Seage et al., 1992). In 2005, it was reported that HIV seropositivity was 6.3% in METH-users compared to 2.1% in nonusers (Buchaz et al., 2005). There is also an increased incidence of HIV encephalitis in METH users (Bell et al., 1998, Bouwman et al., 1998, Urbina et al., 2004, Chang et al., 2005), suggesting increased susceptibility to the neurological effects of HIV (Rippeth, 2004). Both METH dependence and HIV infection have been independently shown to cause brain dysfunction (Carey et al., 2006). Epidemiological evidence shows that METH and HIV have additive deleterious effects on neuropsychological performance (Carey et al., 2006). HIV and METH may act synergistically to cause neuronal cell death (Maragos et al., 2002; Turchan et al., 2001) and exacerbate inflammation (Flora et al., 2003; Conant et al., 2004, Yu et al., 2002). However, the mechanism is not clearly understood.

HIV associated dementia (HIVD) is a chronic neurological disorder that affects 20-30% of HIV infected individuals (McArthur et al., 1993). It has been shown in the HIVD model that infected glial cells undergo activation and release neurotoxic mediators such as cytokines, reactive species, quinolinic acid, prostagladins, arachidonic acid and HIV proteins (Nath and Geiger, 1998). Feline Immunodeficiency Virus (FIV) is a naturally occurring lentiviral infection in cats with similar clinical stages to HIV.
including encephalopathy. Therefore, FIV represents both a clinically relevant and easily manipulated animal model to study HIVE. In the brain, FIV infects macrophages, microglia and to a smaller extent astrocytes. Astrocytes maintain homeostasis and may participate in the FIV-induced neurotoxicity and/or act as a reservoir for FIV infection. It has been shown that FIV predominantly infects microglia/macrophages and astrocytes from in vitro brain-derived cells (Fox and Philips, 2002).

Similar to HIV-1, CXCR4 is used as a co-receptor that mediates viral entry into cat lymphoid cells and is expressed on many cell types. FIV infection of astrocytes has been shown to be dependent on CXCR4 (Nakgaki et al., 2001). CD4 is the primary receptor for HIV and CD4 facilitates efficient interaction of HIV with the entry chemokine receptor CXCR4 to trigger fusion of viral and cellular membranes (de Parserval et al., 2004). Until recently, the primary FIV entry receptor was not known, but in 2004, Shimojima et al., found that CD134 (OX40), an analog to CD4 in humans serves this role in the cat.

Our laboratory has shown that METH enhances cell-associated FIV replication in astrocytes. Feline astrocytes (both primary and cell line G355-5) can be infected by the wild type FIV-MD isolate through cell-to-cell transmission with infected peripheral blood mononuclear cells (PBMC). This transmission depends on a functional CXCR4 receptor (Gavrilin et al., 2002). Others have reported similar results in vitro (Phillips et al., 2000). The purpose of these experiments was to further explore the properties (cell viability and expression of OX40 and CXCR4) of METH enhanced cell-associated infection. Our hypothesis is that METH alters cell proliferation and expression of FIV entry receptors to facilitate cell associated FIV infection.

II. MATERIALS AND METHODS

A. Cell Culture Techniques

i. Peripheral blood mononuclear cells (PBMC)

Peripheral blood mononuclear cells (PBMC) were obtained purified from specific pathogen-free, FIV-tested negative adult cats under approval of the Institutional
Laboratory Animal Care and Use Committee according to the NIH Care and Use of Laboratory Animals Guidelines. Feline PBMC were stimulated with 0.9 µg/ml concanavalin A (ConA, Calbiochem-Novabiochem Corp, San Diego, CA) and 20 U/ml human IL-2 (Hoffmann-La Roche Inc, Nutley, NJ) for 5 days and then infected with 100 TCID₅₀ of FIV-MD per 5x10⁴ cells. The cells were then maintained for an additional 5 days prior to co-culture (Day 10), as described in Gavrilin et al., 2002. PBMC were continually maintained in RPMI/L15 medium containing 10% fetal bovine serum and 1% penicillin/ streptomycin (Gibco, Grand Island, NY). PBMC determined to be greater than 90% infected were used as FIV infected PBMC.

**ii. Astrocyte-PBMC interaction**

In co-culture experiments, 5x10⁴ G355-5 cells were seeded onto #1.5 Borosilicate coverglass chambers (Nalge Nunc International, Naperville, IL) and incubated for 24 h. One hundred thousand FIV infected PBMC were then co-cultured with G355-5 cells for 24 h treated with or without METH. Confocal microscopy (Leica Microsystems) was used to determine FIV infection. After 24 h, cells were fixed and permeabilized with ice-cold methanol (Sigma, St. Louis, MO). The co-cultures were incubated with antibodies directly against CD5-RPE (Southern Biotechnology, Birmingham, AL, 1:50) for 30 min to define the lymphocyte population. Infected cells were determined by staining with FIV positive feline sera (1:400) for 1 h followed by anti-feline FITC-labeled secondary antibodies (United States Biochemical) for 1 h. The cells were then incubated with 1 µg DAPI stain-blue (Molecular Probes, Eugene, OR) for 10 min to define G355-5 cells. Cells were visually examined, counted and images were taken with an inverted fluorescence confocal microscope. Possible drug toxicity in co-culture was assessed by LDH release assay. For larger co-culture experiments, 10⁶ G355-5 cells were seeded in 6 well plates and then co-cultured with 2x10⁶ PBMC in the presence and absence of METH for 24 h.

**B. Co-culture viability assay**

*Measurement of [³H] thymidine incorporation*
Lymphocyte proliferation was determined by thymidine incorporation. PBMCs, $10^5$ cells per well, were cultured in 96 well flat-bottom microtiter plates and stimulated with either vehicle (PBS alone), ConA or METH (1 μM, Sigma, St. Louis, MO) for 24 h. The cultures were pulsed with 1 μCi/ml $[^3]$H-thymidine (Amersham, Arlington Heights, IL) for 18 h. Cells were harvested and $^3$H-thymidine incorporation was measured using a scintillation counter (Packard BioScience).

**ii. Measurement of lactate dehydrogenase (LDH) activity**

Possible drug toxicity in PBMCs was assessed by lactate dehydrogenase (LDH) release. The extracellular release of LDH was used to determine the amount of cell death in cultures. After drug treatment, the culture medium was collected and LDH activity was assessed following manufacturer’s (Sigma, St. Louis, MO) instructions and absorbance measured by a microplate reader at 490 nm. Possible drug toxicity in coculture was assessed by LDH release assay. For larger co-culture experiments, $10^6$ G355-5 cells were seeded in 6 well plates and then co-cultured with $2 \times 10^6$ PBMC in the presence and absence of METH for 24 h.

**iii. Measurement of glutamine synthetase (GS) activity**

GS activity was estimated as previously described in Weber et al., 2002 and Allen et al., 2001. Briefly, GS activity is estimated in cell lysates (0.001M Imidazole and 0.10% Triton-x100) in a reaction mixture containing 50 mM imidazole, 120 mM L-glutamine, 10 mM sodium arsenate, 30 mM hydroxylamine, pH 6.5, 30 μM MgCl₂ and 200 μM ATP (Sigma, St. Louis, MO) at 37°C for 1 h. After protein precipitation, absorbance in supernatant aliquots is read at 490 nm. L-glutamate acid gamma-monohydroxamate standards are run in parallel for quantification.

**C. FIV entry receptor expression**

**i. Detection of CXCR4 and OX40 surface protein**

For flow cytometry measurements, astrocytes, $10^6$ per well, were detached by versene and stained with primary antibody for CXCR4 (clone 44716, R&D Systems,
Minneapolis, MN) or OX40/CD134, (anti-feline CD134, a generous gift from Dr. John Elder) followed by a secondary PE-labeled antibody or FITC-labeled antibody (Southern Biotechnology, Brimingham, AL). Cells stained with IgG2b and rabbit IgG (R&D Systems, Minneapolis, MN) served as negative isotype controls, respectively. Cells were analyzed for 10,000 events on a fluorescence-activated cell sorting (FACS) CALIBUR flow cytometer for determination of the intensity of fluorescence for each labeled sample. Cells positive for CXCR4 and OX40 were expressed as percentage of the total cell population.

**ii. Detection of CXCR4 and OX40 gene transcripts**

Total RNA was extracted from cells using Trizol reagent (Invitrogen, San Diego, CA) and 1.5 ng used for reverse transcription to cDNA and subsequent PCR. Specific primers for feline OX40 were developed forward primer 5’-3’: AGG TTA TGG GAT GGA GAG TCG CT and reverse primer 3’-5’: TAG TTC ACG GCC TCG TTG AAG and the product was 90 base pairs. Primers for feline CXCR4 and GAPDH were previously described (Oonuma et al., 2003 and Caney et al., 2002). PCR products were detected by SYBR green continuously for 40 cycles using LightCycler® 2.5 Instrument (Hoffmann-LaRoche Ltd., Basel, Switzerland). The copy number of target gene expression was expressed as copy number determined from standard curves of known CXCR4 and OX40 gene products and normalized using expression of an internal control.

**III. RESULTS**

**METH increases cell associated FIV infection of G355-5 cells**

In order to understand the role of METH in increased FIV replication in astrocytes (Gavrilin et al., 2002), we specifically evaluated the effect of METH on FIV infection of astrocytes within the first 24 h. The feline astrocytic cell line (G355-5) was co-cultured with FIV infected PBMCs treated with either vehicle or METH (1 µM) for 24 h. Figure 2.1A shows a representative image of cell-associated FIV-infected astrocytes in the absence of METH. At high magnification, FIV-infected astrocytes displayed
punctuate staining pattern, typical of this type of infection (Figure 2.2A). Figure 2.1B shows an increase in cell-associated FIV in astrocyte cultures treated with METH for 24 h. The observed increase in FIV-infected G355-5 cells in the presence of METH was confirmed by cell counting of infected astrocytes using the confocal microscopy (Figure 2.2B). METH (74.8 ± 12.2, mean ± SEM) treated cultures had a significantly greater number of infected cells (p<0.05) than the cultures with vehicle (35.5 ± 0.5, Figure 2.2B). Syncytia formation (Figure 2.3) is common in FIV infection with subsequent cytopathic effects on astrocytes (Billaud and Phillips, 1998). Taken together, this data shows that the presence of METH for 24 h increases cell-associated FIV infection of G355-5 cells.

**FIV infection but not METH treatment increases cell death**

Cell death significantly increased (p<0.01) with FIV infected PBMC (44.1 ± 3.8, mean ± SEM) compared to control PBMC (24.9 ± 1.2, Table 2.1). There is also a significant increase (p<0.05) in cell death in METH-treated FIV infected PBMC (40.9 ± 4.7) compared to control PBMC (29.5 ± 2.4). Cell death was measured using the lactate dehydrogenase (LDH) assay. Thymidine incorporation was also measured in parallel with these cells and the results confirmed the conclusion reached with LDH release. Therefore, cell proliferation did not occur under these conditions (data not shown). There was no statistically significant difference in the METH treated PBMC (29.5 ± 2.4) compared to control PBMC (24.9 ± 1.2).

When PBMC were co-cultured with G355-5 cells whether in the absence or presence of METH, there was a significant increase (p<0.0001) in LDH release of 93.7 ± 6.5 and 102.3 ± 14.6 (mean ± SEM) respectively, compared to PBMC (control, Table 2.1) or G355-5 cells cultured alone (control, Table 2.1). Cell death significantly (p<0.005) increased with FIV infected PBMC co-cultured with G355-5 cells (145.7 ± 15.3) compared to control PBMC co-cultured with G355-5 cells (93.7 ± 6.5). There was a significant increase (p<0.01) in cell death in METH treated FIV infected PBMC co-cultured with G355-5 cells (168.4 ± 21.5) compared to METH treated PBMC co-cultured with G355-5 cells (102.3 ± 14.6). There was no statistically significant difference between PBMC co-cultured with G355-5 cells in the presence of METH (102.3 ± 14.6)
or in its absence (93.7 ± 6.5). Taken together, this data shows that FIV induces cell toxicity but that the presence of METH does not further exacerbate the effect.

**FIV infection but not METH treatment does decrease glutamine synthetase**

GS activity significantly decreased with FIV infected PBMC co-cultured with G355-5 cells compared to control PBMC co-cultured with G355-5 cells. There was a significant decrease in GS activity in FIV infected PBMC co-cultured with G355-5 cells (5.15 ± 0.25, mean ± SEM) compared to control PBMC co-cultured with G355-5 cells (5.58 ± 0.24). There was also a significant decrease in GS activity in METH treated FIV infected PBMC co-cultured with G355-5 cells (4.84 ± 0.38) compared to METH treated PBMC co-cultured with G355-5 cells (5.97 ± 0.12). There was no statistically significant difference between PBMC co-cultured with G355-5 cells in the presence of METH (5.97 ± 0.12) and absence of METH (5.58 ± 0.24). FIV decreased GS activity, but the presence of METH did not further exacerbate the decline in GS activity. The data described thus far explored measurements within the co-culture system and the next experiments will specifically focus on the effects of METH on PBMC cell viability and expression of FIV entry receptors.

