Methamphetamine Alters Blood Brain Barrier Protein Expression in Mice, Facilitating Central Nervous System Infection by Neurotropic Cryptococcus neoformans

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Methamphetamine (METH) is a drug of abuse that is a potent and highly addictive central nervous system (CNS) stimulant. The blood brain barrier (BBB) is a unique interface that in part functions to prevent microbial invasion of the CNS. The effects of METH on brain vasculature have not been studied extensively. We hypothesized that METH alters the BBB integrity, increasing susceptibility to CNS infection. Using a murine model of METH administration, we demonstrated that METH alters BBB integrity and modifies the expression of tight junction and adhesion molecules. Additionally, we showed that BBB disruption accelerates transmigration of the neurotropic fungus Cryptococcus neoformans into the brain parenchyma after systemic infection. Furthermore, METH-treated mice displayed increased mortality as compared to untreated animals. Our findings provide novel evidence of the impact of METH abuse on the integrity of the cells that comprise the BBB and protect the brain from infection.

Keywords. methamphetamine; blood brain barrier; Cryptococcus neoformans.

Methamphetamine (METH) abuse is a major public health problem in the United States. METH is a strong, addictive, central nervous system (CNS) stimulant that mimics the pharmacological effects of cocaine, albeit with longer-lasting effects. The blood brain barrier (BBB) is a unique interface that in part functions to prevent CNS microbial invasion. Cocaine modulates the transmigration of leukocytes by modifying the expression of BBB endothelial cell adhesion molecules. This results in BBB dysfunction and increased cell emigration to the brain, which increases the probability of infection. Although there is substantial evidence of the effects of cocaine on BBB function, the effects of METH on brain vasculature have not been studied extensively.

We have previously demonstrated the detrimental influence of METH on the immune function of hosts in response to a systemic microbial challenge [1]. Here, we investigate the impact of METH on BBB function. On the basis of our earlier work, we hypothesized that METH-induced loss of BBB integrity would increase susceptibility to CNS infection. In this regard, the encapsulated AIDS-associated pathogenic fungus Cryptococcus neoformans is an excellent model organism for the study of CNS susceptibility because of the availability of tools such as specific antibodies and well-established animal models. Annually, C. neoformans causes approximately 1 million cases of meningoencephalitis globally [2]. C. neoformans traverses the BBB at the capillary vasculature [3], either directly [4] or within a macrophage [5]. Although there has not as yet been epidemiological data linking METH abuse with cryptococcosis, large numbers of METH users are
infected with human immunodeficiency virus type 1 (HIV-1) [6], and advanced HIV disease is a major risk factor for cryptococcosis [2]. Using a systemic mouse model of infection, we found that METH causes alterations to BBB protein expression and accelerates fungal cell migration to the brain.

**METHODS**

*C. neoformans* strain H99 (serotype A) was grown in a rotary shaker to stationary phase in Sabouraud broth for 24 hours (30°C).

Individuals who use high doses of METH initially use small amounts of the drug intermittently before progressively increasing the dose [7]. To simulate this pattern, increasing doses (2.5, 5, and 10 mg/kg/day on weeks 1, 2, and 3, respectively) of METH (Sigma) were intraperitoneally administered daily to female C57BL/6 mice (age, 6–8 weeks; NCI) over 21 days. Phosphate buffered saline (PBS)-treated animals were used as controls. On day 21 after initiation of drug or placebo administration, brains were excised, and levels of tight junction (TJ) proteins (occludin and ZO-1) and adhesion proteins (PECAM-1 and JAM-1) in BBBs were analyzed by Western blot. To evaluate whether METH accelerates *C. neoformans* penetration to the CNS due to the permeability of BBB or mortality, METH-treated and control mice were infected intravenously with 10⁶ yeasts after 21 days of drug or placebo administration. Mice were sacrificed every 6 hours, and their brains were removed. For histological analysis, tissues were fixed in 10% formalin for 24 hours, processed, and embedded in paraffin. Twenty-micrometer coronal sections were fixed to glass slides. To determine the number of colony-forming units, brain tissues were homogenized in sterile PBS, plated on Sabouraud agar, and incubated at 30°C for 48 hours, and fungal colony counts were determined. The results were normalized by tissue weights.

The BBB model used in this study consisted of primary endothelial cells isolated from murine cerebral capillaries and glial cells (84% astrocytes, 10% oligodendrocytes, and 6% microglia) cocultured on opposite sides of gelatin-coated tissue culture inserts with 3-µm pores that permit astrocyte processes to penetrate the insert and establish contact with the endothelial cell as described [8]. The intact BBB is highly impermeable to albumin. Therefore, we analyzed the passage of albumin conjugated to Evans blue dye through the BBB in vitro to determine the impact of METH or glucuronoxylomannan (GXM; the major capsular polysaccharide of *C. neoformans*; 10 µM) alone, as well as the effect of a combination of METH (10 µM) and GXM (10 µM), on the integrity of the barrier. Untreated or ethylenediaminetetraacetic acid–treated tissue constructs were used as controls.

