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Ethanol-activated microglial exosomes induce MCP1 signaling mediated death of stress-regulatory proopiomelanocortin neurons in the developing hypothalamus

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Abstract

Background Microglia, a type of resident immune cells within the central nervous system, have been implicated in ethanol-activated neuronal death of the stress regulatory proopiomelanocortin (POMC) neuron-producing β-endorphin peptides in the hypothalamus in a postnatal rat model of fetal alcohol spectrum disorders. We determined if microglial extracellular vesicles (exosomes) are involved in the ethanol-induced neuronal death of the β-endorphin neuron via secreting elevated levels of the chemokine monocyte chemoattractant protein 1 (MCP1), a key regulator of neuroinflammation.

Methods We employed an in vitro model, consisting of primary culture of hypothalamic microglia prepared from postnatal day 2 (PND2) rat hypothalami and treated with or without 50 mM ethanol for 24 h, and an in vivo animal model in which microglia were obtained from hypothalami of PND6 rats fed daily with 2.5 mg/kg ethanol or control milk formula for five days prior to use. Exosomes were extracted and characterized with nanosight tracking analysis (NTA), transmission electron microscopy and western blot. Chemokine multiplex immunoassay and ELISA were used for quantitative estimation of MCP1 level. Neurotoxic ability of exosome was tested using primary cultures of β-endorphin neurons and employing nucleosome assay and immunocytochemistry. Elevated plus maze, open field and restraint tests were used to assess anxiety-related behaviors.

Results Ethanol elevated MCP1 levels in microglial exosomes both in vitro and in vivo models. Ethanol-activated microglial exosomes when introduced into primary cultures of *β*-endorphin neurons, increased cellular levels of MCP1 and the chemokine receptor CCR2 related signaling molecules including inflammatory cytokines and apoptotic genes as well as apoptotic death of β-endorphin neurons. These effects of microglial exosomes on β-endorphin neurons were suppressed by a CCR2 antagonist RS504393. Furthermore, RS504393 when injected in postnatal rats prior to feeding with ethanol it reduced alcohol-induced β-endorphin neuronal death in the hypothalamus. RS504393 also suppressed corticosterone response to stress and anxiety-like behaviors in postnatally alcohol-fed rats during adult period.

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Conclusion These data suggest that alcohol exposures during the developmental period elevates MCP1 levels in microglial exosomes that promote MCP1/CCR2 signaling to increase the apoptosis of β-endorphin neurons and resulting in hormonal and behavioral stress responses.

Keywords Developmental alcohol exposure, Fetal alcohol spectrum disorder, Exosomes, MCP1, CCR2, Microglia, Proopiomelanocortin neuron, Neuronal apoptosis

Background

Fetal alcohol spectrum disorders (FASD) represent the spectrum of defects that includes physical, mental, and behavioral abnormalities. FASD affect around 2–5% of the USA population with an estimated economic burden of 5 billion annually [1, 2]. One of the neuropathological abnormalities of FASD is the loss of stress regulatory proopiomelanocortin (POMC; producing β-endorphin peptide) neurons resulting corticosterone hyper-response to stress and increased anxiety-like behaviors [3–6].

Microglia are the resident immune cells in brain that plays essential role in neuronal development and synaptic pruning while aberrant microglial proliferation and activation are hallmark of various neuroinflammatory and neurodegenerative diseases including FASD [4, 7, 8]. Ethanol-induced microglial activation and neuroinflammation has been considered responsible for β-endorphin neuronal death, however the molecular mechanism of the process remains unclear [4, 5, 9]. Monocyte chemoattractant protein 1 (MCP1), also known as chemokine (CC motif) ligand 2 (CCL2) is primarily expressed by the microglia and reported to be involved in the neuroinflammatory pathway [10, 11]. Human postmortem brains from alcoholics have also indicated higher MCP1 concentration in ventral tegmental area (VTA), substantia nigra (SN), hippocampus and amygdala [12]. C-C motif chemokine receptor 2 (CCR2), a receptor for MCP1, has been found to express by the neurons in cerebral cortex, caudate putamen, globus pallidus, and supraoptic hypothalamic nuclei, amygdala, in the brainstem and cerebellum of adult rats [13]. Moreover, the mice lacking CCR2 have been found to be protected from the detrimental effect of MCP1 overexpression [14, 15]. The MCP1-CCR2 signaling is shown to play an important role in neuroinflammation and have been reported to induce neuronal apoptosis in epileptic brain of mice [16]. In addition, inhibition of MCP1-CCR2 signaling with an pharmacological blocker, RS504393, has been reported to attenuate neuronal apoptosis and alleviate neuroinflammation in subarachnoid hemorrhage (SAH) mice model [17]. These data suggest that MCP1-CCR2 signaling is important in the control of neuronal apoptosis within the central nervous system (CNS).Previous studies have connected microglial derived chemokines, complements and miRNAs in neurotoxicity in various neuroinflammatory diseases [8]. Proteomic analysis of microglia-derived exosomes has identified complement protein, C1a elevated with ethanol exposure which induce death of β-endorphin neurons involving C1q-C3-C4-membrane attack complex [18]. Whether PAE activates chemokine; MCP1 production within microglial exosomes to induce β-endorphin neuronal death is not known and yet to be investigated.

