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Methamphetamine augment HIV-1 Tat mediated memory deficits by altering the expression of synaptic proteins and neurotrophic factors

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Abstract

Methamphetamine (METH) abuse is common among individuals infected with HIV-1 and has been shown to affect HIV replication and pathogenesis. These HIV-1 infected individuals also exhibit greater neuronal injury and higher cognitive decline. HIV-1 proteins, specifically gp120 and HIV-1 Tat, have been earlier shown to affect neurocognition. HIV-1 Tat, a viral protein released early during HIV-1 replication, contributes to HIV-associated neurotoxicity through various mechanisms including production of pro-inflammatory cytokines, reactive oxygen species and dysregulation of neuroplasticity. However, the combined effect of METH and HIV-1 Tat on neurocognition and its potential effect on neuroplasticity mechanisms remains largely unknown. Therefore, the present study was undertaken to investigate the combined effect of METH and HIV-1 Tat on behavior and on the expression of neuroplasticity markers by utilizing Doxycycline (DOX)-inducible HIV-1 Tat (1-86) transgenic mice. Expression of Tat in various brain regions of these mice was confirmed by RT-PCR. The mice were administered with an escalating dose of METH (0.1 mg/kg to 6 mg/kg, i.p) over a 7-day period, followed by 6 mg/kg, i.p METH twice a day for four weeks. After three weeks of METH administration, Y maze and Morris water maze assays were performed to determine the effect of Tat and METH on working and spatial memory, respectively. Compared with controls, working memory was significantly decreased in Tat mice

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DISCLOSURE

All the authors declare no competing financial interests in relation to the work described.

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that were administered METH. Moreover, significant deficits in spatial memory were also observed in Tat-Tg mice that were administered METH. A significant reduction in the protein expressions of synapsin 1, synaptophysin, Arg3.1, PSD-95, and BDNF in different brain regions were also observed. Expression levels of Calmodulin kinase II (CaMKII), a marker of synaptodendritic integrity, were also significantly decreased in HIV-1 Tat mice that were treated with METH. Together, this data suggests that METH enhances HIV-1 Tat-induced memory deficits by reducing the expression of pre- and postsynaptic proteins and neuroplasticity markers, thus providing novel insights into the molecular mechanisms behind neurocognitive impairments in HIV-infected amphetamine users.

Keywords

HIV-1 Tat; methamphetamine; memory deficits; synapsin1; PSD-95; BDNF and p-CaMKII

INTRODUCTION

Despite the availability of combination antiretroviral therapy (cART), HIV-1 infected patients remain at risk of developing HIV-associated neurocognitive disorders (HAND). The prevalence of the most severe form of HAND, HIV-associated dementia (HAD), has declined in the post-cART era [1, 2]. However, patients continue to suffer from minor forms of HAND, namely asymptomatic neurocognitive impairment (ANI) and minor neurocognitive disorder (MND) [3, 4]. Moreover, evidence also indicates a shifting pattern of neurocognitive impairment in HIV patients, from deficits in motor ability, the speed of information processing, and verbal speed in the pre-cART era to deficits in memory and executive function in the post-cART era [5]. HIV-1 infection in the brain is characterized by the presence of activated astrocytes [6], decreased number of neurons [7], alterations in dendritic and synaptic densities [8, 9], decreased expression of synaptic plasticity genes [8], and decreased dopaminergic transporters [10]. The development HAND and pathological changes observed in HIV-1 infected individuals can be attributed to direct and indirect toxic effects of HIV viral proteins (gp120, Nef, Tat, and Vpr) released from infected brain cells such as microglia, perivascular macrophages and astrocytes [11, 12].

HIV-1 transactivator of transcription, Tat, is released early during the infection and is important for a variety of viral functions such as HIV-1 mRNA splicing [13], capping [14–16], translation [17–20], and reverse transcription [21–23]. Tat contributes to neuronal toxicity by various mechanisms including disruption of blood brain barrier (BBB) permeability [24–26], increased oxidative stress [27–31], increased apoptosis [32–34], increase in the release of pro-inflammatory cytokines (CCL5, IL-1 β , IL-6, IL-8, and TNF- α) [35–39], and excitotoxicity through involvement of NMDA receptors, glutamate and calcium influx into cells [40, 41]. Further, HIV-1 Tat has also been shown to suppress long-term potentiation (LTP) [42] and impair spatial learning and memory in both mouse [43, 44] as well as rat models [45, 46].

Illicit drugs such as methamphetamine (METH) and others has been shown to be associated with increased risk of HIV -1 infection [47–49]. METH is a psychostimulant that exerts its

actions by primarily affecting variety of neurotransmitter systems, including dopaminergic [50–52], serotonergic [53, 54], gamma amino butyric acid (GABA)-ergic [55, 56], and glutamatergic systems [57, 58], eventually leading to structural and functional abnormalities in the brain [59, 60]. Consistent with HIV-1 Tat-mediated cognitive impairments, METH use has also been shown to impair learning and memory in the human population and in animal models [61–63]. In view of the existing evidence that Tat and METH affect same brain regions and share similar mechanisms of neurotoxicity, we sought to determine the possible additive/synergistic role of Tat and METH in causing cognitive impairments. There has been several studies addressing the combined effect of methamphetamine and HIV-1 gp120 [64–67]. However, only limited information is available regarding the combined effect of HIV-1 Tat and methamphetamine [68]. Further, there is no information available regarding the role of neuroplasticity genes in regulation of cognitive functions in context of methamphetamine/HIV-1 Tat-mediated cognitive impairments. We utilized HIV-1 Tat transgenic mouse model to assess HIV-1 Tat-mediated cognitive impairments and whether methamphetamine augments Tat-mediated deficits in cognitive functions.

