REGIONAL HETEROGENEITY AND DIVERSITY OF ASTROCYTES IN RESPONSE TO HIV-1 PROTEINS AND MORPHINE

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REGIONAL HETEROGENEITY AND DIVERSITY OF ASTROCYTES IN RESPONSE TO HIV-1 PROTEINS AND MORPHINE

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University

by

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List of Abbreviations

µg..................................................................................................micro gram, $1 \times 10^{-6}$ grams
µL..................................................................................................micro Liter, $1 \times 10^{-6}$ Liters
µM...............................................................................................micro molar, $1 \times 10^{-6}$ molar
AIDS...........................................................................................acquired immune deficiency syndrome
AMPA..........................................................$\alpha$-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate
ANOVA..........................................................analysis of variance
BBB..........................................................................................blood-brain barrier
CNS....................................................................................central nervous system
DOR.........................................................................................$\delta$-opioid receptor
EAAT.......................................................................................excitatory amino acid transporter
ECM.........................................................................................extracellular matrix
GFAP ..................................................................................Glial fibrillary acidic protein
HIV..........................................................................................human immunodeficiency virus
IL...............................................................................................interleukin
IVDUs..........................................................intravenous drug users
K⁺..............................................................................................potassium
KD............................................................................................kilo Dalton
KOR.........................................................................................$\kappa$-opioid receptor
MANOVA..............................................................................multivariate analysis of variance
MCP...........................................................................................macrophage chemoattractant proteins
Mg..............................................................................................milli gram, $1 \times 10^{-3}$ grams
MIP...............................................................................................macrophage inflammatory protein
mL ................................................milli Liter, $1 \times 10^{-3}$ Liters

mm ..............................................................................................milli meter, $1 \times 10^{-3}$ meter

mM................................................................................................ milli molar, $1 \times 10^{-3}$ molar

MOR.............................................................................................. μ-opioid receptor

Na$^+$ ..........................................................................................sodium

NMDA ..........................................................................................N-methyl-D-aspartic acid

NO...........................................................................................nitric oxide

RIPA.......................................................................................... radio immunoprecipitation assay buffer

TNF.........................................................................................tumor necrosis factor
REGIONAL HETEROGENEITY AND DIVERSITY OF ASTROCYTES IN RESPONSE TO HIV-1 PROTEINS AND MORPHINE

Abstract

HIV-infected individuals who abuse opiates have been found to have a higher incidence and a faster progression of HIV encephalitis. Astrocytes, the major support cells in the CNS, are known to play a critical role in the HIV neuropathy. Although astrocytes tend not to be productively infected by the HIV-1 virus, dysregulation of their pro- and anti-inflammatory cytokines/chemokines secretion is usually neurotoxic. Glutamate transport in astrocytes is reported to be impaired as well, which result in extrasynaptic excitatory neurotransmitter accumulation and over stimulation of postsynaptic neurons. It is long known that astrocytes from different brain regions have diverse responses to extracellular stimulants. However, few publications discuss the regional heterogeneity of astrocytes in HIV infected central nervous system. Based on the above information, we hypothesize that astroglia from different brain regions vary in their responses to HIV proteins and the responses could be influenced by co-treatment of morphine. To test this we cultured astrocytes from cerebral cortex, cerebellum and spinal cord, treated them with HIV-1 proteins Tat and gp120 with or without morphine and opioid receptor (mu, delta and kappa receptor) antagonist, naloxone. After 12-18 hours, conditioned medium from each group was analyzed using a Bio-Plex array. Cells from striatal cultures were harvested and lysed; proteins were extracted and evaluated with Western blotting to see whether EAAT2 expression on astrocytes is changed. Results showed that there were significant regional differences among three brain regions in
cytokine/chemokine release, both in their basal secretion and in response to viral proteins. Astrocytes from spinal cord and cerebellum had a significantly higher basal secretion than those from cortical glia. All regions had increased cytokine/chemokine secretion when treated with Tat. Astroglia from the cortex showed the highest overall accumulation of cytokines/chemokines. Astroglia from the spinal cord had a slightly lower response overall, although KC expression was highest than other two groups in response to HIV-1 proteins. Astroglia from the cerebellum had a noticeably low response to Tat compared with those from spinal cord and cortex. However, concurrent morphine administration did not have a synergistic effect. No significant change in cytokines/chemokines release was seen when treated by gp120 with or without morphine. No significant change was found in EAAT2 expression on astrocytes either. In conclusion, astrocytes from different brain regions had different baseline secretion pattern and responses to viral protein. Tat had a noticeable effect in inducing cytokines/chemokines production in astrocytes from all brain regions, while limited change could be found with gp120 and morphine treatments. No significant change was found in EAAT2 expression on astrocytes.
**Introduction**

Although microglia are the main site of HIV productive infection in the CNS, astrocytes are also significant in the neuropathology associated with HIV. Astrocytes provide trophic metabolism and structural support for neurons. They also perform an extremely important role in brain and in HIV infection with cytokines/chemokines secretion to regulate leukocytes migration and proliferation. Astrocytes, contacting with the endothelial cells by the endfeet processes, induce formation of the blood-brain barrier (BBB). During brain inflammation, astrocytes get activated, proliferate and migrate to the injured area, a process which is overall referred to as reactive gliosis. This response leads to astrocyte-dysfunction, including alteration in BBB permeability, which facilitates monocyte/macrophage infiltration into CNS [Mucke and Eddleston, 1993], glutamate transport dysregulation, aberrant cytokines/chemokines production and reduced ability of astrocytes to support neuronal cells [Wang et al, 2003; El-Hage et al, 2005]. Therefore, studying the effects of HIV-1 on astrocytes is important in understanding HIV-associated neuropathology and therapeutic strategies.

Astrocytes are a originally described as a group of stellate shaped cells in the central nervous system, acknowledged by Virchow at late 19th century and early 20th century with the term of ‘Nervenkitt’ to account for ‘a connective substance formed in the brain and spinal cord, in which the nervous system elements are embedded.’ [Kettenmann et al, 1995].

1. Morphology and ultrastructure of astrocytes and astrocytes-related cells in mammals
Astrocytes are developed from the neuroectoderm of the neural tube. They were, under light microscope, described by Cajal in 1913 [Kettenmann and Bruce, 2005] with gold chloride sublimate staining technique, as cells which had stellate appearance and numerous processes terminated on blood vessels and pia mater, containing subcellular particles. Morphologically, astrocytes are further divided into two types: protoplasmic astrocytes in gray matter, which have numerous short, thick and extensive branched processes with spinous projections, and fibrous astrocytes in white matter, which have relatively long, thin and straight processes with few branches [Fedoroff et al, 1986; Ross et al, 2005].

Figure 1 Two types of astrocytes in central nervous system [Leichnetz, 2006]. There are two types of astrocytes: protoplasmic astrocytes and fibrous astrocytes. As shown above, (a) fibrous astrocytes, located in white matter, have long, straight processes, sparsely branched. (b) Protoplasmic astrocytes, located in gray matter, have numerous short and thick processes, with numerous spinous projections.

In the 1950s, astrocytes were identified under electronic microscope [Luse, 1956; Farquhar and Hartmann, 1957] as cells with large and light nuclei, sparsely distributed organelles, giving rise to a ’watery cytoplasm’. There were glycogen particles and
bundles of filaments in both fibrous and protoplasmic astrocytes. The filaments, described as the most characteristic inclusions in the cytoplasm of astrocytes, are intermediate filaments, 6-9 nm in diameter, compacted into bundles 1-2 µm in diameter [Fedoroff et al, 1986]. The major component of the filaments is glial fibrillary acidic protein (GFAP) [Eng et al., 1971], a 49 KD protein [Bignami et al, 1972].

Figure 2. Electron microscopy of astrocytes [Leichnetz, 2006] (a) protoplasmic astrocyte with glycogen granules and (b) fibrous astrocytes with glial filaments.
In recent years, astrocytic cells can be visualized with immunocytochemical markers such as glial fibrillary acidic protein (GFAP), a major component of intermediate filaments, and S-100β or S 100 calcium binding protein β, which specifically expressed in cytoplasm and nucleus of astrocytes [Kettenmann and Bruce, 2005]. The immunoreactivities to the labels of astrocytes would change along with brain maturation and differentiation [Kettenmann and Bruce, 2005]. For example, during CNS development, vimentin and nestin are expressed in immature astroglial cells [Lendahl et al., 1990; Colucci-Guyon et al, 1994]. As the cell matures, glial fibrillary acidic protein (GFAP) expression increases [Liedtke et al, 1996] and vimentin and nestin expression decreases [Zehner and Paterson, 1983]. GFAP then replaces vimentin and nestin, and becomes a reliable marker for differentiated astrocytes [Eddleston and Muckle, 1993, Eng and Ghirnikar, 1994].

After over 100 years of study, astrocytes are considered more and more important in central nervous system (CNS) both physiologically and pathologically rather than just simply occupy the spaces between neurons.

2. Astrocyte functions: the following sections will mainly focus on the roles of astroglia in supporting neurons, neurotransmitter regulation and cytokines/chemokines production in inflammation

2.1 Neuron Support:

Astrocytes are the major glial cell type in the central nervous system. They not only physically support neurons, but also influence neuronal function through the release of neurotropic factors, assist synaptic formation and maintenance, guide neuronal development, regulate neurotransmitter metabolism, and contribute to the integrity of the
blood-brain barrier [Bush et al. 1999; Rubin and Staddon 1999; Sofroniew et al. 1999; Haydon 2000; Ullian et al. 2001]. They are the major source of extracellular matrix (ECM) proteins, adhesion molecules and trophic factors, which are critical in regulating neuron development, maturation and survival [Bernstein JJ et al, 1985; Gonzalez ML et al, 1993]. Receptors for these guidance molecules are present on growth cones and the filopodia of neurons. During interaction with ligands in the extracellular matrix (ECM), they display variant effects such as attraction, repulsion, promoting or inhibition. Therefore, neurites outgrowth during development or after injury depends on the balance of these guidance cues [Powell and Geller, 1999; Ard et al, 1993].

2.2 Glutamate uptake, transport and regulation

Astrocytes play an important role in clearing up the extrasynaptic glutamate neurotransmitter [Danbolt et al, 2001]. Their dysfunction is frequently involved in the pathogenesis of many neurological disorders, such as amyotrophic lateral sclerosis, epilepsy, Alzheimer’s disease, as well as HIV associated dementia [Choi et al, 1988; Hansson et al, 2000; Maragakis and Rothstein, 2001].

Glutamate is known as the major excitatory neurotransmitter in the mammalian CNS. It is implicated in complex neuronal activities such as learning and memory acquisition [Mayer and Westbrook, 1987]. Besides this, glutamate is also a nonessential amino acid, important in cell metabolism. Glutamate has two types of receptors, ionotropic glutamate receptors (iGluRs) and metabotropic glutamate receptors (mGluRs) (Figure 2). iGluRs are ligand gated ion channels, on which, binding of glutamate induces rapid influx of sodium and calcium ions into the postsynaptic neuron. N-methyl-D-aspartate (NMDA), AMPA and kainate receptors are three subtypes of iGluRs. mGluRs
are G-protein coupled receptors (GPCRs), through which, binding of glutamate activate G proteins and subsequently activate or inhibit intracellular signaling pathways [Rosemond et al., 2004]. mGluRs is classified into three groups, Group I mGluRs (mGluR1, 5), Group II mGluRs (mGluR2, 3) and Group III mGluRs (mGluR4, 6, 7 and 8) based on their difference on pharmacological properties [Pin and Acher, 2002]. Excessive activation of glutamate receptors triggers uncontrolled intracellular signaling cascades and results in neuronal excitotoxicity. Therefore, prompt removal of glutamate from extracellular environment becomes crucial to neuron survival [Sheldon et al, 2007].

The high-affinity sodium-dependent glutamate transport was described in brain tissue more than 20 years ago [Balcar et al, 1972; Logan et al, 1972]. The uptake system also transports several glutamate analogues, such as aspartate. Transport of one glutamate molecule is coupled to the cotransport of three sodium ions and one proton and to the countertransport of one potassium ion [Kanner et al, 1987; Bouvier et al, 1992]. The family of glutamate transporters located on the plasma membrane of neuronal and glial cells throughout the CNS. They have five subtypes (EAAT1-5), according to their different distribution and pharmacological properties; each subtype has 8 transmembrane domains, with intracellular carboxyl and amino terminals (Table 1) [O'Shea, 2002].

