Published in final edited form as:

J Neuroimmune Pharmacol. 2013 September ; 8(4): 921–933. doi:10.1007/s11481-013-9468-2.

Enhancement of NMDA receptor-mediated excitatory postsynaptic currents by gp120-treated macrophages: Implications for HIV-1-associated neuropathology

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Abstract

A plethora of prior studies has linked HIV-1-infected and immune activated brain mononuclear phagocytes (MP; blood borne macrophages and microglia) to neuronal dysfunction. These are modulated by N-methyl-D-aspartate receptor (NMDAR) antagonists and supporting their relevance for HIV-1-associated nervous system disease. The role of NMDAR subsets in HIV-1-induced neuronal injury, nonetheless, is poorly understood. To this end, we investigated conditioned media from HIV-1gp120-treated human monocyte-derived-macrophages (MDM) for its ability to affect NMDAR-mediated excitatory postsynaptic currents (EPSC_{NMDAR}) in rat hippocampal slices. Bath application of gp120-treated MDM-conditioned media (MCM) produced an increase of EPSC_{NMDAR}. In contrast, control (untreated) MCM had limited effects on EPSC_{NMDAR}. Testing NR2A NMDAR (NR2AR)-mediated EPSC (EPSC_{NR2AR}) and NR2B NMDAR (NR2BR)-mediated EPSC (EPSC_{NR2BR}) for MCM showed significant increased EPSC_{NR2BR} when compared to EPSC_{NR2AR} enhancement. When synaptic NR2AR-mediated EPSC was blocked by bath application of MK801 combined with low frequency stimulations, MCM retained its ability to enhance EPSC_{NMDAR} evoked by stronger stimulations. This suggested that increase in EPSC_{NMDAR} was mediated, in part, through extra-synaptic NR2BR. Further analyses revealed that the soluble factors with low (<3kD) to medium (3-10kD) molecular weight mediated the observed increases in EPSC_{NMDAR}. The link between activation of NR2BRs and HIV-1gp120 MCM for neuronal injury was demonstrated by NR2BR but not NR2AR blockers. Taken together, these results indicate that macrophage secretory products induce neuronal injury through extra-synaptic NR2BRs.

Keywords

HIV-1gp120; Macrophages; NMDA receptors; EPSC; Neurotoxicity

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Conflict of Interest

The authors declare that they have no competing interests or conflict of interest.

Introduction

The human immunodeficiency virus type 1 (HIV-1) remains a persistent and ever growing health problem affecting the immune, systemic organs and the nervous system (McArthur et al., 2005; Trujillo et al., 2005; Palacio et al., 2012). HIV-1 invades the brain early in the course of infection and provokes mild to progressive cognitive, behavioral and neurological impairments, collectively referred to as HIV-1-associated neurocognitive disorders (HAND) (Antinori et al., 2007). The severity of HAND can vary, from asymptomatic to mild neurocognitive impairment and in its most severe form, a debilitating dementia commonly called HIV-associated dementia or HAD (Antinori et al., 2007; Grant, 2008). Despite dramatic decreases in the severity of all forms of neurological complications of HIV-1 infection by combination antiretroviral therapy (Ances and Ellis, 2007), milder forms of disease remain commonplace (Heaton et al., 2010; Kranick and Nath, 2012; Mothobi and Brew, 2012; McArthur and Smith, 2013). This suggests, all together, that HAND continues to represent a significant health problem (Kranick and Nath, 2012; McArthur and Smith, 2013). To date, there is no specific effective treatment for HAND, mainly because the precise mechanisms underlying HIV-induced neuronal injury remain incompletely understood. Thus, research into the molecular and cellular mechanisms underlying HIV-1-associated neuronal injury towards the development of specific effective therapies are of pressing need.

Evidence abounds that neuronal injury and death in HIV-1-infected brains are linked to metabolic abnormalities (Anderson et al., 2002; Diesing et al., 2002; Palacio et al., 2012). Here, HIV-1-infected and immune competent mononuclear phagocytes (MP; perivascular brain macrophages and microglia) release soluble substances affecting neuronal injury. Such MP-associated neuronal injury and death can be prevented by *N*-methyl-*D*-aspartate (NMDA) receptor (NMDAR) antagonists (Lipton et al., 1991; Pellegrini and Lipton, 1993; Ushijima et al., 1995; Muller et al., 1996; Kaul et al., 2001; Anderson et al., 2004; O'Donnell et al., 2006; Shin et al., 2012) suggesting an involvement of NMDAR in HIV-1 infected MP-induced neuronal injury. However, direct relationships between MP secretory factors and NMDAR activation are not fully understood.

Functional NMDAR are multimeric assemblies composed of at least one obligatory NR1 (Dingledine et al., 1999; Cull-Candy et al., 2001) and two or more NR2 subunits (Behe et al., 1995). The NR1 subunit is ubiquitously expressed throughout the central nervous system (CNS) and contains the glycine-binding site (Kuryatov et al., 1994). The NR2 subunits (A, B, C and D) contain the glutamate-binding site, and their expression is time- and tissue-specific (Monyer et al., 1994). Studies have shown that adult cortex NR2A-containing NMDARs (NR2AR) may preferentially target synaptic sites; whereas, NR2B-containing NMDARs (NR2BR) may locate at extrasynaptic sites (Stocca and Vicini, 1998; Rumbaugh and Vicini, 1999; Tovar and Westbrook, 1999; Mohrmann et al., 2000; Townsend et al., 2003). While NR2AR regulate normal synaptic transmission, NR2BR are associated with neuronal damage (Hardingham et al., 2002; Riccio and Ginty, 2002; Liu et al., 2007). Indeed, activation of NR2BR results in excitotoxicity and neuronal degeneration (Hardingham et al., 2002; Liu et al., 2007). We now hypothesize that soluble factors released from HIV-1-infected and immune activated MP act on neuronal NR2BR, leading to neuronal damage and death. To test this hypothesis, the effects of human monocyte-derived-macrophage (MDM)-conditioned media (MCM) recovered from HIV-1120-treated MDM were studied as the affected NMDAR-mediated excitatory postsynaptic currents (EPSC_{NMDAR}). These were recorded in the CA1 region of rat hippocampal slices. Our results showed that MCM from HIV-1gp120-treated macrophages enhanced the EPSC_{NMDAR}, leading to neuronal injury. The MCM exhibited significant stronger effects on