MATERIALS AND METHODS

Animals

Doxycycline-inducible HIV-1 Tat transgenic mice (C57/BL6J) were obtained from Dr. Kurt Hauser at Virginia Commonwealth University (VCU). These mice express HIV-1 Tat in the brain using a *tet-on* inducible system and GFAP promoter [69–71]. The Tat-Tg mice present many clinical findings of HIV-1 infection, including infiltration of blood cells, neuronal apoptosis, astrogliosis, reduced gray matter density, dendritic degeneration and inflammation [70, 72]. Moreover, deficits in learning and memory have also been observed in Tat-Tg mice [43, 73]. Both male (M) and female (F) mice were used for the experiments and were divided into four groups each: Control M (n=9), Tat M (n=7), METH M (n=9), Tat + METH M (n=7), Control F (n=9), Tat F (n=8), METH F (n=12) and Tat+ METH F (n=8). Mice were administered DOX (6 g/kg) through formulated chow starting at eight weeks of age. DOX was administered for 12 weeks before starting behavioral experiments. Mice were housed in an animal facility in groups of 3–5 per cage and were allowed unlimited access to food and water. Twelve h light/dark cycle was employed by turning on the lights from 6:00 AM to 6:00 PM. Behavioral testing was performed between 8:00 AM and 5:00 PM. All procedures were approved and followed in accordance with UMKC Institutional Animal Care and Use Committee.

Methamphetamine (METH) treatment

To simulate chronic METH use in human abusers, we employed an escalating dosing regimen in mice. METH (Sigma, St. Louis, MO) was dissolved in phosphate buffered saline (PBS) and was administered intraperitoneally twice a day. We escalated the dose of METH stepwise from 0.1 mg/kg to 6 mg/kg over the 7-day duration. After the escalation period, 6 mg/kg METH was given b.i.d for 4 weeks. Control mice received an equivalent volume of PBS intraperitoneally (Fig 1).

Y-Maze

The effect of METH, Tat and Tat+ METH on short term memory was evaluated using a Y-maze. The maze has three interconnected closed arms, that are 12 inches in length each and are 120° from each other. The animal was placed into the end of one of the arms and was allowed to freely explore the maze for 5 minutes. The starting arm positions of mice were chosen at random. The movement of the animal inside the Y-maze was recorded by an overhead camera using ANY-maze (version 4.99z) behavioral tracking software. The arms were thoroughly cleaned with disinfectant and 70% ethanol to eliminate any residual odors of previous mice. The number of arm entries were counted and acted as a marker of locomotor activity. The number of spontaneous alternations, as defined by entry into three different arms in sequence (triad), serves as a measure of working memory. Percentage spontaneous alternations was calculated from number of triads and arm entries using the following equation: % Spontaneous alternations = (number of triads/total number of arm entries-2) X 100.

Morris Water Maze

The Morris water maze is a standard test employing a circular tank and is used to assess spatial learning and memory in rodents. The tank was filled with water and the temperature of the water was maintained between 24–27° C. A circular platform (10 cm in diameter) was placed 1 cm below the surface of the water. In addition, four visual cues were placed in each corner of the tank in order to help mouse locate the platform. The test consisted of five acquisition trials (day 1–5) and a probe trial on day 6. On day 1–5 (acquisition phase), four trials were performed every day and during each trial, mice were released from different starting positions, and given 60 s to locate the platform. The mice were gently guided to the platform if they could not find the platform within 60 seconds. The platform was kept in the same quadrant during the acquisition phase but the platform was removed during the probe trial and mice were allowed to explore the tank. The movement of the mice inside the tank was recorded by using the overhead camera and ANY-maze (version 4.99z) behavioral tracking software. Spatial and learning memory deficits were analyzed by measuring the latency to reach the platform. Time spent in the target quadrant (where the platform was initially located), annulus entries (platform crossings) in the target quadrant and swim speed were also measured.

Real time RT-PCR

Mice were anesthetized with ketamine and xylazine mixture, and brain was harvested after perfusing heart with 7–8 ml of isotonic PBS. Cerebellum was dissected from both the hemispheres after separating pons and medulla. The brain was then hemisected into two hemispheres. The region of the brain rostral to the hippocampus was dissected as the prefrontal cortex, (PFC), which includes motor cortex. Hippocampus was separated from the rest of the brain, and the remaining region was labeled the parietal cortex (PC). Immediately after isolation, these different brain regions were flash frozen in liquid nitrogen. Total RNA was isolated from various brain regions using Qiagen RNeasy® Mini Kit following manufacturer's protocol (Qiagen Inc, Valencia, CA). Briefly, tissues were homogenized in 700 µl of RLT buffer, followed by the addition of 700 µl of 70% ethanol and vortexing for 1

min. The column was then washed with RW1 and RPE buffer, and RNA was eluted in 50 μ l of RNase free water. Hundred ng of RNA was reverse transcribed by real time RT-PCR using the conditions: Reverse transcription at 50°C for 10 min; denaturation at 95°C for 5 min; 45 cycles of denaturation at 95°C for 45 sec, annealing at 54°C and extension at 72°C for 30 sec. 8 μ l of amplified product was mixed with 2 μ l of loading dye, and was loaded into wells of 2% agarose gel. The gel was run at 80 V for 1 h and presence of HIV-1 Tat was confirmed by the identification of band at 220 bp.

Western blot

Protein samples were prepared by homogenizing different brain regions in 500 μ l of RIPA buffer in the presence of protease and phosphatase inhibitors, spun down at 14,000 rpm for 15 min at 4° C and supernatant was collected. Protein concentration was determined using Pierce BCA assay kit (ThermoFisher Scientific, Waltham, MA). An equal amount of protein from different mice in a group was pooled and forty micrograms of each pooled protein sample were electrophoresed on 12% polyacrylamide gel and transferred to polyvinylidene difluoride (PVDF) membrane at 2.5 A for 20 min using Trans-blot Turbo transfer system (Bio-Rad Laboratories, Hercules, CA). PVDF membranes were probed with various primary antibodies, including synapsin 1, synaptophysin, PSD-95, Arg3.1, SHANK2, BDNF, CNTF, p-CaMKII and appropriate secondary antibodies. Proteins were visualized by the addition of BM Chemiluminescence blotting substrate (POD) (Roche, Indianapolis, IN). GAPDH was used as a loading control to normalize the expression of proteins of interest. The bands were quantified by using a gel imaging system (FluorChem E 4.1.4) and Alpha View software (version 3.4.0.0).