Many studies suggested that glutamate uptake in astrocytes is highly affected by neighboring cells or by diffusible factors. For example, direct exposure of astrocytes to IL-1β, a key proinflammatory cytokine, markedly inhibits glutamate transport activity, while IL-1 receptor antagonist was found to be able to reverse the decreased astrocytic glutamate uptake [Ye and Sontheimer, 2002; Hu et al., 2000]. Its mechanism included elevated production of NO and reactive oxygen species [Chao et al., 1995; Hu et al.,
2000], therefore indicating that there exists a crosstalk between chemokine/cytokines interaction and glutamate uptake in astrocytes. Volsky et al also found that glutamate uptake was injured by HIV-1 protein gp120 by transcriptionally downmodulating EAAT2 transporter gene in human astrocytes and attenuate EAAT2 expression [Wang et al, 2003]. Given the fact that when treated with opiates and/or HIV-1 Tat, MCP-1, RANTES and IL-6 were increasingly released from primary striatal astrocytes [El-Hage et al, 2005], we were interested to investigate whether astroglial glutamate transporter expression in astrocytes was also influenced by HIV-1 proteins.
Figure 3: Schematic drawing of a glutamatergic synapse (a) Glutamate activates several different types of glutamate receptors located on the post-synaptic neuron, presynaptic terminal, and the surrounding astrocytes. For simplicity, receptors in this drawing are only shown on the post-synaptic terminal. Glutamate in the synaptic space is cleared by transporters that are located on the postsynaptic termini and on the glial processes. EAAT3 and EAAT4 are primarily expressed on neurons, while EAAT1 and EAAT2 are expressed on astrocytes. Transport of one glutamate molecule is coupled to the cotransport of three sodium ions and one proton and to the countertransport of one potassium ion. (b) The driving force for glutamate transport is consequently membrane potential and the ion gradients, which are built up by Na⁺-K⁺ ATPase.

Table 1 EAAT subtypes, distribution and pharmacology
(Modified from O'Shea, 2002)

<table>
<thead>
<tr>
<th>EAAT subtypes</th>
<th>Major Cell Type</th>
<th>CNS distribution</th>
<th>Competitive inhibitor</th>
<th>Non-competitive inhibitor</th>
</tr>
</thead>
<tbody>
<tr>
<td>EAAT1 (GLAST)</td>
<td>Glia; Transiently in a small number of neurons</td>
<td>Predominantly cerebellar Bergmann glia; but also in glia throughout CNS</td>
<td>L-CCG-III, THA, PDC, 4MG, MPDC, SOS, T4HG</td>
<td>TBOA</td>
</tr>
<tr>
<td>EAAT2 (GLT-1)</td>
<td>Exclusively in glia</td>
<td>Forebrain, cerebellum and spinal cord</td>
<td>L-CCG-III, THA, SOS, T4HG</td>
<td>MPDC, TBOA, 4MG, DHKA, 3MG, KA</td>
</tr>
<tr>
<td>EAAT3 (EAAC1)</td>
<td>Neuron</td>
<td>Throughout CNS</td>
<td>L-CCG-III, THA, PDC, MPDC, SOS</td>
<td>TBOA</td>
</tr>
<tr>
<td>EAAT4</td>
<td>Neuron</td>
<td>Purkinje cell of cerebellum</td>
<td>THA, PDC, αAA</td>
<td>TBOA</td>
</tr>
<tr>
<td>-------</td>
<td>--------</td>
<td>-----------------------------</td>
<td>---------------</td>
<td>------</td>
</tr>
<tr>
<td>EAAT5</td>
<td>Neuron</td>
<td>Rod photoreceptor and bipolar cells of the retina</td>
<td>TBOA, THA, PDC</td>
<td></td>
</tr>
</tbody>
</table>

* L-CCG-III: (2S,3S,4R)-2-(carboxycyclopropyl)glycine; DHKA: l-dihydrokainate; KA: kainate; 3MG: (+/–)-threo-3-methylglutamate; 4MG: (2S,4R)-4-methylglutamate; MPDC: l-antiendo-3,4-methanopyrrolidine dicarboxylate; PDC: l-trans-pyrrolidine-2,4-dicarboxylate; SOS: l-serine-O-sulphate; T4HG: l-threo-4-hydroxyglutamate; TBOA: dl-threo-β-benzylxaspartate; THA: l(–)-threo-β-hydroxyaspartate.

2.3. Secretion of cytokines/chemokines and role in inflammation

Cytokines/chemokines were demonstrated to have important role in progression of many inflammatory diseases in CNS including HIV-associated neuropathy [Clinque et al, 1998; Corasaniti et al, 2001; Zhao et al, 2001]. Astrocytes are potentially important contributors to inflammatory immune responses within the brain. Their functions include antigen presentation, cytokines and chemokines production and secretion [Wisniewski et al. 1989; Tyor et al. 1992; Wesselingh et al. 1993; Giulian et al. 1995; Conant et al. 1998]. Our study mainly focused on their dysfunction in cytokines/chemokines production when exposed to HIV-1 protein with or without morphine and comparison of cytokines/chemokines production among different brain areas.

Inflammation is a nonspecific biological response by the human body to any agents that cause cell or tissue injury. These agents could be physical (i.e. laceration, heat), caustic chemical (i.e. acid or alkali substances) or pathogenic (i.e. bacterium or virus), which induce both local and systematic inflammatory reactions. Local reaction begins with blood vessels dilation and increased vessel permeability. Leukocytes are attracted and migrate through vessel wall to the damage area. Inflammatory response is normally characterized by five signs: swelling or edema, redness, pain, heat and loss of
function. The polymorphonuclear leukocytes and mononuclear cells (monocytes, macrophages) are most important cells in phagocytizing pathogens and cleaning up debris in the injured area. Inflammatory responses in brain tissue are very different from other tissues in the body. The brain was once thought to be an immune privileged organ in which the expression of MHC class II antigen is minimal [Matsumoto et al, 1986], brain-blood barrier (BBB) prevents the passage of large molecules to immune cells from blood into brain. Now this view has been updated by current studies. People recognize that brain could not only receive signals from injured area but also exhibit an array of inflammatory responses which are, however, still different in brain compared with other parts of the body [Kreutzberg GW, 1996; Liu and Hong, 2003; Stret et al, 1999]. For example, because of the existence of BBB, leukocyte recruitment after injury occurs could be delayed, limitations of the skull makes brain edema potentially lethal.

Once stimulated during infection, microglia become activated and release pro-inflammatory cytokines, which in turn stimulates astrocytes to secrete a cascade of additional cytokines/chemokines [Kreutzberg GW, 1996], which are significant components in acute brain inflammation, in repairing damaged tissues, recruiting leukocytes, and increasing capillary permeability. But in chronic infection, uncontrolled and sustained cytokines/chemokines releasing is deleterious [Coussens and Werb, 2002].

Chemokines, named by merging of the term “chemotactic” and “cytokine”, are essential in the inflammatory process in attracting leukocytes to injured tissue. To date, over 40 chemokines have been identified. They are small proteins with molecular weights around 8-10 KD. Most chemokines possess a unique structural domain consisting of four conserved cysteines in N-terminal region [Fernandez and Lolis, 2002]. Chemokines are
subdivided into four families based on the local sequence of N-terminal at the first two cysteine residues. The four chemokine subfamilies are CXC or α-chemokines with one amino acid between two cysteines, CC or β-chemokines with first two cysteines adjacent to each other, CX3C or γ-chemokines with three amino acids between two cysteines and C or δ-chemokines have only two cysteines at N-terminal [Luster, 1998; Rollin, 1997]. CXC and CC families are best characterized among all four families. In CXC family, chemokine could be further divided into two groups, one containing a glutamic acid-leucine-arginine (ELR) preceding CXC sequence near N-terminal and the other group without such sequence. ELR chemokines could attract neutrophils, while those without this sequence act on lymphocytes. Chemokines in CC family generally attract monocytes, eosinophils, basophils and selectively lymphocytes [Luster, 1998; Rollin, 1997]. Chemokine receptors are designated as CXCR1-4 for CXC chemokine receptors, CCR1-8 for CC chemokine receptors, CX3CR1 for one CX3C chemokine receptor. They belong to the family of 7-transmembrane spanning (7-TMS), G-protein-coupled cell surface receptors (GPCR) (Table 2) [Luster, 1998; Rollin, 1997].

Cytokines are soluble proteins or glycoproteins, weighing between 8 to 30 KD, produced by leukocytes and many other types of cells. Cytokines act as intercellular signaling peptides through autocrine, paracrine, or endocrine modes of action. They regulate immune defenses or inflammation responses including regulating lymphocytes migration, proliferation and differentiation, developing and maintaining the inflammatory and immune responses after activated by the stimuli such as injury and infection. Most cytokines have the following four characteristics: pleiotropy (most cytokines are multifunctional), redundancy (some cytokines share the same function), potency (most
cytokines act in low concentration such as in the nanomolar or femtomolar range), and act in cascades and networks. These properties enable them to amplify the immune responses as well as inhibit overactivation, thus maintaining a balanced immune response towards internal or external stimuli [Fitzgerald KA et al, 2001].

Table 2 Chemokine subfamilies and receptors
(modified from Luster, 1998; Rollin, 1997)

<table>
<thead>
<tr>
<th>subfamily</th>
<th>structure</th>
<th>chemokine</th>
<th>receptors</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXC (α)</td>
<td>N CXC C</td>
<td>SDF-1</td>
<td>CXCR4</td>
</tr>
<tr>
<td>CC(β)</td>
<td>N CC C</td>
<td>MIP-1α, MIP-1β, MCP-1, MCP-2, MCP-3, MCP-4, MCP-5, RANTES, Eotaxin</td>
<td>CCR1, CCR5, CCR2, CCR1,2,5, CCR1,2,3, CCR1,2,3, CCR2, CCR1,3,5, CCR3</td>
</tr>
<tr>
<td>CXXX (γ)</td>
<td>N CXXX C</td>
<td>Fractalkine</td>
<td>CX3CR1</td>
</tr>
<tr>
<td>C (δ)</td>
<td>N C C</td>
<td>Lymphotactin</td>
<td>?</td>
</tr>
</tbody>
</table>

Astrocytes are one of the major sources of pro- and anti-inflammatory cytokines/chemokines, including IL-1, IL-6, IL-10, GM-CSF, G-CSF, M-CSF, TNF-α, and TGF-β, and important chemokines, such as RANTES, IL-8 and MCP-1 [Norris et al, 1994; Morganti-Kossmann et al, 1992; Park et al, 2009; Raivich et al, 1999]. The ability to secrete cytokines/chemokines enable astrocytes to serve as integral contributors to development of CNS immune responses. At the earliest stage of many types of infections in the central nervous system, astrocytes detect and respond to pathogens by release pro- and anti-inflammatory cytokines, including IL-6, TNF-α etc [Minagar et al. 2002; Bailey
et al. 2006]. The inflammatory response, although required for pathogen/antigen clearance and damage repair, can also result in collateral damage to the delicate cells present in the CNS such as neurons. For example, MCP-1 is mainly involved in monocytes and lymphocytes recruiting, thus important in HIV encephalitis amplification; IL-1β secreted by astrocytes induced calcium influx and triggered apoptosis cascades in neurons [Yeh et al, 2000; Xiong et al, 1999; Kuang et al, 2009]. Damage to these cells is particularly devastating due to limited regenerative capability [Klegeris et al, 2007]. Therefore, cytokines/chemokines released from astrocytes is a good indicator of inflammatory status of the HIV-infected brain.

2.4 Astrocytes exhibit regional heterogeneity and plasticity

Given the diverse function of the astrocytes and their significant roles both physiologically and pathologically, it is becoming apparent that astrocytes need to modify their characteristics as the environment changes around them throughout life under both physiological and pathological conditions. For example, during neuronal migration and maturation, the morphology of astrocytes changes remarkably. In the early development, astrocytes elongate to form radial fibers, called radial glia, in order to guide immature neurons to migration, while their long processes retract after neurons migrated to their adult positions later during development [Culican et al, 1990; Rakic, 1971, 1985].