enhancing NR2BR-mediated EPSC (EPSC_{NR2BR}), particularly the extra-synaptic NR2BR (exNR2BR) rather than NR2AR-mediated EPSC (EPSC_{NR2AR}). In parallel, NR2BR antagonist blocked MCM-induced neuronal injury, suggesting that MCM induced neuronal injury through NR2BR.

Materials and methods

Animals

Male Sprague-Dawley rats (two- to four-week old) and pregnant female rats (17-18 days in gestation) were purchased from Charles River Laboratories (Wilmington, MA). The male rats were used for preparation of hippocampal brain slices, and the fetal rats were utilized to harvest hippocampal tissues for primary hippocampal neuronal cultures. Animals were housed at a constant temperature (22°C) and relative humidity (50%) under a regular light-dark cycle (light on at 7 AM and off at 5 PM) with free access to food and water. All animal use procedures were strictly reviewed by the Institutional Animal Care and Use Committee (IACUC) of the University of Nebraska Medical Center (IACUC No. 00-062-07).

Chemical and biological reagents

Full-length HIV-1_{MN} gp120 (gp120, produced in baculovirus) were kindly provided by NIH AIDS Research and Reference Reagent Program (Rockville, MD). Ifenprodil, 3-((*R*)-2-Carboxypiperazin-4-yl)-propyl-1-phosphonic acid (R-CPP), and Ro 25-6981 were purchased from TOCRIS Bioscience (Ellisville, MO). Unless otherwise indicated, all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

MCM collection and fractionation

Human monocytes were recovered from peripheral blood mononuclear cells of HIV-1,2 and hepatitis B virus seronegative donors after leukopheresis and purified by counter-current centrifugal elutriation (Gendelman et al., 1988). Cells were obtained under a protocol approved by the University of Nebraska Institutional Review Board. Monocytes were cultured in DMEM supplemented with 10% heat-inactivated human serum, 2mM L-glutamine, gentamicin (50µg/ml), ciprofloxacin (10µg/ml), and macrophage colony-stimulating factor (1000 U/ml). The purity of MDM was >95% of cells by MAC1 staining. After 7 days in culture (37°C, 5% CO₂), MDM were stimulated with HIV-1gp120 (200pM) or lipopolysaccharide (LPS, 1µg/ml, Sigma-Aldrich, St Louis, MO) for 2 h. Then, the culture media was removed and neurobasal media (serum free, Invitrogen, Grand Island, NY) was placed onto MDM for 24 h prior to collection. The cells were then centrifuged at 1,500 rpm and MCM were collected from gp120-, LPS- and control (untreated) MDM. Crude and size fractionated MCM were used in all experiments. The fractionation of MCM was done through centrifuge using Centriplus filters (Millipore, Bedford, MA, molecular weight cutoffs of 10kDa and 3kDa) for 40 min at 3500×g, and filtrates (3-10kDa or < 3kDa fractions) were collected. The portion of supernatants retained by the filter (>10kDa fraction) was resuspended in artificial cerebrospinal fluid (ACSF) in a volume equal to the filtrate volume. The collected crude MCM (from 5 batches) were stored in aliquots at -80°C. On the day of the experiment, MCM was thawed and either used or discarded, unless otherwise indicated. In some experiments, MCM was either boiled for 15 min and cooled down to room temperature before use or incubated with glutamate decarboxylase (GAD, 2 units/1µM glutamate) for 2h at 37°C in order to determine potential effects mediated by proteins or glutamate contained in the MCM groups.

Hippocampal slices and electrophysiology

Rat hippocampal brain slices were prepared as previously described (Xiong et al., 1996). Briefly, animals were anesthetized with isoflurane and decapitated, and brains were quickly removed from the cranial cavity. The brains were placed into an ice-cold (4°C), oxygenated ACSF environment. The hippocampi were dissected free, and transverse hippocampal slices (400 μ m in thickness) were cut using a tissue chopper. The slices were kept in a humidified, oxygenated holding chamber at room temperature for at least 1 h before being transferred into a recording chamber. In the recording chamber, a single slice was fully submerged in continuously perfused ACSF solution at a constant flow rate of 2 ml/min with the use of a peristaltic pump (Rainin Instrument Co., Woburn, MA). The ACSF contained (in mM): NaCl 124.0, KCl 3.0, CaCl_2 2.0, MgCl_2 2.0, NaH_2PO_4 1.25, NaHCO_3 26.0, and glucose 10.0. The ACSF was equilibrated with 95% O_2 and 5% CO_2 and had a pH of 7.4-7.5. The temperature of the perfusion was maintained at $30 \pm 1^\circ\text{C}$ with an automatic temperature controller (Warner Instrument Corp., Hamden, CT).