Microscopic visualizations of tissue sections of the brain were subjected to hematoxylin-eosin staining to examine tissue morphology. Slides were visualized using an Axiosver 40CFL inverted light microscope (Carl Zeiss), and images were photographed with an AxioCam MrC digital camera, using Zen 2011 digital imaging software.

We analyzed the distribution of the TJ proteins in the BBB of METH-treated mice by immunofluorescence. Tissue samples were incubated in primary rabbit polyclonal antibodies overnight at 4°C, washed with PBS, and incubated with FITC-conjugated goat anti-rabbit immunoglobulin G for 1 hour at room temperature. After samples were washed, they were mounted on slides, using anti-fade reagent with DAPI (nuclei marker; Molecular Probes). Additionally, *C. neoformans* and capsular polysaccharide released in tissue were stained using the GXM-binding antibody 18B7. Slides were blocked, and 18B7 (2 µg/mL) was added for 1 hour at 37°C. After slides were washed, FITC-conjugated goat anti-mouse antibody (1:250; 1% bovine serum albumin) was applied for 1 hour at room temperature. Neurons in tissue sections were stained with DAPI and MAP-2 (cell body marker) as described above.

Microscopic examinations of brain sections were performed with a Leica confocal microscope (Germany). Confocal images of blue, green, and red fluorescence were acquired simultaneously, using a multichannel mode. Z-stack images and measurements were corrected using Bio-Rad LaserSharp 2000 software in deconvolution mode.

All data were subjected to statistical analysis using GraphPad Prism 5.0.

**RESULTS**

**METH Reduces Expression of TJ Proteins in a BBB Model**

TJ proteins provide essential structural support to the BBB, playing an important role in maintaining a safe neural microenvironment. We found that METH alters expression of TJ and adhesion molecules (Figure 1A), which help maintain BBB integrity. There was a >55% reduction in occludin, a >40% reduction in ZO-1, a >50% reduction in PECAM-1, and a >25% increase in JAM-1. Results of quantitative analysis are shown in Figure 1B. To confirm these results, immunostaining with occludin-specific monoclonal antibodies (Figure 1C) showed that METH significantly reduces the expression of occludin, causing alterations in the integrity of BBB. Loss of BBB integrity may be a major cause of profound brain modifications, including increasing the susceptibility of the CNS to microbial infections.

**METH Increases BBB Permeability, Promoting *C. neoformans* Migration Into the Brain**

METH-treated mice displayed significant susceptibility to systemic CNS invasion by *C. neoformans* 6 and 12 hours after challenge (Figure 2A). Immunofluorescence staining using capsular-specific antibody 18B7 showed more-numerous and larger brain lesions in METH-treated mice as compared to controls (Figure 2B). Histologically, the tissues from METH-
treated animals contained extensive fungal lesions arranged in biofilm-like formations (ie, yeast cells surrounded by large quantities of fungal polysaccharide) after 12 hours as compared to controls (Figure 2B). The mean area of brain lesions (±SD) on METH-treated infected mice reached 43.85 ± 9.08 µm², whereas the value for control lesions was 8.02 ± 0.61 µm² (P < .001; Figure 2C). Additionally, we documented cryptococcal cells releasing GXM, transmigrating between endothelial cells of a blood vessel, and penetrating brain parenchyma of METH-treated mice 12 hours after infection (Figure 2D). Notably, the yeast cell in the blood vessel traversing to the brain parenchyma displayed more fluorescence than the cell within the brain parenchyma, which is indicative of active GXM production. Additionally, we observed small fragments of GXM around and in proximity to fungal cells within brain parenchyma (Figure 2D). We found a trend toward an increase in the passage of albumin conjugated to Evans blue dye through the BBB in vitro after exposure to either 10 µM of METH or GXM (Figure 2E). However, a combination of METH and GXM produced an additive effect, leading to significant disruption in integrity (P < .001, relative to untreated tissue constructs). Furthermore, METH significantly accelerated the death of C. neoformans–infected mice relative to untreated mice (P < .001; Figure 2F). On day 5 after infection, 100% of METH-treated mice died, compared with none of the untreated mice. On average, METH-treated mice died of C. neoformans infection 3 days after infection, compared with 7 days after infection for untreated mice.

