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Chemokine CCL2 enhances NMDA receptor-mediated excitatory postsynaptic current in rat hippocampal slices –A potential mechanism for HIV-1-associated neuropathy?

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Abstract

Human immunodeficiency virus type 1 (HIV-1)-infected mononuclear phagocytes (brain macrophages and microglial cells) release proinflammatory cytokines and chemokines. Elevated levels of chemokine CC motif ligand 2 (CCL2, known previously as monocyte chemoattractant protein-1) have been detected in serum and cerebrospinal fluid (CSF) of HIV-1-infected individuals and the raised CCL2 in the CSF correlates with HIV-1-associated neurocognitive disorders. To understand how elevated CCL2 induces HIV-1-associated neuropathy, we studied effects of CCL2 on excitatory postsynaptic current (EPSCs) in the CA1 region of rat hippocampal brain slices using whole-cell patch recording techniques. The AMPA receptor (AMPAR)-mediated EPSC ($EPSC_{AMPAR}$) and N-Methyl-D-aspartate (NMDA) receptor (NMDAR)-mediated EPSCs ($EPSC_{NMDAR}$) were isolated pharmacologically. Bath application of CCL2 produced a significant enhancement of the amplitudes of EPSCs, $EPSC_{AMPAR}$ and $EPSC_{NMDAR}$. Further studies revealed that CCL2 potentiated NMDAR subtype NR2A-mediated EPSC ($EPSC_{NR2AR}$) and NR2B-mediated EPSC ($EPSC_{NR2BR}$). To determine the site of action, we recorded spontaneous mini EPSCs (mEPSC) before and during bath application of CCL2. Our results showed that CCL2 decreased inter event interval (IEI) and increased the frequency of mEPSCs without change on the amplitude, suggesting a presynaptic site of CCL2 action. CCL2 was also found to injure primary rat hippocampal neuronal cultures and neuronal dendrites in the CA1 region of hippocampal slices. The CCL2-associated neuronal and dendritic injuries were blocked by a specific NMDAR antagonist or by a CCR2 receptor antagonist, indicating that CCL2-associated neural injury was mediated via NMDARs and/or CCR2 receptors. Taken together, these results suggest a potential role CCL2 may play in HIV-1-associated neuropathology.

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Compliance with Ethical Standards All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of University of Nebraska Medical Center, Omaha, NE, USA.

Competing interests The authors declare that they have no competing interests.

Keywords

chemokine; CCL2; NMDA receptors; EPSCs; hippocampus; HIV-1-associated neuropathy

Introduction

Brain infection with human immunodeficiency virus type-1 (HIV-1) often provokes neurocognitive impairment, termed collectively 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). Although the introduction of combination antiretroviral therapy (cART) has significantly decreased the incidence of HAD, the prevalence of less severe forms of HAND has been on the rise even in the cART era (Heaton et al., 2011; Alfahad and Nath, 2013; Nath, 2015; Watkins and Treisman, 2015). Nevertheless, the precise mechanisms for HAND pathogenesis are still not fully understood. Many studies have shown that HIV-1-infected cells produce soluble immune/inflammatory factors with neurotoxic potential, leading to neural injury and HAND pathogenesis. Among the potential neurotoxic factors is chemokine CC motif ligand 2 (CCL2), formerly known as monocyte chemoattractant protein-1.

CCL2 is a well-characterized β or CC family member of chemokines that plays a crucial role in the recruitment of monocytes into tissues and organs, including the brain (Dhillon et al., 2008; Deshmane et al., 2009). It is produced in response to immune/inflammatory or other nociceptive stimuli by a variety of cell types including, but not limited to, macrophages, microglia, astrocytes and endothelial cells (Deshmane et al., 2009; Yadav et al., 2010; Kim et al., 2014). In addition to its well-established roles in regulating monocyte activation and providing the directional cues for the transmigration of leukocytes, CCL2 also plays an important role in the central nervous system (CNS). In the CNS, CCL2 is expressed in microglial cells, astrocytes and neurons and the levels of expression are often up-regulated in a variety of neuropathological conditions including HAND/HAD. Indeed, elevated levels of CCL2 (5–20ng/ml) have been detected in the cerebrospinal fluid (CSF) of HIV-1-infected individuals (Cinque et al., 1998; Conant et al., 1998; Kelder et al., 1998; Marzocchetti et al., 2005; Shiramizu et al., 2006; Yuan et al., 2013; Thames et al., 2015) and viral infection is one of the main stimuli for CCL2 production (Kim et al., 2014). The elevated levels of CCL2 in the CNS are associated with HIV-1-associated neurological disorders (Marzocchetti et al., 2005; Dhillon et al., 2008; Muratori et al., 2010; Yuan et al., 2013). Nonetheless, how elevated CCL2 induces neuronal injury in HIV-1-infected brain and how the CCL2-mediated neuronal injury contributes to the HAND pathogenesis and progression are still equivocal.

Several lines of evidence indicate that CCL2 is a mediator of excitotoxic brain injury (Sheehan et al., 2007; Yao and Tsirka, 2014). It has been shown that co-administration of CCL2 and a low dose of *N*-methyl-*D*-aspartic acid (NMDA) exacerbate NMDA-induced neural injury and functional inhibition of CCL2 protects against NMDA-induced neurotoxicity, suggesting CCL2 potentiation of NMDA-induced neural injury (Galasso et al.,

2000). We hypothesize that the elevated CCL2 potentiates NMDA receptor (NMDAR)-mediated responses, leading to neuronal injury. To test this hypothesis, we studied effects of CCL2 on excitatory postsynaptic currents (EPSCs) in the CA1 region of rat hippocampal slices. Our results showed that bath application of CCL2 enhanced the NMDAR-mediated EPSCs ($EPSC_{NMDAR}$) via a presynaptic mechanism and incubation of hippocampal neurons and slices with CCL2 resulted in neuronal and dendritic injuries that could be blocked with a specific NMDAR antagonist.