Recently, number of studies have shown that inflammatory cytokines and chemokines are released from microglia packaged in small extracellular vesicles [18, 19]. One of the most studied extracellular vesicles are exosomes [20, 21]. Exosomes are 30 nm to 100 nm lipid bilayer vesicles secreted by the diverse cell types to mediate variety of cellular function including the neuroinflammatory responses within the central nervous system (CNS) [22]. It has been proposed that the exosomes may contribute to the neuron-glia communication [8, 23]. In this study, we determined whether PAE induces apoptosis of β-endorphin neurons and altered stress response through the release of exosomes containing chemokine MCP1 from microglia. We show that PAE causes microglia to release exosomes with elevated level of MCP1 and induces MCP1/CCR2 signaling mediated death of β-endorphin neurons. Additionally, we demonstrate that MCP1-CCR2 antagonist RS504393 prevents β-endorphin neuronal apoptosis, stress hyperresponse and anxiety-like behaviors in PAE rats.

Materials and methods

Animals

Adult Sprague Dawley rats were procured from Charles River Laboratories. All animals were kept under standard lighting conditions (12 h lights on; 12 h lights off) and fed with rodent chow and water ad libitum. Adult animals were bred to generate neonatal animals, which were used in this study. Animal care and treatment were performed in accordance with institutional guidelines, and protocols were approved by the Rutgers Institutional Animal Care and Facilities Committee (approval number 999900286) and complied with National Institutes of Health policy.

In-vivo studies

Postnatal rat pups (PND2; both sexes) were fed by gavage a milk formula containing 11.34% ethanol (v/v; 0.1–0.2 ml/animal; during a period of 1 min), yielding a total daily ethanol dose of 2.5 g/kg (AF), or isocaloric control (PF), or they were left in the litter with their mother (AD) as described by us previously [5, 18, 24]. Gavage feeding

was conducted at 10:00 A.M. and 12:00 P.M. from PND2 to PND6. After feeding, these pups were immediately returned to the litter. RS504393 (Abcam, USA) treatment was given subcutaneously (1 mg/kg; 1 h before the first feeding) daily between PND2-6 [11]. Two hours after the last feeding on PND6, pups (both sexes) were sacrificed and brain tissues were collected and fixed with 4% PFA (2 h), cryoprotected in 30% sucrose (overnight), and cut into 30 µm coronal sections for immunohistochemistry. Another set of pups (with and without RS504393 treatment) were kept with the litters until PND21, weaned, and kept in sex specific cages until PND60. These animals were then used for stress response followed by behavioral studies. Following the behavioral studies these animals were transcardially perfused with ice cold 1X PBS, brains were collected, fixed, cryo-protected in 30% sucrose and cut into 30 µm coronal sections for immunohistochemistry.

Primary microglia culture

Microglia cell culture was prepared from hypothalami of PND 2 male and female rat pups using the method published by us previously [4, 24]. Cells were maintained in DMEM/F12 media with 10%FBS and an antibiotic solution (100 U/ml penicillin, 100 mg/ml streptomycin, and 250 ng/ml amphotericin B). After 12 days, a rotary shaker was used to suspend the cells (175 rpm for 2 h), and the suspended cells (1×10^6 cells/flask) were then plated on poly-ornithine coated T25 flask and fed with DMEM/F12 media with 10% FBS. The purity of the microglial cell culture was confirmed by IBA-1+staining. Prior to alcohol treatment, microglial cells were fed with DMEM/F12 containing serum supplement (30 nM selenium, 20 nM progesterone, 1 µM iron-free human transferrin, 100 µM putrescine, and 5 µg/ml insulin). To assess the effect of acute alcohol treatment on microglia cell cultures, microglia cells were treated with 50 mM ethanol (ETOH) for 24 h. The conditioning medium was harvested for exosome isolation.

Primary POMC neuronal culture

Primary cultures of POMC (β-endorphin) neurons were prepared using the methods described by us previously [18, 25]. Pregnant rats of the Sprague Dawley strain at 16–18 d of gestation were sacrificed, and the fetuses were removed by aseptic surgical procedure. Hypothalami from the brains of these fetuses were dissected out and placed in ice-cold Dulbecco′s Modified Eagle′s Medium - high glucose (HDMEM) media containing an antibiotic solution, 0.1% BSA, HEPES (15 mM) and Sodium Bicarbonate (44 mM) (all from Sigma Millipore). The hypothalamic were washed with HDMEM media at room temperature and mechanically dissociated with 18-gauge sterile needle 3–4 times and strained through 70 µM nylon mesh. The cell suspension was seeded to poly-ornithine (100 µg/ml) coated T-75 flask in HDMEM media with 10% FBS. Media was changed after 48 h, and flask was further incubated for 24 h at 37°C with 7.5% CO2. On day 4, the medium was replaced with HDMEM-containing 10% FBS, 33.6 mg/ml uridine (Sigma Millipore) to reduce the growth of glial cells. On day 5, the culture medium was replaced with HDMEM containing serum supplement with antibiotics. Cells were maintained for the next 2 d with this medium. Cells were passaged three times until the third week when neurosheres started to develop. Cells were then trypsinized and cultured for two weeks in neuroshere formation media (DMEM-F-12, LIF, 0.1 mg/ml; L-glutamine, 10 mM; rat bFGF, 20 ng/ml; MEM amino acid solution, 0.5%; all the chemicals were from Sigma Millipore, except bFGF, which was obtained from R&D Systems). These neurospheres were then differentiated by treating them for two weeks with pituitary adenylate cyclase activating peptide (10 µM; SynPep) and dibutyryl cAMP (10 µM; Sigma Millipore). The differentiated cells were stained for POMC-derived peptide β-endorphin.

