MAJOR ARTICLE

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

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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 10^6 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 Axiovert 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 conceived 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 1*A*), 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 1*B*. To confirm these results, immunostaining with occludin-specific monoclonal antibodies (Figure 1*C*) 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 2*A*). Immunofluorescence staining using capsular-specific antibody 18B7 showed more-numerous and larger brain lesions in METH-treated mice as compared to controls (Figure 2*B*). Histologically, the tissues from METH-

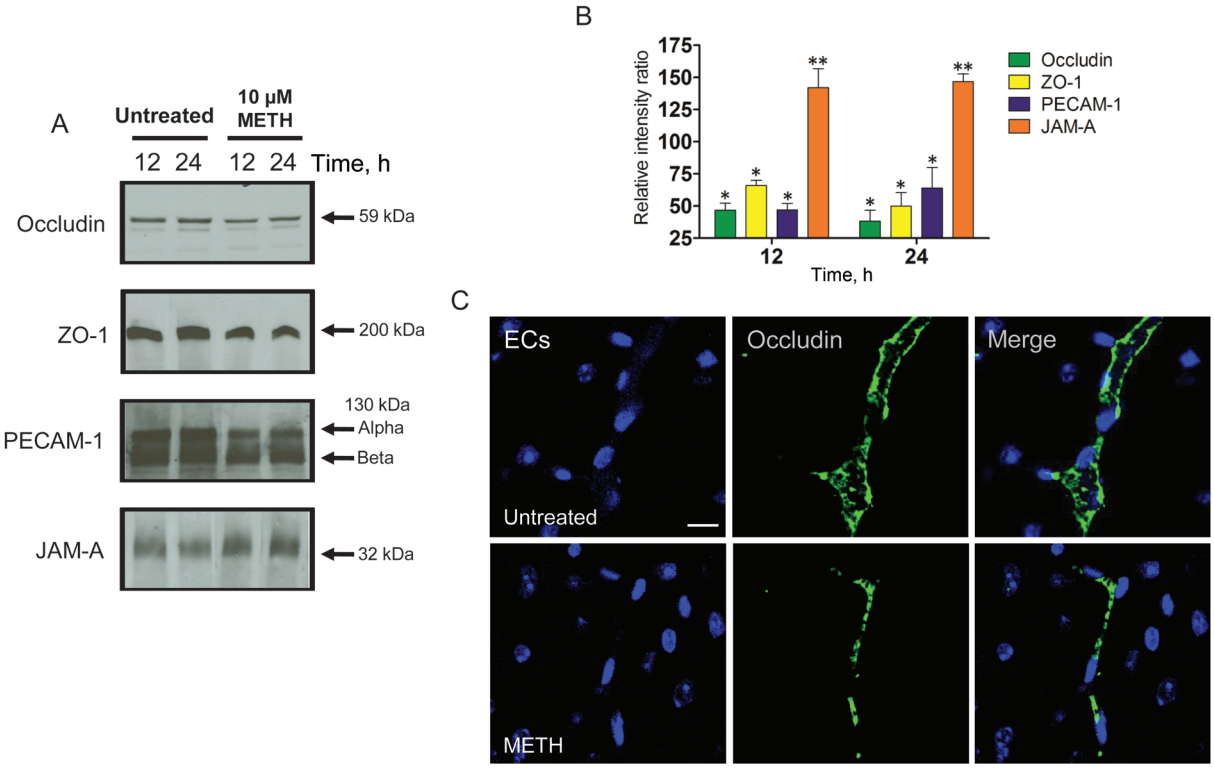

Figure 1. Exposure of murine blood brain barrier (BBB) to methamphetamine (METH) alters tight junction protein (TJ) expressions and distributions in vivo. *A*, Western blot (WB) analysis of brain tissue from untreated (ie, phosphate-buffered saline–injected) or METH-treated C57BL/6 mice was performed to compare the expression of the TJ proteins occludin and ZO-1 and the adhesion proteins PECAM-1 and JAM-A. *B*, Quantitative measurements of individual band intensity in Western blot analysis described in panel *A* for occludin, ZO-1, PECAM-1, and JAM-A, using ImageJ software. Tubulin was used as a control to determine the relative intensity ratio. Bars are the means of 3 independent gel results, and error bars denote SDs. $^{*}P < .001$, for the reduction in the intensity of the band of TJ and adhesion molecules as compared to tubulin; $^{**}P < .001$, for the increase in the intensity of the band of TJ and adhesion molecules as compared to tubulin. *C*, Immunofluorescent staining of brain tissues from untreated or METH-treated animals was done to examine the distribution of occludin. Rabbit polyclonal anti-occludin antibody and DAPI staining were used to label occludin (green fluorescence) and nuclei of endothelial cells (ECs; blue fluorescence). Scale bar, 20 μm. The experiments were performed at least twice with similar results obtained.

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 2*B*). The mean area of brain lesions (±SD) on METH-treated infected mice reached 43.85 ± 9.08 μm^2, whereas the value for control lesions was 8.02 ± 0.61 μm^2 ($P < .001$; Figure 2*C*). 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 2*D*). 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 2*D*). 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 2*E*). 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 2*F*). 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