Materials and Methods

Animals

Fifteen to 35 d old male Sprague-Dawley rats used for experiments were purchased from Charles River Laboratories (Wilmington, MA). Animals were housed at constant temperature (22°C) and relative humidity (50%) under a regular light-dark cycle (light on at 7:00 AM and off at 5:00 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).

Chemicals and reagents

Drugs used in this study were CCL2 (R&D Systems, Minneapolis, MN), picrotoxin, tetrodotoxin(TTX), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), 2-amino-5-phosphonovalerate (APV), (*R*)-3-(2-Carboxypiperazin-4-yl)propyl-1-phosphonic acid (R-CPP), ifenprodil, RS102895, and picrotoxin. Picrotoxin and RS102895 were dissolved in dimethyl sulfoxide (DMSO) and the final DMSO concentration in artificial cerebrospinal fluid (ACSF) was less than or equal to 0.1%. CCL2, TTX, CNQX, APV, R-CPP, and ifenprodil were pre-prepared separately in 1000x stock solutions and stored at −20°C refrigerator, thawed on experimental day just before use and diluted to the test concentrations. Drugs were applied onto brain slices via bath perfusion and added to the culture media. For bath perfusion, the time needed for a drug to reach the chamber was about 1 min. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise indicated.

Primary hippocampal neuronal culture

Hippocampal neuronal cultures were prepared from rat embryos using the methods described previously (Blair et al., 2009). Briefly, female Sprague-Dawley rats with 18–19 days of gestation were anesthetized with isoflurane, and embryonic pups were surgically dissected out and decapitated. Hippocampi were harvested under sterile conditions. The hippocampal tissue was enzymatically dissociated in 0.125% trypsin II (Sigma-Aldrich). Isolated neural cells were placed in poly-D-lysine-coated 35 mm plastic culture dishes containing 2 ml of neurobasal medium to a culture surface cell density of 5×10^5/ml. The cultures were maintained in neurobasal medium supplemented with B27 (2%, v/v, Invitrogen), glutamine (0.5mM) and 1% penicillin/streptomycin for at least 7–10 days before being used for experiments.

Hippocampal brain slices

Hippocampal brain slices (400μm in thickness) were prepared as previously described (Xiong et al., 1996). Young animals were used for preparation of hippocampal slices mainly because the brain tissues from young animals are more resistant to hypoxic/ischemic challenge. Briefly, animals were anesthetized with isoflurane and decapitated. The brain was quickly removed from cranial cavity and placed into an ice-cold (~4°C) oxygenated 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, glucose 10.0 and TTX 0.1μM. ACSF was saturated with 95% O_2 and 5% CO_2 and had a pH of 7.35–7.45. The hippocampi were dissected free, and transverse hippocampal slices were cut using a tissue chopper. The slices were then incubated in oxygenated ACSF at room temperature for at least 1 h before use.

Electrophysiology

During electrophysiological experiments, a single hippocampal slice was transferred into the recording chamber each time and superfused with ACSF at a constant flow rate of 2.5 ml/min. The temperature of the ACSF was maintained at 30°C ± 1°C with an automatic temperature controller (Warner Instrument Corp., Hamden, CT). Whole-cell patch clamp recordings were made from CA1 neurons using a “blind” method. The neuronal cells recorded were voltage-clamed at −70mV and EPSCs were evoked by electrical stimulation of the Schaffer collateral/commissural pathway (0.05 Hz, 40 μs in duration, 30–100 μA in intensity) with a bipolar tungsten electrode and amplified by an Axopatch-1D amplifier (Molecular Devices, Union City, CA). The mini EPSCs (mEPSCs) were recorded in gap-free mode from cells voltage-clamed at −60 mV. The recording electrodes were made from borosilicate glass capillaries (World Precision Instruments, Sarasota, FL) and had a resistance of 6–9 MΩ when filled with pipette solution contained (in mM): K-gluconate 130.0, K-methysulphate 17.5, NaCl 8.0, HEPES 10.0, Mg-ATP 2.0, GTP 0.2, EGTA 0.1, pH 7.25–7.35 (adjusted with KOH), osmolality 292–308 mOsm. Picrotoxin (50μM) was routinely added to ACSF to block the potential inhibition of inhibitory input by CCL2.

Cells with resting membrane potential more negative than −55 mV were used for the study. Each recording trial consisted of an average of three consecutive sweeps. Data were filtered at 1 kHz and digitized at 5 kHz using a Digidata 1322A interface (Molecular Devices). pCLAMP 8.2 software (Molecular Devices) was used for data acquisition and analyses. The series resistance was constantly monitored by delivering a hyperpolarizing voltage pulse during recording and the cells with > 20% changes in access resistance were excluded from the analysis.

Microtubule associated protein 2(MAP-2) staining

Four Sprague-Dawley rats were used in this study. Hippocampal brain slices prepared from each animal was randomly divided into four groups (4 slices in each group) and incubated with either normal ACSF (control), or ACSF contained CCL2 (CCL2, 20ng/ml, the same concentration in the following groups unless indicated), or CCL2 and 50μM AP-V (CCL2+APV), or CCL2 and 10 μM RS102895, a selective CCR2 receptor antagonist (CCL2+ RS102895) at room temperature for 6–8 h. The ACSF contained the above mentioned reagents was continuously oxygenated (95% O_2 and 5% CO_2) during incubation.