A number of morphologically distinct astrocytes classes have been identified in adult CNS system: protoplasmic astrocytes in gray matter, fibrous astrocytes in the white matter, and specialized astrocytes such as Bergmann cells in cerebellum, and Müller cells in retina [Somjen 1988; Garcia-Marin et al. 2007; Kettenmann and Verkharatsky 2008].
They are not only morphologically different, but astrocytes have diverse characteristics as well. For example, astrocytes of the corpus callosum, a large subcortical white matter region, express high GFAP levels, whereas GFAP expression in astrocytes of cerebral cortex and striatum is comparatively lower and sporadic among cells [Bachoo et al. 2004; Lein et al. 2007; Bignami et al. 1972; Ludwin et al. 1976]. EAAT2 is more highly expressed in the hippocampal formation than in the cerebellum, whereas the opposite pattern of expression is observed for EAAT1 (GLAST) [Lehre et al. 1995].

To explain the diversity of receptor expression in astrocytes, McCarthy and coworkers did a series of experiments [Shao Y and McCarthy KD, 1994]. They pretreated the primary astrocyte cultures from identical clone with carbachol, an acetylcholine receptor agonist, 5 min per day for 3 days, found the loss of carbachol response in term of loss of intercellular [Ca^{2+}], while other ligands response (i.e. norepinephrine) still remained. They excluded the possibility of receptor desensitization since it occurred shortly after drug exposure. The delayed loss of response was also unlike receptor down-regulation, which required continuous presence of drug. Instead, they suggested that the external signals played an important role in regulation glial responses. According to the results, they further indicate that during brain maturation, some certain glial responses may be turned off by certain neurotransmitters released from neurons [Shao Y and McCarthy KD, 1994].

Astroglial morphology could also be modulated by neurotransmitters. McCarthy’s study suggested that norepinephrine can stimulate glial stellation through activation of β-adrenergic receptors in the hypothalamus and pituitary gland, while glutamate at
concentration from 50 to 500 \(\mu\text{M}\) can not only block, but also reverse, the isoproterenol-induced stellation [Shao Y and McCarthy KD, 1994].

Therefore, astrocytes can exhibit both morphological and functional plasticity as a consequence of extrinsic signals from neurons and possibly other types of cells. The most famous example of astrocyte plasticity is that which is acquired during inflammation or after neuronal injury. During the early stages of brain injury, astrocytic intermediary metabolism is impaired and protective astrocytic functions, such as glutamate uptake, potassium buffering, or elimination of free radicals, become compromised [Andersson et al., 2001], which is the result of the local environment alteration. During CNS degenerative processes, local astrocytes proliferate, become hypertrophic and undergo alteration in gene expression. This process is called reactive gliosis, driven by various autocrine and paracrine factors secreted locally.

Since there are few articles in the literature discussing the regional heterogeneity of astrocytes in HIV infected CNS, our study may be helpful in clarifying various HIV-associated neuropathologies in different brain region.

3. HIV-1 structure, proteins and associated HIV-associated dementia pathology

Human immunodeficiency virus (HIV) belongs to the primate lentivirus genus of the Retroviridae family. There are two species of HIV known to exist: HIV-1 and HIV-2. HIV-1 is the virus that was initially discovered. It is more virulent, more infective, and is the cause of the majority of HIV infections globally. There are 40 million people worldwide currently infected with HIV-1 [Ances and Ellis, 2007]. The introduction of highly active antiretroviral therapy (HAART) has greatly reduced mortality from HIV, but since the drugs fail to get through brain blood barrier, brain becomes an important
reservoir for virus. Thus the proportion of individuals with HIV neurocognitive impairment—either with minor cognitive motor disorders (MCMD) or neuropsychological impaired—actually has increased. Symptoms can result directly from infection or indirectly by increasing susceptibility to opportunistic infections and HIV-related malignancies. What was once an almost uniformly fatal illness is now a chronic disease requiring long-term medical management [Bell et al, 2006; Brew 2004].

The structure of HIV-1 virus includes a lipid membrane envelope with gp120 on its surfaced anchored by gp41, a cone-shaped capsid core comprising viral protein p24, and two copies of single positive stranded RNA with attached reversed transcriptase. Gp120 and Tat are two HIV-1 neurotoxic proteins. Gp120 is significant for virus entry into host cells. Tat is not a virus structural protein but produced by infected cells, which is essential in virus replication. Both of them are important in HIV-associated neuropathy [DeVita et al, 1997] (Figure 4).

Tat, standing for ‘trans-activator of transcription’, is one of the first genes produced after HIV integration. The 86 amino acids of Tat are encoded by 2 exons. The first exon encodes amino acid 1-72, including proline-rich domain, cysteine-rich domain and basic domain; the second exon encodes 73-86 amino acids, named RGD-containing C-terminal domain. Tat is a potent transactivator of HIV-1. Actually, the first 72 amino acid (Tat1-72) fully possess the transactivating ability. The cysteine-rich region serves as metal-ligands, leading Tat to form a metal-linked dimer to resist proteolytic digestion and therefore is essential for tat function [Garcia et al, 1988; Frankel et al, 1988]. The basic domain is important for nuclear translocation and transactivation [Endo et al, 1989]. Although the 73-86 peptide sequence is not required in Tat transactivating function, the
RGD containing domain involves in binding Tat with α_vβ_3 and α_5β_1 integrins [Barillai et al, 1993], which is one of the important ways for Tat to enter astrocytes. Therefore, we used Tat_1-86, in our study to trigger as much responses from astrocytes as possible. It can be secreted by infected cells at significant level to the extracellular space and remain intact and functional [Westendorp, et al, 1995]. After release, Tat proteins or peptides are capable of entering cells in vitro. Liu suggested that a principal pathway for Tat to enter neurons is through binding it with low-density lipoprotein receptor (LPR) on cell surface, followed by interaction with heparan sulfate proteoglycan (HSPG) and translocation to nuclei [Liu, et al, 2000]. Tat also directly bind to NMDA receptors on neurons, cause calcium influx and induce neuron apoptosis [Li, et al 2008]. Astrocytes uptake Tat by binding it to cell surface integrin α_vβ_5 [Vogel et al, 1993; Ma and Nath, 1997]. Tat can penetrate through plasma membranes by forming membrane invagination that leads to formation of micelles with Tat peptide enclosed. Ultimately Tat is released into cytoplasm, when the basic domain of Tat interacts with heparin sulfate glycosaminoglycan on cell surface [Vivès E et al, 1997]. This property of Tat enables it to be used as media to deliver other proteins or proteins into cells [Wadia and Dowdy, 2005].
In vitro administration of Tat in primary neuron culture was found to cause significant neuron losses [Li et al, 2008]. Bilateral intracerebral injection of Tat could cause developmental problem in neonatal rats, such as delayed onset of developmental sensory-motor reflex, deficits in eye opening and preattentive processes and spatial memory impairments in adulthood rats [Fitting et al, 2008]. In vitro studies showed that there are a variety of mechanisms, including increased [Ca^{2+}] [Haughey et al. 1999], mitochondrial dysfunction [Norman et al. 2007], or by potentiating excitatory amino acid triggered calcium influx and mitochondria dysfunction via NMDA receptors [Haughey NJ et al, 2001] in Tat associated neurotoxicity.

However, an equally important mechanism of Tat-mediated neurotoxicity may be indirect effects mediated by astrocyte dysfunction such as dysregulation of
cytokines/chemokines production. Tat exposure could remarkably increase astrocytes production of IL-6, MCP-1, RANTES, which are significant components in brain inflammation [El-Hage, et al, 2006, 2008].

Tat could modulate cytokine production from astrocytes mainly through the lipopolysaccharide-induced NF-κB signaling pathway [Hua et al, 2002; Kim et al, 2005; Quinones et al, 2008]. As shown in the following figure [El-Hage, et al, 2008], after exposed to Tat, I kappa B kinase (IKK) complex in astrocytes phosphorylates IκB, leading to its degradation. NF-κB, which is constitutively inhibited by its binding to IκB, is subsequently released and translocated from the cytoplasm into the nucleus to induce the expression of genes including TNF-α, IL-6 and IFN-β [El-Hage, et al, 2008].

**Figure 5. NF-κB signaling pathway:** After exposed to Tat, I kappa B kinase (IKK) complex in astrocytes phosphorylates IκB, leading to its degradation. NF-κB, which is constitutively inhibited by its binding to IκB, is subsequently released and translocated from the cytoplasm into the nucleus to induce the expression of genes including TNF-α, IL-6 etc.

Therefore, Tat not only directly causes neuronal death but also changes supportive environment by altering astrocytes function.
The HIV-1 coat protein gp120 is formed from the cleavage of gp160, leaving a gp41 fragment and can be readily shed to become a soluble protein. When HIV-1 virus infects target cells, such as T lymphocytes, monocytes, macrophages, the viral envelope protein gp120 binds to CD4 receptor. The interaction induces gp120 conformational change which exposes gp41, and V3 loop of gp120 to chemokine co-receptors. The chemokine co-receptors include CCR5, CCR3 and CXCR4, depending on different HIV-1 strains. T-tropic viruses infects T-cells via α-chemokine receptor CXCR4 and/or the β-chemokine receptor CCR5. M-tropic viruses infects macrophages/microglia via CCR5 and CCR3, but the α-chemokine receptor CXCR4 may also be involved [He et al, 1997; Oberlin et al, 1996; Ohagen et al, 1999]. With the interaction between gp120, CD4 receptors and chemokine co-receptors, HIV-1 envelope protein fuses with target cell membrane and allows virus capsid to enter the host cell.

Wang et al reported that primary human fetal astrocyte culture had both decrease EAAT2 RNA and protein expression either exposed to HIV-1 or the coat protein gp120 [Wang et al 2003]. Recent studies showed that gp120 participates in HIV-induced neurotoxicity. For example, several studies indicated that gp120 causes rodent neuronal cell death in vitro by interference with vasoactive intestinal peptide (VIP), an endogenous neurotropic substance [Brenneman et al, 1988]. Gp120 also mediated dendritic neurite dystrophy in hippocampus and thus leading to behavioral and learning problems [Glowa et al, 1992].

**4. Drug abuse, opioid receptors and HIV infection in brain**

In 1806, a German pharmacist named Friedrich Sertturner isolated the first active ingredient in opium and named it morphine after the Greek god of dreams, Morpheus.
For centuries, opium derived from secretions of *Papaver somniferum* seedpods has been utilized for analgesic purposes. Along with the increased use of morphine and derivatives of opium for medical purposes, the abuse of opiate drugs has also increased. Naloxone, antagonist to all opioid receptors, is now widely used clinically to counter the effects of opioid overdose, and in scientific research to study the pharmacology characteristics of morphine. There are three well characterized types of opioid receptors found in peripheral and central nervous system, designated as (µ opioid receptor, MOR; κ opioid receptor, KOR and δ opioid receptor, DOR) (Table 3) [taken from Watling, 1998].

**Table 3 Opioid receptors**  
[Revised from Watling, 1998]

<table>
<thead>
<tr>
<th>Name of cloned receptor</th>
<th>µ</th>
<th>δ₁</th>
<th>δ₂</th>
<th>κ</th>
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<tr>
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<tr>
<td>DAMGO</td>
<td>MOR</td>
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<td>DOR</td>
<td>KOR</td>
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<tr>
<td>Endogenous selective agonists</td>
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<td>[Leu²]-Enkephalin</td>
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<td>Naltrindole 5'-isothiocyanate Naltrindole</td>
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<td>nor-BNI</td>
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<td>Receptor selective antagonists</td>
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<td>Naltrexone</td>
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Intravenous drug users (IVDUs) have long been known to have a higher incidence of bacterial and viral infections than non-IVDUs by sharing of contaminated needles [Louria et al., 1967]. According to 2002 national HIV-1 prevalence survey, almost 40% of the total number of individuals who are HIV-1 positive in the United States contacted HIV through intravenous drug use [Center for Disease Control, National HIV-1 [Prevalence Survey, 2002]. Therefore, individuals who are IVDUs are at substantial risk for infection with HIV. The question thus exists whether drug abusers differ from other risk groups in progression of HIV-related disorders.