“Blind” patch recordings were made with microelectrodes (4.0-8.5 M Ω) filled with (in mM): K-gluconate 130.0, K-methylsulphate 17.5, NaCl 8.0, HEPES 10.0, Mg-ATP 2.0, GTP 0.2, EGTA 0.1, pH 7.25-7.35, osmolality 292-308 mOsm. The recording microelectrodes were made from borosilicated glass capillaries (WPI, Sarasota, FL) with inner filaments that allow for quick back-filling. EPSCs and excitatory postsynaptic potentials (EPSPs) were evoked by a constant current stimulation (50-200 μ A, 20 μ sec in duration, 0.05 Hz) of Schaffer collateral fibers using an insulated bipolar tungsten electrode (except for the tip). The stimulation intensity was adjusted to generate approximately 30-40% of the maximal response. The evoked EPSCs or EPSPs were recorded with an Axopatch-1D amplifier (Molecular Devices, Sunnyvale, CA) in the CA1 pyramidal cell layer. To eliminate the possibility that MCM activates inhibitory interneurons, all experiments were conducted in the presence of 50 μ M picrotoxin. To prevent epileptiform activities, a surgical cut was made between CA1 and CA3 areas after the slice had been transferred to the recording chamber. NMDAR-mediated currents were recorded at holding potential of -50 mV in low- Mg^{2+} (0.5 mM), high- Ca^{2+} (2.5 mM) solution containing glycine (1 μ M) and DNQX (10 μ M), and confirmed by reversible APV (50 μ M) blockade. The contributions of NR2ARs or NR2BRs to the EPSCs/EPSPs were assessed using NR2AR high affinity antagonist R-CPP (Feng et al., 2004; Feng et al., 2005) or NR2BR high affinity ifenprodil (Williams, 1993), Ro 2506981 (Fischer et al., 1997), respectively.

A 10 to 15 min control recording was made in each experiment once the adjustment of stimulation parameters had been achieved after rupture of the cell membrane. Each recording trial consisted of an average of three consecutive sweeps. The evoked signals were filtered at 1 kHz and digitized at 2.5 or 5.0 kHz using a Digidata 1440A interface (Molecular Devices). Data were stored on a desktop PC and analyzed off-line using pClamp 8.1 software (Molecular Devices). The peak amplitudes of EPSCs or EPSPs were measured and analyzed. Effects of MCM on resting membrane potential were tested via bath perfusion and recorded in a gap-free configuration. In some cells, membrane resistance was monitored by injection of a hyperpolarizing current pulse every 30 s before, during, and after bath application of MCM and the corresponding voltage changes were recorded and analyzed.

To record field EPSP (fEPSP), the recording electrodes (1.5-4.0 M Ω) filled with ACSF were placed in the CA1 dendrite field, and the fEPSP evoked by electrical stimulation of Schaffer collateral fibers. The initial slope of the fEPSP was measured, analyzed, and expressed as the percentage of the basal level.

Hippocampal neuronal cultures

Hippocampal neuronal cultures were prepared from embryonic Sprague-Dawley rats using the methods described previously (Flavin et al., 1997). Briefly, female rats (Charles River Laboratory) with 18-19 d gestation were anesthetized with isoflurane, and fetus rats were surgically removed and decapitated. Hippocampi were harvested under sterile conditions. The hippocampal tissue was dissociated in 0.125% trypsin II (Sigma-Aldrich). Isolated neural cells were placed in poly-D-lysine-coated 35mm plastic culture dishes containing 2 ml of neurobasal medium to a culture surface cell density of $5 \times 10^5/\text{ml}$ (400-500/mm²). The cultures were maintained in neurobasal medium supplemented with B27 (2%, v/v, Invitrogen), glutamine (0.5mM) and penicillin/streptomycin (100 U) for at least 7-10 days before being used for experiments.

Evaluations of neuronal injury

Experiments were performed in triplicate, and total survival hippocampal neural cells in culture were counted from five different visual fields. MCM were added to hippocampal neuronal cultures (final concentration was 1:30 dilution) for 24 h and then cell injury was assessed by staining with a membrane-impermeable DNA-binding dye propidium iodide (PI, Molecular Probes, Eugene, OR) and counterstaining with membrane-permeable 4',6-diamidino-2-phenylindol (DAPI). More specifically, after incubation with neurobasal medium containing PI (1 $\mu\text{g}/\text{ml}$) for 15 min, cells were washed three times with phosphate buffered saline (PBS), and fixed with 4% paraformaldehyde (in PBS) for 20 min at room temperature. Cells were then permeabilized with 0.2% Triton X-100 (in PBS) for 5 min on ice and washed twice with PBS containing DAPI (0.1 $\mu\text{g}/\text{ml}$). Subsequently, under fluorescent microscopy, red (PI) and blue (DAPI) fluorescent images were captured in order to determine the number of injured cells and the total number of cells, respectively. Five different visual fields per culture dish were evaluated.

Statistical Analysis

Data were analyzed and displayed using Clampfit 8.1 (Molecular Devices, LLC) and Origin@8.0 (OriginLab Corp, Northampton, MA). All data were expressed as mean $\pm$ SEM unless otherwise indicated. Statistical analyses were made via one-way ANOVA or two-tailed *t*-tests. Differences were considered significant if $p < 0.05$.