**FIV-infection decreases expression of lentiviral entry receptors and METH increases receptor RNA expression on PBMC at later timepoints**

FIV infection significantly decreases lentiviral entry primary (OX40) and coreceptor (CXCR4), expression at the RNA and protein levels. OX40 RNA expression is significantly decreased (p<0.001) in FIV-infected PBMC at 6, 12, 24 h (10.13 ± 2.63, 16.5 ± 4.56, 7.5 ± 2.46, percent of control ± SEM), compared to control PBMC (Figure 2.4A). The copy number for the control feline OX40 for 6, 12 and 24 h is 409,250 ± 51,304, 519,463 ± 122,203, 1,923,258 ± 682,840, copy number/50 ng ± SEM, respectively. OX40 surface expression is also significantly decreased (p<0.01) on FIV-infected PBMC at 6, 12 and 24 h (3.13 ± 0.75, 6.09 ± 2.01 and 3.75 ± 1.36, percent of cells ± SEM), compared to control PBMC (29.87 ± 6.33, 33.61 ± 8.7 and 30.84 ± 8.23, Figure 2.4B).
Figure 2.5A shows that CXCR4 mRNA expression is significantly decreased (p<0.01) in FIV-infected PBMC at 6, 12 and 24 h (25.75 ± 2.93, 32.75 ± 2.57, 16.86 ± 8.81, percent of control ± SEM), compared to control PBMCs. The copy number for the feline CXCR4 for 6, 12 and 24 h respectively is the following (138,963 ± 29,806; 121,813 ± 21,963; 194,764 ± 34,392 copy number/50 ng ± SEM). Figure 2.5B shows that CXCR4 surface expression is also significantly decreased (p<0.05) on FIV-infected PBMC at 6, 12 and 24 h (33.97 ± 4.18, 34.39 ± 3.33 and 30.32 ± 4.29, percent of cells ± SEM), compared to control PBMC (68.13 ± 3.38, 70.98 ± 5.04, 66.18 ± 3.69). Overall FIV infection decreases lentiviral entry receptor expression at both the RNA and protein levels.

We also examined the effect of METH on RNA expression of OX40 and CXCR4 at various time points. These studies demonstrate that METH increases RNA expression of lentiviral entry receptors at later time points (Figure 2.6 and Figure 2.7). OX40 RNA expression is significantly increased (p<0.05) in the presence of METH (152 ± 30, 173 ± 22, percent of control ± SEM) as compared to PBMC cultured alone at 12 and 24 h (Figure 2.6A). The copy number for the control feline OX40 for 6, 12 and 24 h respectively is the following (409,250 ± 51,304; 519,463 ± 122,203; 1,923,258 ± 682,840; copy number/50 ng ± SEM). OX40 in FIV-infected PBMC is significantly increased (p<0.05) in the presence of METH (165 ± 36, percent of control ± SEM) as compared to FIV-infected PBMC alone only at 24 h (Figure 2.6B). The copy number for the feline OX40 for 6, 12 and 24 h respectively is the following (42,560 ± 12,313; 54,588 ± 10,379; 70,891 ± 20,354 copy number/50 ng ± SEM). CXCR4 RNA expression is significantly increased (p<0.005) in the presence of METH (197 ± 49, percent of control ± SEM) as compared to PBMC cultured alone only at 24 h (Figure 2.7A). The copy number for the feline CXCR4 for 6, 12 and 24 h respectively is the following (138,963 ± 29,806; 121,813 ± 21,963; 194,764 ± 34,392 copy number/50 ng ± SEM). CXCR4 in FIV-infected PBMC significantly increased (p<0.05) in the presence of METH (143 ± 23, 154 ± 25, percent of control ± SEM) as compared to FIV-infected PBMC alone at 12 and 24 h (Figure 2.7B). The copy number for the feline CXCR4 for 6, 12 and 24 h respectively is the following (30,681 ± 4,647; 36,699 ± 5,503; 24,039 ± 5,210 copy
number/50 ng ± SEM). Thus, METH treatment of PBMC results in increased mRNA for both OX40 and CXCR4 in the presence and absence of FIV.

IV. DISCUSSION

The role that METH plays in the progression of lentiviral neurological disease remains unclear. These studies were specifically designed to investigate the events that occur within the first 24 hours of FIV infection of PBMC and G355-5, in the presence or absence of METH. The current studies show that METH promotes FIV infection within 24 hours of co-culture.

Both HIV-1 and FIV enter the brain early after systemic infection. Both lentiviruses share similar biochemical and structural characteristics and cause similar diseases (Levy, 1993; Billaud and Phillips, 1998). However, the mechanism of how lentiviruses enter the brain is still unclear. The “Trojan horse hypothesis” states that lentivirus-infected peripheral PBMC serve as vehicles able to gain access and interact with resident CNS cells (Gonzalez-Scarano and Martin-Garcia, 2005). Our in vitro model of FIV-infected PBMC attempts to mimic this situation of how a lentivirus might interact with CNS cells such as astrocytes (Peuderiler et al., 1991, Peterson et al., 1994). In the presence of METH, FIV infection of astrocytes increased by 211% within 24 h (Figure 2.2). The number of infected feline astrocytes is not as robust, as previously shown by Gavrilin et al., (2002). However, our study is focused on a shorter period of time. Gavrilin et al., (2002) showed that primary feline astrocytes and the G355-5 cell line behave similarly under these conditions. Interestingly, our results indicate that the effect of METH on viral activity or the cellular environment occurs very early and has a variety of consequences. We used the FIV-Maryland (FIV-MD) strain, one of three viral strains (FIV-PPR and FIV-34TF10) shown to infect astrocytes in culture. FIV-MD and FIV-PPR are strains that cause CNS dysfunction in infected cats. Additionally, FIV-PPR and FIV-34TF10 have been shown to significantly reduce glutamate uptake in infected feline cortical astrocytes (Billaud et al., 2000; Yu et al., 1998). Consistent with these findings,
we found that FIV-MD infection decreased glutamine synthetase activity (glutamine synthetase breaks down glutamate into glutamine) independent of the presence of METH, indicating that there is more glutamate after lentiviral infection (Table 2.2). Both HIV and FIV have been shown to affect glutamate metabolism and glutamate is thought to contribute to the neuropathogenesis of these two lentiviruses (Yu et al., 1998; Kaul and Lipton, 2006).

A number of studies have demonstrated the potentiating effect of METH on HIV in various cell lines and primary cells (Maragos et al., 2002; Turchan et al., 2001, Flora et al., 2003). Ellis et al (2003) demonstrated that the use of METH increases HIV viral load in serum. To study this association and the mechanisms by which this occurs, an experimental animal model was needed. Although an increased HIV load was found peripherally, these results do not imply that the same will be found in the brain, which is considered an isolated compartment. In 2002, Gavrilin et al. showed that METH significantly enhanced cell associated viral replication of FIV-MD in astrocytes, both primary and G355-5 cells. Phillips et al. (2000) confirmed these results in vitro, reporting that METH increased FIV/34TF10 viral replication in G355-5. However, these in vitro results should be interpreted with caution.

Billaud and Phillips (1998) found that the presence of opiates, another commonly abused drug, in vitro also enhanced FIV replication. However, these in vitro results did not translate into in vivo findings. In fact, Barr et al., (2000a) found that opiates significantly delayed FIV progression, decreased viral load and may even serve a neuroprotective role in vivo. But the effect of METH in the progression of FIV has been shown in vivo. Cloak et al., (2004) reported in vivo an interaction between FIV infection and METH treatment on brain metabolism. There is sufficient evidence to suggest a relationship between lentiviral infection and METH treatment to warrant further studies in this area, particularly in vivo.
In addition to the increase in infection, we also observed syncytia formation at 24 h (Figure 2.3). FIV has been shown to be cytopathic to astrocytes in culture by the observation of syncytia formation after 3 days of culture (Billaud and Phillips, 1998). The formation of syncytia \textit{in vitro} in addition to indicating FIV infection is hypothesized to lead to cell death by apoptosis (Ferri et al., 2000, Perfettini et al., 2005). Under these cell culture conditions, we observed cell-associated FIV infection and increased cell death measured by LDH (Table 2.1) and the syncytia formation may have contributed to the observed cell death. This phenomenon is not observed in FIV, but the presence of multinucleated giant cells in the brain tissue of HIV-positive patients is the main neuropathological finding in HIVD and is considered a hallmark of HIV encephalitis (Gonzalez-Scarano and Martin-Garica, 2005). Our co-culture model used activated PBMC that were stimulated by ConA and IL-2. The activation of these PBMC may contribute to the increase in cell death in this model beyond what is expected from the additive cell death between astrocytes and PBMC (Table 2.1). However, treatment with METH did not further exacerbate the amount of cell death. CXCR4 has been shown to play a necessary role in FIV envelope-mediated cell fusion (Garg et al., 2004, Ferri et al., 2000). This reinforces the importance and dependence on CXCR4, the viral coreceptor.

In our studies, we found that FIV infection of PBMCs alone increased lymphocytic cell death. FIV infection has been shown by others to induce apoptosis in feline lymphocytes \textit{in vitro} (Momoi et al., 1996; Johnson et al., 1996). FIV infection, as others have shown, decreases expression of viral receptors (Willett et al., 2006). The fact that these two established observations are confirmed in our studies validates our experimental model. Under these specific conditions, METH did not significantly affect proliferation or surface viral entry receptor expression of Day 10 PBMC (data not shown). METH affected viral entry receptor RNA expression at later time points (12 and 24 h) in PBMC. Comparative flow cytometry analysis studies were done to investigate the cell surface expression of these FIV entry receptors at 6, 12 and 24 h in the presence and absence of METH, however no significant differences were found (data not shown). Therefore, we hypothesize that these changes may not translate into increased surface
protein expression until much later than 24 h. Under different experiment conditions, others have shown that METH can affect lymphocytes. House et al., (1994) demonstrated that the presence of METH in culture with lymphocytes causes suppression of B-cell proliferation, production of IL-2 and enhanced NK-cell activity. Others have found similar results by administering METH \textit{in vivo} and then investigating lymphocytic properties and activity \textit{ex vivo} (Saito et al., 2006, In et al, 2005 and Yu et al., 2002). Therefore, METH may have immunosuppressive properties under certain conditions. PBMC under our experimental conditions were not affected by METH therefore, further investigation of astrocytes may provide insight into possible factors important to the METH induced cell-associated FIV infection. In the next chapter, we isolate the other cell type in this co-culture study and explored the effects of METH on G355-5 cells. We specifically investigated the properties that affect FIV infection including cell proliferation and lentiviral entry receptors.
Figure 2.1. Methamphetamine (METH) increases cell associated FIV infection of G355-5 cells. Confocal microscopy was used to visualize the following: DAPI nuclear staining (blue), CD5+ lymphocyte staining (red) and FIV infection (green). A. G355-5 cells were co-cultured with FIV infected PBMC for 24h. B. G355-5 cells were co-cultured with FIV infected PBMC in the presence of METH, 1 µM, for 24h.
Figure 2.2. METH increases cell associated FIV infection of G355-5 cells.
A. A representative image of high magnification cell associated FIV-infected astrocytes. B. The number of FIV-infected G355-5 cells was determined by counting multiple fields under the confocal microscope. Data is expressed as mean ± SEM of three experiments. *p<0.05 compared to vehicle; Student t-test.
Figure 2.3 Syncytia formation was observed in the co-culture of PBMCs and G355-5 cells. A representative image of syncytia formation under the confocal microscope.
Table 2.1 FIV infection but not METH treatment increases cell death. LDH release was used to estimate cell death. LDH release was measured from PBMC or FIV infected PBMC or G355-5 cells cultured alone. LDH release was also measured in the co-culture of both types of cells in the absence or presence of METH. Data are expressed as mean compared to its respective control ± SEM from three independent experiments. *p<0.05 by student’s unpaired t-test.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control</th>
<th>METH</th>
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<tr>
<td>PBMC</td>
<td>24.9 ± 1.2</td>
<td>29.5 ± 2.4</td>
</tr>
<tr>
<td>FIV PBMC</td>
<td>44.1 ± 3.8*</td>
<td>40.9 ± 4.7*</td>
</tr>
<tr>
<td>G355-5</td>
<td>54 ±6</td>
<td>46 ± 13</td>
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<tr>
<td>G355-5 + PBMC</td>
<td>93.7 ± 6.5</td>
<td>102.3 ± 14.6</td>
</tr>
<tr>
<td>G355-5 + FIV PBMC</td>
<td>145 ± 15.3*</td>
<td>168 ± 21.5*</td>
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Table 2.2 METH does not affect glutamine synthetase (GS) activity however FIV infection does decrease GS activity. GS activity estimates the activity the enzyme that breaks down glutamate to glutamine. GS was measured in the co-culture of both PBMC and G355-5 cells in the absence or presence of METH. Data are expressed as mean compared to respective control ± SEM from three independent experiments. *p-value<0.05 by unpaired t-test.
Figure 2.4 FIV infection decreases expression of OX40 receptors on PBMC. A. mRNA for OX40 in control and FIV-infected PBMC at 6, 12, and 24 h. B. Surface expression of OX40 on control and FIV-infected PBMC at 6, 12 and 24 h. Data are expressed as percent of control or percent of cells expressing OX40 ± SEM compared to control (vehicle alone) from three different experiments. *p<0.05 by nonparametric Mann-Whitney Test.
Figure 2.5 FIV infection decreases expression of CXCR4 receptors on PBMC. A. mRNA for CXCR4 in control and FIV-infected PBMC at 6, 12, and 24 h. B. Surface expression of CXCR4 on control and FIV-infected PBMC at 6, 12 and 24 h. Data are expressed as percent of control or percent of cells expressing CXCR4 compared to control from three different experiments. * p<0.05 by nonparametric Mann-Whitney Test.
Figure 2.6 METH increases OX40 receptor mRNA expression on PBMC at later time points. A. OX40 mRNA expression in PBMC treated with METH at 6, 12 and 24 h. B. OX40 mRNA expression in FIV-infected PBMC treated with at 6, 12 and 24 h. * p<0.05 by nonparametric Mann-Whitney Test.
Figure 2.7 METH increases CXCR4 receptor mRNA expression on PBMC at later time points. A. CXCR4 mRNA expression in PBMC treated with METH at 6, 12 and 24 h. B. CXCR4 mRNA expression in FIV-infected PBMC treated with METH at 6, 12 and 24 h. * p<0.05 by nonparametric Mann-Whitney Test.
CHAPTER 3

METHAMPHETAMINE AND FELINE ASTROCYTES

I. INTRODUCTION

METH is a commonly abused psychostimulant drug. The 2004 National Survey on Drug Abuse and Health (SAMHSA) estimated that 1.4 million people use methamphetamine. This psychostimulant drug alters monoamine release with an overall effect of a heightened monoaminergic transmission. Most of the research literature on METH focuses on the dopaminergic systems (Davidson et al. 2001; Fleckenstein et al., 2000; Riddle et al., 2006). Specifically, METH has been shown to increase levels of synaptic dopamine by inhibiting dopamine reuptake transporters and increasing the release of vesicular dopamine stores (Davidson et al., 2001). High levels of dopamine are thought to be responsible for the acute, as well as chronic physiological and psychological effects of METH. Short-term effects include increased energy and alertness, decreased appetite and feelings of power and invulnerability (Riddle et al., 2006). The long-term consequences are reduction in dopamine transporter activity and the degeneration of dopaminergic nerve terminals (Fleckenstein et al., 2000; Riddle et al., 2006; Colfax and Guzman, 2006).