Some US studies [Nath et al. 2001; Tyor and Middaugh 1999] and a very large number of European cohort studies [Chiesi et al. 1996] both showed that HIV infected drug users have a higher prevalence of cognitive disorders than other groups of HIV infected individuals. Survival time after AIDS diagnosis was also shorter in HIV-I-infected IVDUs, compared with non-drug users, indicating that opiate use exacerbated the HIV infection [Friedman, et al. 1997; Rothenberg, et al, 1987].

While it is clear that some of the accelerated progression to AIDS is due to peripheral effects of opioids on the immune system [Carr et al., 1996; Nyland et al., 1998; Sharp et al., 1998a; Wetzel et al., 2000; McCarthy et al., 2001], we have studied whether there are interactions between HIV or viral proteins and abused opiates directly on the cells of the CNS.

Morphine is the major metabolite of heroin in the CNS. It interacts with the three major opioid receptors and preferentially activates MOR [Sawynok, 1986]. Astrocytes have been found to express all combinations of MOR, DOR, and KOR on their surface [Eriksson et al., 1990; Stiene-Martin and Hauser, 1990; Hauser et al., 1996;
When primary striatum astrocyte cultures were exposed to Tat and morphine, increased chemokines/chemokines (i.e. RANTES, IL-6, MCP-1) were released compared with that treated with Tat alone. Morphine has a synergistic effect on cytokines/chemokines release from striatal astrocytes, which could be prevented by μ-opioid receptor antagonist beta-funaltrexamine or by immunoneutralizing Tat (1-72) or substituting a nontoxic, deletion mutant (Tat delta31-61) [El-Hage, et al 2005], which indicated that the synergistic effect of morphine is mediated by μ-opioid receptor, but this effect requires participation of toxic/functional Tat protein. Further studies suggested that morphine is able to potentiate Tat-induced increases in [Ca^{2+}], at the site upstream of the NF-κB signaling pathway and regulate the Tat-induced cytokine release from astrocytes [El-Hage et al, 2008]

Based on the above information, we hypothesize that astroglia from different brain regions vary in their responses to HIV proteins and the responses could be influenced by co-treatment of morphine. We will further investigate whether EAAT2 expression on astrocytes is altered by exposure to viral protein with or without opiates. Since astrocytes plays a key role in HIV related encephalitis, their regional heterogeneous responses to HIV proteins could help to reveal that there are different susceptibility of different regions in CNS in HIV-related neuropathology.
Materials and Methods

1. Cell Culture

Astrocytes isolated from different brain regions were cultured to investigate cytokines/chemokines produced by them in response to HIV and/or morphine treatment.

0-2 day postnatal ICR mice (Charles River Inc., Charles River, MA) were used for astrocyte culture with our established protocol [El-Hage et al. 2005]. We analyzed N=5 experiments. Each “n” consisted of cells from 2 pups and was sufficient for a 24-well plate. Pups were decapitated, the entire brain was removed and meninges were peeled off. Then we separated the cerebral cortex and cerebellum under a dissecting microscope. Spinal cord tissue was taken from the entire length of the lumbar and thoracic cord. All cultures from different regions were done separately using identical procedure.

Tissues were diced with the tip of scalpels into about 2x2 mm² fine pieces in serum-free Dulbecco’s Modified Eagle’s Medium (DMEM; Invitrogen-Gibco, Carlsbad, CA), dissociated in a solution containing trypsin and DNAse, (0.25% trypsin, 10 µg/ml DNAse) for 30 minutes at 37°C and centrifuged at 1000 rpm for 5 minutes in room temperature. We removed the supernatant and resuspended the cells in DMEM with 10% defined fetal bovine serum (HyClone, Logan, UT), 6% glucose and antibiotics. The tissue was triturated 12-15 times with a 10 ml pipette to allow large pieces of tissue to settle, and filtered top 4 ml through a 135 µm nylon filter. The remaining tissue were triturated with a 5 ml pipette and filtered through 135 µm nylon filter. Filtrates were collected and centrifuged at 1000 rpm for 5 minutes in room temperature. We then resuspended the cells, triturated with 5 ml pipette and filter through a 45 µm nylon filter. Before seeding,
cells were checked under microscope; cell counts were done to make sure that each well was seeded the same cell density of $2 \times 10^5$ cells/cm$^2$. Then cells were plated onto the surface of a poly-L-lysine coated 24 well plate. Every plate was bathed with 1 mg/ml poly-L-lysine for 3 hours to overnight, rinsed with sterile water at least 6 times and leave in the sterile laminar flow hood until completely dry. Usually the plates were coated within two week before cell culture.

It took primary astrocyte culture from striatum, cerebellum and cerebral cortex 7-10 days to reach confluent, while cells from spinal cord usually took 14 days to reach 98% confluent and be ready to be used in the experiments.

2. Immunohistochemistry:

Immunohistochemistry was used to determine the primary cell culture composition. Cells were fixed for 30 min with 4% paraformaldehyde at room temperature, and blocked for an additional 30 min with 1.5% goat serum and 0.1% Triton x-100 in PBS (pH 7.4). Cells were incubated in primary antibody mouse anti glia fibrillary acidic protein (GFAP) (1:200, Millipore, Temecula, CA, USA) or rabbit anti Iba-1 (2 µg/ml Wako, Osaka, Japan) for 24 hours at 4°C, followed by fluorochrome-conjugated secondary antibodies goat anti mouse IgG Alexa 488 (1:500, Invitrogen, Carlsbad, CA, USA) or goat anti rabbit IgG Alexa 594 (1:500, Invitrogen). After the final wash, the cells were mounted on glass slides with ProLong™ Antifade mounting media containing DAPI (Sakura Finitek USA, Torrence, CA) to stain nuclei. Non-specific staining was determined by omission of the primary antibody from the immunostaining procedure, while double-staining procedures were conducted sequentially and employed secondary antibodies from different species.
Images of immunostained cells were acquired using a fluorescence microscope equipped with a digital camera (Zeiss).

3. BCA Protein Assay

Protein standards were prepared with one albumin standard (BSA) ampule, which was diluted into 9 vials. The 9 vials contained BSA with concentration ranged from 0 µg/ml to 2000 µg/ml. BSA standards and samples were placed in a 96 well plate. 200 µl of BCA working reagent (prepared from 50 parts of BCA reagent A and 1 part of BCA reagent B) was then adding to each well. After mixing thoroughly, the plate was covered and incubated at 37°C for 30 minutes. After the plate was cooled to room temperature, it was read at 562 nm on plate reader. Absorbance measurement of blank standard was corrected by subtracting from each individual standard and unknown sample replicates. Then standard curve was prepared by plotting the measurement for each corrected BSA standard. With this standard curve, the protein concentrations of the samples could be determined.

4. HIV-1 Protein and Opiate Treatments

There are 11 treatment groups in our experiment: control, Tat only, Tat + morphine, Tat + morphine + Naloxone, gp120 only, gp120 + morphine, gp120 + morphine + Naloxone, Tat + gp120, Tat + gp120 + morphine, Tat + gp120 + morphine + Naloxone only, morphine only. Drug or HIV-1 proteins were administrated in accordance with our previous studies [El-Hage et al. 2005, Singh et al. 2004, and El-Hage et al. 2006]. Tat1-86 (IIIB strain; ImmunoDiagnostics, Woburn, MA) is production from Escherichia coli system, which is purified by reverse phase HPLC and ion affinity to purity over 99%. Gp120 (IIIB strain; ImmunoDiagnostics) is a product from a baculovirus expression
system and purified to over 95% by immune-affinity chromatography. Tat1-86 and gp120 were administrated at concentration of 100 nM and 500 pM respectively for 18 hours to produce maximum effects on cytokine output but have less autocrine or paracrine secretory effects that complicate interpretation.

5. Intracellular [Ca\(^{2+}\)] assay

Activity of gp120 and the morphine preparations was evaluated with ratiometric intracellular [Ca\(^{2+}\)] assay. Cortical astroglia were cultured in 96 well plates. At 80-90% confluence, the culture was incubated in 10\(\mu\)M fura-2/AM (Molecular Probes, Eugene, OR) solution (diluted in Hank’s Balanced Salt Solution with 10mM Hepes buffer (pH 7.2) for 45 min at 35°C). It was then washed twice and incubated for another 30 min at same temperature. Ratiometric Ca\(^{2+}\) were measured at 340- and 380-nm excitation and 510-520 nm emission wavelengths [Gryniewicz et al. 1985] in the fluorescent microplate NOVO Star reader equipped with an integrated injector (BMG Labtech, Durham, NC). Measurements were done twice on 6 samples per treatment before and after morphine (500 nM) or gp120 (500 pM) injection in each well. Data are presented as the mean of fura-2 ratio 340 / 380-nm ± standard error of mean (SEM).

6. Bio-Plex Assays

Our analysis was performed on the following cytokines/chemokines: IL-1\(\beta\), GM-CSF, IL-9, IFN-\(\gamma\), TNF-\(\alpha\), RANTES, KC, MIP-1\(\alpha\), MCP-1, Eotaxin and MIP-1\(\beta\). In initial experiments, 23 cytokines and chemokines were assayed including IL-1\(\alpha\), IL-1\(\beta\), IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12 (p40), IL-12(p70), IL-13, IL-17, Eotaxin, G-CSF, GM-CSF, IFN-\(\gamma\), KC, MCP-1, MIP-1\(\alpha\), MIP-1\(\beta\), RANTES, and TNF-\(\alpha\). But nearly half of these cytokine are not observed consistently in any treatment group.
Therefore, we picked the ones which showed dynamic response either in vehicle groups or in treatment groups to continue the experiment.

Bio-Plex assay was performed with a mouse cytokine assay system (Bio-Plex; Bio-Rad, Hercules, CA). Standard curves were prepared by sequentially four-fold diluted recombinant cytokine standards. The assays were performed in the light-protected 96-well microplate-format Bio-Plex assays (Bio-Rad) at room temperature. 50μl multiplex bead working solution was first added to all wells and removed by vacuum filtration, and then the wells were washed with 100μl of Bio-Plex wash buffer twice. After that, diluted samples or blanks were shaken at 300 rpm in room temperature. 30 minutes later, beads were washed with Bio-Plex wash buffer again, incubated with detection antibody and streptavidin-PE respectively to detect and visualize fluorescence signals.

125 μl of Bio-Plex assay buffer was used to measure the fluorescence intensity of the beads. Fluorescence measurement of blank standard was subtracted from each individual standard and unknown sample to eliminate background. Then standard curve was prepared by plotting the measurement for each corrected recombinant cytokine standards. With this standard curve, protein concentration of samples could be determined. Samples were measured and blank values were subtracted from all readings.

7. Western Blotting

To further investigate whether glutamate transport in astrocytes would be impaired with morphine and/or HIV viral protein treatment, we did western blotting to assay the expression of EAAT2 on primary striatal astrocyte culture. As stated above, primary striatal astrocytes were cultured and treated with the same protocol as cells from other brain region. 18 hours following HIV-1 proteins or morphine treatment, cells were
harvest and lysed in ice-cold RIPA buffer, which included 50mM NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50mM Tris, pH 8.0 with protease inhibitor cocktail (Roche, Indianapolis, IN). Cell lysates were resolved by SDS–PAGE on 4-20% Criterion Precast Gels (Bio-Rad) and transferred onto a 0.2μm Amersham Hybond™-P Hydrophobic polyvinylidene difluoride (PVDF) membrane (GE Healthcare). The membranes were incubated in 5% (w/v) skim milk in T-TBS (0.1% Tween-20 in Tris Buffered Saline) for 1 hour at room temperature (RT) and exposed to rabbit anti EAAT2 (1:1000, Abcam, Cambridge, MA, USA) overnight at 4 °C. Membrane was washed in T-TBS three times and for 15 minutes each time. Bound antibodies were detected by appropriate horseradish peroxidase conjugated secondary antibodies anti rabbit IgG HRP conjugated (1:1000, GE health). The membrane was washed in T-TBS again three times and for 15 minutes each time, and then exposed to mouse anti-β-Actin-peroxidase antibody (1:1000, Sigma) for 1 hour under room temperature. We used enhanced chemiluminescence, detected and assayed the intensity of each band with Kodak Digital Science 1D Image Analysis System (Eastman Kodak, Rochester, NY). Bands in each group were normalized to β-actin then compared to the control of each group.