Exosome isolation from primary microglial cell culture medium

Exosome isolation from microglial cells was carried out using a procedure described by us previously [18]. Microglial cell culture media was collected after the treatment and centrifuged at 2000 g for 30 min to remove cells and debris. The supernatant containing the cell-free culture media was then transferred to a new tube and passed through a 0.2 µm syringe filter. Cell-free culture media were further centrifuged again at 300 g and 3000 g for 10 min and then at 10,000 g for 30 min. The exosome isolation reagent (500 ul) from Invitrogen (Catalog #4478359) was then added to 1 ml of cell-free culture media. This mixture was thoroughly mixed by vertexing and pipetting until became a homogeneous solution. The tubes were then incubated at 4 °C overnight. Next day, the tubes were centrifuged at 10,000 g for 1 h at 4 °C. The supernatant was discarded from the tube. The exosomes pellet was re-suspended in 100 µl of 1XPBS and used for biochemical measurements.

Exosome preparation from mediobasal hypothalamus (MBH) microglia

Microglial cells were prepared from mediobasal hypothalami of three neonatal pups using Optiprep density gradient method [24, 26]. The purified microglia were resuspended in DMEM/F12 media (1 ml) and gentle triturated multiple times. Gentle trituration of purified microglia release exosomes in the extracellular space. Cells were pelleted by centrifugation at 2000 g for 15 min

and the supernatant was used for the exosome isolation as described previously [18].

Nanosight tracking analysis (NTA) of exosomes

The Nanosight LM10 was used to analyze the size distribution and concentration of exosomes as described previously [18]. The exosome samples were diluted at 1/1000 with PBS and were injected in the sample chamber with 1 ml sterile syringes (BD, USA) until the liquid reached the tip of the nozzle. This unique technology utilizes the properties of both light scattering and Brownian motion to obtain the size distribution and concentration measurement of particles in liquid suspension. A laser beam is passed through the sample chamber, and the particles in suspension in the path of this beam scatter light in such a manner that they can easily be visualized via a 20 X magnification microscope onto which a camera is mounted. The camera operates at 30 frames per second, capturing a video file of the particles moving under Brownian motion. The software tracks many particles individually and, using the Stokes-Einstein equation, calculates their hydrodynamic diameters. All measurements were performed at room temperature.

Western blot analysis for MCPIP1 protein level

The exosomes lysate (20 µg) and β-endorphin neuronal lysate (25 µg) samples prepared in RIPA buffer (Sigma) were separated by 4-20% SDS-PAGE and transferred overnight to immobilon-P PVDF membranes. Membranes were blocked with 5% milk for 1 h and incubated with primary antibody for Tsg101 (1:1000; ab125011, Abcam), Alix (1:1000; ab275377, Abcam), Iba1 (1:1000; ab178846, Abcam), Cytochrome C (1:1000, A4912, ABclonal Technology), Calnexin (1:1000, PA5-34754, Invitrogen), GM130 (1:1000, A11408, ABclonal Technology) and MCPIP1 (1:1000,ab97910, Abcam) for 18 h at 4 °C in blocking buffer. Membranes were then washed and incubated with peroxidase-conjugated secondary antibody (1: 5000) for 1 h. Afterward membranes were washed and then incubated with ECL Western blot chemiluminescence reagent (Pierce). The membrane was imaged with iBright 1500 (Invitrogen).

Transmission electron microscopy

Exosome samples were prepared for inspection as described previously [18]Briefly, exosomes were mounted on copper grids (FCF400-Cu-50 Formvar/Carbon 400 Mesh, Cu from Electron Microscopy Sciences) and then fixed by 1% glutaraldehyde in cold DPBS for 5 min to stabilize the immunoreaction. It was then washed in sterile distilled water and contrasted with uranyl-oxalate solution at pH 7 for 5 min. Finally, it was embedded by methyl cellulose-UA for 10 min on ice. A JEOL 1010 TEM was used to image exosome samples at a voltage of 80 kV.

Chemokine multiplex immunoassay

Exosome pellet were resuspended in ProcartaPlex™ Cell Lysis Buffer (EPX-99999-000) and used for ProcartaPlex multiplex immunoassay using Luminex® xMAP® (multianalyte profiling) technology. The Rat Chemokine 8-Plex ProcartaPlex Panel (EPX080-30121-901) enables the study of the immune response by analyzing 8 protein targets in a single well. Protocols described by the manufacturer were followed and data were analyzed with the LuminexTM instrument (MAGPIX® xPONENT®, Life Technologies Corporation, Grand Island, New York, NY, USA). Median fluorescence index (MFI) and presented.

MCP1 quantification with ELISA

Rat JE/MCP-1 DuoSet ELISA (R&D Biosystems, Minneapolis, MN, USA) was used to estimate MCP1 levels in exosome lysate prepared from primary microglia cells and microglia isolated from MBH tissue. Briefly, exosome pellet was lysed in ProcartaPlex™ Cell Lysis Buffer (EPX-99999-000) and supernatant (20 µl) was used for ELISA analysis for MCP1 as per the manufacturer instructions.

Nucleosome assay for neuronal apoptosis

β-Endorphin cells were cultured (1×10^4/well) in a 24-well plate in a neuronal medium. The cells were then exposed to medium containing exosomes ($\sim1\times10^7$ particles/well) from microglia (treated with or without 50 mM ethanol +/- 10 µM RS504393) for a period of 24 h. For in-vitro study, we evaluated toxicity of RS504393 on β -endorphin neurons for 10 µM, 20 µM, 50 µM and 100 µM (data not provided). We found that 10 µM of RS504393 cause no toxicity and death of β -endorphin neurons and therefore selected 10 µM of RS504393 as dose for in-vitro studies. The cells were then lysed with nucleosome lysis buffer and used for nucleosome assay using the ELISA kit (Sigma Millipore, catalog #11774425001) for determination of β-endorphin neuronal apoptosis.

qPCR

Real-time RT-PCR was carried out in 384-well plates in a final volume of 10 µL with 1:20 dilution of cDNA (25 ng/µL) in Power SYBR® Green PCR Master Mix (Applied Biosystems™) using the comparative CT (ΔΔCT) standard run on a the QuantStudio™ 7 Flex Real-Time PCR System (Applied Biosystems™, Wilmington, DL). The primers were designed using Primer 3 (https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). Data were analyzed using QuantStudio™ Real-Time PCR (Applied Biosystems™, Wilmington, DL) software to calculate relative quantification (RQ values). The primers used are listed in Supplementary Table S1; three genes were used as housekeeping control genes: GAPDH, ATCB, and HPRT1.