Results

MCM increases EPSC in CA3 to CA1 synapses

To examine whether activated macrophages alter synaptic transmission in the brain, we tested the effects of MCM on Schaffer collateral fibers to CA1 synapses in rat hippocampal slices *in vitro*. EPSC, evoked by electrical stimulation of Schaffer collateral fibers, were recorded in CA1 neurons in the presence of picrotoxin (50 μM , a GABA_A receptor antagonist) in the perfusate. Bath perfusion of hippocampal slices incubated with MCM for 5–10 min produced an increase of EPSC amplitude in a dilution (concentration)-dependent manner (Figure 1). At 1:50 dilution (v/v), MCM had no significant effects on EPSC ($103.3 \pm 9.7\%$ of control, $n=6$). However, at dilutions of 1:5 and 1:1, it produced a significant increase of the EPSC (Figure 1), with average peak EPSC amplitudes of $247.3 \pm 18.5\%$ of control ($n=10$, Figure 1B, 1C) and $312.6 \pm 19.1\%$ of control ($n=6$, Figure 1C), respectively. In comparison with the EPSC amplitude recorded during bath application of untreated MCM ($115.3 \pm 8.6\%$ of control, $n=8$), the differences were statistically significant ($p < 0.001$), indicating MCM enhancement of evoked EPSCs in Schaffer collateral fibers to CA1 synapses in the hippocampus. The MCM-induced increase of EPSCs peaked at 10-15 min after MCM reached recording chamber and washout usually took 20-30 min. MCM

produced a significant enhancement of the EPSC at a dilution of 1:5, this dilution was used throughout this study. Application of LPS-treated MCM as a positive control also produced a significant ($p < 0.001$) enhancement of EPSC amplitude, with an average peak amplitude ($279.6 \pm 9.4\%$ of control; $n=4$, data not shown). These results suggest that the HIV-1gp120- or LPS-treated MDM release soluble substances resulting in an increase of EPSC. In addition, bath application of MCM had no significant effect on neuronal resting membrane potential as observed under gap-free recording. Membrane input resistance showed no significant change when monitored before, during and after bath application of MCM by injecting a hyperpolarizing current pulse and recording resultant voltage changes ($n=14$, data not shown).

Enhancement of EPSC_{NMDAR} by MCM

Studies have shown that neuronal injury induced by conditioned media recovered from HIV-1-infected macrophages can be blocked by NMDAR antagonists (Giulian et al., 1990; Giulian et al., 1996; Jiang et al., 2001; O'Donnell et al., 2006), suggesting the involvement of NMDARs in HIV/MDM-induced neuronal injury. However, the direct relationship between MCM and NMDAR activation has not yet been investigated. To this end, we tested if MCM activates NMDARs by examining the effects of MCM on EPSC_{NMDAR} in the presence of a specific AMPA receptor antagonist DNQX ($10\mu\text{M}$) in the ACSF that contained a low Mg^{2+} (0.5mM), high Ca^{2+} (2.5mM) and glycine ($1\mu\text{M}$). Under these experimental conditions, bath application of MCM significantly increased the EPSC_{NMDAR} evoked by electrical stimulation of the Schaffer-collateral fibers, with average peak amplitude of $210.9 \pm 11.2\%$ of control (Figure 2, $n=10$, $p < 0.001$). The MCM-induced increase of EPSCs was blocked by $50\mu\text{M}$ APV, a specific NMDAR antagonist, demonstrating MCM increase of EPSC_{NMDAR} by NMDAR in the CA1 region of rat hippocampal slices. Bath application of MCM was also found to increase NMDAR-mediated excitatory postsynaptic potentials (EPSP_{NMDAR}) recorded under current clamp. Before and during bath application of MCM, the average EPSP amplitudes were $99.5 \pm 4.2\%$ and $130.7 \pm 5.9\%$ of basal level ($n=4$), respectively. The difference was statistically significant ($p < 0.01$, Figure 3).

MCM had stronger effects on enhancing EPSC_{NR2BR} than EPSC_{NR2AR}

Studies have shown that NR2AR and NR2BR are the predominant NR2 subunits in the hippocampus (Monyer et al., 1994; Kim et al., 2005; Chen et al., 2007). To evaluate the contributions of NR2ARs and NR2BRs to MCM-induced increase of EPSC_{NMDAR}, we examined NR2BR antagonist Ro 25-6981 ($1\mu\text{M}$), ifenprodil ($10\mu\text{M}$, which has a high affinity for NR2BRs with an $\text{IC}_{50}=0.34\mu\text{M}$ and $\text{IC}_{50}=146\mu\text{M}$ for NR2ARs) (Williams, 1993), and NR2AR antagonist R-CPP ($1\mu\text{M}$), which has ~7-fold greater selectivity for NR2ARs compared with NR2BRs (Feng et al., 2004; Feng et al., 2005), on their ability to block MCM-induced increase of EPSC_{NMDAR}. In the presence of ifenprodil (or Ro 25-6981) or R-CPP in the perfusate, bath application of MCM elicited an increase of EPSC_{NMDAR} to $149.9 \pm 19.2\%$ of control ($n=8$, $p < 0.05$ vs control) and $221.7 \pm 17.0\%$ of control ($n=8$, $p < 0.001$ compared to control), respectively (Figure 4). The difference between the peak amplitudes of EPSC_{NR2BR} and EPSC_{NR2AR} was statistically significant ($p < 0.05$, Figure 4C), demonstrating that MCM had much stronger effects on enhancing EPSC_{NR2BR} than EPSC_{NR2AR} (Figure 4).

The effects of MCM on NR2BRs were further tested on fEPSPs in hippocampal slices. As shown in Figure 5, bath application DNQX ($10\mu\text{M}$, to block AMPA receptor-mediated fEPSP) decreased fEPSP to $30.9 \pm 5.9\%$ of control level ($n=6$). In the presence of DNQX in the perfusate, addition of MCM (1:5 dilution) to the bath increased fEPSPs from $30.9 \pm 5.9\%$ of control level to $75.1 \pm 7.3\%$ of control level ($n=6$, $p < 0.001$). Further addition of

ifenprodil (10 μ M) to the perfusate significantly reduced the MCM-associated increase of fEPSPs, suggesting MCM enhancement of fEPSPs via NR2BRs.