Brain to body weight ratio

Mice were weighed and humanely euthanized to collect the brain. Brain weight was recorded and brain to body weight ratio was calculated from brain weight to body weight and multiplying by 100.

Statistical analyses

Statistical analyses were performed using GraphPad® Prism and SPSS Software (version 23) (IBM, Armonk, NY). Y maze parameters (% spontaneous alternations and arm entries), water maze parameters (escape latency, swim speed, time spent in target quadrant and annulus entries) and fold change of various proteins were analyzed using three-way ANOVA (with sex, Tat expression and METH treatment as factors). The primary analysis was followed by Tukey's post hoc test to determine significant group differences. Values are expressed as mean $\pm$ SE and $p < 0.05$ was considered statistically significant. The results are presented including F values for significant differences whereas F values are not presented for non-significant effects. The gender difference is calculated as conversion of response into fold difference for randomly selected set of control and experimental mouse and fold response was then subjected to one way ANOVA to calculate the significance followed by tukey's post-hoc test for multiple comparisons.

RESULTS

Effect of HIV-1 Tat and METH on working memory

HIV-1 infection alone is associated with a decline in working memory [74, 75] which is further exacerbated among HIV-1 positive individuals using illicit drugs [76]. However, only limited knowledge is available about the combined effects of HIV-1 Tat and methamphetamine on memory and cognition. Therefore, we employed Y-maze and Morris water maze assays to assess effects of the HIV-1 Tat and METH on working and spatial memory, respectively. The study involved both male and female mice as to assess whether there was gender difference. During the escalating METH regimen, HIV-1 Tat-Tg and non-Tg (control) mice were injected twice daily for 7 days with PBS or escalating doses of METH (starting with 0.1 mg/kg and increasing to 6.0 mg/kg). METH treatment did not cause any significant change in the mouse body weight (Suppl. Fig. 1A, 1B). The expression of HIV-1 Tat mRNA in different brain regions was confirmed by RT-PCR (Suppl. Fig. 2). Examination of RT-PCR product on agarose gel confirmed the expression of Tat mRNA in the hippocampus, parietal cortex, prefrontal cortex and cerebellum. As expected, the Tat mRNA was not expressed in the different brain regions of control mice.

Working memory in Tat and METH treated mice was evaluated by calculating the % spontaneous alternations. As compared to control, a significant [$(df_{sa}3, df_{error}34)$ $F=5.395$ $p=0.004$] decrease in the % spontaneous alternations was observed in male Tat+ METH group ($p=0.003$) (Fig. 2A). Further, the the tat+METH group showed significantly reduced % spontaneous alterations as compared to Tat only group ($p=0.05$). However, Tat expression alone did not affect the percent spontaneous alterations compared to the control group in both the sexes ($p=0.320$). In addition, there was a non-significant decrease in the % spontaneous alterations in METH-treated male ($p=0.753$) and female ($p=0.485$) mice compared to the control groups (Fig. 2A, 2B). In male mice, a non-significant ($p=0.376$) decrease in % spontaneous alternations was observed in Tat + METH group compared to the METH-treated group (Fig. 2A). Additionally, there were no significant differences in the number of total arm entries made by mice in any group in both sexes (Fig. 2C, 2D) indicating that general locomotor function was not altered and did not influence the effects of HIV-1 Tat and/or METH on spontaneous alternations. Finally there was no gender difference observed in % spontaneous alterations (supplementary Fig. 3).

Effect of HIV-1 Tat and METH on spatial learning and memory

The combined effect of HIV-1 Tat and METH on spatial learning and memory was evaluated using Morris water maze assay. Data were normally distributed in all the groups between sexes (Shapiro Wilk test; $p>0.05$ for all groups). Both male and female mice in control group learned to find the escape platform quickly compared to treated groups. Escape latencies were 38.2 ± 3.6 sec on the day (D) 1 compared to 14.6 ± 1.9 sec on D5 for male control mice; and 39.2 ± 3.1 sec on D1 compared to 13.1 ± 1.4 sec on D5 for female control mice (Fig. 3A, 3B). Both male and female mice expressing HIV-1 Tat required longer times to find the escape platform [$(df_{treatment}3, df_{error}620)$ $F= 13.183$, $p=2.36E-8$ for males; and $(df_{treatment}3, df_{error}708)$ $F= 20.401$, $p=1.09E-12$ for females)]. Likewise, METH treatment also resulted in longer times to find the escape platform but reached to statistical significance only in

females ($p=7.00E-06$). The Tat mice treated with METH took significantly longer time to reach the hidden platform compared to control groups in both the sexes ($p=1.89E-08$ for males and $p=5.23E-13$ for females). However, Tat+METH group showed significant difference only with METH ($p=0.015$) and not with the Tat group among males, and no significant difference was observed between METH+Tat and either METH or tat group among females. We also determined the swim speed of the animals over the course of acquisition trial (Fig. 3C, 3D). Average swim speed of the animals between different groups remained consistent over the duration of acquisition trial, indicating that treatment did not affect the swim speed of the animals. No significant differences were observed in the swim speeds of the animals between the sexes. The gender effect on spatial learning and memory is shown in supplementary Fig. 5. The male mice showed significantly reduced acquisition ($F_{5,947}=10.28$, $p=1.29E-09$) when exposed to METH ($p=2E-5$) as compared to female mice (supplementary Fig. 4). There was no other significant difference observed between 2 sexes when mice were exposed to HIV-1 Tat and/or METH.