8. Statistics

Bio-Plex assay data were presented with Bio-Plex Manager software, version 4.0. Since a few data of the original cortex (~10.9%) and spinal cord (~4.6%) samples had readings out of the upper range, we diluted the samples from the above areas in 1:1 ratio and analyze them again with the same procedure. As for the data below the lower limits, they were replaced by the lowest detectable values to avoid the situation of missing data points in the ANOVA analysis. Data concerning the basal release (pg/ml) of individual
chemokines/cytokines from untreated astrocytes is presented as raw numbers, while data of treated samples, both the original and the diluted values, are transformed as the percentage of the control level of cytokine expression. Data were analyzed with multivariate analysis of variance (MANOVA) techniques (SPSS 2008, Version 16.0 for Windows, SPSS Inc.) to compare general trend of cytokines/chemokines across brain regions, different treatment groups and brain region vs. HIV-1 proteins interaction. Therefore, data were analyzed in three treatment group: Tat (2 levels: no, yes), gp120 (2 levels: no, yes), morphine (2 levels: no, yes), and three brain region group (3 levels: spinal cord, cerebellum, cortex). Further post-hoc testing using the Bonferroni correction factor was performed if there were significant ANOVA or MANOVA main effect to determine specific treatment effects.
Results

1. Astrocyte cultures: morphology and immunohistochemistry observation, and protein assay

(1) Astrocyte culture:

Astrocytes were grown in medium containing 10% serum. Astrocytes from different brain regions needed different lengths of time in the medium to become confluent. It took 7-10 days to reach this level of confluence for cultures derived from cerebellum and cortex, while 10-14 days were needed for cells from spinal cord.

(2) Morphology and Immunohistochemistry:

However, all cells seemed to have the same morphology when viewed by phase microscopy. They are cells with an irregular round to oval shape, relatively light cytoplasm, and numerous processes extended and attached to plates (Figure 6a). To determine the culture purity, astrocytes were immunostained with antibodies against glial fibrillary acidic protein (GFAP) (astrocyte marker) and Iba-1 (microglia cell marker) (Figure 6b, c). Cultures derived from all of the regions contained more than 98% astrocytes, with less than 1% microglia as determined (Figure 7a).
Figure 6. Phase image and immunostaining of primary striatal astrocyte culture  (a) Phase image of primary striatal astrocyte culture; (b) Primary astrocytes were fixed and immunostained with the astrocyte marker GFAP antibody (green), the microglia marker Iba-1 antibody (red), and DAPI staining nuclei (blue) to evaluate the purity of the culture. (c) microglial culture immunostained with an antibody to the microglia marker Iba-1 (red), and DAPI staining of nuclei (blue) to prove that the antibody we used works on microglia cells.

(3) Protein assay:

Since our main purpose is to compare chemokines/cytokines released from astrocytes of each brain region, it is important to know the number of cells in each culture. Given the fact that astrocytes from each brain region are similar in basic feature and morphology, protein concentration assay is a feasible and reliable way to achieve the purpose. BCA protein assay was performed on confluent cultures from each brain region. Although they took varied time to get confluent, the protein concentration at the end of
the growth period was similar (Figure 7b), and this is significant for interpreting the
cytokines/chemokines assays because the possible difference of analytes thus comes from
inherent characteristics of astrocytes or HIV-1 proteins/ morphine treatment but not just a
different number of cells producing different amount of analytes.

Figure 7. Primary astrocyte culture purity and protein concentration: (a): Cultures
derived from all of the regions contained more than 98% astrocytes (GFAP), with less
than 1% microglia (Iba-1); (b). BCA protein assay showed that protein concentration of
astrocytes from different brain region was similar.
(4) Intracellular [Ca$^{2+}$] assay

To evaluate the biological activity of the prepared morphine and gp120, intracellular [Ca$^{2+}$] was measured with a standard ratiometric fura-2 AM assay. As shown in Figure 8, morphine (500 nM) and gp120 (500 pM) administration induced a significant increase in the 340/380 ratio, which indicated intracellular calcium elevation within cells. Intracellular [Ca$^{2+}$] increased more quickly in morphine-treated astroglial cultures than in gp120 treated cultures, although the two treatments reached the similar fura-2 ratios after 5 minutes incubation.

Figure 8. Intracellular [Ca$^{2+}$] measurement with a standard ratiometric fura-2/AM assay. 80-90% confluent primary astrocytes were cultured in 96 well plates and loaded with 10 µM fura-2/AM. Ratiometric Ca$^{2+}$ was measured before and after morphine and gp120 administration. Results were presented with mean of 340/380 ratio±SEM. The two-way ANOVA (treatment and time) showed that both treatments (morphine and gp120) have significant increased 340/380 ratio compared with vehicle group. N=6 independent samples from each treatment group.
2. Basal Release of Chemokines/Cytokines

Cytokines/chemokines release from untreated (control) cells was analyzed using Bio-Plex. Statistical analysis (one-way MANOVA) showed that primary cultured astrocytes from all three brain regions secreted almost all of the 12 chemokines/cytokines except IL-9 in the cortical glia, and IFN-γ and TNF-α from the cerebellar glia. These were not detected by the Bio-Plex assay, and were either not secreted or were below the limits of detection of this assay.

The regional heterogeneity of released chemokines/cytokines was also significant \( F(24, 4) = 11.94, p \leq 0.05 \) according to one-way MANOVA analysis. GM-CSF \( F(2, 12) = 5.77, p \leq 0.05 \), INF-γ \( F(2, 12) = 5.25, p \leq 0.05 \), TNF-α \( F(2, 12) = 12.65, p \leq 0.01 \), RANTES \( F(2, 12) = 4.88, p \leq 0.05 \), MCP-1 \( F(2, 12) = 5.94, p \leq 0.05 \), and IL-9 \( F(2, 12) = 8.50, p \leq 0.01 \) were the analytes contributing to the regional diversity of cytokines/chemokines basal release. Astrocytes from cerebellum and spinal cord released more analytes than those from cerebral cortex, while the cytokines/chemokines levels were similar between cerebellum and spinal cord. (see Figure. 9).
Figure 9. Basal release of cytokines/chemokines from astrocytes of cortex, cerebellum and spinal cord. Five independent, primary astrocyte cultures from each brain region were used. Statistical analysis (one-way MANOVA) and post-hoc Bonferroni’s testing showed that GM-CSF, IFN-γ, RANTES, MCP-1, IL-9, and TNF-α were significantly different among the three brain regions; *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001. All data in the figure are expressed as the mean ±S.E.M.
3. Analysis of brain region-treatment interactive effects

(1) **Brain region effects**: All data were normalized as the percentage of basal level of analytes within each brain region group. To investigate the effects of brain regions on cytokines/chemokines release from astrocytes, data from all treatment groups within the same brain region group were collected and compared among three brain regions. 4-way MANOVA [3 (cortex, cerebellum, spinal cord) x 2 (±Tat) x 2 (±gp120) x 2 (±morphine)] showed that a significant brain region effect \( F(24, 262) = 11.08, p \leq 0.001 \) existed for all chemokines/cytokines except IL-1β, IFN-γ, and KC (Figure 10a). Cortical astrocytes secrete higher levels of IFN-γ, TNF-α, IL-6, MIP-1α, Eotaxin, MIP-1β, MCP-1, and IL-9 than cerebellum; spinal cord astrocytes secreted more IFN-γ, KC, MIP-1α, RANTES, and MCP-1 than cerebellar astrocytes (Figure 10a).

Figure 10. Regional heterogeneity of cytokines/chemokines secretion from astrocytes treated with Tat. Five independent primary astrocyte cultures from each brain region were used.
a. Astrocytes from different brain regions secreted significantly different levels of all analytes except IL-1β (see Table 4).

1 Post-hoc analysis of analytes between cortex and cerebellum revealed that cortical astrocytes secreted significantly higher amounts of all analytes than those in cerebellar cultures \(p < 0.001\), except for IL1-β, IFN-γ, and KC.

2 Post-hoc analysis of analytes between cerebellum and spinal cord revealed that spinal cord astrocytes secreted significantly higher amounts of IFN-γ \(p \leq 0.001\), KC \(p \leq 0.001\), MIP-1α \(p \leq 0.023\), RANTES \(p \leq 0.001\), and MCP-1 \(p \leq 0.01\) than those in cerebellum cultures.

3 Post-hoc analysis of analytes between cortex and spinal cord revealed that cortical astrocytes secreted significantly higher levels of IFN-γ \(p \leq 0.01\), TFN-α \(p \leq 0.001\), IL-6 \(p < .001\), MIP-1α \(p \leq 0.001\), eotaxin \(p \leq 0.001\), MIP-1β \(p \leq 0.001\), MCP-1 \(p \leq 0.019\), and IL-9 \(p \leq 0.001\) than those in spinal cord cultures.

b. Astrocytes in Tat-treated groups secrete significantly higher levels of all analytes than those in non-Tat treated groups \(** p \leq 0.01, ***p \leq 0.001; Table 4\).

c-e. 4-way MANOVA \([3 \text{ (brain region)} \times 2 (\pm \text{Tat}) \times 2 (\pm \text{gp120}) \times 2 (\pm \text{morphine})]\) and post-hoc analysis using the Bonferroni correction factor showed that there was a significant interactive effects between brain region and Tat \(F(24, 262) = 8.42, p \leq 0.001\) (Table 5), except IL-1β. Among three brain regions, cortical astroglia had the most obvious response to Tat treatment, while cerebellar astrocytes had the least response to Tat.

(2) Tat vs. gp120 vs. morphine effects

i. Tat effects.

As shown in Figure 10b, The 4-way MANOVA showed that primary astrocyte cultures treated with Tat secreted significantly higher levels of chemokines/cytokines except IL-1β compared with the non-Tat treated groups \(F (12, 130) = 27.81, p \leq 0.001\) (Figure. 10b, Table 4).

ii. Gp120 effects.

As shown in Figure 11a, cytokines/chemokines secreted from gp120 treated astrocytes collected from all three brain region were not significantly different from astrocytes in non-gp120-treated groups.

iii. Morphine effects.
As shown in Figure 11b, cytokines/chemokines secreted from morphine treated astrocytes collected from all three brain regions did not show a significant difference from astrocytes in non-morphine-treated group.

![Graph](image)

**Figure 11. Cytokines/chemokines secreted from gp120 or morphine treated astrocytes collected from all three brain region.** (a) one-way MANOVA indicated that cytokines/chemokines secreted from gp120-treated astrocytes collected from all three brain regions were not significantly different from astrocytes in non-gp120-treated group (n=5 for astrocytes from each brain region); (b) one-way MANOVA indicated that cytokines/chemokines secreted from morphine-treated astrocytes collected from all three brain regions were not significantly different from astrocytes in non-morphine-treated groups (n=5 for astrocytes from each brain region)

### 4. Brain region x Tat Interactive Effects

4-way MANOVA [3 (brain region) x 2 (±Tat) x 2 (±gp120) x 2 (±morphine)] analysis showed that astrocytes from different brain regions had a significantly different response to Tat treatment \(F (24, 262) = 8.42, p \leq 0.001\). This was true for the release of all cytokines/chemokines except IL-1β (Figure. 10 c-e and Table 5). Post-hoc Bonferroni analysis (Table 6), further indicated that astroglia from the cortex had the highest release of cytokines/chemokines in response to Tat (see also Figure. 10 c-e and Table 6).
Astrocytes from spinal cord had a slightly lower response than those from cortex, except that KC release from spinal cord astrocytes was the highest among all regional groups (Figure. 10 c-e; Table 6). Cerebellar astroglia had the lowest response to Tat among three brain regions, although their basally released amounts were quite high. However, interactive effects between all other regional or treatment factors such as Tat and morphine, Tat and gp120, gp120 and brain region, or gp120 and morphine, morphine and brain region did not show a significant difference.