Evidence for MCM activation of exNR2BR

The location of NMDAR may affect its function. Survival promoting signals derived from synaptic NMDAR, which consist of predominant NR2A; whereas, a cell-death signal comes from extra-synaptic NMDAR, which contain mostly NR2B (Tovar and Westbrook, 1999; Hardingham et al., 2002; Liu et al., 2007). To determine if MCM enhancement of EPSC_{NR2B} was mediated via exNR2B, we blocked synaptic NMDAR with an irreversible, use-dependent NMDAR channel blocker MK801 (25 μ M) applied to the slices via bath perfusion. During 5-10 min bath perfusion of brain slices with MK801, repeated low frequency (0.05Hz) stimulations were delivered to Schaffer collateral fibers to evoke glutamate release and activate synaptic NMDAR, creating an environment for MK801 to block these synaptic NMDAR. After MK801 blockade of synaptic NMDAR, we increased stimulation intensities from 40-50 μ A to 100-140 μ A to elicit exNR2B-mediated EPSC (EPSC_{exNR2B}). As shown in Figure 6, MCM retained its ability to enhance EPSC_{NMDAR} after the synaptic NMDAR were blocked by MK801. Such enhancement was blocked by a specific NR2B blocker Ro 25-6981 (Figure 6) and ifenprodil (data not shown), suggesting the MCM enhancement of EPSC_{NMDAR} via exNR2BRs.

NMDAR antagonists attenuate MCM-induced neurotoxicity

Immune activated macrophages release soluble neurotoxins that lead to neuronal injury (Cotter et al., 1998; Xiong et al., 2000). We previously demonstrated that MCM induces neuronal apoptosis in rat primary hippocampal neuronal cultures (Hu et al., 2009). To address whether MCM-mediated increase of EPSC_{NR2A} and/or the EPSC_{NR2B} is associated with neuronal injury, we studied protective effects of NR2A and NR2B antagonists on MCM-associated challenges in cultured rat hippocampal neurons. The hippocampal neuronal cultures were treated with MCM, MCM and ifenprodil, MCM and R-CPP, and MCM, ifenprodil and R-CPP for 24 h. The cell viability was assayed using PI/DAPI staining. The PI/DAPI staining revealed that addition of MCM to the culture media (1:5 dilution) reduced the percentage of cell survival to $68.5 \pm 3.5\%$. In comparison with the percentage of survival cells observed in hippocampal neuronal cultures treated with CMCM (Ctrl, as 100%), the difference is statistically significant (Figure 7, $n=3$, $p<0.01$), demonstrating that MCM induces neuronal injury *in vitro*. Addition of NR2A and NR2B antagonists alone or together protect hippocampal neurons from MCM-associated challenge. When treated with MCM and ifenprodil, MCM and R-CPP, or MCM, ifenprodil and R-CPP for 24 h, the percentages of survival cells were $93.5 \pm 5.3\%$ ($p<0.01$), $71.4 \pm 5.5\%$ ($p>0.05$), and $92.0 \pm 3.6\%$ ($p<0.01$, Figure 7), respectively, showing NR2B antagonist significantly protected hippocampal neurons from MCM-associated challenges. These results suggest that NR2BR are mainly involved in MCM-induced neuronal injury.

Active components underlying MCM-mediated EPSC_{NMDAR} alterations

Activated macrophages secrete a number of putative neurotoxic substances including but not limited to cytokines, chemokines, excitatory amino acids, etc. To examine the active component(s) involved in the mediation of enhancement of EPSC_{NMDAR}, we separated the soluble molecules in MCM into three different fractions (<3kDa, 3-10kDa, >10 kDa) using centrilplus filters (Millipore). As shown in Figure 8, bath application of <3kDa or 3-10kDa fraction of MCM, but not >10kDa fraction, produced significant increase of the amplitude of EPSC_{NMDAR}. The average peak amplitude was $227.9 \pm 15.4\%$ of control level ($n=9$, $p<0.001$) or $243.1 \pm 9.3\%$ of control level ($n=8$, $p<0.001$), respectively. In contrast, the average peak amplitude during bath application of >10 kDa fraction was $115.1 \pm 7.3\%$ of control level, which is not significant when compared with control ($n=6$, $p>0.05$). These

results suggested that the active components in MCM were most likely those with molecular weight <10 kDa. This suggestion was further supported by the results demonstrating heat (boiled) denature of the proteins contained in the MCM failed to diminish increases in EPSC_{NMDAR}. Moreover, pre-treatment of MCM with GAD for 1h at 37°C, to degrade glutamate released possibly by activated macrophages (Jiang et al., 2001), did not alter the ability of MCM to increase EPSC_{NMDAR} with an average peak amplitude of $241.2 \pm 15.0\%$ of control level ($n = 7$, $p < 0.001$). These results indicate proteins and glutamate, which may be present in the MCM, may not be the main components in the mediation of enhancement of EPSC_{NMDAR}. To ensure the enzymatic activity, we added GAD to a glutamate cocktail containing L-glutamate (10μM) and glycine (1μM) and tested its effects on EPSCs. In the absence of GAD, the glutamate cocktail induced an increase in the amplitude of EPSCs. In contrast, the increase was significantly inhibited after addition of GAD (data not shown). Taken together, our results revealed that soluble substances with low-to-medium molecular weights are the active components responsible for MCM-increases in EPSC_{NMDAR}.