A probe trial was conducted 24 h after the acquisition trial to evaluate the effect of HIV-1 Tat and METH on reference memory. Animals from control group from both sexes learned the platform location better than animals from other groups, indicated by the time spent in the target quadrant (20.09 ± 1.24 sec for males vs 22.18 ± 1.58 sec for females) (Fig. 3E, 3F). There was a significant decrease in the time spent by the animals [$\{(df_{\text{treatment}}3, df_{\text{error}}32), F=9.175$ $p=1.58E-04$ for males} and $\{(df_{\text{treatment}}3, df_{\text{error}}37), F=3.984$ $p=0.015$ for females] in the target quadrant from Tat group ($p=0.004$ in males and $p=0.030$ in females) and Tat + METH group ($p=2.70E-04$ in males and $p=0.032$ in females). The Tat+METH group animals showed significantly compromised spatial memory compared to METH group among males ($p=0.015$) but not among females (Fig. 3E, 3F). Furthermore, data from the number of platform crossings showed similar results that align with the time spent in target quadrant (Fig 3G, 3H). These data suggest that reference memory in mice was impaired by HIV-1 Tat but not by METH.

Effect of HIV-1 Tat and METH on expression of synaptic proteins and neurotrophic factors

Milder forms of neurocognitive impairments mediated by HIV-1 have been demonstrated to cause changes in synapse organization without neuronal death suggesting that alterations in the levels of synaptic proteins be responsible for neurocognitive impairments [9]. Therefore, we sought to determine the expression of various synaptic proteins in response to Tat and METH. Mice were euthanized and brain to body ratio was calculated (Suppl. Fig. 5A, 5B). There was no significant difference in the brain weights between different treatment groups in both sexes. We performed western blot to determine the effect of HIV-1 Tat and METH on expression of two pre-synaptic proteins (synaptophysin and synapsin 1), three postsynaptic proteins (PSD-95, Arg3.1 and SHANK2) and p-CaMKII.

Effect HIV-1 Tat and METH on pre-synaptic proteins

To determine the effect of Tat and METH on synaptic plasticity, we examined the expression of synapsin 1 and synaptophysin which are two important pre-synaptic proteins in different regions of the brain (Fig. 4A–H). Synaptophysin plays a significant role in formation of synapse and vesicle endocytosis [77, 78]. As compared to the control group, the expression

of synaptophysin was significantly [(df_{treatment}3, df_{error}36) F= 4.447, p=0.009] decreased ($41 \pm 4.5\%$) in parietal cortex of Tat + METH male mice (p=0.008) and hippocampus (p=0.008) (Fig. 4A, 4C). However, other groups did not show any significant change in synaptophysin level in any regions of the brain of male mice tested for synaptophysin. Further, the female mice did not show any significant change in 4 brain regions after exposure with HIV-1 Tat or Meth or HIV-1 Tat and Meth.

Synapsin 1 is another pre-synaptic protein present in the cytoplasmic surface of synaptic vesicles of nerve terminals, and is involved in regulation of synapse formation and maintenance of synaptic plasticity [79–81]. The synapsin increased significantly in hippocampus after HIV-1 Tat exposure [(df_{treatment}3, df_{error}32) 10.825, p=4.60E-05 and post hoc p=4.10E-04] and cerebellum after HIV-1 Tat+METH exposure [(df_{treatment}3, df_{error}32) 10.825 p=4.60E-05 post hoc p=1.34E-04] of male mice. It did not change significantly in any other regions of brains after any exposure. On the other hand Among female mice the Tat exposure did not cause any significant change in any of the brain regions tested whereas Meth exposures caused significant decrease ($35 \pm 9.8\%$) [(df_{treatment}3, df_{error}32) 11.634 p=2.60E-05 with post hoc 8.00E-05] in hippocampus but not in other regions (Fig. 4F, 4H). The cumulative METH and Tat exposure caused significant synapsin 1 increase ($35 \pm 9.8\%$) in cerebellum [(df_{treatment}3, df_{error}32) 11.634 p=2.60E-05, and post hoc p=8.00E-05] with no significant change in other regions (Fig. 4F, 4H).

Effect on HIV-1 Tat and METH on postsynaptic proteins

Postsynaptic density protein 95 (PSD-95) belongs to the family of membrane associated guanylate kinase. Previous studies have indicated the key role of PSD-95 in learning and memory [82–84]. Therefore, we determined the expression levels of PSD-95 in response to METH treatment and/or HIV-1 Tat induction (Fig. 5A–D). In males, a non-significant decrease in the expression of PSD-95 was observed in the prefrontal cortex ($20 \pm 3.0\%$) of Tat group and parietal cortex ($21 \pm 4.2\%$) of METH group (Fig 5A, 5C). Nevertheless, male mice exposed to METH and Tat showed significant reduction in PSD95 level [(df_{treatment}3, df_{error}24) F=8.497, p=0.001 post hoc p=0.001]. The PSD95 decrease exposed to METH and Tat as compared to controls was found to be $40 \pm 11.9\%$ (p=0.047), $38 \pm 1\%$ (p=0.005) and $31 \pm 10\%$ (p=0.05) in parietal cortex, hippocampus and prefrontal cortex, respectively (Fig. 5A, 5C). Further, the PSD95 in this group was significantly reduced as compared to Tat only (p=0.003) as well as METH only group (p=0.019). The PSD-95 expression in female exposed to Tat and METH also showed significant decrease [(df_{treatment}3, df_{error}30) F=6.255, p=0.002] and this decrease was significant in hippocampus as compared to control group ($46 \pm 5.8\%$ reduction, p=0.002) as well as in hippocampus (p=0.008) and prefrontal cortex (p= 3.15E-05) as compared to Tat exposed group (Fig. 5A, 5C).