**Table 4. F-values for 4-way MANOVA analysis on the chemokines/cytokines released from primary astrocyte culture**

<table>
<thead>
<tr>
<th>Dependent Variable</th>
<th>Brain Region Main Effect $F (2, 141)$</th>
<th>Tat Treatment Main Effect $F (1, 141)$</th>
<th>Brain region x Tat Treatment interaction $F (2, 141)$</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>1.98</td>
<td>41.95***</td>
<td>1.43</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>18.44***</td>
<td>126.00***</td>
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</tr>
<tr>
<td>IFN-γ</td>
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<td>18.56***</td>
<td>4.45*</td>
</tr>
<tr>
<td>TNF-α</td>
<td>9.31**</td>
<td>12.53**</td>
<td>9.13**</td>
</tr>
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<td>IL-6</td>
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<td>15.57**</td>
</tr>
<tr>
<td>KC</td>
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<td>23.73***</td>
<td>9.14**</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>42.85***</td>
<td>121.06***</td>
<td>33.15***</td>
</tr>
<tr>
<td>RANTES</td>
<td>11.10***</td>
<td>44.91***</td>
<td>10.97**</td>
</tr>
<tr>
<td>EOTAXIN</td>
<td>32.23***</td>
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</tr>
<tr>
<td>MCP-1</td>
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<tr>
<td>MIP-1β</td>
<td>50.98***</td>
<td>137.34***</td>
<td>43.56***</td>
</tr>
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</table>

*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001
Table 5. *F*-values for 3-way MANOVA analysis on chemokines/cytokines released from Tat-treated

<table>
<thead>
<tr>
<th>Dependent Variable</th>
<th>DF</th>
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<th>Cerebellum</th>
<th>Spinal Cord</th>
</tr>
</thead>
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<tr>
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<td>IFN-γ</td>
<td>(1, 47)</td>
<td>65.17***</td>
<td>1.85</td>
<td>6.26*</td>
</tr>
<tr>
<td>TNF-α</td>
<td>(1, 47)</td>
<td>10.24**</td>
<td>0.36</td>
<td>12.38**</td>
</tr>
<tr>
<td>IL-6</td>
<td>(1, 47)</td>
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<td>19.22***</td>
<td>13.97**</td>
</tr>
<tr>
<td>KC</td>
<td>(1, 47)</td>
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</tr>
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<td>33.01***</td>
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<td>15.33***</td>
</tr>
<tr>
<td>MCP-1</td>
<td>(1, 47)</td>
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<td>31.73***</td>
<td>13.93**</td>
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<tr>
<td>IL-9</td>
<td>(1, 47)</td>
<td>111.06***</td>
<td>23.50***</td>
<td>21.68***</td>
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</tbody>
</table>

*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001
DF = degrees of freedom
Figure 12. Levels of 12 chemokines/cytokines expressed in each treatment group from all three brain region. Analytes are divided into three groups to according to their different range of values as shown in three graphs (a-c) for each brain region. Tat induced significantly higher production of cytokines/chemokines in astrocytes from each brain region, while morphine (alone), gp120 (alone) or morphine and gp120 co-treatment did
not show statistically significant effects. M = morphine; Nal = naloxone. Values are expressed as mean± S.E.M. n=5 independent cultures from each brain region.

1 Tat has significant effect for all 12 chemokines/cytokines (see Table 5 for F values and significance level)

2 Tat has significant effect for all chemokines/cytokines, except for IFN-γ and TNF-α (see Table 5 for F values and significance level)

Cortex: Tat significantly increased release of all 12 analytes. Co-treatment with gp120 and/or morphine did not significantly alter this effect. Gp120 and/or morphine treatment without Tat did not affect analyte release. Significance values are given in Table 5.

Cerebellum: Tat significantly increased release of all 12 analytes except IFN-γ and TNF-α. Co-treatment with gp120 and/or morphine did not significantly alter this effect. Gp120 and/or morphine treatment without Tat does not have effect on analyte release. Significance values are given in Table 5.

Spinal Cord: Tat significantly increased release of all 12 analytes. Co-treatment with gp120 and/or morphine did not significantly alter this effect. Gp120 and/or morphine treatment without Tat did not affect analyte release. Significance values are given in Table 5.

Table 6. Post-Hoc Analysis for the Brain Region x Tat Interaction Effect on Percent Cytokine/Chemokine Levels* Using the Bonferroni Correction Factor.

<table>
<thead>
<tr>
<th></th>
<th>Cortex</th>
<th>Cerebellum</th>
<th>Spinal Cord</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
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<tr>
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<td>Tat</td>
<td>No Tat</td>
</tr>
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<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Spinal Cord</td>
<td>No Tat</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>GM-CSF</td>
<td></td>
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<tr>
<td>Cortex</td>
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<td>Tat</td>
<td>X</td>
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<td>Cerebellum</td>
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<td>X</td>
<td>X</td>
</tr>
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<td>No Tat</td>
<td>X</td>
<td>X</td>
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<tr>
<td>IL-9</td>
<td></td>
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<tr>
<td>Cortex</td>
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<td>Tat</td>
<td>X</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>No Tat</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Spinal Cord</td>
<td>No Tat</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>IFN-γ</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cortex</td>
<td>No Tat</td>
<td>Tat</td>
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<td>X</td>
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<tr>
<td>Spinal Cord</td>
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<td>X</td>
</tr>
<tr>
<td>TNF-α</td>
<td></td>
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<td>Tat</td>
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<tr>
<td>Cortex</td>
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<td>Tat</td>
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<tr>
<td>Cerebellum</td>
<td>No Tat</td>
<td>X</td>
<td>X</td>
</tr>
</tbody>
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5. Evaluation of the effect of Tat, gp120, and opiates on EAAT2 expression by striatal astrocytes

We confirmed that striatal astrocytes in all groups, including those treated with Tat/gp120 and/or opiates, expressed glial glutamate transporter (EAAT2) (Figure 13). With the current antibody (rabbit anti EAAT2, 1:1000, Abcam), we routinely detected four bands of different molecular weight on all membranes. The bands were detected at approximately 180KD, 65KD, 45KD, and 18KD. Similar bands existed when we used another antibody: guinea pig anti-glial glutamate transporter (1:1000, Millipore). Statistical analysis (ANOVA) showed that none of the treatments significantly affected EAAT2 expression as compared to control ($p>0.05$). MANOVA analysis showed that EAAT2 expression did not differ among treatment groups ($p>0.05$). Therefore, neither HIV-1 protein nor opiate has a significant affect on levels of EAAT2 expression.
Figure 13. Expression of the EAAT2 (glutamate transporter) in murine striatal primary astrocytes in culture (a) Western-blotting image of EAAT2 (glutamate transporter) expression in murine striatal primary astrocytes in culture. N=5 independent culture. 15 μg total protein was loaded in each lane from different treatment groups, and subjected to immunoblotting using antibodies to EAAT2 and β-actin as described in
Materials and Methods. EAAT2 was expressed in every treatment group. The EAAT2 antibody detected four bands of different molecular weight, including bands at 180KD, 65KD, 45KD, and 18KD. C=control, T=Tat, M= morphine, G=gp120, N=naloxone, antagonist of mu opioid receptor (b) The intensity of each band was measured using Kodak Digital Science 1D Image Analysis System (Eastman Kodak, Rochester, NY). Bands in each group were normalized to their controls and β-actin bands and then graphed. All values are expressed as a percentage of control± S.D. ANOVA and MANOVA analysis showed that EAAT2 expression did not differ among any group ($p>0.05$).
Discussion

Astrocyte dysfunction plays a key role in HIV associated neuropathy. Astrocytes are known to be a significant source of pro- and anti-inflammatory cytokines/chemokines in CNS [Norris et al, 1994; Morganti-Kossmann et al, 1992; Park et al, 2009; Raivich et al, 1999]. They are essential in removal of extrasynaptic glutamate and limiting neuronal excitotoxicity [Danbolt et al, 2001; Ye and Sontheimer, 1996; Hu et al., 2000; Wang et al, 2003]. Since they are known to be highly diversified among different brain regions [Shao Y and McCarthy KD, 1994; Somjen 1988; Garcia-Marin et al. 2007; Kettenmann and Verkharatsky 2008], we cultured astrocytes from cerebellum, cerebral cortex and spinal cord, treated them with HIV proteins Tat and/or gp120 with or without morphine co-treatments to analyze whether the treatments affected cytokines/chemokines secretion from astrocytes. We also investigated the expression of EAAT2, the major astroglia glutamate transporter, in striatal astrocytes to evaluate whether exposure to HIV-1 proteins with or without morphine would affect the expression of EAAT2.

According to the previous work of our lab and other literature [Hahn et al, 2010; El-Hage et al, 2008a; El-Hage et al, 2008b; Zhao et al, 2007; Bush et al, 2007; El-Hage et al, 2005; El-Hage et al, 2006; Khurdayan VK et al, 2004], we treated primary astrocyte cultures with Tat1-86 at a concentration of 100 nM to optimally induce the production of inflammatory factors [Bansal et al, 2000]. We chose 500 nM morphine in order to mimic the situation that occurs in chronic drug abusers. Injection drug abusers are at high risk of being infected with HIV through needle sharing, and have been shown in numerous studies to have a higher incidence of HIV-associated neuropathology [Nath et al, 1999;
Concha et al, 1997; Bouwman et al, 1998; Bell et al, 1998]. This population is the major reason that we study the synergistic effect of morphine and HIV viral proteins on HIV neuropathology. Morphine concentration in chronic drug abuser is 2.5-100-fold higher than the acute therapeutic concentration (100 nM) [Lötsch et al, 1998; Wolff et al, 1996]. According to the previous article [Gurwell et al, 2001], morphine at concentration of 500 nM could both mimic morphine concentration in brains of drug abusers and have maximum effects on cytokines/chemokines secretion from astrocytes. The 18-hour time point was chosen according to the previous studies to get the most representative primary response to the presence of viral proteins with or without opiates. After 24 hours, cytokines/chemokines secreted extracellularly can feedback to alter the pattern of cytokines/chemokines release from astrocytes, and this can mask the initial responses of the cells [El-Hage et al, 2005].

1. Basal release of cytokines/chemokines in different brain regions

Our results showed that almost all 12 cytokines/chemokines analyzed could be found in the supernatant of untreated astrocyte cultures derived from different brain regions. This is consistent with many other studies [Achour and Pascual, 2010; Zhang et al, 2010; Deverman and Patterson, 2009], indicating that cytokines and chemokines are both constitutively secreted by cells of the brain, and may be involved in normal physiologic functions such as neuronal development and differentiation. Neurons and glial cells produce various types of cytokines/chemokines during brain development to act both as neurotropic factors to promote cell survival and as signals to trigger apoptosis and remove cells which fail to perform appropriate functions [Deverman and Patterson, 2009]. For example, during neural migration, gp130 family cytokines participate in
maintaining and supporting the renewal of radial glial cells; RANTES can regulate astrocyte proliferation in forebrain by inducing IFN-γ binding to receptors on astrocytes in the first trimester [Bakhiet et al, 2001].

Since all of the experimental procedures, including primary astrocyte cultures, Tat/gp120 and morphine treatment are done using the same protocol, the regional heterogeneity in basal release of cytokines/chemokines most probably resulted from the intrinsic properties of astrocytes from different brain regions. Although this is an in vitro study, cells derived from different brain regions still have intrinsically diversified characteristics. Although cells in our study were all cultured from 0-2 days postnatal mice and seeded at the same cell density, astrocytes from cerebellum, cortex and striatum took 7-10 days to reach confluent, while those from spinal cord took about 14 days to be confluent. According to Sturrock [Sturrock, 1976], there is a regional difference in duration of astrocyte differentiation. For example, in mouse forebrain, astrocyte differentiation takes about 10-15 days [Sturrock, 1974, 1976, 1980], whereas in spinal cord the process is much shorter [Fedoroff et al, 1986]. Therefore, we speculate that astrocytes from spinal cord may be more differentiated than those from the other three regions when they were removed from brains.

Given their diverse intrinsic characteristics and somewhat different stages of differentiation, it is not surprising that basal cytokines/chemokines release from astrocytes derived from different brain regions is significantly different.

2. The effect of Tat to increase cytokines/chemokines production from astrocytes
Although the basal levels of chemokines/cytokines secreted by astroglia from different regions are obviously different, some common findings could be reported amongst the three regions we examined. Firstly, compared to the non-Tat treated group, Tat exposure tremendously increased cytokines/chemokines secretion.