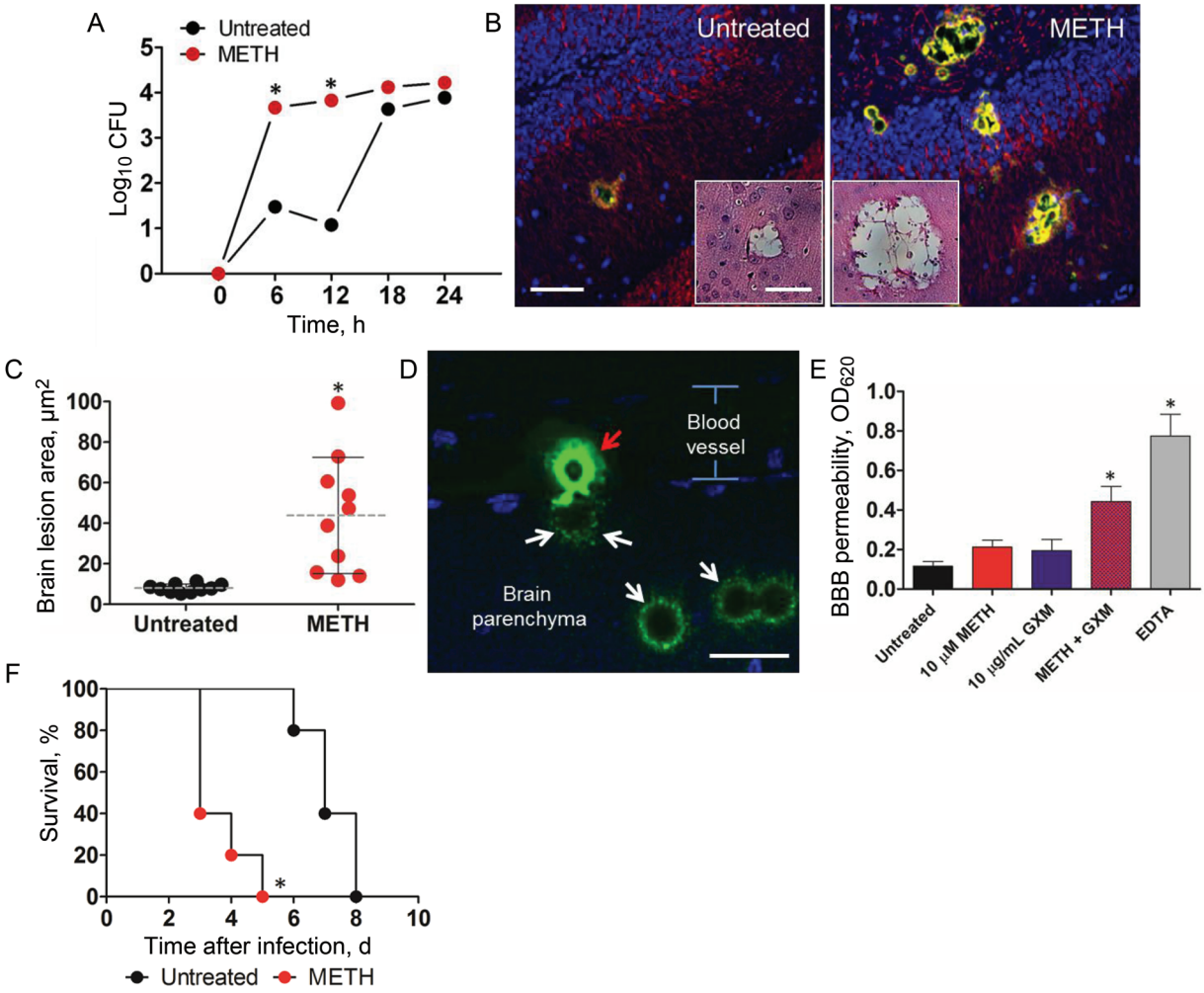

Figure 2. Methamphetamine (METH) enhances *Cryptococcus neoformans* migration into the central nervous system (CNS) of C57BL/6 mice after systemic infection. *A*, Brain fungal burden in METH-treated mice infected intravenously with 10^6 *C. neoformans* is significantly higher than that in untreated (ie, phosphate-buffered saline–injected) mice (n = 3 per time point; n = 12 per group). This experiment was performed twice with similar results. **P* < .001, by analysis of variance, with Bonferroni correction. CFU, colony-forming units. *B*, Immunofluorescent staining of brain lesions (cryptococcomas) caused by *C. neoformans* in untreated or METH-treated animals. Capsular-specific monoclonal antibody 18B7 (monoclonal antibody 18B7; yellow) was used to label fungal cells. MAP-2 (red) and DAPI (blue) staining were used to label the cell bodies and nuclei of neurons, respectively. Scale bar, 5 µm. Insets show histological analysis of untreated or METH-treated C57BL/6 mice 12 hours after cryptococcal infection. Representative hematoxylin-eosin–stained sections of the brain lesions are shown. Scale bar, 5 µm. *C*, Area analyses of brain lesions caused by *C. neoformans* strain H99 cells were performed in tissue sections of untreated or METH-treated mice (n = 3 per group). The areas of 10 brain lesions per condition were measured using ImageJ software. Each circle represents a single lesion. Dashed lines and error bars denote the mean value of 10 measurements and SDs, respectively. **P* < .001, by the *t* test. *D*, Confocal microscopy of cryptococcal cells releasing glucuronoxylomannan (GXM) and penetrating across a blood vessel's endothelial cells to the brain parenchyma of METH-treated mice. FITC-labeled monoclonal antibody 18B7 was used to label capsular polysaccharide (green) in fungal cells. DAPI (blue) staining was used to label nuclei of endothelial cells. The red arrow indicates a cryptococcal cell traversing endothelial cells in a blood vessel. White arrows designate cryptococcal cells inside the brain parenchyma. Scattered green dots indicate released GXM. Scale bar, 10 µm. *E*, In vitro BBB permeability to albumin conjugated to Evans blue dye after treatment with 10 µM of METH, GXM, or a combination of METH and GXM. Untreated or ethylenediaminetetraacetic acid (EDTA)–treated cells were used as controls. EDTA enhances BBB penetration. **P* < .001, by analysis of variance, with Bonferroni correction. The experiments were performed twice with similar results. *F*, Survival differences of METH-treated and untreated C57BL/6 mice after systemic intravenous infection with 10^6 *C. neoformans* (n = 5 per group). **P* < .001, by the log-rank (Mantel–Cox) test.

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

among METH users creates an additional layer of complexity in understanding METH's harmful effects on the brain. In recent years, METH is increasingly being recognized as a damaging agent not only to the neuronal environment but also to the cerebral vasculature.

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 meningitis.

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 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 meningoencephalitis. 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 Ca^{2+} 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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Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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