DISCUSSION

Selective activation of NR2BRs mediates neuronal death under a variety of conditions, few have investigated the subunit specific effects of NMDAR activation in HIV-1-associated neuronal injury. In the present study, we found that conditioned media, recovered from HIV-1gp20-treated human monocyte-derived macrophages, enhances EPSC_{NMDAR} in the CA1 region of rat hippocampal slices. This was demonstrated by assays of bath perfusion of hippocampal slices with MCM that produced a concentration-dependent increase of electrically evoked EPSC_{NMDAR}. The effects of MCM were further tested on EPSC_{NR2AR} and EPSC_{NR2BR} isolated pharmacologically. The results showed that MCM increased both EPSC_{NR2AR} and EPSC_{NR2BR}, with a much stronger effect on EPSC_{NR2BR}. The MCM-induced enhancement of EPSC_{NR2BR} appeared to be mediated at least in part via exNR2BRs because the enhancement was still observed after synaptic NMDARs (namely NR2ARs) were blocked by bath application of MK801 (25μM, an open NMDA channel blocker) during 10-15 min low frequency (0.05Hz) of the Schaffer collateral fibers. These results suggest MCM enhancement of EPSC_{NMDAR} via NR2BRs, most likely the exNR2BRs.

MP play a pivotal role in immune and inflammatory responses. They are key cells in orchestrating the pathophysiology of many neurological disorders including HAND. In HIV-1-infected brain, the infected and immune activated macrophages produce a variety of soluble bioactive substances including, but not limited to, pro-inflammatory cytokines, chemokines, quinolinic acid, arachidonic acid and its metabolites, and viral proteins (gp120 and *Tat*), which can injure or kill neurons and contribute to the neuropathogenesis of HAND. It has been shown that the numbers of immune competent macrophages, rather than absolute levels of virus *per se*, are the major predictors for dementia (Glass et al., 1995). Importantly, the abundance of macrophages in the brain appears to better correlate with HAND than the extent of brain infection (Gendelman et al., 1994; Wiley and Achim, 1994; Glass et al., 1995). These findings suggest that in the late-stage of viral infection, an increase in trafficking of macrophages to the brain may be associated with the development of HAND (Gartner, 2000). How infected and/or immune-activated macrophages mediate neuronal injury and cognitive dysfunction in HAND remains an area of considerable research interest. Many studies have shown that HIV-1-associated neuronal injury or death can be attenuated or prevented by NMDAR antagonists (Pellegrini and Lipton, 1993; Ushijima et al., 1995; Muller et al., 1996; Kaul et al., 2001; Anderson et al., 2004; O'Donnell et al., 2006; Shin et al., 2012), suggesting an involvement of NMDARs in macrophage-associated neuronal injury. However, few have so far investigated a direct activation of NMDARs by the MCM recovered from virus infected and/or immune activated macrophages. We have previously shown that MCM, recovered from HIV-1-infected MDM,

activates engineered NR1a/NR2B receptors expressed in *Xenopus* oocytes. In this study, we provided experimental evidence showing MCM activates native NMDARs in a mammalian brain tissue, and the MCM-induced activation of NMDARs underlies MCM-associated neuronal injury. This is, to our knowledge, the first report demonstrating MCM enhancement of EPSC_{NMDAR} in hippocampal brain slices.

It is well documented that in adult brains NR2ARs preferentially target to synaptic sites; whereas, NR2BRs predominately target to extrasynaptic sites (Stocca and Vicini, 1998; Rumbaugh and Vicini, 1999; Tovar and Westbrook, 1999; Mohrmann et al., 2000; Hardingham et al., 2002; Townsend et al., 2003). While NR2ARs are necessary for normal synaptic transmission, NR2BRs are considered to be associated with neuronal injuries under pathological conditions (Hardingham et al., 2002; Riccio and Ginty, 2002; Liu et al., 2007). In the present study, we demonstrated that MCM had much stronger effects on enhancing EPSC_{NR2BR} than EPSC_{NR2AR}. Further, the MCM retains its ability to enhance EPSC_{NR2BR} while synaptic NR2ARs were blocked by a low frequency stimulation of Schaffer-collateral fibers in the presence of MK801 in the perfusate (Figure 6). The MCM enhancement of EPSC_{NR2BR} under such a condition could be mediated via exNR2BRs because the EPSC_{NR2BR} evoked by a stronger stimulation (more glutamate release and spill over synaptic cleft) was enhanced by MCM and blocked by a specific NR2BR antagonist Ro 25-6981. Combined with the neurotoxicology data showing MCM-induced neuronal injury was significantly blocked by NR2BR antagonist ifenprodil (Figure 7), but not by R-CPP, an antagonist with a high affinity for NR2ARs (Feng et al., 2004; Feng et al., 2005), our results suggest that MCM increases neuronal EPSC_{NR2BR} leading to neuronal injury, at least in part, via exNR2BRs. These results are consistent with the findings reported by other investigators demonstrating activation of neuronal NR2BRs causes neuronal injuries (Hardingham et al., 2002; Riccio and Ginty, 2002; Liu et al., 2007). Since activation of NR2BRs is believed to be associated with neuronal injury, the increase of EPSC_{NR2BR} by MCM may imply that in HIV-1-infected brains the infected and/or immune-activated macrophages release soluble substances leading to neuronal injury by acting on exNR2BRs.