Previous work indicated that Tat modulated MCP-1, IL-6 and TNF-α production from astrocytes mainly through NF-κB signaling pathway [El-Hage, et al 2005; Hua et al, 2002; Kim et al, 2005; Quinones et al, 2008]. NF-κB, an essential transcription factor, is significant in regulating gene transcription for immune and inflammatory responses [Baeuerle et al, 1996; Wang et al, 1998; Zhai et al, 2004]. The NF-κB family is comprised of five members, RelA (p65), RelB, c-Rel, p105/p50, and p100/p52. They all have the Rel homology domain, which can form dimmers and translocates to the nucleus. When NF-κB is activated, IκB, bound to NF-κB and acts as an inhibitor, is ubiquitized and degraded, which masks the nuclear localizing signals (NLS), and thus unbound NF-κB translocates to nucleus and modulates the transcription of specific gene to regulate the cytokines/chemokines secretion [Israël et al, 2000; Ghosh et al, 2002]. Tat can phosphorylate IκB by activating the I kappa B kinase (IKK) complex in astrocytes, leading to IκB degradation, and thus increasing the rate of p65/ NF-κB translocation to nucleus and subsequently inducing the gene expression of TNF-α, IL-6 and MCP-1 [El-Hage et al, 2008]. Tat_{1-72} (100 nM) exposure significantly elevated intracellular calcium in astrocytes within seconds and increased the release of inflammatory cytokines MCP-1, RANTES, and IL-6. Chelating [Ca^{2+}]_i with BAPTA/AM prevented Tat induced p65/ NF-κB translocation to nucleus and cytokine production, while artificially increase [Ca^{2+}]_i exacerbated the cytokine release [El-Hage et al , 2008 ]. Some other pathways are also
involved in Tat-induced cytokines/chemokines production. For example, Tat was also found to activate MAPK signaling pathway by phosphorylating ERK1/2 and inducing production of MCP-1, IL-8 and IP-10 (IFN-γ inducible protein). UO126, a highly potent and specific ERK1/2 inhibitor, partially reduced MCP-1 secretion, totally aborted IL-8 production, but did not affect IP-10 mRNA, while SB202190, a p38 MAPK inhibitor, suppressed the Tat-induced IP-10 production [Kutsch et al, 2000].

Besides that, cytokines could be induced by each other through a network of signaling cascades. For example, CCL5/RANTES was involved in Tat-induced MCP-1 production in astroglia. In CCL5 (-/-) mice, glia cells were less likely activated by Tat administration compared with wild type mice, and there was a significant decline in CCL2/MCP-1 production [El-Hage et al, 2008]. The interaction among cytokines/chemokines, however, makes our study on the induction effect of Tat or morphine more complicated. That is why we choose the 18-hour time point, to minimize the possibility for cytokines/chemokines to interact with each other.

Nath et al suggested that Tat induces cytokine production in astrocytes with a ‘hit and run’ pattern. Exposure to Tat (100 ng/ml) for 5 minutes is sufficient to trigger a maximal amount of IL-6 secretion from astrocytes [Nath et al, 1999], and the elevated IL-6 level could last for a few hours even though Tat itself was degraded [Jones et al, 1998]. Since IL-6 production in astrocytes was NF-κB dependent, it could be that IL-6 itself or other factors in this pathway activate the NF-κB pathway and form a positive feedback loop between NF-κB and cytokines/chemokines [Lowenthal et al, 1989; Nath et al, 1999].

Consistent with other studies [El-Hage, 2006; El-Hage, 2008; Lowenthal et al, 1989], our studies showed that Tat induced significant responses in
cytokines/chemokines release from astrocytes derived from different brain regions. It further revealed that there were great regional differences in how astrocytes responded to Tat. This may result from different calcium signaling and/or NF-κB signaling pathways in astrocytes among different brain regions [Matyash et al, 2009]. The astroglial regional heterogeneity in response to Tat may also be because Tat interacts with different receptors or internalization-related proteins (i.e. low-density lipoprotein receptor-related protein, NMDA, αβ5 integrin). These receptors or internalization-related proteins likely have regional heterogeneity in term of their expression. Astrocytes are known to have diverse responses to certain neurotransmitters because of their heterogeneity in receptor expression among different brain regions [Matyash et al, 2009]. For example, when treated with glutamate, Bergmann glia in cerebellum had a doubly rectifying AMPA current mediated by AMPA receptors [Muller et al, 1992; Burnashev et al., 1992], while a large population of astrocytes in the supraoptic nucleus of hippocampus do not respond because they lack functional glutamate receptors [Schipke et al, 2001].

Interestingly, astrocytes in cortex with lower basal release had higher response to Tat, and vice versa in cerebellum. We speculate that the total capability or reservoir to produce cytokines/chemokines might be similar in astrocytes from cerebral cortex, cerebellum and spinal cord. Since the basal release in cerebellum is already high, when treated with Tat, astrocytes in cerebellum do not have as much reserve response as those in cerebral cortex. Therefore, the Tat-induced cytokines/chemokines production could also be affected by their physiological basal release.

3. HAD associated chemokines and cytokines
In our result, all 12 analytes were elevated following Tat treatment. Being consistent with previous studies [Clinque et al, 1998; Corasaniti et al, 2001; Zhao et al, 2001], cytokines, such as IL-1, IL-6, IL-10, GM-CSF, G-CSF, M-CSF, TNF-α, and TGF-β, and important chemokines, such as RANTES, IL-8, MCP-1 were important in progression of HIV-associated neuropathy in enabling astrocytes to detect and respond to pathogens [Minagar et al. 2002; Bailey et al. 2006].

For example, HIV-associated inflammation is sustained by virus replication in monocytes, which are mainly recruited by monocytes chemoattractant protein-1 (MCP-1). MCP-1 is a member of CC or β-chemokine family. CCL2 has been characterized as a human homolog of MCP-1, based on its monocyte chemoattractant properties [Van Coillie et al, 1999]. MCP-1/CCL2 could be secreted by a variety of cells including endothelia, fibroblasts, microglia, astrocytes, monocytes, epithelial and smooth muscle cells either constitutively or after inflammatory stimuli [Barna et al, 1994; Brown et al, 1992; Cushing et al, 1990; Standiford et al, 1991]. MCP-1/CCL2 is one of the well-studied chemokines because of its important role in immune modulator functions, such as attracting monocytes, and influencing macrophage migration [Rollin, 1996; Sarafi et al, 1997; Gendelman et al, 2008]. SIV-infected macaques with moderate-to –severe encephalitis had significant higher CCL2 levels in CSF than in plasma as early as 28 days after SIV infection through blood, which indicates that CCL2 could be a sensitive diagnosis marker in SIV associated encephalitis [Zink et al, 2001]. CCL2 level in the cerebrospinal fluid was significantly elevated in HIV-1 encephalitis patients and its level in plasma correlated with virus load of HIV-1 infection.
TNF-α and IL-1β are two pro-inflammatory cytokines, which play important roles in neuronal injury and death in HIV-associated dementia. IL-1β is expressed by a wide variety of cells such as monocytes, macrophages, astrocytes and lymphocytes. Studies showed that, in brain tissue of HAD patients, the mRNA and protein expression of IL-1β significantly increased, and lateral cerebral ventricle injection of gp120 enhanced IL-1β expression in neocortex of rat [Corasaniti et al, 2001]. IL-1β activates astrocytes to produce TNF-α and iNOS via the NF-κB signaling [Zhao et al, 2001]. TNF-α itself could also induce the production of iNOS in co-operation of IL-1β. iNOS leads to the formation of NO, which is toxic to neurons, increases the BBB permeability and facilitates monocyte and macrophage entry into brain [Chao et al, 1996; Nottet et al, 2005]. IL-1β potentiates NMDA receptor-mediated intracellular calcium increase [Yeh et al, 2000] and induces the secretion of intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), and E-selectin by astrocytes and endothelial cells to facilitate monocyte infiltration into CNS [Collins et al, 1995; Lee et al, 1998; Winkler and Beveniste, 1998]. IL-1β induced N-methyl-D-aspartic acid receptors (NMDARs) activation on neurons resulted in Ca^{2+} influx and p38 MAPK activation, mitochondrial Ca^{2+} overload and release of cytochrome C, free radical generation and reactive oxygen species, caspase activation and apoptosis [Yeh et al, 2000; Xiong et al, 1999; Kuang et al, 2009; Epstein, 1998].

The level of TNF-α was found to be elevated in the brain tissue of HAD patients. Exposure to HIV-1 proteins in vitro caused elevated production of TNF-α [Tyor et la, 1992; Yeung et al, 1995]. Similar to IL-1β, TNF-α increase BBB permeability and induced secretion of adhesive molecules to facilitate monocyte and macrophage entry.
migration [Collins et al, 1995; Lee et al, 1998; Winkler and Beveniste, 1998]. Wang et al indicate that TNF-α was involved in glutamate uptake inhibition in CNS of HAD patients by decreasing the expression of glutamate transporters, EAAT1 and EAAT2 [Wang et al, 2003]. Together with its induction in L-cysteine release from macrophages and microglia [Piani and Fontana, 1994], TNF-α caused accumulation of excessive excitatory amino acid in the extracellular spaces and contributed to glutamate-induced excitotoxicity. Furthermore, TNF-α altered astrocyte function by stimulating proliferation and production of pro-inflammatory cytokines [Saha and Pahan, 2003], which would in turn activate microglia cells to secrete more pro-inflammatory cytokines including TNF-α itself, thus creating a vicious positive feedback cycle and enhancing the likelihood of neuronal death. Therefore, dysfunction of cytokines/chemokines production in astrocytes treated with HIV-1 protein can clearly contribute to HIV-1 induced neurotoxicity.

4. Effect of morphine co-treatment with HIV-1 proteins on cytokines/chemokines production in astrocytes

As the major metabolite of heroin in brain, morphine is often used to study the effect of opiate exposure in the CNS. Morphine alone is generally not considered to be toxic to most neurons, but combined with HIV proteins, it can significantly exacerbate their toxic effect [Gurwell et al, 2001]. Interestingly, morphine displayed a paradoxical neurotoxic or neuroprotective effect depending on cell types, opioid receptor types or target tissues. In SH-SY5Y neuroblastoma cells, mu opioid receptor activation had protective effect by activating antiapoptotic effectors downstream to PI-3-K-dependent signaling pathway, while in neurons, administration of morphine and Tat could significantly decrease their viability [Polakiewicz et al, 1998; Gurwell et al, 2001]. The
heterogeneous effects of morphine are caused by the fact that opioid receptor molecular signaling pathways are highly diversified depending on different cell types and target tissues.

Morphine enhances the Tat induced pro-inflammation and anti-inflammation cytokines/chemokines release from astrocytes derived from the striatum. In the previous studies of our lab, it was found to synergistically exacerbate the release of MCP-1, RANTES, TNF-α and IL-6 from striatal astrocytes treated with Tat1-72, and this effect could be blocked by beta-funaltrexamine, a µ-opioid receptor antagonist. It did not occur when a deletion mutant, nontoxic TatΔ31-61 was used instead of Tat1-72 [El-Hage et al 2005, El-Hage et al 2006, El-Hage et al 2008]. This study indicated that the synergistic effect of morphine and Tat to cytokines/chemokines production is MOR dependent. Previous studies showed that opioid-induced calcium influx into cells occurs through several pathways including voltage-dependent L-type calcium channel activation, and Ca\textsuperscript{2+}-dependent Ca\textsuperscript{2+} release from dantrolene-sensitive intracellular stores [Hauser et al, 1996]. Therefore, El-Hage et al suggested that opioids enhanced the effect of Tat in cytokines/chemokines production in astrocytes by increasing intracellular calcium and thus potentiating Ca\textsuperscript{2+}-dependent NF-κB signaling pathway. Their hypothesis was proved by an experiment showing that chelating [Ca\textsuperscript{2+}], with BAPTA/AM could block p65/ NF-κB translocation into nucleus and the subsequent cytokines/chemokines release, conversely bathing astrocytes in excess extracellular Ca\textsuperscript{2+} accelerated translocation of p65/ NF-κB and leaded to more release of MCP-1, IL-6 and TNF-α [El-Hage et al 2008].

In our results, the effect of morphine to induce cytokines/chemokines release from astrocytes was not significantly different from the non-morphine groups, which is
consistent with the previous studies that activation of mu opioid receptor alone was not sufficient to activate NF-κB signaling pathway [El-Hage et al, 2008]. However, our results did not show this synergistic effect of morphine and Tat in cytokines/chemokines release from primary astrocyte cultures of cerebral cortex, cerebellum and spinal cord. μ-opioid receptors are widely distributed throughout the brains, including cerebral cortex, striatum, cerebellum and spinal cord [Watling, 1998]. In our study, exposing astrocytes from cerebral cortex with 500 nM morphine remarkably increased [Ca^{2+}], 5 min after exposure [Fitting et al, 2010]. Therefore, the current results could not be explained by the lack of μ-opioid receptor expression.