Then hippocampal slices were fixed in 4% paraformaldehyde for 24 h followed by dehydrations in 15% and 30% sucrose overnight, respectively. The slices were then embedded in Optimal Cutting Temperature (OCT) compound and stored in −80°C freezer. Serial frozen sections (30μm in thickness) were then cut and the dendritic arbors were labeled with rabbit anti rat antibodies against MAP-2 (Chemicon, Temecula, CA). The second antibody was goat anti rabbit IgG (Invitrogen, Molecular probes). Sections were viewed with an E800 Nikon Eclipse microscope (Nikon, Japan), and images were captured with a MagnaFire digital camera and software (Optronics, Goleta, CA).

Measurement of neuronal cell viability

Experiments were performed in triplicate and total survival hippocampal neural cells in culture were determined by Hoechst 33342 (Sigma) staining and counted from five different visual fields. CCL2, CCL2+CNQX(10μM), CCL2+AP-V(100μM), CCL2+CNQX(10μM) +AP-V(100μM) were added to hippocampal neuronal cultures for 24 h, respectively, and then the cultured cells were incubated at 37°C with H 33342 (5μg/ml) for additional 30min, washed with PBS 3 times, and fixed for 15 min with 4% paraformaldehyde. After fixation, cultures were gently washed 3 times and photographed under a fluorescence microscope. Cells with bright blue fragmented nuclei, representing condensation of chromatin, were identified as injured cells and analyzed quantitatively by cell counting.

Data Analyses

Data were analyzed and displayed using ClampFit 8 (Molecular Devices, Mini Analysis Program (Synaptosoft Inc.,Decatur, GA) and Origin 8.5 (Northampton, MA). All numerical data were expressed as mean ± SEM unless otherwise indicated. Statistical significance was determined using ANOVA, two-tailed Student's *t*-tests, or the Kolmogorov-Smirnov-test. The level of significance was determined at $p < 0.05$.

Results

CCL2 Enhancement of NMDA receptor- and AMPA receptor-mediated EPSCs in the CA1 region of hippocampal slices

In a previous study, we demonstrated that CCL2 (20ng/ml) enhanced EPSCs recorded in the CA1 region of rat hippocampal slices in a concentration-dependent manner (it had no apparent effect at concentrations of 0.2ng/ml and 2ng/ml) (Zhou et al., 2011). In consistent with previous results, we found that bath application of CCL2 (20ng/ml) produced an increase of EPSCs to 194.82±13.05% of basal level (n=17, $p<0.05$; Fig.1 A, D). As the hippocampal excitatory synaptic transmission is mainly mediated by AMPA receptors and NMDA receptors, we further examined the effects of CCL2 on pharmacologically isolated AMPA receptor-mediated EPSCs ($EPSC_{AMPAR}$) and NMDA receptor-mediated EPSCs ($EPSC_{NMDAR}$) in the CA1 region of rat hippocampal slices.

To record $EPSC_{AMPAR}$ and $EPSC_{NMDAR,}$ the cells were held at −70 mV and −50mV, respectively. Our results showed bath application of CCL2 significantly increased the $EPSC_{AMPAR}$ to 142.35±15.57% of basal level (M±SD, n=9, $p<0.05$, Fig.1 C, D) in presence of AP-V (50μM) in normal ACSF. In contrast, application of CCL2 significantly increased

$EPSC_{NMDAR}$ to 155.60±22.12% of basal level (M±SD, n=13, $p<0.05$; Fig.1 B, D) in the presence of 10 μM CNQX and 1μM glycine in Mg^{2+} free ACSF. These results indicate that CCL2 enhanced both $EPSC_{AMPAR}$ and $EPSC_{NMDAR}$ in the hippocampus. Although CCL2 exhibited a stronger effect on $EPSC_{NMDAR}$ than $EPSC_{AMPAR}$, the difference is not statistical significant ($t_{(20)}=0.11$).

Effects of CCL2 on subtype NMDA receptor-mediated EPSCs in CA1 region of hippocampal slices

Studies have shown that NR2A receptors (NR2AR) and NR2B receptors (NR2BR) are the major subtypes of NMDARs in the hippocampus (Monyer et al., 1994; Kim et al., 2005; Chen et al., 2007). To evaluate the effects of CCL2 on NR2AR-mediated EPSCs ($EPSC_{NR2AR}$) and NR2BR-mediated EPSCs ($EPSC_{NR2BR}$), we recorded $EPSC_{NR2AR}$ in the presence of NR2BR antagonist ifenprodil in the perfusate (10μM, which has a high affinity for NR2BRs with an IC_{50}=0.34μM and IC_{50} =146μM for NR2ARs) (Williams, 1993) and $EPSC_{NR2BR}$ in the presence of NR2AR antagonist R-CPP in the perfusate (1μM, which has ~7-fold greater selectivity for NR2ARs compared with NR2BRs) (Feng et al., 2004; Feng et al., 2005). In the presence of ifenprodil or R-CPP in the perfusate, bath application of CCL2 increased $EPSC_{NR2AR}$ to 160.97 ± 18.32% of control level (n=10, $p<0.05$ vs control) or $EPSC_{NR2BR}$ to 137.19 ± 12.09% of control level (n=12, $p<0.05$ vs control), respectively (Fig. 2). CCL2 seems to have a stronger effect on enhancing $EPSC_{NR2AR}$ than $EPSC_{NR2BR}$, but the difference was not statistically significant (Fig. 2, $t_{(22)}=0.28$).