Activity regulated cytoskeleton-associated protein (Arg3.1) is an essential immediate early gene, involved in various memory processes, including formation of spatial memory and fear memory [85]. In Tat + METH groups, the expression of Arg3.1 was significantly decreased in the parietal cortex by $31 \pm 8.3\%$ (p=0.025) and $41 \pm 6.0\%$ (p=0.003) among males [(df_{treatment}3, df_{error}28), F= 3.12, p=0.042] and females [(df_{treatment}3, df_{error}31) F=5.236, p=5.00E-04], respectively. The prefrontal cortex region in this group also showed significant

decrease in both males ($22 \pm 1.6\%$, $p=0.038$) and females ($32 \pm 1.8\%$, $p=0.012$) as compared to control (Fig. 5E–H). Further the Arg3.1 decrease was found to be significant in this group in parietal cortex ($p=7.80E-5$), hippocampus ($p=2.80E-6$), and prefrontal cortex ($p=6.80E-6$) as compared to HIV-1 Tat only group as well. There were some insignificant decrease also observed for Arg3.1 level as HIV-1 tat caused $14 \pm 4.4\%$ reduction in parietal cortex ($p > 0.05$) of female Tat mice (Fig. 5F, 5H). Meth only exposure caused statistically insignificant reduction ($p > 0.05$) in parietal cortex ($22 \pm 12.8\%$) of female mice (Fig. 5F, 5H); and in parietal cortex ($23 \pm 14.2\%$), hippocampus ($17 \pm 2.5\%$) and prefrontal cortex ($21 \pm 1.8\%$) of male mice (Fig. 5E, 5G).

SHANK2, a member of postsynaptic protein family, plays a vital role in synaptogenesis by facilitating the attachment of glutamate receptors to NMDA receptors [86]. We evaluated the expression of SHANK2 in different brain regions by western blot analysis (Fig. 5I–L). Expression of SHANK2 was significantly ($p < 0.05$) decreased in the prefrontal cortex of male [(df_{treatment}3, df_{error}40), $F=4.244$, $p=.011$] and female [(df_{treatment}3, df_{error}36), $F=15.828$, $p=9.99E-07$] mice. The male mice exposed to Tat and Tat+METH showed $24 \pm 4.3\%$ ($p=0.049$) and $28 \pm 2.7\%$ ($p=0.007$) reduction respectively in prefrontal cortex region whereas female mice [(df_{treatment}3, df_{error}36) $F=15.828$ $p=9.99E-07$] showed significant decrease in prefrontal cortex region only when they were exposed to Tat+METH ($18 \pm 0.9\%$ reduction, $p=6.00E-06$). The cerebellum region of the brain showed consistent and statistically significant increase in all the 3 groups when female mice were exposed to Tat ($p=8.00E-06$), METH ($p=6.00E-06$) and Tat+METH ($p=8.00E-09$) as shown in Fig. 5J and 5L.

Effect of HIV-1 Tat and METH on the expression of neurotrophic factors

Neurotrophic factors perform critical roles in synaptic plasticity, neuronal development, survival and proper function. Decrease in the expression of various neurotrophic factors has been observed in HIV infected individuals with encephalitis [87]. Previous studies have explored the role of neurotrophic factors and neurotrophin signaling in HIV-1 Tat and METH-mediated effects [88–90]. In order to determine the combined effect of HIV-1 Tat and METH on the expression of neurotrophic factors, we analyzed the BDNF and CNTF expression by western blot in different brain regions (Fig. 6A–H). The antibody used for the study recognize two BDNF isoforms in western blotting with only one being mature form [91]. Therefore, we quantified only the mature BDNF band as it is known to promote LTP through interaction with Trk receptors [86, 92–96]. We determined the expression of brain-derived neurotrophic factor (BDNF) and ciliary neurotrophic factor (CNTF) in prefrontal cortex, parietal cortex, hippocampus and cerebellum.

The BDNF expression was significantly altered in all 4 regions of both male [(df_{treatment}3, df_{error}32), $F=4.791$ $p=.007$] and female [(df_{treatment}3, df_{error}32) $F=11.233$ $p=3.40E-05$] mice. The Tat and METH exposure significantly reduced the BDNF expression by $24 \pm 11\%$ ($p=0.038$) and $23 \pm 5\%$ ($p=0.026$) in parietal and prefrontal cortex of male mice. Whereas Tat+METH exposure reduced the BDNF expression in all 4 brain regions of male mice. The reduction was $48 \pm 11\%$ ($p=0.015$), $30 \pm 3.3\%$ ($p=0.029$), $32 \pm 7.2\%$ ($p=0.042$) and $46 \pm 10\%$ ($p=0.032$) in parietal cortex, hippocampus, prefrontal cortex and cerebellum,

respectively (Fig. 6A, 6C). Among female mice, the METH exposure caused significant decrease in parietal ($24 \pm 11\%$ decrease, $p=0.038$) and ($23 \pm 5\%$ decrease, $p=0.026$) prefrontal cortex. Similar to male mice, the Tat+METH exposure also caused reduction in BDNF expression in all 4 brain regions of female mice. The reduction was $44 \pm 11\%$ ($p=1.10E-05$), $18 \pm 3\%$ ($p=0.029$), $32 \pm 7.2\%$ ($p=0.027$) and $22.0 \pm 4.5\%$ ($p=0.045$) in parietal cortex, hippocampus, prefrontal cortex and cerebellum, respectively (Fig. 6B, 6D). We also determined the expression of CNTF in different brain regions of both male and female mice (Fig. 6E–H). However, no significant differences were observed in any of the treatment groups in both the sexes.

Effect of HIV-1 Tat and METH on the expression of p-CaMKII

Calcium/Calmodulin-dependent protein kinase II (CaMKII) is highly expressed protein in the brain and its activation results in the induction of LTP [97]. Therefore, we measured the effect of HIV-1 Tat and/or METH on the expression of CaMKII by western blot. We specifically measured the phosphorylated form of CaMKII on Thr 286, which is an active form. In male mice [(df_{treatment}3, df_{error}28) $F=5.145$, $p=0.010$], the HIV-1 Tat significantly decreased the expression of p-CaMKII in by $19 \pm 1.9\%$, $41 \pm 5\%$ and $31 \pm 20\%$ in parietal cortex ($p=0.035$), prefrontal cortex ($p=0.012$) and cerebellum ($p=0.042$), respectively (Fig. 7A, 7C). It was reduced by METH alone or METH+Tat exposure in all the 4 brain regions significantly (Fig. 7A, 7C). Similarly, the female mice also showed significant change in p-CaMKII expression [(df_{treatment}3, df_{error}32) $F=4.256$ $p=0.030$]. Meth treatment caused significant reduction in all 4 regions. However, only HIV-1 Tat exposure did not cause any significant change in p-CaMKII expression (Fig. 7B and 7D).