More recent studies found that METH may have other effects that extend beyond the dopaminergic system (Davidson et al., 2001 and Cadet et al., 2003). At high concentrations, METH is a potent neurotoxin causing neurodegeneration and gliosis. There is growing evidence that glutamate may contribute to the development of METH-induced toxicity. METH enhances glutamate release in the brain in vivo, and ionotropic and metabotropic glutamate receptor antagonists are protective against METH
neurotoxicity (Davidson et al, 2001). The exact mechanism(s) of METH-induced neurotoxicity is not fully elucidated.

At lower concentrations, METH has different effects than at high concentrations, and it is therefore important to investigate these differences. For example, at lower concentration, METH has been shown not only to be a substrate for the monoaminergic transporter but also is able to bind receptors such as the sigma (Itzhak, 1993, Nguyen et al., 2005) and trace amine receptors (Miller et al., 2005 and Bunzow et al., 2001). Studies have also shown that METH can alter cellular immune responses, causing decreased cytotoxic T cell activity, increased macrophage secretion of mediators, increased cytokine levels (House et al., 1994; Iwasa et al., 1996, In et al., 2004 and Theodore et al., 2006) and activation microglia and astrocytes (Thomas et al., 2004 and Thomas et al., 2005). These METH-induced effects are different depending on the concentration of the drug.

In these experiments we investigated the effect of various concentrations of METH on the astrocytic line G355-5. Specifically, we characterized the effect of different concentrations of METH on cell viability and morphology, to provide insight into the effect of METH on astrocytes. Furthermore, we investigated the mechanisms by which METH could induce astrocytotoxic or proliferative effects such as glutamatergic indices and expression of lentiviral receptors respectively.

II. MATERIALS AND METHODS

A. Cell culture
G355-5 cells (feline astrocyte cell line, American Type Culture Collection, Rockville, MD, CRL-2033) were maintained in McCoy’s modified medium containing 10% fetal bovine serum and 1% pencillin/streptomycin (Gibco, Grand Island, NY). G355-5 cells were seeded for 24 h before experiments.

B. Drug Treatment
One hundred thousand to $10^6$ cells were used and treated with various concentrations of METH ($10^{-9}$M to $10^{-3}$M) for 1-3 days as indicated. 20 µM of NBQX was co-administered with METH ($10^{-4}$M) in the toxicity experiments. In all of the toxicity experiments, the concentration of METH used was 10mM. The optimal lower concentration that was used for the proliferation and lentiviral receptor studies was 1 µM of METH. METH was dissolved in PBS which served as the vehicle. Prior to experimentation, the cells were examined for changes in morphology under an inverted light microscope (Leica Microsystems).

C. Astrocyte viability assay

i. Measurement of astrocyte cell number and viability

Cell number/well were estimated by trypan blue dye exclusion. Astrocyte cell viability and proliferation were estimated by the tetrazolium compound MTT (3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide, Sigma, St. Louis, MO) assay. MTT, 0.5 mg/ml, was added to cells 3 h before the end of 24 h treatment with METH, 1µM, or vehicle. The product of MTT conversion, formazan, was solubilized with 0.04 M HCl (Fisher ChemAlert, Fairlawn, NJ) and absorbance measured on a microplate reader at 570 nm.

ii. Measurement of lactate dehydrogenase (LDH) activity

Toxicity in PBMCs was assessed by lactate dehydrogenase (LDH) release. The extracellular release of LDH was used to determine the amount of cell death in cultures. After drug treatment, the culture medium was collected and LDH activity was assessed following manufacturer’s (Sigma, St. Louis, MO) instructions and absorbance measured on a microplate reader at 490 nm.

D. Glutamatergic Indices

i. Measurement of Glutamate (GLU) Content

GLU in the cells and medium were estimated by HPLC-ED, derivatization by o-phthalaldehyde in 0.1 M sodium tetraborate containing 2-mercaptoethanol for 4 min at
room temperature. Homoserine was used as an internal standard and GLU standards were run in parallel for quantification.

ii. Measurement of glutamine synthetase (GS) activity

GS activity was estimated as previously described in Weber et al., 2002 and Allen et al., 2001. Briefly, GS activity is estimated in cell lysates (0.001M Imidazole and 0.10% Triton-x100) in a reaction mixture containing 50 mM imidazole, 120 mM L-glutamine, 10 mM sodium arsenate, 30 mM hydroxylamine, pH 6.5, 30 μM MgCl₂ and 200 μM ATP (Sigma, St. Louis, MO) at 37°C for 1 h. After protein precipitation, absorbance in supernatant aliquots is read at 490 nm. L-glutamate acid γ-monohydroxamate standards are run in parallel for quantification.

E. Lentiviral entry receptor expression

i. Detection of CXCR4 and OX40 gene transcripts

Total RNA was extracted from cells using Trizol reagent (Invitrogen, San Diego, CA) and 1.5 ng used for reverse transcription to cDNA and subsequent PCR. Specific primers for feline OX40 were developed forward primer 5’-3’: AGG TTA TGG GAT GGA GAG TCG CT and reverse primer 3’-5’: TAG TTC ACG GCC TCG TTG AAG and the product was 90 base pairs. Primers for feline CXCR4 and GAPDH were previously described (Oonuma et al., 2003 and Caney et al., 2002). PCR products are detected by SYBR green continuously for 40 cycles using ABI Prism 7700 Sequence Detection System. The target gene expression was expressed as copy number determined from standard curves of known CXCR4 and OX40 gene products and normalized using expression of an internal control.

ii. Detection of CXCR4, CCR5 and OX40 protein

1. Detection of CXCR4 intracellular and extracellular staining by immunohistochemistry was used to determine the localization of CXCR4 receptor on the surface or in the cytosol of astrocytes. Cells were fixed with 2% paraformaldehyde with or without permeabilization in ice-cold methanol. Then they were incubated with CXCR4
antibodies (R&D clone 44716, 1:100) for 1 h followed by FITC-labeled secondary antibodies (Santa Cruz) for ½ h. Cells were visually examined and images were taken with an inverted fluorescence microscope (Leica Microsystems).

2. Detection of CXCR4, OX40 and CCR5 surface protein
For fluorescence-activated cell sorting (FACS), the astrocytes, $10^6$ per well, were detached by versene and stained with primary antibody for CXCR4 or CCR5 (R&D Systems, Minneapolis, MN) or OX40/CD134, (anti-feline CD134, a generous gift from Dr. John Elder) followed by a secondary PE-labeled antibody or FITC-labeled antibody (Southern Biotechnology, Birmingham, AL). Cells stained with IgG2b and IgG (R&D Systems, Minneapolis, MN) served as negative isotype controls respectively. Ten thousand events were analyzed on a FACS CALIBUR flow cytometer for determination of the intensity of fluorescence for each labeled sample. Cells positive for CXCR4 and OX40 were expressed as a percentage of the total cell population.

3. Detection of CXCR4 total protein
This method has been previously described in Duchemin et al. 2002. Briefly, cell pellets were sonicated in lysis buffer (5 mM NaCl, 1 mM Tris, pH 8, 1% Triton X-100, 10% glycerol, 0.1M PMSF, 1µg/ml aprotinin, Sigma, St. Louis, MO). After 30 min on ice, the insoluble material was removed by centrifugation at 15,000g for 10 min at 4°C. 10 µL of the lysate was used for protein determination (Bradford, Bio-Rad Laboratories). The remaining lysate was boiled in Laemmli sample buffer and equal amounts of lysate protein, 20 µg, was separated by electrophoresis on 12% sodium dodecyl sulfate (SDS)-PAGE polyacrylamide gel, in parallel with pre-stained benchmark weight markers (Invitrogen, Carlsbad, CA). Separated proteins were transferred to a nitrocellulose membrane, and blots incubated for 30 min in TBS-Tween containing 5% dry milk as a blocking agent, sequentially followed by incubation with CXCR4 (1:2000 with 2%BSA, Chemicon) or actin (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA) antibody and horseradish peroxidase-conjugated secondary antibody HRP (1:2000, Santa Cruz Biotechnology, Santa Cruz, CA). The bound antibodies were detected by enhanced
chemiluminescence (Amersham, Arlington Heights, IL). The density of the band of interest on x-rays is determined by image analysis (Metamorph) based on calibrated standards.

4. Detection of SDF-1ω Protein
SDF-1ω levels were measured using an enzyme linked immunosorbent assay (ELISA) following manufacture’s instructions (R&D systems, Minneapolis, MN). Briefly, the kit was a quantitative sandwich enzyme immunoassay using a capture monoclonal specific antibody and detection polyclonal antibody. The colorimetric substrate solution is added for color development followed by the stopping solution. Within 30 minutes, absorbance was measured on a microplate reader at 450 nm.

III. RESULTS

METH increases G355-5 cell number in a concentration dependent manner
We found that METH exposure causes an increase in G355-5 cells as measured by trypan blue dye exclusion. We then investigated the optimal concentration of METH which induces an increase in astrocyte cell number. Figure 3.1 shows a significant increase (p<0.05) in cell number at $10^{-7}$ and $10^{-6}$M concentration of METH (143 ± 8, 178 ± 10, percent of control ± SEM) as compared to control. We observed that $10^{-3}$M METH induced significant toxicity in G355-5 cultures.

High concentration of METH induces toxicity evident through changes in cell morphology, increased cell death and glutamate content
Figure 3.2 shows the difference in cell morphology between G355-5 cells treated with vehicle and $10^{-4}$M METH by light microscopy. The G355-5 cells treated in $10^{-4}$M METH cultures are less abundant and full of vacuoles compared to the cells in the presence of vehicle alone. Table 3.1 further confirms the cell morphology results using biochemical measures. Cell viability, determined by the MTT assay, was significantly decreased (p<0.05) in the presence of $10^{-4}$M METH (0.57 ± 0.04, mean OD value ±
SEM) as compared to vehicle (0.79 ± 0.08). Cell death measured by LDH release significantly increased (p<0.05) in the presence of METH (28.49 ± 2.07, percent of LDH ± SEM) as compared to vehicle (4.29 ± 0.82). Figure 3.3 shows representative images demonstrating the toxicity of the high concentration of METH (10^{-7}M). As early as 6 h (Figure 3.3B), there is evidence of cell loss and by 24 h (Figure 3.3C) many more cells ~50% are lost. At 48 h (Figure 3.3D), the cells have almost completely detached from the culture plate. These differences are profound when compared to the control (vehicle, Figure 3.3A).

Figure 3.4A shows that this high concentration of METH (10^{-4}M) increases the release of glutamate. This increase in glutamate in the presence of METH (2580.94 ± 136.95) is statistically significant (p<0.01) compared to vehicle (383.46 ± 48.30). The addition of the AMPA antagonist, NBQX, to METH (366.26 ± 16) attenuates the increase in glutamate. METH exposure significantly decreases (p<0.01) GS activity (7.78 ± 0.51) by 26% compared to vehicle (10.54 ± 0.19, Figure 3.4B). The addition of NBQX (11.94 ± 1.18) also attenuated the decrease in GS activity in the presence of METH. Therefore, glutamate and GS activity may be involved in METH induced toxicity in G355-5 cells under these conditions.

**METH increases G355-5 cell proliferation**

We further explored the observation that at lower concentrations METH exposure causes an increase in G355-5 cell number. Figure 3.5 shows that the optimal increase in cell number found at 24 h after treatment with METH and the cell number significantly increased (p<0.002) after treatment with METH (10.07x10^5 ± 0.13x10^5 with mean cell count/well ± SEM) compared to vehicle alone (5.6x10^5 ± 0.05x10^5). The cell number continued to increase with time in culture but the difference between vehicle control and METH-treated cell, at 3 and 7 days was not statistically significant. Table 3.2 shows that METH increases G355-5 cell proliferation using a number of measures. Cell number significantly increased (p<0.002) after treatment with METH (10.07x10^5 ± 0.13x10^5, mean cell count/well ± SEM) compared to vehicle alone (5.6x10^5 ± 0.05x10^5) for 24 h as measured by trypan blue dye exclusion. Confirmation of this result was by MTT assay.
where the cell viability significantly increased (p<0.0001) in the presence of METH (1.03 ± 0.01, mean OD value ± SEM) as compared to vehicle alone (0.79 ± 0.08). No differences were noted in LDH release in the absence or presence of low concentration of METH. Thus in response to exposure of G355-5 cells to 10^-6M METH we also observed increased proliferation but no cell death.