Immunofluorescence for β-endorphin of primary neuronal cultures

Neurosheres (1×10^4) were seeded and differentiated to β-endorphin neurons in 8-well chamber slides. Two weeks after differentiation, neurons were added with exosomes ($\sim1\times10^7$ particles/well) isolated from microglia and activated with or without ethanol or vehicle and with and without pre-treatment (3 h prior to exosome addition) with RS504393 (10 µm/ml) for a period of 24 h. The cells were then washed twice with 1X PBS and fixed for 15 min with 4% paraformaldehyde, washed and permeabilized for 10 min with 1X PBS with 0.2% Triton X-100, washed and blocked for 1 h at room temperature with 5% BSA in 1X PBS with 0.05% Tween-20. Cells were then incubated overnight at 4°C with the rabbit anti-β-endorphin in 1% BSA in PBS-T (0.05% Tween-20) (1:1000; Peninsula Laboratories). Next days, cells were washed three times with 1X PBS and incubated for 1 h with anti-rabbit Alexa-488 secondary antibody (1:500, Abcam). Cells were then with DAPI (1:1000) for 5 min, washed with 1X PBS and mounted with ProLong™ Gold Antifade Mountant (Thermofisher Scientific, USA). Fluorescent images were captured with EVOS M5000 Imaging System (Invitrogen, USA). Fluorescent intensity was measured using ImageJ software (National Institutes of Health).

Immunohistochemistry localization of *β*-endorphin neurons in tissue sections

Serial coronal sections of OCT embedded brains (PND6 and PND60-PND80) were made using a Leica cryostat at 30 µm in thickness from stereotaxic plates 19 to plates 23 (bregma-2.3 to -4.3 mm) spanning the arcuate nucleus. These sections were mounted on Superfrost Plus glass slides (VWR). The sections were washed in PBS twice followed incubation in blocking buffer (2.5% normal horse serum in PBS with 0.4% Triton X-100) at room temperature for 1 h. The sections were subsequently washed in PBST (0.05% Triton X-100) and incubated overnight at 4 °C with the rabbit anti-β-endorphin (1:1000; Peninsula Laboratories). After the primary antibody incubation, samples were washed in PBST, and then sections were incubated with an AlexaFluor-488 donkey anti-rabbit secondary antibody (1:500; Thermo Fisher Scientific). Sections were stained with DAPI (1:1000) for 5 min, washed with 1XPBS and mounted with ProLong™ Gold Antifade Mountant. These sections were then imaged with EVOS M5000 Imaging System. Cell counting was performed using ImageJ software (National Institutes of Health).

Corticosterone response to restraint stress

Corticosterone response to restraint stress at various time points were determined in male and female rats (on day of diestrus) at PND 60–80. Animals were restrained by placing them in a transparent plexiglass restrainer for 1 h (between 12:00 and 14:00 h). Time-dependent changes of cortisol before (0 min), during (30 min, 60 min), and after restraint (120 min) were determined. Female rats were restrained on the day of diestrus to avoid the influence of the fluctuating levels of steroid on the corticosterone response. Plasma corticosterone levels were measured using corticosterone ELISA kit (Enzo Life Sciences, Farmingdale, NY, USA) following manufacturer's instructions.

Elevated plus maze

Elevated plus maze (EPM) test was conducted to assess anxiety-related behaviors as described previously [27, 28]. The EPM consisted of four arms (50 cm L × 10 cm W), two arms closed by non-transparent walls (~40 cm high) and two open arms without walls that were joined by a central square (10 cm × 10 cm). Rats were transported to the behavioral testing room under cover 1 h prior to testing to allow acclimation to the testing environment. Rats placed on the junction of the open and closed arms were allowed to freely explore the maze for 300 s. The time spent in the open and closed arms was recorded using ANY-Maze video tracking software. Proper care was taken to avoid any sudden noise or disturbance. The apparatus was cleaned with 75% ethanol after each trial.

Open field test

Open field test was conducted to determine exploratory and anxiety-related behaviors in the adult rats as described by us previously [28]. The open field comprises of a black plastic board measuring 90 cm (L) × 90 cm (W) × 40 cm (H) surrounded by black plastic walls with a height of 50 cm. Rats were transported to the room under cover 1 h prior to testing to allow acclimation to the testing environment. The rats were placed in the arena and allowed to explore it for 300 s. The movements of animals were recorded by ANY-Maze video tracking software and stored on a computer. Proper care was taken to avoid any sudden noise or disturbance. The apparatus was cleaned with 75% ethanol after each trial.

Statistical analysis

Results are expressed as mean ± SEM ($n=6$–8). *t*-test was used to analyze the differences between two groups, and one-way ANOVA with Newman Keuls post hoc analysis was used to analyze the differences between multiple groups. Two-way analysis of variance with Tukey's multiple comparison post hoc test was used when two variables were compared. $p<0.05$ was considered significant. Data were analyzed using Prism 9.0 (GraphPad Software).