CCL2 increased the spontaneous occurrence of mEPSCs in the CA1 region of the hippocampal slices

To evaluate the site of action for CCL2 in the enhancement of EPSCs, we recorded spontaneous miniature excitatory postsynaptic currents (mEPSCs) in the hippocampal CA1 pyramidal neurons in the absence and presence of CCL2 in the perfusate. When applied through bath perfusion, CCL2 (20ng/ml) significantly increased spontaneous mEPSCs occurrence from 9.01±1.12 counts/30s before (control) to 14.58 ± 2.97 counts/30s during CCL2 application (CCL2) (Fig. 3, $p<0.05$, n=9). In contrast, CCL2 had no apparent effect on the amplitude of spontaneous mEPSCs. The average amplitudes before and during bath application of CCL2 were 8.54±1.64pA and 7.84±1.99 pA, respectively ($p>0.05$, n=9, K-S test, Fig.3 B, D). These results suggest that CCL2 enhancement of EPSCs via a presynaptic mechanism. Analysis of the inter-event interval (IEI) revealed that CCL2 decreased the IEI duration from 1462.42 ± 136.05 ms in control (before bath persufion of CCL2) to 784.71 ± 31.18ms during bath perfusion of CCL2 (CCL2, $p<0.05$, n=9). The decrease of IEI following CCL2 treatment suggest an increase of either functional synapses and/or probability of synaptic quantal release.

CCL2 induced hippocampal neuronal injury

As over-activation of NMDA receptors causes neuronal injury and our results showed CCL2 increased $EPSC_{NMDAR}$, we further examined the effects of CCL2 on cell viability in cultured rat hippocampal neurons. To mimic its chronic action under diseased conditions, CCL2 (20ng/ml) was added to neuronal cultures for 24 h before analysis of cell viability. As shown in Fig 4, the amount of cellular survival in the control group was defined as survival

rate 100%. Addition of CCL2 to the culture media substantially enhanced the number of bright blue fragmented nuclei showing condensation of chromatin for injured neurons, and produced a significant reduction on neuronal viability (67.5±2.7%) in comparison with the control group which was 100% ($p<0.05$, n=3). The CCL2-induced reduction of neuronal viability was attenuated by pretreatment of the neuronal cultures with AP-V, CNQX or AP-V+CNQX (AP-V group: 96.5±3.1%; CNQX group: 78.6±2.2%; AP-V+CNQX group: 99.5±2.9%; vs. CCL2 group: 67.5±2.7%, $p<0.05$, n=3), demonstrating that CCL2 induces neuronal injury mainly via both NMDA and AMPA receptors.

Attenuation of CCL2-induced loss of MAP2 from CA1 dendrites by an NMDAR antagonist or a CCR2 receptor antagonist

To further examine CCL2 injury of hippocampal neurons via NMDA receptors, we assayed its detrimental effect on rat hippocampal brain slices and evaluated the protective effects of AP-V (50μM) and RS102895 (10μM, a specific CCR2 receptor antagonist). Incubation of hippocampal slices with CCL2 (20ng/ml) for 6 – 8 h produced a significant loss of MAP-2 in the CA1 region of the hippocampal slices as revealed by an evident reduction of immunoreactivity for MAP2 (Fig. 5). Notable difference in MAP-2 staining was visible in apical dendrites. As MAP2 is localized in the neuronal dendritic compartment and it is considered a marker of structural integrity(Di Stefano et al., 2001; Hoskison and Shuttleworth, 2006; Hoskison et al., 2007), the loss of dendritic MAP2 suggests a detrimental effect of CCL2 on neuronal dendrites in the CA1 region of the hippocampal slices. In contrast, addition of AP-V or RS102895 to the incubation solution, which was the ACSF, significantly increased MAP-2 expression in the CA1 region, demonstrating that blockade of NMDARs or CCR2 receptors attenuated CCL2-induced loss of MAP2 (Fig. 5). Densitometry analyses revealed the expression levels of MAP-2 in the CA1 dendrite region were 81.1±2.4% or 93.8±1.9% of control (untreated) when the slices were treated with CCL2+ AP-V or CCL2+RS102895, respectively. In comparison with the MAP-2 expression level detected on CCL2-treated slices (43.2±3.0% of control) (Fig. 5 B), the differences were statistically significant, indicating blockade of NMDARs or CCR2 receptors attenuated CCL2-induced neuronal dendritic injury in the CA1 region of the hippocampus.

Discussion

CCL2 is one of the most studied chemokines of the CC chemokine family and plays an important role in the CNS, in addition to its well established role in the immune system (Deshmane et al., 2009; Reaux-Le Goazigo et al., 2013). CCL2 and its cognate receptor CCR2 are expressed in the CNS and elevated levels of CCL2 have been detected in the cerebrospinal fluid (CSF, 5–20ng/ml) (Cinque et al., 1998; Conant et al., 1998; Kelder et al., 1998; Marzocchetti et al., 2005; Shiramizu et al., 2006; Yuan et al., 2013; Thames et al., 2015) and brain of HIV-1-infected patients with HIV-1 encephalitis, implicating CCL2 may be associated with HAND pathogenesis. Indeed, clinical studies have revealed that elevated levels of CCL2 do correlate with neuropathology seen in patients with HAND (Cinque et al., 1998; Conant et al., 1998; Kelder et al., 1998; Ragin et al., 2006), even in the era of cART (Yuan et al., 2013; Thames et al., 2015). How does the raised CCL2 in the CSF and brain causes HAND remains to be determined. In the present study, we tested our hypothesis that

elevated CCL2 potentiates NMDA receptor-mediated responses resulting in neuronal injury. We found that CCL2 enhanced $EPSC_{AMPAR}$ and $EPSC_{NMDAR}$ in the CA1 region of rat hippocampal slices. The CCL2-associated enhancement of EPSCs was mediated through a presynaptic mechanism as CCL2 decreased IEI duration and altered spontaneous mEPSC occurrence (frequency) without significant influence on the amplitude. Further studies revealed that CCL2 enhanced both $EPSC_{NR2AR}$ and $EPAC_{NR2BR}$, and such enhancements were blocked by a specific NR2AR blocker R-CPP and a specific NR2BR blocker ifenprodil, respectively, suggesting that CCL2 interacts with NMDA receptors. Attribution of CCL2-medied enhancement of $EPSC_{NMDAR}$ to neuronal injury was demonstrated by experimental results that the CCL2-associated neural injuries both in primary neuronal culture and hippocampal slices were attenuated by a specific NMDA receptor antagonist or a specific CCR2 receptor antagonist, suggesting a detrimental role CCL2 may play in the CNS, especially when its expression levels are upregulated under neurological conditions(Cinque et al., 1998; Ragin et al., 2006; Yuan et al., 2013).