**METH increases de novo synthesis of CXCR4 on G355-5 cells**

METH significantly increases (p<0.01, p<0.01, p<0.02) CXCR4 RNA expression at 3, 6 and 12 h after addition of METH to culture (213 ± 74, 139 ± 36, 152 ± 50, percent of control ± SEM) compared to vehicle alone (Figure 3.6). Figure 3.7 shows representative images of CXCR4 intracellular (Figure 3.7a) and extracellular staining (Figure 3.7b) of G355-5 cells. These results indicate that CXCR4 is expressed both internally and on the external surface of the astrocytic cell line. Figure 3.8B is a representative histogram demonstrating that METH increases CXCR4 surface expression after 24 h in G355-5 cells. METH (26.4 ± 2.5, 35 ± 1.7 percent of cells ± SEM) significantly increases (p<0.05, p<0.02) the percent of G355-5 cells expressing CXCR4 compared to vehicle alone (figure 3.8; 19.8 ± 1.1, 19.7 ± 2.3) at both 12 and 24 h, as measured by flow cytometry, as measured by flow cytometry (Figure 3.8A). Figure 3.9 shows a dramatic increase (p<0.05, p<0.01) in CXCR4 protein expression by Western blot confirming the results observed with flow cytometry (Figure 3.8). This increase was due to the presence of METH (121 ± 10, 157 ± 10, percent of control ± SEM) at 12 h and 24 h as compared to control alone. Overall, these results indicate that METH increased the synthesis of viral entry co-receptor, CXCR4 on G355-5 cells, and that this effect is significantly elevated at 12-24 hours.

**METH does not affect OX40 expression but does increase surface CCR5 expression on G355-5 cells**

After we found that METH did affect CXCR4 expression, we investigated whether METH had an effect on other chemokine receptors, specifically other lentiviral entry receptors. METH does not affect cell surface expression of OX40 on G355-5 cells
at 6, 12 and 24 h (Table 3.3), nor does it affect OX40 RNA expression these same time points (data not shown). Overall, OX40 expression on G355-5 cells was not affected by exposure to METH. However, METH (33.42 ± 5.77, mean percent of cells ± SEM) significantly increased (p<0.05) the percent of G355-5 cells expressing CCR5 compared to vehicle alone (20.27 ± 1.45, Table 3.4) at 24 h.

**METH increases SDF-1α in medium of G355-5 cells**

In Table 3.5, METH exposure significantly increases (p<0.05) SDF-1α in the medium (60 ±12, mean ± SEM) of G355-5 cells compared to the vehicle (18 ± 5) at 24 h. This contributes to the overall increase in total SDF-1α of G355-5 cells in the presence of METH (70 ± 13) compared to vehicle (46 ± 6) at 24 h. In our studies, we found an increase in SDF-1α in the medium at 24 h after METH exposure (Table 3.5).

**G355-5 cells expresses vimentin, not glial fibrillary acidic protein (GFAP)**

A representative image of G355-5 cells expressing intermediate filament vimentin (Figure 3.10). G355-5 cells did not express GFAP staining (data not shown).

**IV. DISCUSSION**

METH acts as an extrinsic factor that can affect different properties of astrocytes. Previous *in vitro* and *in vivo* studies have investigated the effect of METH on both microglia and astrocytes and found that both are activated during METH-induced neurotoxicity (Thomas et al., 2004, Kawasaki et al., 2006). Our first set of studies focused on a toxic concentration of METH. We demonstrated that 10⁻⁴M METH caused cell death measured by several different techniques. We found an increase in the release of LDH, a measurement of cell death (Table 3.1). We also observed a decrease in cell number by counting using trypan blue dye exclusion (Figure 3.1) and visual inspection with an inverted light microscope (Figure 3.2 and Figure 3.3). The decrease in cell number was confirmed by the MTT cell viability assay which showed a lower OD value in the presence of METH (Table 3.1). The decrease in cell number is consistent with
many studies that have shown that such high concentrations (millimolar) of METH causes cell death (Stadlin et al., 1998, Lau et al., 2000 and Cadet et al., 2000). In our studies, we observed vacuole formation in G355-5 cells that were treated with METH. The formation of vacuoles is thought to contribute to METH neurotoxicity, but the mechanism is still unclear (Cubells et al., 1994). A limitation of these studies is that we used a very high concentration of METH due to the presence of 10% FBS in these cultures.

Other studies in the literature reported similar effects with concentrations of METH as low as 2 or 4mM. These studies utilized different cell types including immortalized mesencephalic cells or cultured primary mouse astrocytes. Further, these studies either utilized no FBS or less than 10 percent FBS to maintain the cell cultures (Stadlin et al, 1998; Cadet et al, 2000). When we attempted to replicate the current studies with lower concentrations of FBS, we did not see the same robust results (data not shown).

In addition, it has been shown that METH can induce the release of glutamate (Stadlin et al., 1998). Both NMDA and mGlu5 receptor antagonists attenuate METH-induced neurotoxicity in vivo. METH causes an increase in glial fibrillary acidic protein (GFAP) and gliosis in vivo suggesting astrocyte involvement. METH can gain access to astrocytes by utilizing the monoaminergic transport. Increased drug concentration into the cells might alter their function and cause astrocytotoxicity. In turn, toxic mediators released by reactive astrocytes could have additional detrimental effects on neuronal survival. Glutamate is a strong candidate for such a mediator. Astrocytes play an important role in neuronal glutamate recycling and have been implicated in excitotoxicity. Furthermore, both ionotropic (AMPA) and metabotropic (mGlu3 and mGlu5) glutamate receptors are present on astrocytes (Seiger and Steinhauser, 2001, Schools and Kimelberg, 2001). Therefore, the release of glutamate after METH exposure has been one of many mechanisms implicated in neurotoxicity (Davidson et al., 2001). In agreement with other studies, we demonstrated an increase in the release of glutamate
after exposure to METH (Figure 3.4). Stadlin et al., (1998) found in mouse cortical astrocytes a 48% reduction in GS activity after 48 h of treatment with 4 mM METH. Similarly, we found a 26% reduction in GS activity after 24 h of treatment with 10 mM METH. Moreover, we found that NBQX, a glutamate receptor antagonist, was able to attenuate these METH-induced differences including the secretion of glutamate into the medium and decrease in GS activity. Others have shown that NBQX may be neuroprotective against the toxic effects of METH (Lockhart et al., 2005). Taken together, these results indicate that glutamate may play a role in METH induced toxicity in astrocytes. However more studies need to be done to further explore this relationship in a different animal model using primary astrocytes. This may represent a better model to mimic the in vivo condition than an immortalized cell line that is highly resistant to cell death.

Our studies also focused on nontoxic concentrations of METH (10^{-6}M). First, we demonstrated that METH treatment increased G355-5 proliferation (Figure 3.1, Figure 3.5 and Table 3.2). We also observed that this concentration did not change G355-5 cell morphology (Figure 3.5). This is consistent with many studies showing that METH can induce astrogliosis in vitro and in vivo (Pu and Vorhess, 1993, Pu et al., 1994, Fukumara et al., 1998, Stadlin et al., 1998). Second, METH treatment induced up-regulation of CXCR4 on G355-5 cells. FIV infection of feline astrocytes has been shown to be primarily dependent on the coreceptor, CXCR4 (Nakagaki et al., 2001). Our results are consistent with the hypothesis that FIV infection of astrocytes is dependent on CXCR4, because the expression of the primary FIV entry receptor OX40 did not change (Table 3.3). Interestingly, another chemokine receptor, CCR5, expression was also increased after treatment with METH (Table 3.4). CCR5 has been implicated as a coreceptor for lentiviral entry, but not in the FIV model. These results suggest that the observed effect of METH on CXCR4 expression is not unique to this chemokine receptor. Moreover, the results lead us to suspect an underlying mechanism that modulates multiple chemokine receptors. METH did increase FIV infection in G355-5 cells which is correlated with the up-regulation of CXCR4 expression. This METH-induced increase seems to be due to
the *de novo* synthesis of CXCR4 as indicated from the RNA and protein results. These results from this chapter and the previous chapter demonstrates that METH does affect viral entry receptors differently depending on the receptor and cell type. METH has an indirect effect on astrocytic cellular factors involved in the regulation of lentivirus replication, including increasing expression of cell surface antigen and coreceptor, CXCR4. To our knowledge, this is the first study to investigate the effects of METH on the expression of FIV entry receptors.

CXCR4 plays a paradoxical role in the brain. CXCR4 has been shown to play an essential role in brain development and neurogenesis. Yet CXCR4 can also cause neuronal injury and death (Kaul et al., 2007) in pathological conditions such as lentiviral infection. Bajetto et al., (2001) found that SDF-1α, the natural ligand for CXCR4, can act via CXCR4 as a growth factor and directly stimulate the proliferation of cultured astrocytes. In our studies, we found an increase in SDF-1α in the medium at 24 h after METH exposure (Table 3.5). It is possible that SDF-1α complexed with CXCR4 could be causing or exacerbating the gliosis that occurs in HIV-1 and FIV in the presence of METH. However, more studies are needed to better understand the relationship between proliferation and METH exposure. Our findings suggest that METH promotes astrocytic proliferation and *de novo* synthesis of CXCR4 on G355-5 cells.

Taken together, we determined that METH affects several aspects important to early FIV infection. These studies lead us to fundamental questions about the effects of METH on astrocytes, specifically the expression of CXCR4. A feline astrocyte cell line was appropriate to use for our first group of studies to ask basic questions about cell viability and receptor expression. Although G355-5 cells yielded valuable information in our previous experiments, we found these cells did not express glial fibrillary acidic protein (GFAP) (data not shown) a commonly used marker for astrocytes. G355-5 cells did express vimentin, indicating that they are immature cells (Pixley et al., 1984, Figure 3.10). In the next chapter, we further explore the effect of METH on CXCR4 expression in primary rat astrocytes and study the underlying mechanism of this effect.
Figure 3.1 METH increases cell number in a concentration dependent manner. Cell number was estimated by trypan blue dye exclusion. G355-5 cells were treated with various concentrations of METH (10^{-9} to 10^{-3} mM). *p<0.05 compared with vehicle, student t-test.
Figure 3.2 High concentration of METH causes changes in G355-5 cell morphology and number. Representative light microscope images are shown under various conditions. A. G355-5 cells treated with vehicle (control) at 24 h. B. G355-5 cells treated with $10^{-4}$M METH at 24 h. The formation of vacuoles are present in these cells (arrows). The inserted image gives a closer view of the vacuoles.
Table 3.1 High concentration of METH induces toxicity in G355-5 cells. The MTT assay was used to estimate cell number and viability. LDH release was used to estimate cell death. Cell viability was measured in G355-5 cells in the absence and presence of $10^{-4}$M METH at 24 h. Data is expressed as mean ± SEM compared to control (vehicle) from three independent experiments. *p<0.05 by Student’s t-test. Cell death as measured in G355-5 cells in the absence and presence of $10^{-4}$M METH at 24 h. Data is expressed as percent of LDH release and adjusted for protein content ± SEM compared to control (vehicle) from three independent experiments. *p<0.05 by non-parametric Mann-Whitney Test.

<table>
<thead>
<tr>
<th></th>
<th>Vehicle</th>
<th>METH</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTT (OD/well ± SEM)</td>
<td>0.79 ± 0.08</td>
<td>0.57 ± 0.04*</td>
</tr>
<tr>
<td>LDH Release (% of OD/mg of prot ± SEM)</td>
<td>4.29 ± 0.82</td>
<td>28.49 ± 2.07*</td>
</tr>
</tbody>
</table>
Figure 3.3 High concentration of METH as early as 6 h changed cell morphology and within 48 h caused G355-5 cell death. A. G355-5 cells in the presence of the vehicle at 24 hr. B. G355-5 cells treated with $10^{-4}$M of METH at 6h. C. G355-5 cells treated with $10^{-4}$M of METH at 24 h. D. G355-5 cells treated with $10^{-4}$M of METH at 48 h.
Figure 3.4 High concentration of METH increases the glutamate content and decreases glutamine synthetase (GS) activity in the media of G355-5 cells. Glutamate was measured in media by HPLC and GS activity estimates the activity the enzyme that breaks down glutamate to glutamine. A. Glutamate was measured in G355-5 cells under various conditions including in the absence and presence of METH and/or NBQX. Data is expressed as mean ± SEM and *p<0.05 according to student t-test. B. GS activity was measured in G355-5 cells under various conditions including in the absence and presence of METH and/or NBQX. Data is expressed as mean ± SEM and *p<0.05 according to student t-test.
Figure 3.5 METH increases cell number at 24 h and does not change cell morphology. A. Representative light microscopy image of G355-5 cells treated with vehicle at 24 h. B. Representative light microscopy image of G355-5 cells treated with 10^{-6} M of METH at 24 h. Cell number was estimated by trypan blue exclusion. C. G355-5 cells were treated with 10^{-6} M for various time periods (1, 3 and 7 days). *p<0.05 compared with vehicle, student t-test.
Table 3.2 METH increases cell number in G355-5 cells. Typan blue dye exclusion and MTT was used to estimate cell number and viability. LDH release was used to estimate cell death. Typan blue exclusion, MTT and LDH release were measured from G355-5 cells cultured in the absence and presence of METH for 24 h. Data is expressed as mean ± SEM compared to its respective control from three independent experiments. * p<0.05 by Student’s t-test.
Figure 3.6 METH increases *de novo* synthesis of CXCR4 on G355-5 cells. CXCR4 RNA expression of G355-5 cells in the presence and absence of METH at 3, 6, 12 and 24h. Data is expressed as percent of control ± SEM of 8 independent experiments. * p<0.05 versus vehicle alone; non-parametric Mann-Whitney Test.
Figure 3.7 Immunostaining of CXCR4 on G355-5 cells. Representative images are of immunofluorescent staining. A. Intracellular staining of CXCR4 B. Extracellular staining of CXCR4.
Figure 3.8 METH increases surface expression of CXCR4 on G355-5 cells. A. CXCR4 surface expression on G355-5 cells was measured using flow cytometry in the presence and absence of METH at 6, 12 and 24 h. Data is expressed as percent of cells ± SEM of 3 independent experiments p<0.05 versus vehicle alone, non-parametric Mann-Whitney Test. B. One representative histogram of CXCR4 surface expression on G355-5 cells in the presence (bolded line) and absence (dotted line) of METH and isotype (thin line) control at 24 h.
Figure 3.9 METH increases CXCR4 protein expression on G355-5 cells. CXCR4 protein expression of G355-5 cells was analyzed by Western blot of cell lysates, in the presence and absence of METH for 3, 6, 12 and 24 h. Data was quantitatively evaluated through optical densitometry of CXCR4 relative to β-actin content. Data is expressed as percent of vehicle ± SEM of 10 independent experiments. * p<0.05 versus vehicle alone; non-parametric Mann-Whitney Test.
<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Surface Expression of OX40 (Percent of Cells ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>6</td>
<td>21.1 ± 0.8</td>
</tr>
<tr>
<td>12</td>
<td>18.9 ± 1.8</td>
</tr>
<tr>
<td>24</td>
<td>17.5 ± 2.7</td>
</tr>
</tbody>
</table>