DISCUSSION

Short- and long-term METH abuse damages multiple organ systems; however, none is more prominently affected than the brain. METH neurotoxicity disrupts normal neuronal communication in the dopaminergic system and induces neuroinflammation due to glial activation [9]. METH neurotoxicity...
manifests as severe cognitive deficits, such as memory loss and psychotic behavior. Because METH use also occurs by injection and because behavior changes include a propensity to engage in precarious sexual behavior, METH users are at high risk of contracting HIV-1 [10]. One of the consequences of HIV-1 infection is the development of HIV-1 encephalitis, which is characterized by monocyte migration across the BBB. The increased prevalence of HIV-1 infection and other infections
METH alters mouse BBB, aiding fungal CNS infection

Although much is known about the devastating effects of METH on neuronal and glial function, the effect of METH on the brain endothelium is less well characterized [11]. Recent findings suggest that impairment of GLUT1 at the brain endothelium by METH may contribute to energy-associated disruption of TJ assembly and loss of BBB integrity [12]. Interestingly, BBB compromise present in HIV-1 encephalitis [13] occurs in vivo after METH administration [14]. The main goal of the present study is to provide evidence that METH alters BBB function through direct effects on endothelial cells. Our experiments show enhanced BBB permeability, alterations in expression of cell membrane-associated TJ and adhesion proteins, and increased neurotropic C. neoformans migration across endothelial cells of METH-treated animals.

The BBB represents a complex cellular system consisting of brain microvascular endothelial cells (BMVECs), pericytes, perivascular microglia, astrocytes, and basal lamina [15]. BMVECs form a unique, tightly interconnected cellular monolayer. Special characteristics of BMVECs include the presence of TJs. TJs in the BBB are composed of an intricate combination of transmembrane and cytoplasmic proteins linked to an actin-based cytoskeleton that allows TJs to form a tight seal while still remaining capable of rapid modulation and regulation. TJ formation and disruption is a process involving complex interactions between different TJ proteins and can be modulated by HIV-1 proteins [16]. Similarly, METH induced capsular polysaccharide release by C. neoformans and disruption of TJ and adhesion proteins, which are important in maintaining the integrity of the BBB.

METH abuse by HIV-1–infected individuals results in exacerbated neurodegenerative changes, suggesting that METH acts as a cofactor in HIV-1 neuropathogenesis [17, 18]. Similar to HIV-1 viral proteins such as gp120, C. neoformans GXM may cause toxicity to dopaminergic neurons, and this toxicity is synergistic with METH, which also acts on the dopaminergic system [19]. The neurotransmitter dopamine is involved in the regulation of several BMVEC functions, and the fact that both METH and GXM act on the dopaminergic system suggests that they may either alone or synergistically regulate the TJ and adhesion molecules’ modulation in endothelial cells in vivo, thereby compromising BBB integrity and exacerbating cryptococcal menigitis.

The major molecular components of TJs include the transmembranous and structural proteins occludin, JAM, and claudins and the submembranous peripheral ZO proteins [20]. ZO proteins are essential for targeting TJ structures, and they are linked to the actin cytoskeleton and related signal-transducing mechanisms, which are critical for TJ function [21]. JAM proteins are not connected to ancillary proteins of the cytoplasm, but they affect passage of cells when endothelial or mononuclear cells are activated [20, 22]. TJs and ZO are highly sensitive to microenvironments and respond to inflammatory cytokines in vitro, resulting in an alteration in the subcellular localization and dissociation of the occludin/ZO complex, which is associated with BBB impairment [23, 24]. We evaluated the TJ proteins occludin and ZO-1 and the adhesion proteins PECAM-1 and JAM-1, which are relevant in maintaining BBB integrity.

Infectious agents can enter the CNS early during infection and can penetrate normal BBB, one of the functions of the barrier is to protect the brain from microbial invasion. Loss of BBB integrity followed by a microbial infection is recognized as a major cause of profound brain alterations in METH users [25]. We investigated whether METH can alter the permeability of murine BBBs resulting in increased susceptibility to CNS infection in vivo. METH causes profound defects in the integrity of BBB in vivo, increasing permeability and facilitating transmigration of C. neoformans. A number of studies show that C. neoformans accesses brain parenchyma [3–5], suggesting that the yeast must cross the brain microvasculature to cause meningoen cephalitis. However, the ability of C. neoformans to transmigrate into parenchyma across microvasculature is significantly facilitated by METH, which, consequently, increases mortality. In this study, we showed by immunohistochemistry analysis that the yeast cells release GXM to facilitate transmigration. It is possible that GXM release may also be influenced by Ca2+ sequestration from endothelial cells on the BBB, leading to GXM-mediated BBB disruption and enhanced fungal invasion [26]. Likewise, C. neoformans can use a “Trojan horse” mechanism [5] to cross the brain vasculature by transmigrating within macrophages, which is also consistent with our model.

In conclusion, METH causes profound defects in the integrity of the mouse BBB in vivo, increasing permeability and facilitating transmigration of microbes to the CNS. METH-induced alterations to the molecules responsible for maintaining the integrity of the BBB provide an explanation for the susceptibility of METH abusers to brain infection by HIV and other pathogens.

Notes

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