NMDAR is a ligand-gated cation channel that comprises one of the major subclasses of glutamate receptors (Studzinski et al., 2015). It has a presumed role in excitatory synaptic transmission, learning, memory, and nociceptive pathways (Huo et al., 2015; Wang et al., 2015). Excessive stimulation of the NMDAR induces neuronal injury via an excitotoxic mechanism (Bonfoco et al., 1995; Prentice et al., 2015). Studies have shown that CCL2 potentiates NMDA-induced neural injury in the hippocampus and enhances AMPA and NMDA receptor currents in spinal neurons (Galasso et al., 2000; Gao et al., 2009). In a manner consistent with these aforementioned studies, we found that CCL2 enhanced AMPA and NMDA receptor currents in the CA1 region of rat hippocampal slices and induced neural injury on primary rat neuronal cultures which was blocked mainly by a specific NMDAR antagonist AP-V. Incubation of hippocampal slices with CCL2 produced neuronal dendritic injury in the CA1 region which was also blocked by AP-V. It has been demonstrated that NR2A and NR2B are the two major subtypes of NMDARs in the hippocampus; our further investigation revealed that CCL2 enhanced $EPSC_{NR2AR}$ and $EPSC_{NR2BR}$. As activation of NR2BRs is believed to induce excitotoxicity and neuronal injury, the CCL2 enhancement of $EPSC_{NR2BR}$ may underlie CCL2-induced neuronal and dendritic injuries observed in this study. It is not clear at present whether CCL2 enhancement of $EPSC_{NR2AR}$ contributes to CCL2-induced neural injury. Considering the role that NR2ARs play in the regulation of normal synaptic transmission, the enhancement of $EPSC_{NR2AR}$ by CCL2 may reflect the modulatory effect of CCL2 on synaptic transmission in the hippocampal slices as observed in our previous study (Zhou et al., 2011).

It is worth pointing out that in this study we focused on CCL2-mediated enhancement of $EPSC_{NMDAR}$ although the $EPSC_{AMPAR}$ was also increased by CCL2 (Fig. 1). The reason for focusing on NMDARs is because activation of NMDARs and resultant Ca^{2+} influx is a well-established mechanism for neuronal excitotoxicity (Fan et al., 2014; Parsons and Raymond, 2014; Fujikawa, 2015). Though bath application of CCL2 produced a transient enhancement of $EPSC_{NMDAR}$, such an enhancement may exist during a chronic disease condition like brain infection with HIV-1 leading to over-activation of NMDARs, increase of intracellular Ca^{2+} concentration, and consequently neuronal injury. Thus, the CCL2-associated potentiation of $EPSC_{NMDAR}$ may underlie HIV-1-associated neuropathology in HIV-1-

infected brain since activation of NMDARs is believed to induce excitotoxicity. It is also possible that activation of AMPARs could contribute to CCL2/HIV-1-associated neuronal injury as AMPAR-mediated excitotoxicity has been observed on hippocampal neurons (Ohno et al., 1998; Tomita et al., 2007). We did observe that addition of AMPA receptor antagonist CNQX (10μM) to neuronal culture attenuated CCL2-associated neuronal injury as detected by Hoechst stain (Fig. 4C), though the potency of CNQX was less potent than NMDAR antagonist AP-V. These results suggest that the CCL2-associated neuronal injury was largely mediated by potentiation of NMDAR-mediated effect although AMPAR was also involved in CCL2-associated neuronal injury.

In summary, we have demonstrated that CCL2 enhanced $EPSC_{AMPAR}$ and $EPSC_{NMDAR}$ recorded in the CA1 region of rat hippocampal slices via a presynaptic mechanism. The enhancement of $EPSC_{NMDAR}$ might be associated with CCL2-associated neural injury because the CCL2-associated neural injury was attenuated either by a specific NMDAR antagonist or by a specific CCR2 receptor antagonist, suggesting NMDAR and CCR2 receptor were involved in CCL2-associated neural injury. As elevated levels of CCL2 have been detected in the CSF and brain of HIV-1-infected patients with HAND, the CCL2-mediated enhancement of $EPSC_{NMDAR}$ and resultant neural injury may have implications for the pathogenesis of HIV-1-associated neurological disorders such as HAND.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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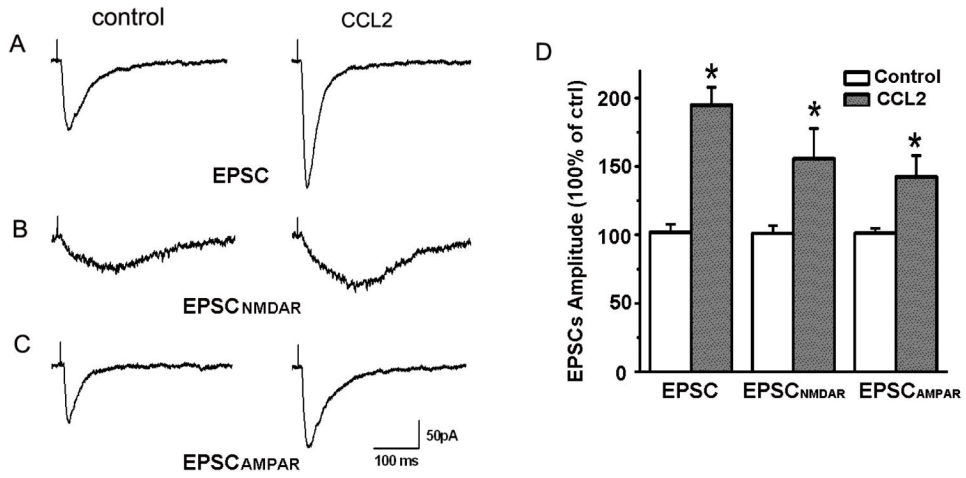