Table 3.3 METH does not affect OX40 expression on G355-5 cells. Surface expression of OX40 on G355-5 cells at 6, 12 and 24 h. Data is expressed as mean ± SEM of 3 independent experiments.
### Table 3.4

METH increases surface expression of CCR5 on G355-5 cells. Surface expression of CCR5 on G355-5 cells at 6, 12 and 24 h. Data is expressed as mean ± SEM of 3 independent experiments. * p<0.05 versus vehicle alone; non-parametric Mann-Whitney Test.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Control</th>
<th>METH</th>
</tr>
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<tbody>
<tr>
<td>6</td>
<td>19.3 ± 1.74</td>
<td>21.54 ± 3</td>
</tr>
<tr>
<td>12</td>
<td>19.78 ± 0.66</td>
<td>23.12 ± 1.16</td>
</tr>
<tr>
<td>24</td>
<td>20.27 ± 1.45</td>
<td>33.42 ± 5.77*</td>
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</tbody>
</table>
Table 3.5 G355-5 cells were treated with METH, $10^{-6}$M, or vehicle for 24 h. SDF-1 expression was estimated by ELISA. This data represents the amount of SDF-1 present in the cells and medium. *p<0.05 compared with vehicle, N=12 in two separate experiments.

<table>
<thead>
<tr>
<th>SDF-1 (pmol/µg of prot ± SEM)</th>
<th>Media</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>18 ± 5</td>
<td>46 ± 6</td>
</tr>
<tr>
<td>METH</td>
<td>60 ± 12*</td>
<td>70 ±13*</td>
</tr>
</tbody>
</table>
Figure 3.10 Immunostaining of vimentin to characterize G355-5 cells. A representative image demonstrating the staining of intermediate filament, vimentin on G355-5 cells.
CHAPTER 4

METHAMPHETAMINE AND PRIMARY RAT ASTROCYTES

I. INTRODUCTION

During the last decade, chemokines and their receptors have emerged as important signaling molecules in neuronal migration, cell proliferation and synaptic transmission. In addition, chemokines are involved in inflammation, degeneration and death of a number of cell types in the CNS. Astrocytes are the most abundant CNS cells and no longer considered passive bystanders for neuronal function. They express neurotransmitter receptors, transporters and ion channels, produce neurotrophic factors, cytokines and chemokines, and are an integral part of the blood brain barrier. The expression of chemokines and their receptors indicate that astrocytes might be important in the modulation of these signaling molecules.

In the previous chapter, we found that METH increased CXCR4 expression on G355-5 cells (feline astrocyte cell line). This observation raised many questions including what was the underlying mechanism of this METH induced effect. Specifically, how does METH influence cell surface properties (such as receptors or transporters) of astrocytes to cause changes? And what possible intracellular events (signaling molecules) could METH affect to cause an end result, increased in CXCR4 surface expression? To begin to answer these questions, we need a well-developed and established animal model to approach both immunological and neurological aspects. We also need a model that better mimicks *in vivo* situation than an established cell line. Therefore, for studies of mechanism, we selected to use primary rat astrocytes. Moreover, CXCR4 receptor expression and regulation has been studied in primary rat astrocytes (Bajetto et al., 1999 and 2001).
CXCR4 and CCR5 chemokine receptors are both expressed by astrocytes and serve as co-receptors for HIV infection. Astrocytes display low level HIV infection, but undoubtedly contribute to the pathogenesis of HIV encephalopathy (Kaul and Lipton, 1999). METH is a long recognized risk factor for HIVD; however, the mechanism(s) by which METH promotes increased HIVD is not well understood. Our laboratory has shown that METH enhances lentiviral infectivity of astrocytes via the CXCR4 chemokine receptor, suggesting that the stimulant might act at the site of the entry of the virus. The studies in this chapter explores the hypothesis that METH, or other stimulants, enter astrocytes and alter the synthesis and release of chemokines and the expression of chemokine receptors. Furthermore, we explore possible intracellular signalling mechanisms (ROS, NO, Ca\(^{2+}\) and NFκB) that may contribute to the regulation of the CXCR4 receptor.

II. MATERIALS AND METHOD

A. Cell culture Techniques

\textit{i. Preparation of primary rat cortical astrocytes}

Cultures of cortical type-1 astrocytes were obtained by methods previously described (McCarthy and De Vellis, 1980). This method has been further adapted by Meucci et al., 1998 and Bajetto et al., 2001. Two to 4 day old Sprague-Dawley rats (Harlan, Indianapolis, IN) were decapitated. The brains were then removed under aseptic conditions and cerebral cortices dissected in Dulbecco’s minimum essential medium (DMEM, Gibco, Grand Island, NY). The tissue is chopped into 3mm pieces using a McIlwain tissue chopper (Mickle Laboratory Enigneering Co., Surrey, United Kingdom) and digested with 0.25% trypsin, 1 mg/ml papain and 1 mg/ml collagenase (Sigma, St.Louis, MO) for 30 min at 37°C. The tissue is then serially rinsed three times with DMEM containing 10% fetal bovine serum (FBS), 100 IU/ml penicillin, 100 ug/ml streptomycin (Gibco, Grand Island, NY). After washing, the tissue was then mechanically dissociated using a 1 ml pipette to obtain a single cell suspension in DMEM. The larger pieces are allowed to settle to the bottom and the supernatant was
then removed and transferred to a fresh tube. Cells were centrifuged at 1000 rpm for 10 mins and then the medium was carefully aspirated and the pellet resuspended in fresh medium. This was repeated with leftover tissue to obtain as many cells as possible. Cells are grown in flasks with medium changed after 24 hours and thereafter every 3 days. After the cultures reached confluence, cells were shaken overnight and replated into culture wells. The cultures were identified as astrocytes by glial fibrillary acidic protein (GFAP) staining and >90% of the cells were GFAP positive. Cells were exposed to 7.5 nM L-leucine-methyl ester for 24 h to further purify the astrocyte cultures (Kaul and Lipton, 1999 and Bajetto et al., 2001). Cell number was estimated using trypan blue dye exclusion.

**ii. Drug Treatment**

One hundred thousand to 7.5x10^5 primary astrocytes were used after 48 h of plating, for various experiments and treated with various concentrations (0.01 to 100 µM) for 3-72 h as indicated. The optimal concentration of METH that was used for the CXCR4 receptor studies was 10 µM. METH was dissolved in phosphate buffered saline (PBS, Gibco, Grand Island, NY) and PBS was used as the vehicle. Cells were exposed to psychostimulants in addition to METH, including amphetamine or cocaine (Sigma, St. Louis, MO) for 24 h. For co-treatment studies, inhibitors were added 30 min prior to METH. Various inhibitors were administered alone or co-administered with METH for CXCR4 experiments. The following inhibitors were used for various experiments: monoamine transporter inhibitors (mazindol, GBR, fluoxetine and despiramine, Sigma, St. Louis, MO), ROS inhibitor (Trolox, Calbiochem, La Jolla, CA) and NFkB inhibitor (SN50, Calbiochem, La Jolla, CA). Prior to experimentation, the cells were examined for changes in morphology under an inverted light microscope and representative images were taken (Leica Microsystems, Germany).

**B. CXCR4 receptor expression**

**i. Detection of CXCR4 gene transcripts**
Total RNA was extracted from cells using Trizol reagent (Invitrogen, San Diego, CA) and 5 µg used for reverse transcription to cDNA. cDNA were used for the reverse transcriptase (RT) PCR reaction or real-time PCR. The cDNA used for RT-PCR to detect CXCR4, SDF-1 and the housekeeping gene actin used primers for CXCR4, SDF-1 and actin as previously described. The PCR procedure consisted of one cycle of 92°C for 45 sec, 58°C for 40 sec, 72°C for 30 sec and a final cycle of 72°C for 2 min for 42 cycles. PCR products (10 or 12 µl) were separated by electrophoresis in 1% agarose gel containing ethidium bromide, detected with a UV transilluminator, stained with SYBR green and analyzed using a phosphoimager. CXCR4 (Rn00573522_s1) and actin (Rn00667869_m1) mRNA expression was also detected using real-time PCR following manufacturer’s instructions (TaqMan Expression assays, Applied Biosystems). Briefly, actin was used as an internal control and FAM dye was detected by Applied Biosystems 7500 Real-time PCR System.

ii. Detection of CXCR4 protein expression

1. Detection of CXCR4 surface protein- flow cytometry

For FACS, the astrocytes, 750,000 cells per well, were detached by versene and stained with primary antibody, anti-CXCR4 (1:200, clone 44716, R&D Systems, Minneapolis, MN) followed by a secondary PE-labeled antibody (1:200, Southern Biotechnology, Birmingham, AL). Cells stained with IgG2b (R&D Systems, Minneapolis, MN) served as a negative respective isotype control. Cells were analyzed (10,000 events) on a FACS CALIBUR flow cytometer for determination of the intensity of fluorescence for each labeled sample. Cells positive for CXCR4 were expressed as percentage of the total cell population. To confirm these results, a modified method previously described in Odemis et al., 2002 was used with alternative antibodies (Santa Cruz sc-6190, goat polyclonal CXCR4 C-20).

2. Detection of CXCR4 protein- SDF-1 Binding Assay

SDF-1 binding methods are previously described (Hesselgesser et al., 1998). Briefly, whole cells were incubated with 1 nM of 125I-SDF-1 (Amersham Biosciences,
Piscataway, NJ) in the presence or absence of SDF-1, 1µM, in binding buffer (phosphate-buffered saline, 5 mM EDTA, and 0.25% bovine serum albumin, Sigma) for 1 h at 4°C (surface receptors) or 37°C (intracellular receptors, Zoon et al., 1983, Dohlman et al., 1991). The reaction was terminated by harvesting and washing the cells. Radioactivity was counted in whole cell preparations (surface sites) or following solubilization (intracellular sites) using a gamma counter.

iii. Cell Signalling

1. Measurement of intracellular Nitric Oxide (NO)
A nitrate assay was used following manufacturer’s instructions (Promega, Madison, WI). Briefly, the Griess Reagent System is based on a chemical reaction that uses sulfanilamide and N-1-naphthylethylenediamine dihydrochloride (NED) under acidic (phosphoric acid) conditions, and detects NO₂. After addition of NED solution, the mixture is incubated for 5-10 mins protected from light. After 30 mins, absorbance is measured on a microplate reader at 530 nm.

2. Measurement of intracellular Calcium (Ca²⁺) Influx
Intracellular Ca²⁺ concentration was measured using methods previously described (Bajetto et al., 1999 and Kim and Saffen, 2005). Cells were loaded with 2 µM fura2-AM (Calbiochem) in Kerbs-Ringer/HEPES buffer (NaCl 125 mM, KCl 5 mM, KH₂PO₄ 1.2 mM, HEPES 10 mM, MgSO₄ 1.2 mM, CaCl₂ 2 mM, glucose 6 mM, pH 7.4) as a buffer for astrocytes. After 30 min, cells were washed with Ca²⁺-free buffer and dispensed into culture well plates for fluorescence measurements. Fura-2-loaded cells were stimulated with 340 nM light at 0.5s intervals and fluorescence was recorded at 510 nM. All measurements were performed at room temperature to avoid probe compartmentalization. Changes in intracellular fluorescence were measured in individual cells or small groups of cells using a calcium imaging system on a Leica inverted microscope. Hardware and data collection were performed using OPENLAB3 imaging software (Improvision, Lexington, MA).
Detection of SDF-1 protein

SDF-1α enzyme linked immunosorbent assay (ELISA) was used following manufacturer’s instructions (R&D systems, Minneapolis, MN). Briefly, the kit was a quantitative sandwich enzyme immunoassay using a capture monoclonal antibody and detection polyclonal antibody. The colorimetric substrate solution is added for color to develop and then the reaction stopped. Within 30 mins, absorbance is measured by a microplate reader at 450 nm.

III. RESULTS

METH increases CXCR4 expression on primary rat astrocytes in a concentration and time dependent matter.