These pre and postsynaptic proteins as well as CaMKII were compared in both male and female mice for gender difference. The results of these analyses are shown in supplementary Fig. 6. The fold difference was calculated by pairing individual control mouse with corresponding treatment mouse and ratio was calculated. The results are presented as mean +SE for each pre/post synaptic proteins and CaMKII. There was no gender difference observed in Arg3.1, and CNTF in any of the 4 brain regions. However, METH exposed male mice showed significant reduction in BDNF expression in cerebellum as opposed to female mice [(df_{treatment}3, df_{error}30) $F=7.814$ $p=0.026$]. Likewise HIV-1 Tat exposed male mice showed significant reduction in CaMKII expression in cerebellum [(df_{treatment}3, df_{error}26) $F=9.085$, $p=0.003$], PSD95 in prefrontal cortex [(df_{treatment}3, df_{error}26) $F=5.655$, $p=0.05$] and synaptophysin [(df_{treatment}3, df_{error}34) $F=3.337$, $p=0.008$] as opposed to female mice. Conversely, the female mice showed significantly reduced SHANK2 expression in prefrontal cortex [(df_{treatment}3, df_{error}30) $F=22.438$, $p=1.37E-4$] and synapsin expression in hippocampus [(df_{treatment}3, df_{error}34) $F=2.508$ $p=0.001$] after HIV-1 Tat exposure. The synapsin expression was also significantly reduced in female mice in hippocampus after METH ($p=0.001$) and METH+Tat exposure ($p=0.03$) as shown in Supplementary Fig.-6.

DISCUSSION

METH users have an increased risk for HIV-1 infection and development of neuropsychological impairments [98]. In addition, there is significant decrease in the

number of interneurons in the frontal cortex of HIV-1 positive individuals with a history of METH use [99]. HIV-1 viral proteins, including HIV-1 Tat and gp120, are known to play a major role in the development of neurological impairments in HIV-1 positive individuals [61, 64, 73, 100]. In the present study, we sought to determine the effect of escalating, dose regimen of METH on neurocognition in adult HIV-1 Tat Tg mice. As previously reported, escalating doses of METH did not induce hyperthermia (data not shown), and is known to cause distinct behavioral changes in mice [64, 66]. Previous studies have shown various degree of behavioral impairments of METH in HIV-1 Tg mice [64–68, 101, 102]. However, the studies on behavioral effects of METH in HIV-1 Tat Tg mice are sparse [68]. In the present study, we utilized Y maze and Morris water maze assays to test the effect of METH on working and spatial memory in HIV-1 Tat Tg mice, respectively. Our Y-maze results demonstrated a significant deficit in working memory as indicated by the decrease in % spontaneous alternations in HIV-1 Tat transgenic mice that were treated with METH. Treatment with METH showed a trend towards reduced percent spontaneous alterations. There was no difference in percent spontaneous alterations based on the sex. Further, results from Morris water maze indicated that both HIV-1 Tat and METH treatment resulted in impaired spatial learning. However, combination of HIV-1 Tat induction and METH treatment resulted in longest times to reach the escape platform during the acquisition trial. There was no significant effect of METH on the time spent in target quadrant and number of platform crossings in probe trials, in both wild type and HIV-1 Tat transgenic mice. These results indicate that METH treatment did not affect reference memory. Results from Y maze and Morris water maze suggest that executive function is more compromised in HIV-1 + METH group. These results are similar to the results from human studies indicating poor working memory in HIV-1 positive individuals and METH users [75, 103].

Proper synaptic integrity is required for transmission of information and thereby achieving synaptic plasticity. Various synaptic proteins, located either presynaptically or postsynaptically, are responsible for the maintenance of synaptic plasticity and consolidation of memory [104]. We evaluated the expression of synaptophysin and synapsin 1 as markers of pre-synaptic integrity, whereas PSD-95, Arg3.1 and SHANK2 were evaluated as markers of postsynaptic integrity. Synaptophysin is a pre-synaptic protein located abundantly in the synaptic vesicles and is involved in the regulation of synaptic vesicle endocytosis, synapse formation and long-term potentiation [78, 105]. Learning and memory deficits have demonstrated in synaptophysin knockout mice [106]. Loss of synaptophysin have been shown to correlate with cognitive impairment in Alzheimer's disease patients [107], mouse [108–110] models, and in aged animals [111–113]. Furthermore, restoration of synaptophysin levels has been shown to improve memory performance [109–112]. In the present study, the expression levels of synaptophysin were significantly reduced by HIV-1 Tat and METH in parietal cortex of both males and females, and in hippocampus of females. The results are in line with previous reports indicating decreased synaptophysin expression HIV-1 and METH exposure [114–116]. The expression levels of another pre-synaptic protein, synapsin 1, were also significantly reduced in the parietal cortex and hippocampus of Tat mice and Tat+ METH treated mice of both the sexes. Synapsin 1 belongs to the family of pre-synaptic proteins which are involved in the formation and maintenance of synapse by regulating the organization and abundance of pre-synaptic vesicles [117, 118]. Reduced

expression levels of synapsin 1 have been demonstrated in HIV-1 infection and AD [119–121]. Chronic METH administration also resulted in decreased expression of synapsin 1 in rats [122]. Furthermore, synapsin 1 has been shown to enhance stress-related memory in hippocampus via MAPK-activation [123] indicating that the reduced expression of synapsin 1 might result in the depletion of synaptic vesicles in the releasable pool thereby affecting synaptic function and plasticity [124, 125] and ultimately resulting in memory impairments. Taken together, decreased expression of pre-synaptic markers observed in this study might very well represent long-lasting changes that might play a vital role in altered neuronal plasticity and enhanced neurocognitive impairments in HIV-1 infected individuals who abuse METH.