Figure 1. CCL2 enhancement of EPSCs in the CA1 region of hippocampal slices

Panels A–C are representative EPSCs recorded during control (left) and bath perfusion of CCL2 (right) in the absence (A) or presence of AMPA receptor antagonist CNQX (B) or NMDA receptor antagonist AP-V (C) in the perfusate. Note the bath perfusion of slices with CCL2 produced an enhancement of EPSCs, $EPSC_{NMDAR}$ and $EPSC_{AMPAR}$. Panel D shows the average amplitudes of EPSCs, $EPSC_{NMDAR}$ and $EPSC_{AMPAR}$ expressed as percentages of corresponding controls. Note that CCL2 significantly enhanced EPSCs (n=17), $EPSC_{NMDAR}$(n=13) and $EPSC_{AMPAR}$ (n=9). *$p < 0.05$, vs control, $^{\#}p < 0.05$ vs CCL2.

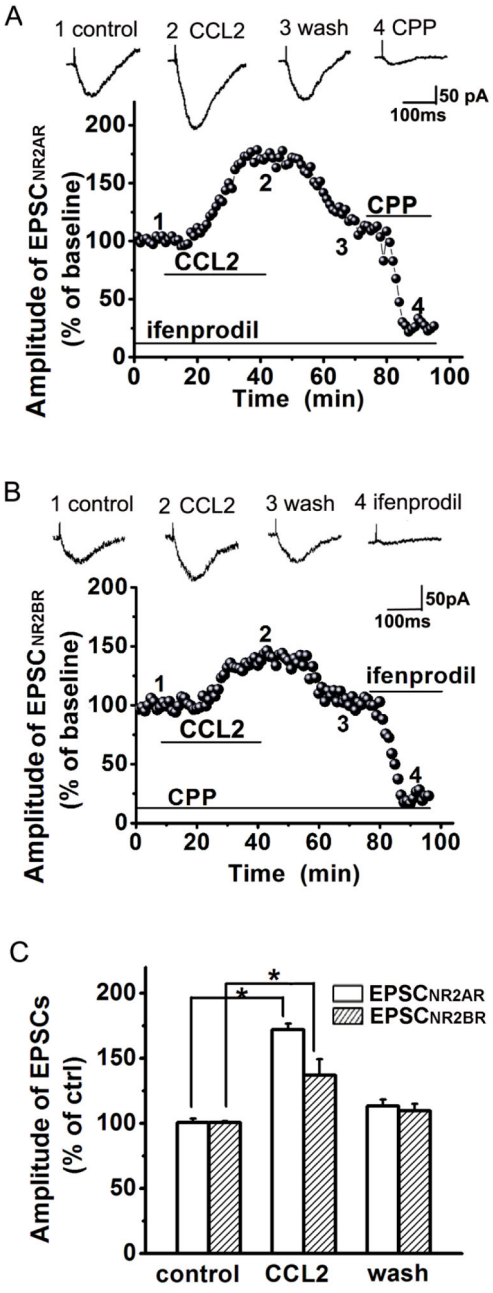

Figure 2. Enhancement of $EPSC_{NR2AR}$ and $EPSC_{NR2BR}$ by CCL2

Panels A and B illustrate the time course and amplitude (% of baseline) of the $EPSC_{NR2AR}$ and $EPSC_{NR2BR}$ recorded, in the presence of ifenprodil (Panel A, to block NR2BR, n=10) and R-CPP (Panel B, to block NR2AR, n=12), from two different neuronal cells in the CA1 region of two different slices taken from the same animal in response to constant current stimulation of Schaffer-collateral fibers (80μA, 40μS, 0.05Hz). Each data point plots the average of three consecutive EPSCs. Note that bath application of CCL2, as indicated a horizontal bar, increased the $EPSC_{NR2AR}$ (A) and $EPSC_{NR2BR}$ (B). Above each time course graph are representative individual $EPSC_{NR2AR}$ (A) and $EPSC_{NR2BR}$ (B) taken from

different time points as marked by numbers 1, 2, 3, and 4, respectively. The CCL2-induced increase of $EPSC_{NR2AR}$ or $EPSC_{NR2BR}$ was almost completely blocked by a specific NA2AR antagonist R-CPP (A) or a specific NR2BR blocker ifenprodil (B). Panel C is a bar graph exhibiting the average amplitudes of $EPSC_{NR2AR}$ or $EPSC_{NR2BR}$ before (control), during (CCL2) and post (Wash) bath application of CCL2. CCL2 enhanced both $EPSC_{NR2AR}$ or $EPSC_{NR2BR}$. All experiments were carried out in the presence of CNQX (10μM) in the perfusate and the cells were voltage clamped at −50mV. *$p<0.05$.