Primary rat astrocytes were identified as by GFAP staining. Isolation of primary astrocytes produced greater than 90% GFAP positive cells in three independent experiments (91.78% ± 1.59, mean ± SEM). Figure 4.1 shows a representative image of GFAP expressing cells, Hoescht nuclear staining and the overlay to count GFAP positive cells. The primary cortical rat astrocytes were treated with various concentrations (0.1 to 100 µM) of METH for 24 h and expression of CXCR4 was measured by flow cytometry using CXCR4 antibody. METH (1 and 10 µM concentrations; 143 ± 7, 151 ± 6, respectively percent of control ± SEM) exposure maximally increased (p<0.05) the percent of primary rat astrocytes expressing CXCR4 compared to vehicle alone (Figure 4.2). METH (10 µM) also significantly increased (p<0.05) CXCR4 surface expression at 12 (127 ± 8, percent of control ± SEM) and 24 h (147 ± 5) compared to vehicle alone (Figure 4.3).

METH increases the number of surface but not intracellular CXCR4.

Measurement of radiolabeled SDF-1 (natural ligand to CXCR4) binding was used to assess CXCR4 expression on primary rat astrocytes. Table 4.1 shows that in the presence of METH (10 µM) for 24 h, primary rat astrocytes increase (p<0.05) ^125^I-SDF-1 binding on the cell surface (547 ± 37, mean ± SEM) compared to vehicle alone (314 ±
However, there was no significant difference between the intracellular binding of $^{125}$I-SDF-1 to METH treated versus vehicle treated astrocytes. Overall, the increase in the total amount of binding to CXCR4 after exposure to METH is attributed to an increase in the cell surface CXCR4 and not to the intracellular stores of CXCR4 for 24 h (655, total mean) compared to vehicle (425). This result suggests de novo synthesis of CXCR4 receptors and not redistribution of internal stores to the cell surface.

**METH increases CXCR4 RNA expression at 3 and 6 h on primary rat astrocytes.**

Similar to our findings in G355-5 cells, we found that METH increased CXCR4 mRNA expression at 3, 6 and 12 h compared to vehicle. Figure 4.4 is a representative image of the CXCR4 RNA expression at 3, 6, 12 and 24 h in the presence and absence of METH. This result was confirmed by real-time PCR. METH significantly (p<0.05) increased CXCR4 mRNA expression at 3 h (8.11 ± 2.87, fold increase ± SEM) and 6 h (4.32 ± 1.53) compared to respective control (6.59 ± 2.33, 2.4 ± 0.85, Figure 4.5). There was an increase at 12 h but the difference was not statistically significant.

**Nitric oxide (NO) and calcium (Ca$^{2+}$) did not increase after treatment with METH.**

Two intracellular signaling molecules that have been involved in chemokine signaling and astrocytes are nitric oxide and calcium. There was no effect on nitric oxide levels after 2 h of METH (10 µM) exposure in primary rat astrocytes (Figure 4.6). There was a significant increase in nitric oxide after exposure of astrocytes to LPS (2.5 ng/ml) which served as a positive control. Intracellular Ca$^{2+}$ in primary rat astrocytes was not affected after stimulation with METH (10 µM). Figure 4.7B shows Ca$^{2+}$ levels during a representative experiment, astrocytes were exposed to METH. Figure 4.7A shows Ca$^{2+}$ in astrocytes stimulated with levels ATP, which served as a positive control. Taken together, this data shows that NO and Ca$^{2+}$ are not involved in intracellular signalling in METH-induced changes of astrocytes.

**ROS is involved in METH-induced increases in CXCR4 expression in primary rat astrocytes.**
Reactive oxygen species (ROS) have also been implicated in intracellular signaling in chemokines as well as astrocytes. Furthermore, Trolox (200 µM) was shown to inhibit this METH induced increase in ROS (data not shown). This indicates that METH causes an increase in ROS levels in primary rat astrocytes. These results were further confirmed by using a pharmacological inhibitor of ROS, Trolox (Park et al., 2004). Trolox is also called vitamin E and a free radical scavenger (Park et al., 2003). We found that METH (6.18 ± 2.87, Fold Increase ± SEM) significantly (p<0.05) increases CXCR4 mRNA expression at 3 h compared to its respective control (0.32 ± 2.33 and 0.08 ± 0.72; Trolox alone). Furthermore, we found that Trolox (0.17 ± 0.68) inhibited the METH-induced increase in CXCR4 mRNA expression (Figure 4.8). In this set of experiments, we again found that METH (192 ± 8, percent of control ± SEM) significantly (p<0.05) increased the CXCR4 expression on primary rat astrocytes compared to vehicle (99 ± 18) alone (Figure 4.9), and that Trolox (117 ± 10) could inhibit the METH-induced increase in CXCR4. Figure 4.10A shows a representative histogram of the METH induced increase in CXCR4 expression. Figure 4.10B shows a representative histogram of Trolox inhibition of the METH-induced increase in CXCR4 expression.

**NFκB is involved in METH-induced increases in CXCR4 expression in primary rat astrocytes.**

The results thus far suggest that METH causes de novo synthesis of CXCR4. It has been shown that transcription factor, NFκB, increases the synthesis of CXCR4 (Helbig et al., 2003). It has also been shown that METH can increase NFκB expression (Lee et al., 2001). Furthermore, that METH may induce ROS to then activate NFκB because researchers have shown that METH without ROS cannot activate NFκB (Asanuma and Cadet, 1998). Therefore, NFκB is a likely factor to be involved in the METH induced increase in CXCR4 expression. We used a pharmacological inhibitor of NFκB, SN50, which is a cell-permeable peptide that has a nuclear localization sequence that inhibits translocation of that NFκB active complex into the nucleus (Lin et al., 1995, Lee et al., 2001). We found that SN50 (100 µg/ml, 0.13 ± 0.53, fold increase ± SEM)
significantly (p<0.05) inhibited the METH-induced (6.18 ± 2.87) increase in CXCR4 mRNA expression at 3 h (Figure 4.11). We found that SN50 (119 ± 31, percent of control ± SEM) inhibited METH-induced increase in CXCR4 surface expression on primary rat astrocytes (Figure 4.12). Figure 4.13 is a representative histogram of SN50 inhibition of the METH-induced increase in CXCR4 expression. Taken together, this data showed that NFκB may of the transcription factors involved in the METH-induced changes in astrocytes.

**Monoamine transport inhibitors prevent METH induced increase in surface expression of CXCR4 on primary rat astrocytes.**

Astrocytes have been shown to express monoamine transporters for: dopamine (DAT), serotonin (SERT) and norepinephrine (NERT) (Inazu et al., 1999, 2001 and 2003). METH is known to utilize these monoamine transporters to gain access into cells. Pharmacological inhibitors have been shown to inhibit specific monoamine transport function (Table 4.2). Mazindol (MAZ) (100 ± 5, percent of control ± SEM), fluoxetine (FLU) (103 ± 3) and despiramine (DES) (87 ± 6) all significantly (p<0.05) inhibited the METH-induced (137 ± 4) increase in surface expression of CXCR4 (Figure 4.14). Taken together, these data show that monoamine transporters may be one route METH uses to gain access to astrocytes.

**Other psychostimulants do not have the same effect as METH on the surface expression of CXCR4 on primary rat astrocytes.**

Amphetamine (AMPH) and cocaine (COCA) are drugs belonging to the same class of psychostimulant drugs as METH. However, they did not have the same effect on cell surface CXCR4 expression on primary rat astrocytes. Figure 4.15 shows a representative histogram of 3 different experiments with a clear shift in mean channel fluorescence of METH treated astrocytes compared to vehicle alone. Figure 4.15B is a representative histogram of astrocytes treated with AMPH (10 µM) that showed a slight but not a significant shift in the mean channel fluorescence compared to vehicle alone. Figure
4.16C is a representative histogram of astrocytes treated with COCA (10 µM) that showed no shift in the mean channel fluorescence compared to vehicle alone.

**METH increases SDF-1 expression at 24 h on primary rat astrocytes.**

Primary rat astrocytes in the presence of METH (1581 ± 177) significantly (p<0.05) increased their secretion of SDF-1 in the medium compared to vehicle (575 ± 20) alone (Table 4.3). In our studies, we found that there was an increase in RNA expression of SDF-1 (Figure 4.16) and SDF-1 protein was released in the medium (Table 4.3) of astrocytes after 24 h treatment of METH.

**IV. DISCUSSION**

CXCR4 is a chemokine receptor of importance because of its role as a coreceptor in lentiviral infection, specifically HIV-1. CXCR4 is constitutively expressed in non-hematopoietic organs such as the lung, heart and brain. In the brain, various neural cell types express CXCR4 including neurons, microglia and astrocytes. CXCR4 is one of the best characterized chemokine receptors in the CNS (Patel et al., 2006). Although astrocytes are a major source of chemokines in the CNS, the details of the regulation of chemokine expression is not known. A potential function of CXCR4 on astrocytes is involvement in neural migration, differentiation, and survival (Lazarini et al., 2003). Another function may be the recruitment of both astrocytes and leukocytes to specific regions of the brain during pathophysiological conditions. The studies in this chapter expand upon the findings of the previous chapter which reported that a psychostimulant drug, such as METH, can have multiple effects on the brain including modulation of chemokine receptors. These studies confirm the results from the feline astrocyte cell line, G355-5 that METH increases the surface expression of chemokine receptor, CXCR4. These results were confirmed using primary astrocyte cultures (Figure 4.1). In these experiments, we found that METH increases CXCR4 surface expression in a concentration (Figure 4.2) and time dependent (Figure 4.3) manner. Ten µM and 1 µM of METH significantly increased the expression of surface CXCR4, with the maximal effect at 10 µM. Between 6-72 h, METH (10 µM) increased CXCR4 surface expression at 12
and 24 h, with maximal effect at 24 h. The concentration of METH is higher than the concentration used in the cell line but, the maximal effect is similar at 24 h. The higher concentration of METH was used because there were more consistent results than with the lower one. Overall these results demonstrate that the METH-induced increase in CXCR4 expression is a consistent finding between two different animal model systems.

Studies have shown that modulation of the cell surface expression of chemokine receptors is either due to inhibition of receptor internalization, increased translocation of receptors from intracellular pools to the cell surface (receptor compartmentalization/recirculation) or enhanced receptor synthesis (gene expression) (Ruibal-Ares et al., 2004, Murdoch, 2000). We found evidence that the METH-induced increase in CXCR4 cell surface expression levels could be due to the stimulation of RNA synthesis. Figure 4.4 and Figure 4.5 demonstrate an increase in CXCR4 mRNA expression in primary rat astrocytes after 3 and 6 h in the presence of METH. This is also confirmed by binding assay which showed that METH increases the overall binding of CXCR4 in primary rat astrocytes compared to vehicle alone (Table 4.1). An alternate possibility is that METH could affect RNA stability. CXCR4, like many cytokine receptors have an A-U-rich elements serve as target motifs for degradation by specific RNases (Shaw and Kamen, 1986 and Gupta et al., 1998). However, additional studies will be needed to determine whether this increase in CXCR4 RNA synthesis is due to transcription of NFκB or RNA stability. Additionally, another possibility by which METH could regulate CXCR4 cell surface expression is by affecting receptor compartmentalization. This possibility seems less likely based on results of the binding experiments (Table 4.1). There was no difference between the internal stores of CXCR4 receptors in the presence and absence of METH. There was a difference in surface expression of CXCR4 between METH and vehicle-treated cells. This difference was due to a 1.5 fold increase in CXCR4 on METH-treated cells compared to vehicle alone. These both mRNA and protein studies indicate that receptor compartmentalization is not a factor, but synthesis of additional CXCR4 receptors.
The detrimental effects of METH are hypothesized to be due to the increase in monoamines, specifically dopamine, in the extracellular space between neurons. Much of this effect is attributed to the ability of METH to be transported into neurons via monoamine transporters. Astrocytes express monoamine transporters (Hanson, 1985, Schools and Kimelberg, 2001). Our data suggests that METH is transported via monoamine transporters to cause increased CXCR4 expression. Pharmacological inhibitors of monoamine transporters prevented the METH-induced increase in surface expression of CXCR4 on primary rat astrocytes (Figure 4.14). Other studies have shown that SERT and NERT play important roles in preventing METH-induced effects (Rothmann et al., 2001). We found similar results, via, that pharmacological inhibitors of SERT (FLU) and NERT (DES) were better at preventing METH-induced increase in CXCR4 expression.

The increase in CXCR4 cell surface expression could be due to many possible mechanisms including external factors or activation of receptors such as trace amine receptors (Miller et al., 2005 and Bunzow et al., 2001) or sigma receptors (Itzhak, 1993, Nguyen et al., 2005). These factors could cause autocrine and/or paracrine signaling to modulate CXCR4 cell surface expression. Some factors that been shown to modulate CXCR4 cell surface expression include IL-6, cAMP, IL-1β and tumor necrosis factor-alpha (TNF-α) on astroglioma cells (Odemis et al., 2002, Oh et al., 2001, Han et al., 2001). Therefore, the METH-induced increase in CXCR4 expression may be an indirect effect, causing the release of intermediary factor(s) to increase CXCR4 receptor expression.

Once METH is inside the cell, there are many possible intracellular signaling molecules such as nitric oxide (NO), calcium (Ca^{2+}) and reactive oxygen species (ROS) that could be activated by METH, its metabolites or some other intermediary factor. All these signaling molecules have been implicated in METH use and CXCR4/SDF-1 signaling (Bajetto et al., 2001, Davidson et al., 2001). NO has been found to be involved in CXCR4/SDF-1 induced activity (Cherla et al., 2001) and inhibition of NO has been
shown to be neuroprotective to METH (Kawasaki et al., 2006; Kita et al., 2003). Primary rat astrocytes NO level after the administration of METH were not significantly different compared to vehicle alone (Figure 4.6). Ca\(^{2+}\) has been shown to be an important signaling molecule within astrocytes specifically for cell-to-cell communications (Rose and Konnerth, 2001; Scemes and Giaume, 2006). It has been shown in astrocytes that CXCR4 can induce Ca\(^{2+}\) mobilization after SDF-1\(\alpha\) stimulation (Bajetto et al., 1999a and Bajetto et al., 1999b; Bajetto et al., 2001). However, our studies show that Ca\(^{2+}\) influx also did not change after administration of METH (Figure 4.7). This indicates that METH is not directly stimulating CXCR4 signaling in conjunction with or similar to SDF-1\(\alpha\). However, this type of experiment measures immediate Ca\(^{2+}\) influx and does not measure overall concentration of Ca\(^{2+}\). The latter would require more experiments to elucidate the specific relationship between METH and Ca\(^{2+}\).