Results

Ethanol activated microglia release exosomes with elevated level of MCP1/CCL2

Exosomes isolated from hypothalamic microglia grown in primary culture and treated with or without 50 mM ethanol or prepared from hypothalami of neonatal rats fed with control (AD and PF) or ethanol diets (AF) were analyzed with NTA, TEM and western blotting for initial characterization. In consistent with our previous findings [18]. NTA data of exosomes prepared from microglia in primary cultures show particle sizes between 50 and 80 nm (Fig. 1A, B), a size range defined for exosome [29]. Also, ethanol treatment did not change the mean particle size but elevated the particle concentration of exosomes in primary microglia cultures. As shown previously [18], NTA analysis revealed the exosome prepared from neonatal hypothalamic microglia of AD, PF and AF rats had particle size range between 57 and 74 nM. Microglial exosome collected from AF rats MBH showed higher particle concentrations than those in MBH of PF and AD rats. Determination of exosome concentration prepared from the primary microglia culture as well as from neonatal hypothalamic microglia of AD, AF and AF revealed thatethanol treatments cause primary microglia in culture to release a higher number of exosomes (Fig. 1C). Furthermore, alcohol-fed male neonatal rats show elevated

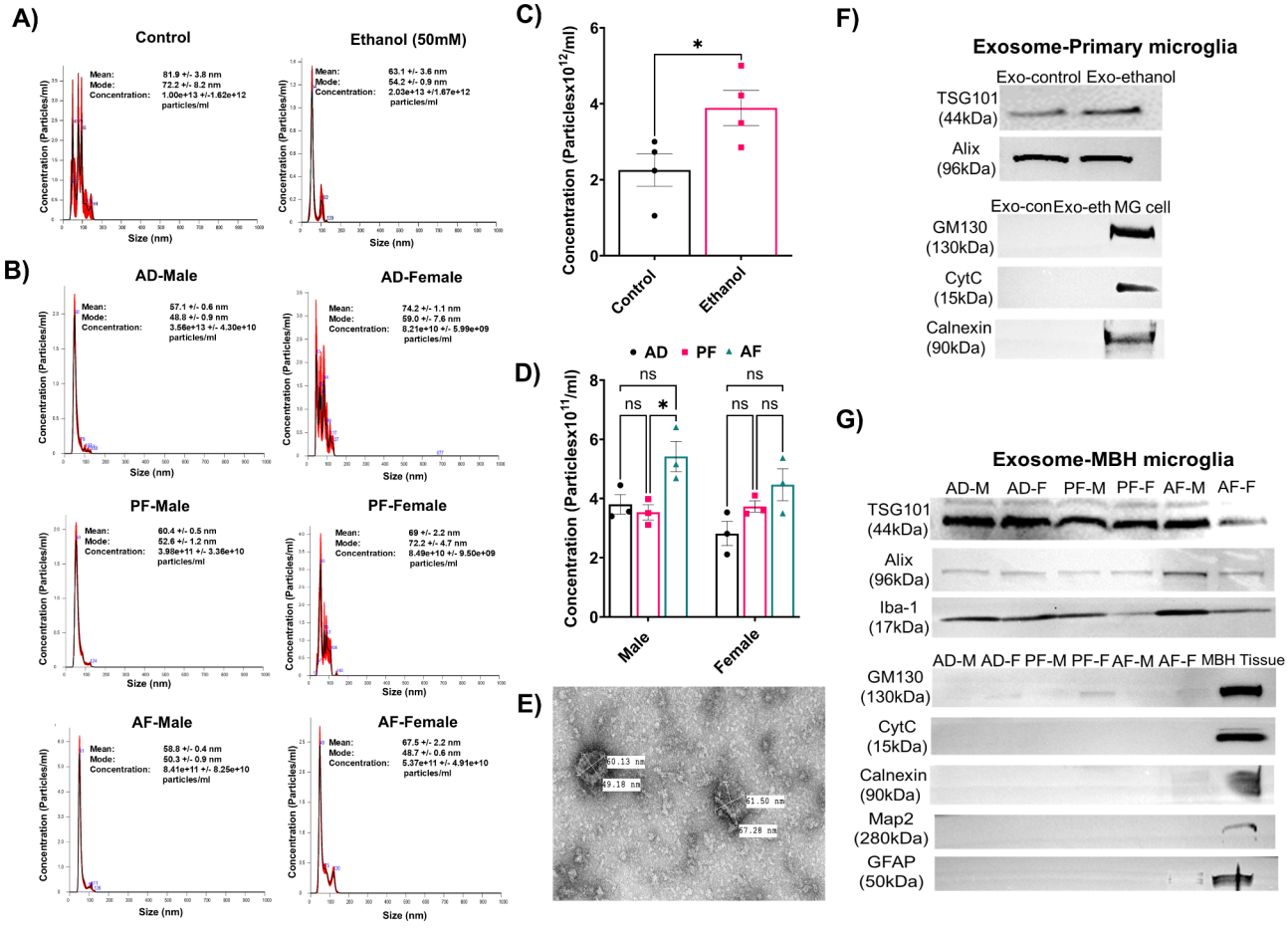

Fig. 1 Characterization of exosomes derived from microglia treated with or without ethanol. Nanoparticle tracking analysis **(A, B)**, Mean ± SEM data of exosome concentration **(C, D)**, Transmission electron microscopy **(E)**, and Western blot verification **(F)** of exosomes prepared from media of hypothalamic microglial cultures treated with vehicle (Control) or 50 mM ethanol (Ethanol) for 24 h, or **(G)** from microglia of mediobasal hypothalamus (MBH) tissue obtained from PND 6 male or female rats, which were fed daily from PND 2–6 with ethanol-containing milk formula (AF), pair-fed isocaloric milk formula (PF), or left undisturbed in the litter with mother (AD). For nanoparticle tracking analysis, the calculated size distribution is depicted as a mean (black line) with SE (red shaded area). Mean particle size, mode particle size, and concentration of particles in exosomes are shown in each tracking analysis graphs. Ethanol -induced changes in exosome concentration in primary microglia in culture and MBH microglia in tissue preparation are shown in panel **C** and **D**, respectively.Representative images of exosomes from MBH microglia of AD rats under TEM **(E)**. The width and length of each exosome are represented on the individual exosome. Representative bands for the protein levels of exosome proteins TSG101 and Alix, mitochondria marker cytochrome c, Golgi apparatus marker GM130, and endoplasmic reticulum marker calnexin in exosomes from primary microglia and MBH microglia as determined by Western blot analysis **(F, G)**. Data shown in Fig. 1C are mean ± SEM ($N = 4$) and were analyzed with unpaired t-test while the data shown in Fig. 1D are mean ± SEM ($N = 3$) and were analyzed using two-way analysis of variance with Tukey's multiple comparison post hoc test. *, $p < 0.05$; **, $p < 0.01$

number of exosomes but alcohol-fed female neonatal rats don't show significant difference in number of exosome particles (Fig. 2D). TEM data confirm that microglial exosome had smaller particle size (~49–67 nM) (Fig. 1E). Western blots data of exosome from primary microglia and MBH microglia detected the positivity for exosome markers Tsg101 and Alix, while negativity for Golgi marker GM130, mitochondrial marker Cytochrome C and ER marker Calnexin, neuronal marker MAP2 and astroglia cell marker GFAP (Fig. 1F, G). Immunoblotting data also show positivity for microglial marker Iba1 for exosomes prepared from MBH microglia (Fig. 1G).