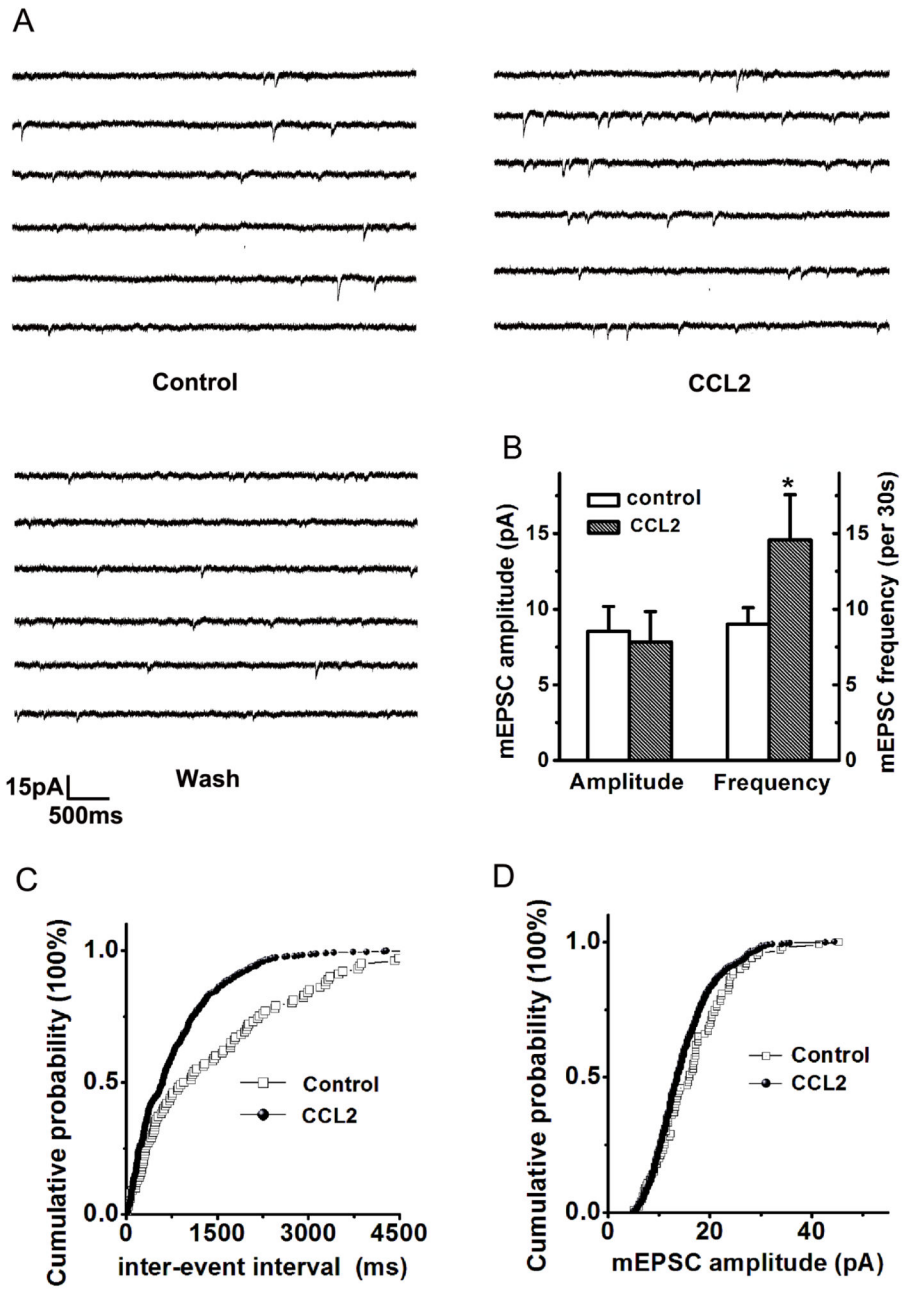

Figure 3. CCL2 Enhancement of EPSCs via a presynaptic mechanism

Panel A shows the example traces of spontaneous mEPSCs recorded from a neuronal cell in the CA1 region of a hippocampal slice. Bath application of CCL2 significantly increased frequency of spontaneous mEPSCs without apparent effect on the amplitude (Panel B, n=9). Panel C exhibits cumulative distribution of mEPSCs inter-event interval (IEI) showing that CCL2 significantly decreased the IEI, indicating a significant increase in mEPSC frequency during bath application of CCL2 ($p<0.05$ *vs* control, n=9). A representative amplitude cumulative histogram is shown in panel D, showing no significant change (Kolmogorov-Smirnov-test) on mEPSC amplitude during bath application of CCL2.