Another signaling molecule that may play a role after METH treatment is ROS. The administration of antioxidants or free radical scavengers (vitamin E) or transgeneic overexpression of CuZn-SOD has been shown to attenuate METH-induced toxicity (Wagner et al., 1986; Quinton et al., 2006 and Cadet et al., 1994). Therefore, with high concentrations of METH, it was found that ROS plays an important role. The ROS inhibitor, Trolox (vitamin E, Figure 4.8 and Figure 4.9), prevented the METH-induced increase in expression of CXCR4. Trolox gave consistent results in the prevention of increased production of ROS and the increase in surface expression of CXCR4. More studies are needed to elucidate the particular ROS pathway involved in this METH induced increase in CXCR4 expression in astrocytes.

Nuclear factor \(\kappa\) B (NF\(\kappa\)B) is a transcription factor, composed of a p50/p65 heterodimer complexed to inhibitor \(\kappa\)B under unstimulated conditions (Asanuma and Cadet, 1998). The activation of this transcription factor is initiated by the dissociation of \(\kappa\)B via its phosphorylation and activation of NF\(\kappa\)B causing its translocation into the nucleus where it binds to various target genes. NF\(\kappa\)B has been shown to be expressed in both neurons and glial cells. One of the NF\(\kappa\)B target genes is CXCR4 (Kukreja et al.,
We hypothesized that METH induces the production of ROS which is responsible for the activation of NFκB and subsequent expression of CXCR4 by rat astrocytes. ROS has been shown to activate NFκB to induce expression of other chemokines in astrocytes (Park et al., 2004 and Park et al., 2003). In our studies, we found that NFκB inhibitors prevented the METH induced increase in CXCR4 mRNA and surface protein expression (Figure 4.11 and Figure 4.12). Both ROS and NFκB have been shown to play a role in METH-induced toxicity (Asanuma et al., 1998, 2002; Hirata and Cadet, 1997). Asanuma and Cadet (1998) were the first to demonstrate that METH-induced ROS generation can activate striatal NFκB DNA-binding and moreover, may play a role in the drug’s induced neurotoxicity. Our data expanded upon this observation by demonstrating that METH at a lower concentration can induce ROS and NFκB and may further induce the upregulation of CXCR4 on the astrocyte surface.

One of the limitations of these studies is the use of CXCR4 R&D (mAB 172) antibody clone 44717. It has been suggested by the manufacturer that this antibody is not specific to rat CXCR4 (Baribaud et al., 2001). We originally used this antibody for consistency with our previous feline studies and for continuity decided to use the antibody for the rat experiments. We also did not question the reactivity with rat astrocytes because repeatedly we were able to distinguish between the isotype and the antibody labeled astrocytes. We have also found that others have used this antibody to recognize rat CXCR4 (Peng et al., 2004). However, recognizing the limitations of these results, we decided to confirm the results using another antibody shown to be specific for rat CXCR4 in flow cytometry experiments (Odemis et al., 2002). We were able to confirm the specificity of the expression of CXCR4 with this polyclonal antibody. However, due to high background staining with polyclonal antibody; we were not able to obtain consistent results (data not shown).

These findings indicate that this observation is specific to METH within the group of psychostimulants (Figure 4.15), because neither amphetamine (AMPH) nor cocaine
(COCA) caused upregulation of CXCR4. AMPH caused a shift in the surface expression of CXCR4, but it was not statistically significant. It is plausible that AMPH would have a similar effect since its chemical structure is similar to METH. COCA did not have a significant effect. These findings were reconfirmed by Hu et al., 2006, who found that COCA in similar concentrations induced a down-regulation of CXCR4 on neural precursor cells.

There is growing evidence that SDF-1, the only natural ligand to CXCR4, may act as a growth factor (Bajetto et al., 2001). Primary rat astrocytes in the presence of METH increased their secretion of SDF-1 (Table 4.3) and mRNA expression of SDF-1 (Figure 4.16). However, more studies are needed to fully understand the relationship between the receptor and its natural ligand. Usually when there is an increase in SDF-1, it causes endocytosis of the receptor (Tarasova et al., 1998). However, we did not observe this result perhaps because the level of SDF-1 present was too low to cause endocytosis or there is a separate mechanism occurring to increase SDF-1 and CXCR4 expression. Bajetto et al. (2001, 1999), have hypothesized that CXCR4/SDF-1 may play a role in the generation of gliosis, a hallmark of pathological diseases. There is a possibility that both METH and HIV might modulate SDF-1 production or modulate/interact with CXCR4 expression to generate proliferative signals to cause gliosis (Okamoto et al., 2005). Beyond toxicity related consequences, there may be consequences to these astrocyte reactive/proliferative signals. Therefore, conditions that may readily occur with METH use may further exacerbate the detrimental effects of HIV.

Taken together, in the primary rat astrocyte model, METH (10 µM) increases surface CXCR4 expression and this increase seems to be due to de novo synthesis of CXCR4 similar to the feline cell model. From the data, we also concluded that both ROS and NFκB are involved in the METH-induced effect on astrocytes. This all indicates a possible pathway in which METH can cause changes in astrocytes. In addition, the data indicates that METH (10 µM) increased NFκB transcription, which could cause more global changes within the CNS including modulation of other cytokines and chemokines and their receptors.
Figure 4.1 Representative image of GFAP staining to characterize primary rat cortical astrocytes. A. Immunostaining of GFAP B. Hoechst staining for the nucleus C. Overlay of both GFAP and Hoechst staining images to be able to identify astrocytes. Cells were counted from three independent experiments and greater than 90% of the cells were GFAP positive.
Figure 4.2 METH at 1 and 10 µM concentrations maximally increases CXCR4 surface expression on primary rat cortical astrocytes. Cells are treated with various concentrations (0.1 to 100 µM) of METH for 24 h. CXCR4 expression was determined by flow cytometry. Data was expressed as percent of control ± SEM in 4-8 independent experiments. *p<0.05 Mann-Whitney Test.
Figure 4.3 METH (10 μM) maximally increases CXCR4 surface expression at 12 and 24 h on primary rat cortical astrocytes. CXCR4 expression was determined by flow cytometry. Data was expressed as percent of control ± SEM in 4-8 independent experiments. *p<0.05 Mann-Whitney Test.
<table>
<thead>
<tr>
<th>Treatment</th>
<th>CXCR4 Receptors (fmol/mg prot ± SEM)</th>
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<tbody>
<tr>
<td></td>
<td>Cell Surface</td>
</tr>
<tr>
<td></td>
<td>Cell Cytosol</td>
</tr>
<tr>
<td>Vehicle</td>
<td>314 ± 16</td>
</tr>
<tr>
<td>METH, 10 µM</td>
<td>547 ± 37</td>
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</table>

Table 4.1 METH increases the number of surface CXCR4 receptors but, not intracellular receptors. Astrocytes were treated with METH, 10 µM, for 24 h and $^{125}$I-SDF-1α, 1 nM, binding was measured on whole astrocytes as in Methods. *p<0.05 compared to vehicle. N=7-8 for surface and N=3 for cytosol CXCR4 binding.
Figure 4.4 METH increases CXCR4 mRNA expression at 3, 6 and 12 h on primary astrocytes. CXCR4 RNA expression of primary cortical astrocytes was measured in the presence and absence of METH at 3, 6, 12 and 24 h. A representative image of the RNA expression is shown above. Actin, housekeeping gene was used as an internal control.
Figure 4.5 METH increases CXCR4 mRNA expression at 3 and 6 h on primary astrocytes. Primary rat astrocytes were treated with either METH (10 µM) or vehicle alone for 3, 6, 12 and 24 h. Astrocyte mRNA expression was measured by real-time PCR. Data is expressed as fold increase (mean exponent of delta delta threshold, expDDCT ± SEM) of 3-4 independent experiments. *p<0.05 confidence intervals were compared to respective vehicle alone.
Figure 4.6 METH (10 µM) had no effect on nitric oxide (NO) production in primary rat astrocytes at 2 h. The amount of nitrates was used to estimate the concentration of NO. LPS shows increased NO in astrocytes and serves as a positive control. Data is expressed as mean SEM of three independent experiments. *p<0.05 student t-test.
Figure 4.7 Stimulation of primary rat astrocytes with METH (10 µM) does not immediately increase the concentration of calcium (Ca$^{2+}$). Representative graphs of Ca$^{2+}$ concentration over time are shown above. Primary astrocytes were loaded with Fura2M 30 min prior to experimentation and the fluorescence measured Ca$^{2+}$. The stimulation of primary rat astrocytes with ATP increases calcium signaling and is used as a positive control. METH does not similar result in three independent experiments.
Figure 4.8 Trolox prevents the METH induced increase in CXCR4 mRNA expression on astrocytes. Primary rat astrocytes were treated with either METH (10 µM) or Trolox (200 µM) or vehicle alone for 3h. Astrocyte mRNA was measured by real-time PCR. Data is expressed fold increase (mean exp DDCT) ± SEM of 3 independent experiments. *p<0.05 confidence interval test, compared to respective control (vehicle alone).
Figure 4.9 Trolox prevents the METH induced increase in CXCR4 surface expression on astrocytes. Primary rat astrocytes were treated with either METH (10 µM) or Trolox (200 µM) or vehicle alone for 24 h. CXCR4 expression was measured by flow cytometry. Data was expressed as percent of control ± SEM of 3-7 independent experiments. *p<0.05 compared to vehicle alone, Mann-Whitney Test.
Figure 4.10 Trolox prevents the METH induced increase in CXCR4 surface expression on astrocytes. Primary rat astrocytes were treated with either METH (10 µM) or Trolox (200 µM) or vehicle alone for 24 h. CXCR4 expression was measured using flow cytometry. A. A representative histogram of CXCR4 expression in the presence of METH (pink), vehicle (green) and isotype control (light blue) at 24 h. B. A representative histogram of CXCR4 expression in the presence of METH (pink), vehicle (green) and METH+Trolox (orange) at 24 h.
Figure 4.11 SN50 prevents the METH induced increase in CXCR4 mRNA expression on primary rat astrocytes. Primary rat astrocytes were treated with either METH (10 µM) or SN50 (100 µg/ml) or vehicle alone for 3h. Astrocyte mRNA was measure by real-time PCR. Data is expressed as fold increase (mean exp DDCT ± SEM) of 3 independent experiments. *p<0.05 confidence interval compared to respective control (vehicle alone).
Figure 4.12 SN50 prevents the METH induced increase in CXCR4 surface expression on primary rat astrocytes. Primary rat astrocytes were treated with either METH (10 µM) or SN50 (100 µg/ml) or vehicle alone for 24 h. CXCR4 expression was measured by flow cytometry. Data was expressed as percent of control ± SEM of 3-5 experiments. *p<0.05 Mann-Whitney Test.
Figure 4.13 SN50 prevents the METH induced increase in CXCR4 surface expression on primary rat astrocytes. Primary rat astrocytes were treated with either METH (10 μM) or SN50 (100 μg/ml) or vehicle alone for 24 h. CXCR4 expression was measured using flow cytometry. A. A representative histogram of CXCR4 expression in the presence of METH (pink), vehicle (green) and isotype control (light blue) at 24 h. B. A representative histogram of CXCR4 expression in the presence of METH (pink), vehicle (green) and METH + SN50 (orange) at 24 h.
Table 4.2 Monoamine transporter inhibitors

<table>
<thead>
<tr>
<th>Name of Inhibitor</th>
<th>Monoamine Transporter Specifically Inhibits</th>
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<tbody>
<tr>
<td>Mazindol (MAZ)</td>
<td>DAT and NERT</td>
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<tr>
<td>GBR12389 (GBR)</td>
<td>DAT</td>
</tr>
<tr>
<td>Fluoxetine (FLU)</td>
<td>SERT</td>
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<td>Despramine (DES)</td>
<td>NERT</td>
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"Name of Inhibitor Monoamine Transporter Specifically Inhibits"
Figure 4.14 Monoamine transporter inhibitors (MAZ, FLU and DES) prevent METH induced increase in surface expression of CXCR4 on primary rat astrocytes at 24 h. CXCR4 expression was measured by flow cytometry. Data was expressed as percent of control ± SEM of 3-10 experiments. *p<0.05 Mann-Whitney Test.
Figure 4.15 Other psychostimulants do not have a similar effect on METH on the surface expression of CXCR4 on astrocytes. CXCR4 surface expression on astrocytes was measured using flow cytometry. A. A representative histogram of CXCR4 expression in the presence of METH (pink), vehicle (green) and isotype control (light blue) at 24 h. B. A representative histogram of CXCR4 expression in the presence of AMP (purple), vehicle (green) or isotype control (light blue) at 24 h. C. A representative histogram of CXCR4 expression in the presence of COCA (blue), vehicle (green) or isotype of control (light blue) at 24 h.
Table 4.3 METH increases release of SDF-1 in primary cortical rat astrocytes.
Astrocytes were treated with METH, 10 µM or vehicle for 24 h and SDF-1 expression was estimated by ELISA. The table summarizes the amount of SDF-1 (mean ρmol/µg of prot ± SEM) present in the cells and media. *p<0.05 compared with vehicle, N=12 in two separate experiments.