Rat Chemokine 8-Plex ProcartaPlex Panel was used to measure the changes in the levels of various chemokines in exosomes released from primary microglial cultures following 50 mM ethanol treatment for a period of 24 h. The results indicate ethanol treatment significantly elevated the level of interferon-gamma-induced protein 10 (IP-10), monocyte chemoattractant protein-1 (MCP-1/CCL2), monocyte-chemotactic protein 3 (MCP3) and macrophage inflammatory protein-1 alpha (MIP-1α/CCL3) in microglial exosomes (Fig. 2A). Since MCP1 roles in alcohol effects has been our primary interest, we validated our Chemokine-Plex data using ELISA assay. Results show that development exposure to ethanol induces hypothalamic microglia to release exosomes with elevated level of MCP1 both in primary cultures (Fig. 2B) and in postnatal male and female rats in vivo (Fig. 2C). We did not find any significant sex differences in exosome MCP1 level in response to ethanol [Interaction: $F(2, 6)=1.152$; $p=0.3771$].

Ethanol activated microglial exosomes increases β-endorphin neuronal apoptosis in vitro

Next, we determined if MCP1 participates in the apoptotic action of ethanol-activated microglial exosomes on β-endorphin neuronal cells in primary cultures. The involvement of MCP1 in ethanol's apoptotic action was determined by evaluating the ethanol-activated microglial exosomes' effects on β-endorphin neuronal apoptosis in the presence and absence of a MCP1 blocker RS504393 which acts on CCR2 receptor. Changes in nucleosome levels as a measure of apoptotic cellular death and immunofluorescence intensity of β-endorphin were used to measure neuronal apoptosis. As shown in Fig. 3A, ethanol-activated microglial exosomes significantly elevated nucleosome levels in β-endorphin neurons in culture which was prevented by pretreatment with RS504393. Figure 3B and C demonstrate that ethanol activated exosome reduces the β-endorphin neuronal cell number in cultures as demonstrated by fluorescence intensity measurements. RS504393 pretreatment prevented ethanol activated exosome effects on β-endorphin neuronal cell numbers. These data provide in vitro evidence for a role of exosome-derived MCP1 in ethanol induced killing of β-endorphin neurons.

Since our in vitro studies identified a role of MCP1 in alcohol apoptotic action on β-endorphin neurons, we determined if the MCP1-CCR2 blocker RS504393 is effective in preventing postnatal alcohol effects on β-endorphin neurons. Data show that postnatal alcohol-fed rats (AF) had lower number of β-endorphin neurons in the hypothalamus of both sexes as compared to those in control-fed (AD and PF) rats on 6 days after birth (Fig. 4A-C). The pretreatment of RS504393 prior to alcohol feeding was able to prevent postnatal alcohol effects on lowering the number of β-endorphin neurons in developing hypothalamus. We did not find any significant difference between the sexes in terms of the effect of alcohol with or without RS504393 on the of number of β-endorphin neurons [Interaction: $F(5, 84)=0.5201$, $p=0.76$].

We also tested if the prenatal alcohol effects with or without RS504393 on β-endorphin neurons persist during the adult period. Data show that, like in postnatal rats, adult rats which were given alcohol-diet during the early postnatal period, show reduced number of β-endorphin neurons in the hypothalamus as compared to those in AD and PF rats (Fig. 4D-F). Pretreatment of RS504393 prior to alcohol feeding was able to prevent postnatal alcohol effects on lowering the number of β-endorphin neurons in hypothalamus. There were no sex differences between the effect of alcohol with or without RS504393 on the of number of β-endorphin neurons [Interaction: $F(5, 84)=0.8600$, $p=0.5115$]. These data suggest that activation of MCP1-CCR2 signaling might be responsible for the reduced number of β-endorphin neurons in alcohol fed pups.