Opioid receptors are regionally diversified among different brain regions. For example, striatal astrocytes were found to express a higher density of MOPr than the cultures derived from cerebellum and cerebral cortex [Steine-Martin, et al. 1998; Ruzicka et al, 1995], which might be due to cell-cycle or developmental sensitive expression. Astrocytes from different brain regions also exhibited different changes in [Ca^{2+}] in response to μ opioid agonist, which might be associated with variability in G-protein-signaling pathways [Eriksson et al., 1990, 1992; Piros et al., 1995]. Therefore, the failure of raising [Ca^{2+}] to certain extent by morphine perhaps result in absence of change in cytokines/chemokines production by astrocytes co-exposed to Tat and morphine in culture. Regional difference in extent of attenuation of MOR-mediated G-protein activity in astrocytes when chronically administrated with morphine among spinal cord, cerebral cortex and striatum was also reported. This regional heterogeneity indicated that multiple mechanisms may exist in regulating morphine-mediated effects among different CNS regions [Sim-Selley et al, 2007]. The differences between present results and previous
results from our laboratory [El-Hage et al. 2006; El-Hage, et al, 2008] most likely are due to regionally specific responses of the astroglia. It is quite interesting that astroglia from the striatum, which is extremely vulnerable to the effects of HIV-1, are also the only astroglia thus far examined that appear to be sensitive to the synergistic effects of Tat and morphine [El-Hage et al, 2008].

Our project was designed to explore the general trend of cytokines/chemokines release across different brain regions and treatment groups. Therefore, the statistical analysis could overlook a small but biologically significant change of individual cytokine/chemokine release in the Tat and morphine co-treatment group. However, the results clearly showed that the greatest effect on cytokines/chemokines release was due to Tat alone, and not to Tat and morphine co-treatment.

5. Effect of gp120 on cytokines/chemokines production in astrocytes

HIV-1 enters CD4 positive cells (e.g., microglia, macrophages, T-lymphocytes) when gp120 interacts with CD4 receptor, followed by the binding of gp120 and chemokine receptors or HIV-1 co-receptors such as CXCR4 and CCR5. In central nervous system, gp120 shed from HIV-1 infected microglia can directly interact with CXCR4 and CCR5 on CD4-negative cells such as astrocytes or neurons [Persidsky and Gendelman, 2003; Kaul et al., 2005]. Gp120 elevated NO production and the level of malondialdehyde, a lipid peroxidation by-product and oxidative stress marker, but in the meantime, suppressed intracellular glutathione (GSH), a factor critical in maintaining the cellular redox balance. Thus, gp120 treatment increased oxidative stress in astrocytes [Ronaldson and Bendayan, 2006].
Studies demonstrated that HIV-1 gp120 is involved in inflammatory response through cell surface chemokine receptors (e.g., CXCR4 and CCR5) [Wu et al., 1997; Koller et al., 2002]. Different virus strains have different preferences for chemokine co-receptors, although the tropism is not absolute. T-tropic viruses that infect T-cells preferentially utilize α-chemokine receptor CXCR4 while M-tropic viruses that infect macrophages/microglia preferentially utilize CCR5 as a co-receptor [He et al, 1997; Oberlin et al, 1996; Ohagen et al, 1999]. Different strains of gp120 have different effects on proinflammatory cytokine and nitric oxide (NO) production. Glia cells treated with gp120 IIIB (a T-tropic, R4 preferring strain) had a dose-dependent release of NO, TNF-α, IL-6, but no IL-1α and IL-1β; whereas, only IL-6 was increasingly released from astrocytes exposed to gp120 SF2, a strain that in purported to be dual-tropic [Kong et al, 1996].

Types of chemokine receptor are closely associated with the effect of gp120 in dysregulating astrocytes function and triggering neurotoxicity. Bathing the rat cerebral cortex astrocytes with HIV-1g96ZM651 gp120 (1.0 nM), which is R5 tropic, and 3A9, the anti-CCR5 antibody, did not produce any cytokines, while the same strain of gp120 and anti-CXCR4 antibody co-treatment still triggered significant cytokines production from astrocytes compared with the control group [Ronaldson and Bendayan, 2006]. Therefore, CCR5 might be the major co-receptor to mediate Gp120-induced cytokines/chemokines production from astrocytes.

Different chemokine receptors also mediated different neurotoxicity. Previous literature suggested that gp120 from X4-preferring HIV-1 strains could not induce neuronal death in CXCR4-deficient cerebrocortical neuronal cultures, while dual tropic
gp120SF2 triggered even greater neurotoxicity in CCR5 knockout neuronal cultures compared to wild-type or CXCR4-deficient neuronal cultures, which indicates that CXCR4 mediated the primarily neurotoxic effect while CCR5 could be partly neuroprotective depending on the HIV-1 strain [Kaul et al, 2005].

However, in our study, gp120 did not have a statistically significant main effect on cytokines/chemokines production in astrocytes with or without opiates. Gp120 exposure on primary cerebral cortex astrocytes triggered significant intracellular calcium elevation [Fitting et al, 2010], which indicated that the gp120 used in the study had biological activity and that the astrocytes we cultured had HIV-1 co-receptors (e.g. CXCR4, and /or CCR5) expressed on the cell surfaces as well. Therefore, the absence of cytokines/chemokines secretion from astrocytes could not be explained as the problem of gp120 bioactivity or that cells we used did not have receptors that interacted with the gp120. Since we conducted an exploratory study, and examined our results for main effects, it is possible that we would overlook a smaller but biologically significant effect of gp120 on release of individual cytokine/chemokine.

Some of our results may be due to the strain (IIIB) of gp120 we used in the experiment. Gp120 IIIB prefers to utilize the CXCR4 receptor [Kaul, M et al 2007]. Another study suggested that CCR5, not CXCR4, mediates increased production of certain cytokine/chemokine in rat astrocytes as measured by ELISA [Ronaldson and Bendayan, 2006]. But it is somewhat difficult to compare our studies due to different experimental conditions, and due to the analytic methods employed. However, given that our purpose was to compare among different brain regions and treatment groups, the result clearly showed that gp120 IIIB did not have a main effect on cytokines/chemokines.
release from primary astrocyte cultures derived from any of the brain regions. Thus the overwhelming cytokine/chemokine response was to Tat.

6. Effect of HIV-1 protein and/or morphine on EAAT2 expression in astrocytes

Because of their central role in regulating glutamate concentration in neuronal synapses and preventing excitotoxicity, dysfunction in EAATs or dysregulation of their expression could be involved in the pathological mechanism of many neuronal diseases. In SIV-infected macaques, both EAAT1 and EAAT2 expression were decreased and correlated with progression of disease; in the meantime, extracellular glutamate concentration in the putamen was increased [Bossuet et al. 2004; Meisner et al, 2007].

EAATs are regulated in many ways. Since the transport of the glutamate molecule is coupled with potassium, sodium and proton [Kanner et al, 1987; Bouvier et al, 1992], the driving force for glutamate transport is membrane potential and the ion gradients, which are built up by Na⁺-K⁺ ATPase. Therefore, factors that impair Na⁺-K⁺ ATPase would possibly influence glutamate transport. For example, during brain hypoxia or ischemia, lack of ATP slows the work of the Na⁺/K⁺ pump; accumulation of sodium inside the cell and potassium outside the cell decreases the transmembrane gradients for [Na⁺],[K⁺], which leads to depolarization of the cell membrane and eventually causes the glutamate transporters to operate in reverse by releasing glutamate, instead of taking up glutamate from extracellular spaces [Takahashi M et al, 1997; Rossi et al, 2000; Allen et al, 2004; Grewer et al, 2008 ]. Deficient glutamate uptake or reverse transport thus overstimulates glutamate receptors and leads to neural excitotoxicity [Choi, 1992; Choi et al, 1990; Olney, 1990]. Gp120 was shown to accelerate Na⁺:H⁺ exchange, leading to intracellular alkalinization and activation of pH-sensitive K⁺ permeability. These changes
in turn inhibited glutamate uptake activity [Patton et al, 2000]. Several other studies [Wang et al, 2003; Meisner et al, 2008] further proved that the expression of glutamate transporter EAAT2 was reduced in human astrocytes exposed to HIV-1 or gp120.

Glutamate transporter-associated proteins (GTRAP) are other factors that modulate glutamate transport activity. Increasing expression of GTRAP3-18 protein, the EAAC1 associated protein and vitamin A responsive protein homologue of rats, caused progressive decrease in glutamate transport specifically through EAAT3. This inhibition was not caused by lowering EAAT3 expression on cell membranes or altered protein trafficking, but by decreasing EAAT3 affinity to glutamate [Lin et al, 2001]. GTRAP41, an actin binding protein and GTRAP48, interacting with Rho GTPase, could both enhance glutamate transport by increasing Vmax and stabilizing EAAT4 expression on cell membranes [Jackson et al, 2001].

EAATs are regulated through interaction between endogenous factors and protein kinases, such as protein kinase C (PKC) [Lortet et al, 1999], protein kinase A (PKA) [Lortet et al, 1999] and phosphatidylinositol 3-kinase (PI3K) [Zelenkaia et al, 2000; Sims et al, 2000]. For example, epidermal growth factor (EGF) could increase GLT-1 mRNA transcription, protein expression translation and glutamate transport activity in cultured astrocytes through PI3K activation, while platelet-derived growth factor (PDGF) induces a translocation of EAAC1 from intracellular compartments to the cell surface [Zelenkaia et al, 2000; Sims et al, 2000]. Pituitary adenylate cyclase-activating polypeptide (PACAP), a neuron-derived peptide, enhanced EAAT2 activity by activating PKA, and promoted EAAT1 expression by activating both PKA and PKC pathways [Figiel and Engele, 2000].
However, we did not see any significant change in EAAT2 expression on astrocytes when treated with HIV-1 protein gp120/Tat and/or morphine. Compared with other related studies [Wang et al, 2003], we used striatal astrocytes from mice of postnatal 0-2 days, while astrocytes they used were derived from human fetal brain 14-19 weeks gestational age. We already know that glutamate transporters of different species are very different, and the expression are under tremendous changes during development. Therefore, it is possible that human embryonic glutamate transporters are more sensitive to the HIV-1 protein [Shibata et al, 1997].

Since we did not evaluate the ability of our cells to handle a glutamate challenge, it is still possible that although the level of EAAT2 expression in our studies did not show a significant change, the transport function was impaired. To test this possibility would require studies of glutamate uptake assay, in which the cell-associated radioactivity is determined in cell lysates after incubated in L-[H]-glutamic acid solution for designated time [Wang et al, 2003].

Microglia are another key player in EAAT2 expression. Microglia activation was closely associated with dysfunction of EAAT2 in HIV-infected brain [Meisner et al, 2007]. Elevated glutamate levels in the CSF of macaques during simian immunodeficiency virus (SIV)-infected animals were found to be related with the increased production in macrophages and microglia [Koutsilieri et al, 1999]. Immune activation was increased in SIV-infected asymptomatic rhesus monkeys and even higher in AIDS animals, which negatively correlated with expression of EAAT1 and EAAT2 [Meisner et al, 2007]. Since our astrocyte culture was highly pure ( <1% microglia), the lack of microglia activation could be a factor in the stability or our EAAT2 expression.
The lacking of other resident CNS neuronal cells such as neurons, oligodendrocytes and progenitor cells could also contribute. Since cross-talk among these resident cells is physiologically and pathologically significant. For example, astrocytes could increase the survival rate of neurons treated with lipopolysaccharide (LPS) compared with that in isolated neuron cultures [Li et al, 2009]. Therefore, interactions among resident neuronal cells in brain when infected with HIV-1 virus or proteins with our current in vitro primary astrocytes culture could easily be overlooked. However, the highly pure isolated astrocyte culture provides us an excellent model to investigate the role of astrocytes in HIV-1 associated neuropathy, although we need to be very careful when relating our conclusions to the HIV-infected CNS.
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