<table>
<thead>
<tr>
<th></th>
<th>Media</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>575 ± 20</td>
<td>974 ± 18</td>
</tr>
<tr>
<td>METH</td>
<td>1581 ± 177*</td>
<td>2103 ± 155*</td>
</tr>
</tbody>
</table>
Figure 4.16 METH increases and SDF-1 RNA expression. Primary cortical rat astrocytes were treated with METH, 10 µM, or vehicle for 3, 6, 12 or 24 h, and SDF-1 mRNA measured by PCR. A. METH increased SDF-1 mRNA expression at 24 h. B. The housekeeping gene, actin was used as an internal control. Representative images are shown. N=3 separate experiments.
CHAPTER 5

DISCUSSION

METH at a low concentration promotes cell-associated FIV infection. Gavrilin et al., (2004) demonstrated that METH maximally increases FIV transcription at 14 days and expression of viral protein, p24 in G355-5 cells. Gavrilin et al., (2004) also showed that FIV infected PBMC are necessary for the infection of feline astrocytes (primary and G355-5 cells). These results served as the basis for our experiments. In our studies, we further demonstrated that within 24 h at the early stages of infection and contact between PBMC and G355-5 astrocytes, METH promoted FIV infection of G355-5 astrocytes as observed by microscopy (Chapter 2). The 24 h time period was established due to the short-lived effects of METH on G355-5 proliferation. In chapter 2 in addition to the infection studies, we also investigated the effect of METH on lymphocytes. However, we did not observe any effect of METH on PBMC within the specific conditions and parameters investigated in our experiments. This chapter established that there is a relationship between METH and FIV infection that warranted further study.

The focus of the majority of studies then moved to the effect of METH on astrocytes (Chapter 3). One of the factors that influences lentiviral infection is the expression of the lentiviral entry receptors. In Gavrilin et al (2004), they observed that viral infection was dependent on CXCR4. Other studies have confirmed that FIV infection of astrocytes is dependent on CXCR4 (Nakagaki et al., 2001). Multiple studies examined the effect of a range of concentrations of METH on G355-5 cells, including toxicity studies where at millimolar (mM) levels, METH was observed to cause
gliotoxicity. METH is toxic to astrocytes and causes cell death and other morphological changes including the presence of vacuoles. Moreover, these studies also implicated that glutamate may play a role in toxicity, as observed by others (Stadlin et al., 1998). However, the studies of interest were the lower concentration of METH, because we did find increased CXCR4 expression and proliferation. The interest stemmed from: 1) the implications of the interaction of METH and FIV and 2) the use of METH at physiologically relevant concentrations which caused cellular changes.

To further investigate the METH induced cellular changes, we decided to change animal models to rats for numerous reasons including using an established animal model for primary culture of CNS astrocytes to better study the effects of METH exposure. The primary rat astrocyte model provided an opportunity to identify possible mechanisms by which METH initiates/utilizes to cause these cellular changes. Our data provides a plausible mechanism that underlies the METH-induced effect on astrocytes, specifically the involvement of ROS and NFκB. Most of the work in the field of METH and HIV has focused either on toxic levels of METH and/or the specific toxic effects on neural cells. Our studies investigated this problem from a different perspective.

I. Future studies: METH and HIVD

METH use has been shown to be a risk factor for HIV infection (Page-Shafer et al., 1997, Buchbinder et al., 2005, and Seage et al., 1992). There is also an increased incidence of HIV encephalitis in METH users (Bell et al., 1998, Bourman et al., 1998, Urbina et al., 2004, Chang et al., 2005), suggesting susceptibility to the neurological effects of HIV-1(Rippeth et al., 2004). Epidemiological evidence shows that METH and HIV have additive deleterious effects on neuropsychological impairment (Carey et al., 2006). Both METH dependence and HIV infection have been independently shown to cause brain dysfunction. However, there are some epidemiologic data that show no association between drug abuse and increased HIV-1 disease progression (Barr et al., 2000). This outlines the complex issues with studying the interaction of METH and HIV dementia. Therefore, the following are some factors to consider in conflicting
epidemiological studies including: properly controlled epidemiologic studies in the drug
abuser population are extremely difficult to conduct, as surreptitious use of drugs, co-
infection with other pathogens, time of infection, route of infection, virus strain, genetic
factors, malnutrition, stress, environmental factors, dose of drug, frequency of drug use,
and degree of compliance with investigator’s instructions are all confounding variables
(Barr et al., 2000). There needs to be more study to better identify these issues in the
human population to better mimic these conditions in the laboratory.

HIV and METH may act synergistically to cause neuronal death (Maragos et al.,
2002, Turchan et al., 2001) and exacerbate inflammation (Flora et al., 2003, Conant et al.,
2004, Yu et al., 2002). It has been shown that lentiviruses can induce neuropathology in
the absence of substantial amounts of virus replication (Barr et al., 2000). To better study
the effects of lentiviruses on neuropathogenesis, FIV presents both a clinically relevant
and easily manipulated animal model. Our co-culture experiments demonstrated that
METH increased cell-associated FIV infection of astrocytes which paralleled the short-
lived of effects of METH on two very important parameters of G355-5 cells: proliferation
and CXCR4 expression. In future co-culture experiments, similar conditions should be
tested within 24 h to investigate inhibitors (monoamine transporters, ROS and NFκB)
which would likely hinder the METH-induced increase in FIV associated infection. The
temporal sequence of events may be important, so studies investigating the sequence of
FIV infection and METH and alternating patterns of drug administration should be
explored, since they may produce different results. One of the major advantages of the
feline model is the possibility to investigate \textit{in vitro} findings \textit{in vivo}. Future studies
should include investigating the effect of METH and FIV \textit{in vivo}. Also, it would be
interesting to investigate whether inhibitors would decrease infection and/or
neuropathogenesis \textit{in vivo}. It has already been shown that FIV infected cats express
CXCR4 on astrocytes (Kiorala et al., 2000).

Another possibility is to continue with rodent studies as the model to study the
interaction of HIV and METH. One of the difficulties with the rodent model is that there
is no lentiviral model that exists for HIV in the rodent species. Primary rat culture studies could continue to be investigated using mixed cultures including other neural cells such as microglia and neurons, or incorporating HIV viral proteins such as Tat, Nef, Vpr and the Env proteins gp120 and gp41 to cause neuronal injury (Kaul, 2008). In addition to viral proteins that may exacerbate METH-induced neuronal injury, it may also be interesting to investigate other neurotoxic factors such as TNF-α. TNF-α has been implicated in the neuropathology of lentivirus infections (Barr et al., 2000). Another option for these studies that has recently become available possibility is due to the development of is a mouse model which enables systemic HIV-1 infection, immune responses and neuroinvasiveness (Potash et al., 2005).

Animal studies are also needed that accurately model the addict’s pattern of drug use (Barr et al., 2000). Human METH use usually results from longer-term exposure of low doses or brief exposure to higher doses. This idea of “sensitization” is an enhanced physiological and behavioral response to a low dose of METH after prior exposure to low intermittent doses of this drug. This sensitization causes subtle alterations in brain morphology and motivation (early stages of abuse). To study these changes, it should also be considered that METH has a shorter half life than in a rat (less than an hour) than human (6-34 hours) and adjust the concentration of METH accordingly.

II. Future studies: METH and CXCR4 on astrocytes

CXCR4 plays a paradoxical role in the brain. CXCR4- SDF-1 receptor-ligand axis plays an essential role in brain development and neurogenesis (Kaul, 2008). Yet CXCR4 is also predominantly involved in HIV entry and associated neuronal damage (Kaul, 2008). Inflammation of the brain leads to reactive gliosis which is common in HIV dementia, a process characterized by proliferation and migration of astroglia and the subsequent formation of a glial scar. It has been suggested that chemokines play a role in the reactive process. The exact role of SDF-1 remains elusive. However, SDF-1 activates distinct signaling pathways in astroglia and promotes proliferation (Bajetto et al., 2001). SDF-1 may be involved in reactive gliosis in HIVD (Okamoto et al., 2005). We have
shown that METH modulates CXCR4 and SDF-1 expression in astrocytes, even though the exact mechanism has not been elucidated and further studies are still needed to further understand the relationship. An important observation is the gradual increase in expression of the OX40 and CXCR4 receptors on cells over time in vitro. Therefore, the lack of difference in receptor expression between the treated and vehicle at later time points may be due to saturation or outside the limits of the measurement. There needs to be further studies to understand the previous observation. We hypothesize that this may be yet another mechanism induced by METH to exacerbate the pathological condition of HIV dementia. Further studies are needed to understand if METH potentiates HIV-induced gliosis. Our studies into SDF-1 were limited and future studies should concentrate on the temporal pattern of the increase in CXCR4 expression relative to the increase in SDF-1 expression. Studies have shown that SDF-1 causes the endocytosis of CXCR4 and the effect of the ligand temporally on the receptor needs to be further elucidated (Tarasova et al., 1988). However, SDF-1 could play a potentially important role in HIV-related neurotoxicity, since HIV dementia patients have increased SDF-1 expression in both neurons and astrocytes (Rostasy et al., 2003). Also, Zhang et al., 2003 found that in the presence of MMP-2, SDF-1 is cleaved into a neurotoxic molecule.

The exact binding site for METH in astrocytes and/or how METH enters the cells remains unclear. Astrocytes have monoaminergic transporters and METH is a substrate for these transporters. We demonstrated that METH induced effects (increase in CXCR4 expression) are inhibited by pharmacological blockade of monoamine transporters (Chapter 4). However, this does not rule out the possibility that METH may bind to receptors such as the sigma (Itzhak, 1993, Nguyen et al., 2005) and more recently, trace amine receptors (Miller et al., 2005 and Bunzow et al., 2001). Sigma receptors have been shown to be present on astrocytes (Prezzavento et al., 2007 and Ben-Ami et al., 2006). Recently cocaine, another psychostimulant, has been shown to enhance HIV infection by activating sigma receptors and modulating the expression of both CXCR4 and CCR5 in PBMC (Roth et al., 2005). This finding raises the possibility that METH may act at another site and warrants further investigation into the contribution of sigma receptors on METH effects in astrocytes. Since both CXCR4 and sigma receptors are GPCR (G-
protein-coupled receptors) and there is growing evidence that the heterodimerization of GPCR can regulate its activity (Percherancier et al., 2005), the sigma receptor should be closely examined.

METH may also play a role in the modulation of immune molecules. We have shown that NFκB peptide inhibitor attenuated METH-induced expression of CXCR4. The administration of METH has been shown to activate NF-κB (Lee et al., 2001). It has been shown by others that NF-κB regulates directly the up-regulating the expression of CXCR4 (Helbig et al., 2003). Transcription of NF-κB has been shown to be involved in the upregulation of many cytokines and chemokines, suggesting that METH activation of NF-κB may cause a more global effect modulating other chemokines and cytokines.

METH has been shown to increase expression of cytokines such as TNF-alpha and IL-1 beta (Nakajima et al., 2004, Flora G et al., 2002, Yamaguchi et al., 1991). Thomas et al., (2004) has confirmed using oligonucleotide microarrays increased expression of many cytokines and chemokines. Astrocytes may play a role in the secretion of these cytokines. The mechanism underlying METH-induced astrocytic secretion is unclear at this time. METH could be taken up by astrocytes across the astrocytic plasma membrane (via transporter), and result in astrocytic secretion. Not only NF-κB transcription factor may play a role, but other transcription factors such as COX-2 have been shown to be involved in METH-induced toxicity (Asanuma et al., 2003, Thomas et al., 2005). It has also been shown that inflammatory cytokines such as TNF-alpha, INF-gamma and IL-1 attenuate CXCR4 expression (Odemis et al., 2002, Busillo et al., 2006). Future studies should focus on the involvement of cytokines and chemokines after acute and chronic methamphetamine administration.

Of the different classes of psychoactive drugs that have a potential for abuse, opiates are the best studied to date in the context of lentivirus pathogenesis. As mentioned before, morphine has been shown to increase FIV and HIV replication in vitro. Morphine has also been shown to increase another HIV coreceptor, CCR5 gene expression (Mahajan et al., 2002) in astrocytes and CXCR4 on neuronal cells (Patel et al., 2006). Cocaine, part of the same class of psychostimulants, has been reported to increase
both CXCR4 and CCR5 expression (Roth et al., 2005). Hence, our finding represents an example of a commonly abused drug regulating coreceptor expression and consequent enhancement of HIV infection.

Neurogenesis in the adult brain is thought to be associated with memory formation and learning plays an essential role in drug abuse dependence (Narita et al., 2006). There is growing evidence that substances of abuse, including METH alter neurogenesis and gliogenesis, associated with their changes in neurobehavioral effects including drug rewarding dependence and drug seeking behaviors (Hu et al., 2006, Narita et al., 2006, Manadyam et al., 2007). However, these results have been conflicting. Manadyam et al., 2007 found that METH abuse decreased medial prefrontal cortex gliogenesis which can contribute to compulsive drug seeking behavior. Narita et al., 2006 found that METH activates cortical purified astrocytes and astrocyte-related soluble factors enhance the development of METH-induced rewarding effects via the Jak/STAT pathway. This reinforces that astrocytes directly participate in rewarding effects induced by METH, adding to the evidence that METH affects astrocytes in addition to neurons.

**Conclusion:**

Taken together, what this data adds to the literature is that METH, a frequently abused drug, causes an increase in CXCR4 which may potentiate HIV infection, particularly in the nervous system. These data also identify potential therapeutics/interventions for METH users that are potentially at risk of HIV infection. This is an exciting area that stretches across many fields including virology, pharmacology, immunology and neuroscience and is truly interdisciplinary in nature. It brings about the exciting possibility that METH can have effects on the chemokine receptor system and raises the possibility that chemokines may play a novel role in the brain.
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