MCP1/CCR2 activates apoptotic pathway in β-endorphin neurons in vitro

Next, we determined the changes in the levels of various genes known to be involved in neuronal apoptosis *in* β-endorphin neurons in primary cultures when they were challenged with ethanol-activated microglial exosomes. Gene expression analyses indicate that ethanol-activated microglial exosome induces apoptotic genes (*Caspase 3*, *Caspase 8* and *Bax*) in *β*-endorphin neurons (Fig. 5A). The expression of inflammatory genes, *IL-1β*, *IL6* and *Tnf-β* along with *CCL2* (*MCP1*) was also elevated in β-endorphin neurons when they were exposed to ethanol activated microglial exosomes (Fig. 5B). The expression of important transcriptional genes (*MCPIP1* and signaling molecules (*Gsk3β*, *Myd88*, *NFkβ1*, *Stat3*, *MAPK1*) known to control cellular apoptosis and inflammation were also determined. We found that the *MCPIP1* gene level was only increased in *β*-endorphin neurons when ethanol activated microglial exosomes were added (Fig. 5C).

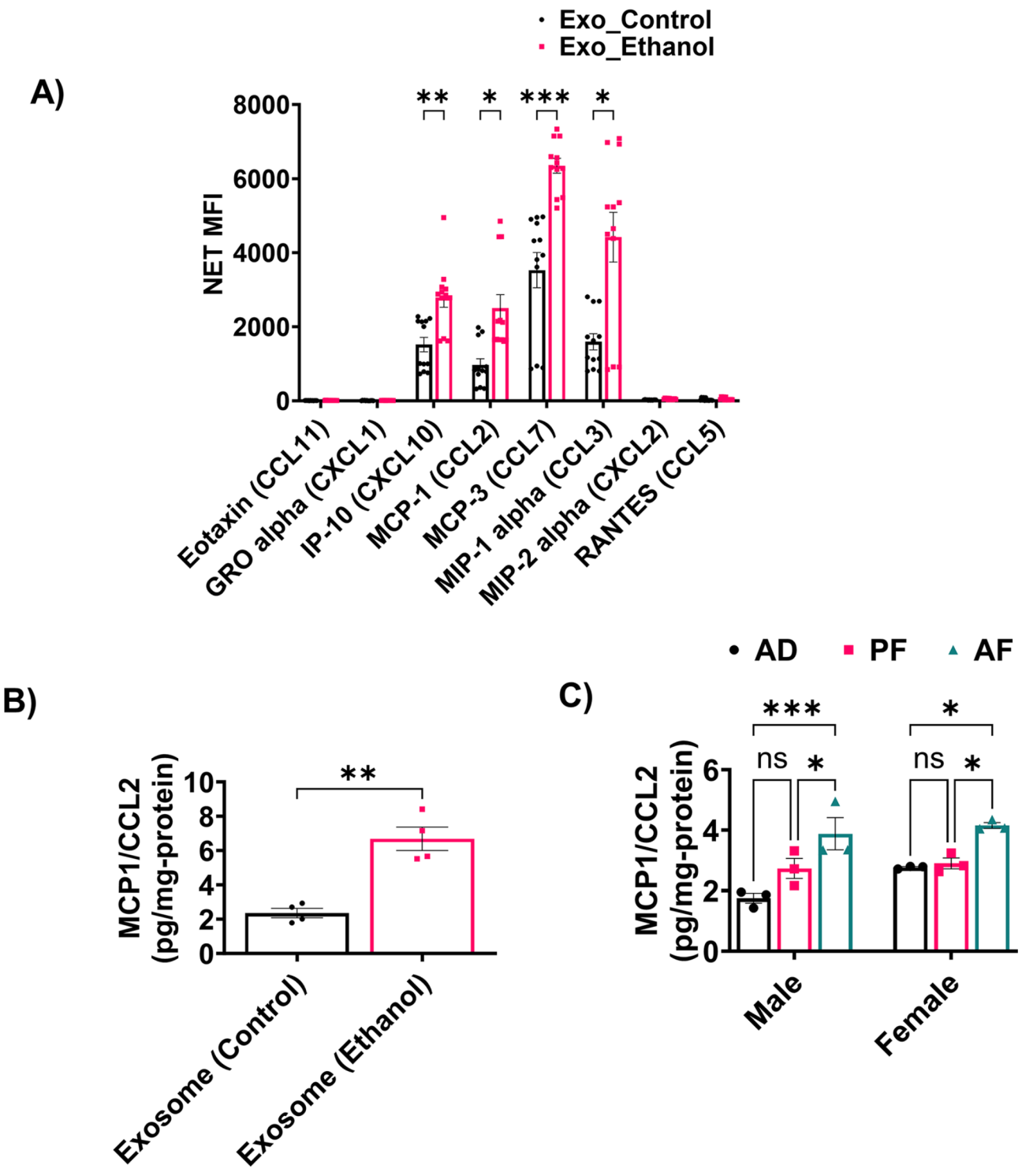

Fig. 2 Chemokine profile of exosomes derived from microglia treated with or without 50 mM ethanol. Rat Chemokine 8-Plex assay was used to determine the percentage changes of various chemokines in exosomes released from microglial cells in primary cultures following ethanol treatment for 24 h **(A)**. Data are mean ± SEM values of three independent experiments. ELISA assay was used to validate the ethanol-induced changes in MCP-1 levels in exosomes of primary microglia **(B)**. Data are mean ± SEM values of three independent experiments and were analyzed using t-test. **, $p < 0.01$. ELISA assay was used to determine the levels of MCP in exosomes of microglia from pooled three MBH tissues obtained from control (AD, PF) or alcohol-fed (AF) neonatal male (M) and female (F) rats **(C)**. Data are mean ± SEM ($N = 3$) and were analyzed using two-way analysis of variance with Tukey's multiple comparison post hoc test. *, $p < 0.05$; **, $p < 0.01$