Owing to its critical role in regulation of excitatory synapses in brain [126], we determined the expression levels of PSD-95 as a marker for postsynaptic plasticity. PSD-95 expression was unaltered in most brain regions upon HIV-1 Tat induction or treatment with METH. However, significant difference in the expression of PSD-95 was observed in Tat+METH of both sexes in different brain regions. Decrease in the expression of PSD-95 compromises the ability of glutamate receptors to connect with the intracellular signaling pathways [126] and this might lead to synaptic weakening and long-term depression [127, 128]. The role of PSD-95 in memory has been demonstrated in various studies showing that PSD-95 downregulation/deletion/knockdown impairs spatial learning, fear memory, conditional taste aversion and simple operant associative learning [82, 83, 129–131]. Also, up-regulation of PSD-95 improves memory performance [132, 133]. Another postsynaptic protein, Arg3.1/Arc, is also known to be involved in the maintenance of synaptic plasticity and memory [134–137] via NMDA receptor activation [138] and, regulation of AMPA receptor trafficking [139], Notch signaling [140] and ERK/MAPK activation [141]. Furthermore, Arg3.1 knockout mice have been shown to perform poorly in water maze task indicating impaired spatial memory [134]. In the current study, HIV-1 Tat and METH significantly decreased expression of Arg3.1 in the parietal cortex of both sexes, but not in the other brain regions. Also, there was further reduction in the expression of Arg3.1 in the parietal cortex and prefrontal cortex of Tat+ METH group. Our findings are in line with previous reports indicating reduced expression of Arg3.1 in response to HIV-1 and METH exposure [8, 142]. The expression of SHANK2 was also significantly decreased in the prefrontal cortex of HIV-1 Tat mice treated with METH. SHANK2 belongs to the family of postsynaptic density proteins, and contributes to the excitatory synaptic transmission of NMDA and mGluRs by binding with PSD-95/GKAP/Homer complex [86, 143]. SHANK2 mutant mice display a variety of behavioral deficits including anxiety and, impaired spatial learning and memory [144, 145]. However, restoring NMDA function improved the behavioral outcomes [145]. Taken together, it can be concluded that decreased levels of postsynaptic proteins, PSD-95, Arg3.1 and SHANK2, observed in our study might be responsible for impaired spatial and working memory through compromised synaptic strength and transmission.

Different types of neurotrophic factors exist in the brain with diverse functions on neuronal plasticity, survival and development [146, 147]. Altered expression of neurotrophic factors are associated with neurological disorders, including HIV-1 [87, 148]. One of the most important neurotrophic factor with regards to memory is BDNF. BDNF has been shown to play a vital role in synaptic plasticity and memory through the development of LTP [149,

150], regulation of synapse formation [151] and dendritic growth [152]. BDNF activity has been demonstrated to play a major role in memory formation through a variety of learning tasks in humans [153] and rodents [154–162]. Furthermore, alteration in the BDNF activity have also been observed in diseases with cognitive decline [163–165]. In our current study, HIV-1 Tat did affect the BDNF expression levels whereas METH significantly decreased the expression of BDNF in different brain regions. However, previous studies have shown that METH induces the expression of BDNF in multiple brain regions in rats [166, 167]. A plausible explanation for this observation could be a shorter duration of METH administration [168] or METH administered to postnatal mice [169], as opposed to the long-term METH treatment protocol that we have employed. The increase in the levels of BDNF can be a compensatory mechanism to overcome the neurotoxic effects of METH. A significant decrease in the expression levels of BDNF was observed in all brain regions of HIV-1 Tat mice treated with METH, and can explain the memory deficits seen in these mice. Administration of BDNF can therefore be used as a therapeutic approach to counteract the memory deficits associated with HIV-1 and METH. Although CNTF has also been implicated synaptic plasticity, learning and memory [170–172], we found no significant differences in the expression of CNTF in any of the brain regions of both sexes. Results from our current study indicate that decreased expression of BDNF plays a crucial role in HIV-1 and METH induced neurocognitive deficits.

Previous studies have shown the vital role of CaMKII in LTP [173] and memory [174, 175] by modulating the expression and conductance of AMPA and NMDA receptors [176]. As autophosphorylation of CaMKII at Thr 286 is essential for its activation and subsequent LTP induction, the expression levels of p-CaMKII were analyzed as a marker of synaptic strength and plasticity [176, 177]. The expression of p-CaMKII was found to be significantly decreased in HIV-1 Tat mice, METH-treated mice and HIV-1 Tat mice treated with METH. Previous studies have also demonstrated reduced expression of p-CaMKII after SIV exposure [178], and acute and chronic METH treatment [179]. The decline in the expression levels of CamKII might also affect the function of synapsin 1 and thereby affecting the vesicle number in the releasable pool [80].

In conclusion, administration of METH to HIV-1 Tat transgenic mice exacerbated the deficits in spatial learning and memory characterized by decreased spontaneous alternations in Y maze and increased latency time to reach the escape platform in Morris water maze. We correlated the changes in learning by measuring expression of various synaptic markers and neurotrophic factors that contribute to neuroplasticity and memory formation. HIV-1 Tat transgenic mice that were treated with METH showed significant decrease in the expression of synaptic markers and neurotrophic factors. Future studies will be directed to identify the molecular mechanisms that are involved in the altered expression of synaptic markers and neurotrophic factors by HIV-1 Tat and METH. Furthermore, future studies will also focus on the inflammatory markers, structural changes of neurons and use of therapeutic agent to overcome the cognitive deficits mediated by HIV-1 Tat and METH.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- METH augmented Tat mediated memory deficits in Tat transgenic mice
- METH administration to Tat mice significantly affected the expression of synaptic proteins
- Tat mice given METH showed significant decrease in BDNF expression
- Tat mice given METH showed significant decrease in p-CaMKII expression

Figure 1. Schematic representation of the experimental timeline

Six-week-old male and female WT mice and HIV-1 Tat mice were administered doxycycline for 12 weeks before starting administration of METH as described in materials and methods. Animals were subjected to behavioral testing by Y maze and Morris water maze assays on week 24. Animals were euthanized at week 25 and different brain regions were harvested to identify the expression of various synaptic proteins and neurotrophic factors.