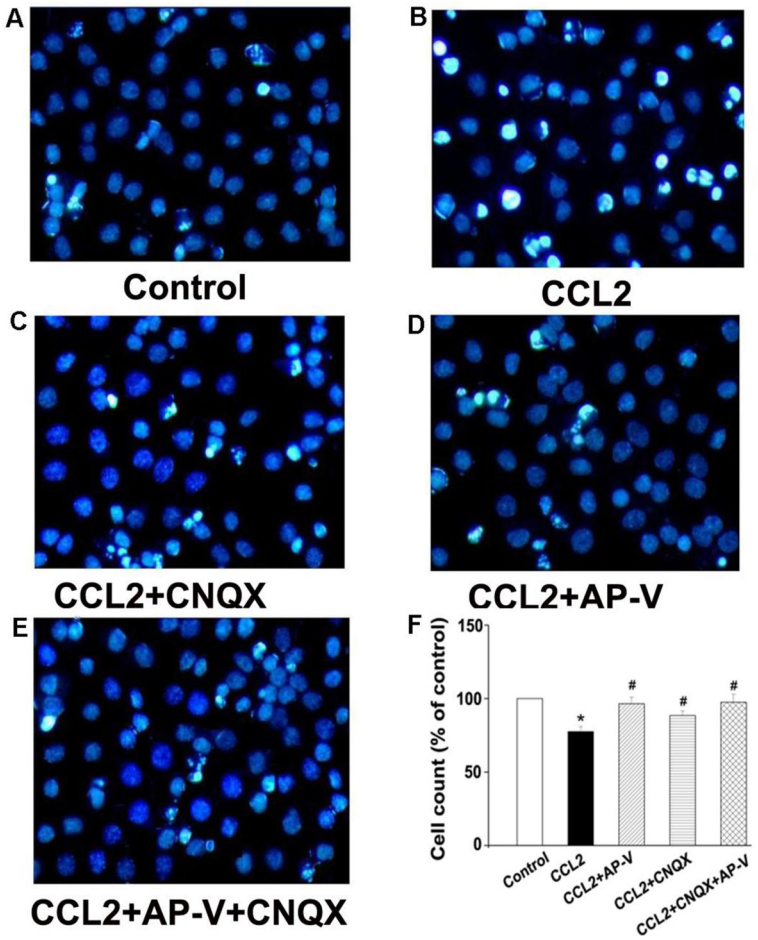

Figure 4. Attenuation of CCL2-induced neuronal injury by Hoechst staining

Panels A–E are primary hippocampal neurons untreated (control) or incubated with CCL2, CCL2+CNQX, CCL2+AP-V, or CCL2+AP-V+CNQX as indicated. The injured cells were quantified after staining with Hoechst 33342. Survival rates were calculated by counting Hoechst-stained cells (bright blue) and total cells from five different visual fields in each dish contained cultured neurons are shown in panel F. CCL2 significantly decreased neuronal survival rates and the CCL2-induced reduction of survival rates were blocked by a NMDA receptor antagonist AP-V and by an AMPA receptor blocker CNQX as well. Addition of CNQX did not further improve the neuronal survival rates under existence of CCL2 and AP-V in the culture media, suggesting the CCL2-induced neuronal injury was largely mediated via NMDA receptors. $^{*}p<0.05$ *vs* control, $^{\#}p<0.05$ *vs* CCL2. Experiments were done in three triplicates. Objective magnification: 40×

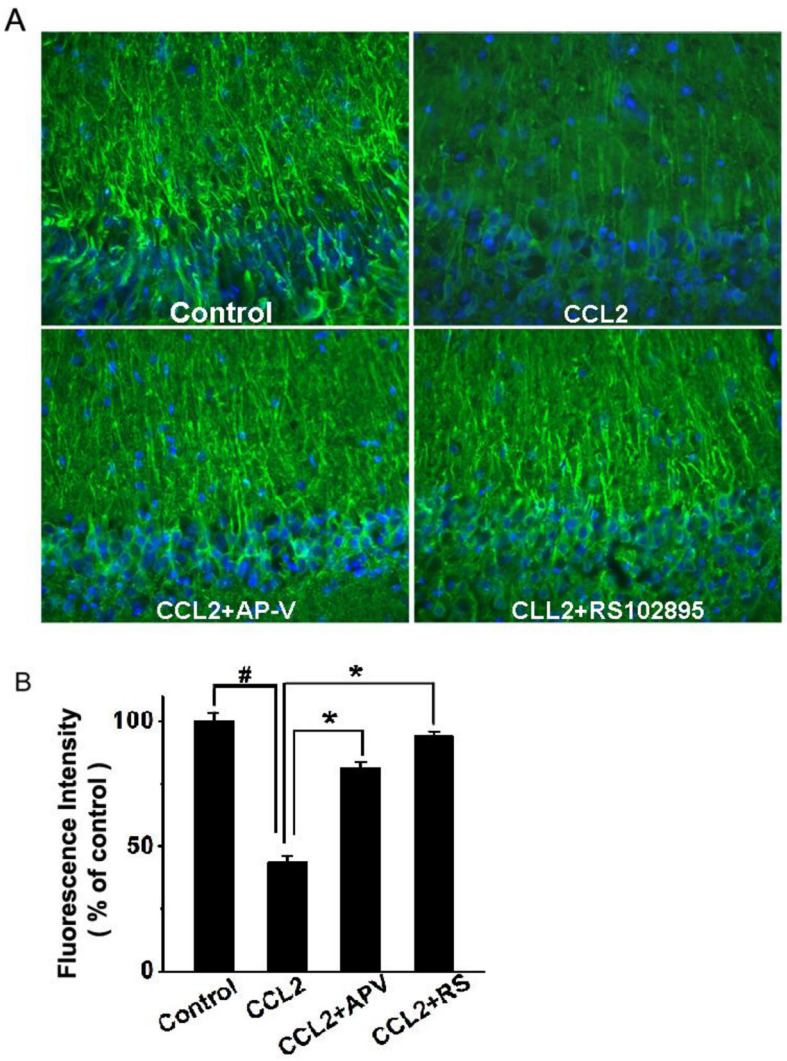

Figure 5. Attenuation of CCL2-induced dendritic injury in the CA1 region of hippocampal slices as revealed by MAP-2 staining

Panel A shows the representative sections of the CA1 region immunostained with antibodies to MAP-2 in different experimental conditions as indicated. The same region of the CA1 was selected in all samples. MAP-2 expression was reduced in the CCL2-treated group (CCL2) as compared with the untreated group (control). The CCL2-induced reduction of MAP-2 expression was attenuated by either a NMDA receptor antagonist AP-V (CCL2+AP-V), or a CCR2 receptor antagonist RS102895 (CCL2+RS102895), demonstrating that CCL2 induces dendritic injury via NMDA receptor and CCR2 receptor which are expressed in the hippocampus. Quantification of fluorescence intensities using ImageJ software is shown in panel B. Note that CCL2 significantly reduced the fluorescence intensity and such a reduction was significantly attenuated by AP-V (CCL2+AP-V) or RS102895 (CCL2+RS). Values are expressed as mean±SE from five independent experiments. # $p < 0.05$ vs control, * $p < 0.05$ vs CCL2 group. Objective magnification: 20×