As HIV-1-infected and/or immune activated MDM release a number of substances, it is not clear which substance(s) is(are) responsible for the activation of NMDARs. To identify potential active components mediating the increase of EPSC_{NMDAR}, we fractionated MCM through Centrplus filters and found that the MCM-induced increase of EPSC_{NMDAR} was mainly mediated by low (<3Kda) to medium (3-10kDa) molecular weight fractions. These results are consistent with previous reports describing the presence of low molecular weight neurotoxins in the supernatants recovered from HIV-infected MDM (Giulian et al., 1996) and their involvement in HIV-1 infected MDM-associated neurotoxicity (O'Donnell et al., 2006). It has been shown that HIV-infected MDM release glutamate (Jiang et al., 2001) and elevated levels of glutamate were detected in the CSF and plasma of patients with HAND (Ferrarese et al., 1997; Gurwitz and Kloog, 1997). Addition of pyruvate and glutamate pyruvate transaminase to remove glutamate from HIV_{Jago}-infected MDM supernatants attenuated HIV/MDM-induced neurotoxicity (O'Donnell et al., 2006). Interestingly, our results showed the addition of GAD to MCM did not abolish the MCM-induced increase of EPSC_{NMDAR}, suggesting that glutamate might not be the main active factor in the MCM. The mechanisms for such a discrepancy remain to be determined. It may reflect a secretory variation of MDM in response to different activation stimuli, HIV infection vs gp120 treatment. As >10kDa fraction showed no significant effects on enhancing EPSC_{NMDAR} and heat (boiled) denaturation of MCM failed to alter its ability in enhancing EPSC_{NMDAR}, we want to rule out the potential effects mediated by proteins and “narrow-down” the active components to <10kDa fractions. However, what the exact active components are remain to be determined. Recent studies from our laboratory showed that gp120-treated human glial cells produce high levels of D-serine, an NMDAR co-agonist that binds to glycine-binding

sites of NMDAR, and bath application of D-serine produced an enhancement of EPSC_{NMDAR} in rat hippocampal slices (unpublished observations). We postulate that D-serine might be one of the active components within the MCM. Nevertheless, future studies using comparative proteomic approaches could help to identify and characterize these factors as described by Boukli and her colleagues (Boukli et al., 2012).

Biological significance of macrophage-conditioned media activation of NR2BRs remains to be elucidated. Previous studies have shown that brain regions (e.g., the hippocampus) enriched in NR2BRs are injured in both excitotoxic insults and HIV-1 brain infection (Everall et al., 1999; Haughey et al., 2001; Lynch and Guttman, 2002; Sa et al., 2004; Waxman and Lynch, 2005; Heng et al., 2009), while other regions such as the cerebellum, which express relatively high levels of NR2CR and low levels of NR2BR, are commonly spared in both conditions (Lynch and Guttman, 2002). As macrophage and microglia activation is a striking neuropathological feature in many neuroimmune and neuroinflammatory disorders, the enhancement of EPSC_{NR2BR} (especially the EPSC_{exNR2BR}) by MCM may represent a common pathway in the pathogenesis of neurodegenerative disorders including, but not limited to, Huntington disease (Li et al., 2004; Heng et al., 2009), Parkinson's (Nash et al., 2000; Quintana et al., 2012; Riquelme et al., 2012) and Alzheimer's disease (Li et al., 2011; Liu et al., 2012) and HAD (Xiong et al., 2003; O'Donnell et al., 2006; Jing et al., 2010).

In summary, our results provide an *in vitro* experimental evidence that HIV-1gp120-stimulated macrophages release soluble substances leading to neuronal injury by enhancing EPSC_{NMDAR} in the CA1 region of rat hippocampal slices. The MCM enhanced EPSC_{NR2BR} more than EPSC_{NR2AR}. Moreover, MCM-induced neuronal injury was blocked by NR2BR antagonists. This suggested the involvement of neuronal NR2BR in MCM-associated neuronal injury. The MCM-induced enhancement of EPSC_{NR2BR} appeared to be mediated at least in part via exNR2BRs since the EPSC_{NR2BR} evoked by stronger stimulation during MK801 blockade of synaptic NMDARs (namely NR2AR) was increased by MCM and blocked by a specific NR2BR antagonist Ro 25-6981. These results may implicate one of the potential mechanisms underlying the neuropathogenesis of HIV-1 infection.

Acknowledgments

The authors thank Ms. Robin Taylor for reading the manuscript. The authors also thank Ms. Julie Ditter, Ms. Robin Taylor and Ms. Johna Belling for their excellent administrative support and to two anonymous reviewers for their constructive criticisms and comments. This work was supported by NIH grants R01 NS06878(HX), R01 NS036126 (HEG) and National Institute on Minority and Health Disparities (8G12MD007583-27) to the Universidad Central del Caribe (UCC).

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Figure 1.

MCM increase of EPSCs in the CA1 region of rat hippocampal slices. **A.** The time course illustrating bath application of MCM (indicated by a horizontal bar) increased the EPSC amplitudes recorded in a CA1 neuron. The graph plots amplitudes of the EPSCs evoked in response to constant current stimulation of Schaffer-collateral fibers (150 μ A, 0.05Hz). Each point in this graph is an average of 3 consecutive EPSCs. Above the time course are representative EPSCs taken from different time points as indicated by numbers 1, 2 and 3. Note the significant increase of evoked EPSC amplitude following bath application of MCM. The experiment was performed in the presence of 50 μ M picrotoxin to block GABA_A-mediated inhibitory postsynaptic potentials. Holding potential was -70 mV. **B.** Summary data showing normalized EPSC peak amplitudes after perfusion of MCM or untreated MCM. Note that MCM significantly increased EPSCs; whereas, untreated MCM had no apparent effect. **C.** MCM produced an enhancement of EPSCs in a dilution (concentration)-dependent manner. As shown in the figure 1C, MCM increased EPSCs significantly at 1:5 dilution, and this dilution (dose) was adopted in this study. *** $p < 0.001$ vs. Ctrl.

Figure 2.

Enhancement of NMDAR-mediated EPSCs (EPSC_{NMDAR}) by MCM. **A.** The time course showing bath application of MCM increased EPSC_{NMDAR} amplitudes in a CA1 neuron. The representative current traces taken from different time points as indicated by numbers 1, 2 and 3. Note that bath application of MCM enhanced EPSC_{NMDAR}. **B.** A summary bar graph depicting MCM induced a significant enhancement of the EPSC_{NMDAR}. Experiments were done in the presence of 10 μ M DNQX and 1 μ M glycine in the ACSF contained low-Mg²⁺, high-Ca²⁺. Holding potential was -50mV (***) $p < 0.01$ vs Ctrl).