Figure 2. Impaired working memory in METH treated HIV-1 Tat mice

Six-month-old male (panels A and C) and female (panels B and D) WT mice and HIV-1 Tat mice were injected intraperitoneally either PBS or METH for 3 weeks, and were tested for working memory in Y maze assay. The graphs in panels A and B represent percentage spontaneous alternations for each group. The number of arm entries made by the mice in each group are shown in panels C and D. All values are represented as mean $\pm$ SEM. Three-way ANOVA with Tukey's post hoc test for multiple comparisons was performed to calculate the statistical significance. * denotes p-value < 0.05 compared to control group and ! denotes p-value < 0.05 compared to METH-treated group.

Figure 3. Impaired spatial learning and memory in METH treated HIV-1 Tat mice

Six-month-old male (panels A, C, E and G) and female (panels B, D, F and G) WT mice and HIV-1 Tat mice were administered either PBS or METH for 3 weeks and were tested for escape latency in Morris water maze assay (A, B). The escape latencies (in sec) were measured in a 5-day acquisition trial (day 1–5). The curves represent average on each of the 5 days of acquisition trial. The results are shown as mean escape latencies in seconds ($\pm$ SEM) to the platform using solid line with solid circle (control group), dotted line with open circle (Tat group), solid line with solid triangle (METH group) and dotted line with triangle (Tat+ METH group). Swim speed (m/s) of mice was calculated on each day of the acquisition trial (C, D). On day 6, each mouse was subjected to a 60 sec probe trial and time spent in target quadrant is shown in seconds (E, F) and total number of annulus entries (platform crossings) is shown in panels G and H were calculated. All values are represented as mean $\pm$ SEM. Three-way ANOVA with Tukey's post hoc test for multiple comparisons was performed to calculate the statistical significance. * denotes p-value < 0.05 compared to control group and ! denotes p-value < 0.05 compared to METH-treated group.

Figure 4. Altered expression of pre-synaptic proteins in METH treated HIV-1 Tat mice
Six-month-old male (panels A, C, E and G) and female (panels B, D, F and H) WT mice and HIV-1 Tat mice were administered either PBS or METH for 3 weeks. Different brain regions were isolated as described in materials and methods. Expression of synaptophysin (A, B) and synapsin 1 (E, F) were measured in different brain regions through western blot. Protein band intensities were quantified and were normalized to GAPDH. The bar graphs represent synaptophysin (C, D) and synapsin 1 (G, H) fold change (mean $\pm$ SEM) from three independent replicates. Three-way ANOVA with Tukey's post hoc test for multiple comparisons was performed to calculate the statistical significance and * denotes p-value < 0.05 compared to WT controls.

Figure 5. Altered expression of post-synaptic proteins in METH treated HIV-1 Tat mice
Six-month-old male (panels A, C, E, G, I and K) and female (panels B, D, F, H, J and L) WT mice and HIV-1 Tat mice were administered either PBS or METH for 3 weeks. Different brain regions were isolated as described in materials and methods. Expression of PSD-95 (A, B), Arg3.1 (E, F) and SHANK2 (I, J) were measured in different brain regions through western blot. Protein band intensities were quantified and were normalized to GAPDH. The bar graphs represent PSD-95 (C, D), Arg3.1 (G, H) and SHANK2 (K, L) fold change (mean $\pm$ SEM) from three independent replicates. Three-way ANOVA with Tukey's post hoc test for multiple comparisons was performed to calculate the statistical significance. * denotes p-value < 0.05 compared to WT controls; # denotes p-value < 0.05 compared to HIV-1 Tat mice and ! denotes p-value < 0.05 compared METH-treated mice.

Figure 6. Altered expression of neurotrophic factors in METH treated HIV-1 Tat mice
Six-month-old male (panels A, C, E and G) and female (panels B, D, F and H) WT mice and HIV-1 Tat mice were administered either PBS or METH for 3 weeks. Different brain regions were isolated as described in materials and methods. Expression of BDNF (A, B) and CNTF (E, F) were measured in different brain regions through western blot. Protein band intensities were quantified and were normalized to GAPDH. The bar graphs represent BDNF (C, D) and CNTF (G, H) fold change (mean $\pm$ SEM) from three independent replicates. Three-way ANOVA with Tukey's post hoc test for multiple comparisons was performed to calculate the statistical significance. * denotes p-value < 0.05 compared to WT controls; # denotes p-value < 0.05 compared to HIV-1 Tat mice and ! denotes p-value < 0.05 compared METH-treated mice.

Figure 7. Altered expression of p-CaMKII in METH treated HIV-1 Tat mice

Six-month-old male (panels A, C) and female (panels B, D) WT mice and HIV-1 Tat mice were administered either PBS or METH for 3 weeks. Different brain regions were isolated as described in materials and methods. Expression of p-CaMKII (A, B) was measured in different brain regions through western blot. Protein band intensities were quantified and were normalized to GAPDH. The bar graphs represent p-CaMKII (C, D) fold change (mean $\pm$ SEM) from three independent replicates. Three-way ANOVA with Tukey's post hoc test for multiple comparisons was performed to calculate the statistical significance. * denotes p-value < 0.05 compared to WT controls and # denotes p-value < 0.05 compared to HIV-1 Tat mice.