Figure 3. MCM increase of NMDAR-mediated excitatory postsynaptic potentials (EPSP_{NMDAR}). **A.** An averaged time course recorded from CA1 neuronal cells ($n=4$) illustrating that bath application of MCM increased the EPSP_{NMDAR}, which was in parallel with the effects of MCM on the EPSC_{NMDAR}. The representative voltage traces taken from time points *a* and *b* are shown above the time course. The superimposed voltage traces exhibited a remarkable increase of EPSP_{NMDAR} amplitude. Bar graph (**B**) shows a significant ($p < 0.01$) increase of EPSP_{NMDAR} amplitude by MCM. Experiments were carried out in the same conditions as described in Figure 2.

Figure 4.

MCM increased EPSC_{NMDAR} via NR2B-containing NMDARs (EPSC_{NR2BR}). **A**, **B** and **C** are examples showing the effects of MCM on pharmacologically isolated EPSC_{NR2AR} (**A** and **B**) and EPSC_{NR2BR} (**C**) respectively. The current traces shown in **A**, **B** and **C** are individual EPSC_{NR2AR} (**A** and **B**) and EPSC_{NR2BR} (**C**) taken from the time points as indicated by numbers accordingly. Note that MCM had a significant stronger effect on increasing EPSC_{NR2BR} than EPSC_{NR2AR}. As shown in **D**, bath application of MCM produced an increase of EPSC_{NR2AR} and EPSC_{NR2BR} to $149.9 \pm 9.2\%$ and $221.7 \pm 91.0\%$ of control, respectively. The difference is statistically significant ($p < 0.05$, $n=8$), suggesting that MCM increase of EPSC_{NMDAR} was mainly mediated via NR2BRs. * $p < 0.05$, *** $p < 0.001$, # $p < 0.05$. Holding potential: -50 mV.

Figure 5.

MCM enhanced NMDAR-mediated field EPSPs (fEPSPs). The fEPSPs, evoked by constant current stimulation of Schaffer-collateral fibers, were recorded in the CA1 dendritic field. The initial slope of the fEPSPs was measured and expressed as the percentage of the basal level (Ctrl). As shown in the figure, bath application DNQX ($10\mu\text{M}$) decreased fEPSP to $30.9 \pm 5.9\%$ of control level ($n=6$). In the presence of DNQX in the perfusate, addition of MCM (1:5 dilution) to the bath increased fEPSPs from $30.9 \pm 5.9\%$ of control level to $75.1 \pm 7.3\%$ of control level ($n=6$, $p < 0.001$). The MCM-mediated increase of fEPSPs was blocked by ifenprodil, an NR2BR antagonist, indicating MCM acts on NR2BRs. *** $p < 0.001$.

Figure 6.

MCM increases EPSC_{NMDAR} via extra-synaptic NR2BRs (exNR2BRs). **A.** EPSC_{NMDAR} was recorded in the presence of DNQX (10 μ M) in the bath solution. Addition of MK801 (25 μ M) to the perfusate almost completely blocked the EPSC_{NMDAR}. Increase of stimulation intensities, started from the downward arrow, resulted in re-appearance of EPSC_{NMDAR}, which was most likely mediated via exNR2BRs. Bath application of MCM (as indicated by a horizontal bar) led to an increase of EPSC_{NMDAR} and this MCM-associated increase of EPSC_{NMDAR} was blocked by subsequent bath application of Ro 25-6981, a specific NR2BR blocker (n=5), suggesting MCM activation of exNR2BRs. **B.** EPSC_{NMDAR} was isolated as described in **A.** Addition of MK801 inhibited EPSC_{NMDAR} and stronger stimulations (started from the downward arrow) evoked EPSC_{NMDAR}. Application of Ro 25-6981 (as indicated by a long horizontal bar) blocked the EPSC_{NMDAR} evoked by stronger stimulations and abolished the effect of MCM on enhancement of EPSC_{NMDAR}, indicating again that MCM enhances EPSC_{NMDAR} via exNR2BRs.

Figure 7.

Neurotoxic activity produced by MCM in hippocampal neuronal cultures and NR2BR antagonist exerted a significant protective effect against MCM-induced neurotoxicity. **A.** Ifenprodil or ifenprodil with R-CPP, rather than R-CPP alone, protected MCM-induced neurotoxicity in cultured hippocampal neurons. Nuclear morphology and membrane integrity of hippocampal neurons were evaluated by fluorescent dyes PI (red) and DAPI (blue). **B.** Summary data showing the cell count from five different visual fields under fluorescent microscopy (n=10), note that NR2BR antagonist ifenprodil significantly protected hippocampal neurons from MCM-associated challenge, suggesting involvement of NR2BRs in MCM-induced neuronal injury. ** $p < 0.01$ vs Ctrl. ## $p < 0.01$ vs MCM

Figure 8.

The active components underlying MCM increase of EPSC_{NMDAR} are substances with low-to-medium molecular weight. Soluble factors with molecular weight >10kDa had no significant effect on EPSC_{NMDAR}. Heat (boiled) denature of proteins contained in MCM did not alter its ability to enhance EPSC_{NMDAR}. Enzymatic degradation of glutamate that may be present in MCM did not change MCM enhancement of EPSC_{NMDAR}, indicating glutamate was not the factor for the mediation of MCM-associated increase of EPSC_{NMDAR}. *** $p < 0.001$ vs Ctrl.