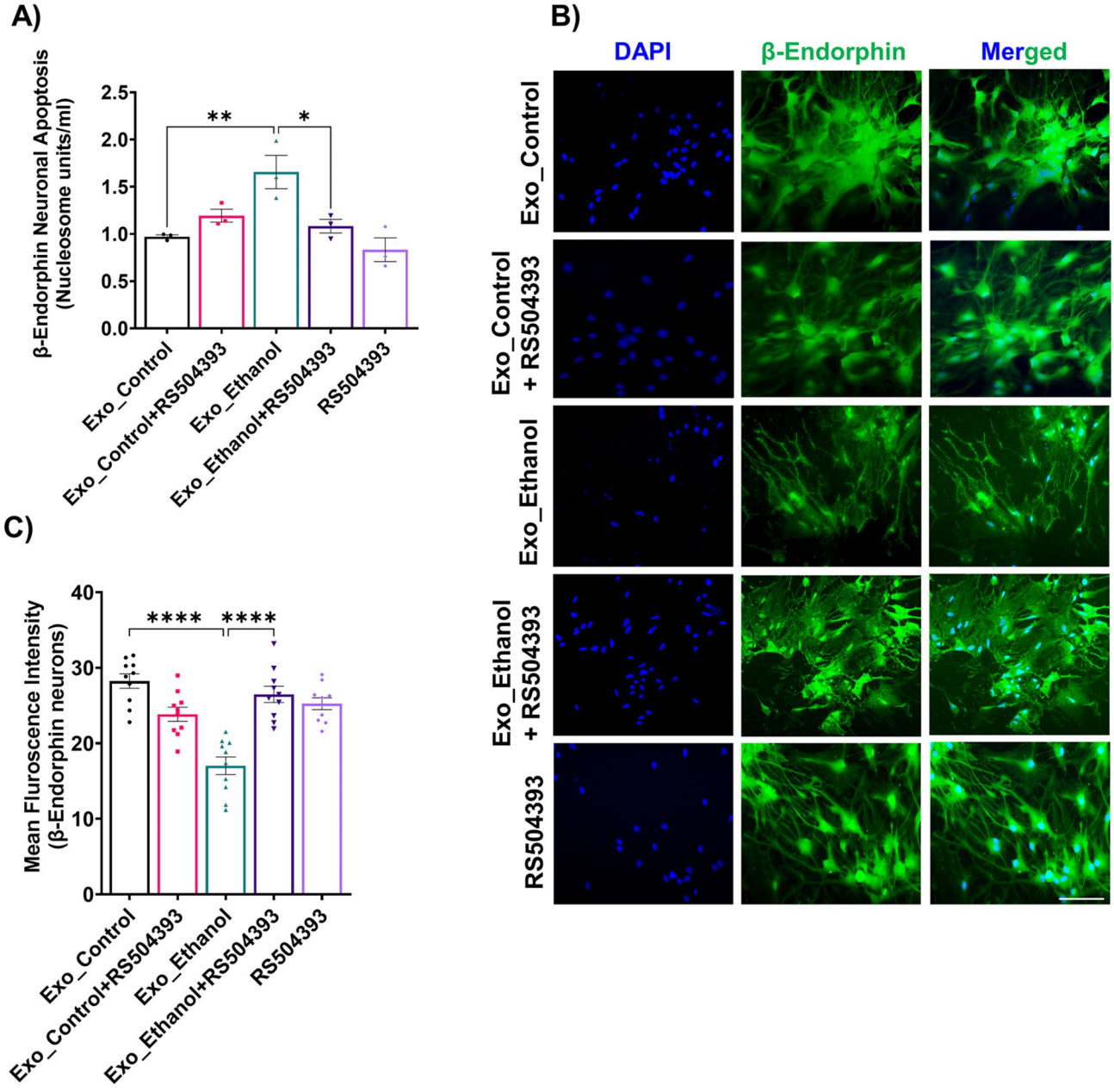

Fig. 3 MCP-1 antagonist RS504393 prevents β-endorphin neuronal apoptosis induced by ethanol-activated microglial exosomes in cultures. β-endorphin neuronal apoptosis was determined by measuring the nucleosome unit **(A)** or by measuring mean fluorescence intensity **(B, C)** of β-endorphin neurons in primary cultures following incubation with exosomes obtained from control culture (Exo_Control) or with Exo_Control + RS50493, with 50 mM ethanol treatment (Exo_Ethanol), or with Exo_Control + RS50493. RS504393 (10μM/ml) was applied 3 h prior addition of exosomes. Representative images are shown, β-endorphin neuron are stained Green. Nucleus are stained with DAPI (blue). Scale bar represents 100 μm. Histograms represent mean ± SEM values of mean fluorescence intensity for β-endorphin neurons and are analyzed using using one-way ANOVA followed by Newman-Keuls post hoc test. Significant differences between the groups are marked with *, $p < 0.05$; **, $p < 0.01$; ****, $p < 0.0001$

Western blot data also confirmed ethanol activated exosome action on MCPIP1 protein levels (Fig. 5D). Pretreatment with RS504393 significantly reduced the levels of MCPIPI in β-endorphin neurons treated with ethanol activated microglial exosomes. These results suggest that ethanol induced β-endorphin neuronal death may involve MCP1-CCR2-MCPIP1 signaling mechanism.

Improved stress and behavioral responses in alcohol fed adult rats treated with RS504393

Prenatal alcohol exposure has been known to cause disturbance in HPA axis function via causing the death of stress-regulatory β-endorphin neurons in developing brain [30]. Cognitive impairment in the offspring is the result of alteration in the HPA function [31]. In previous

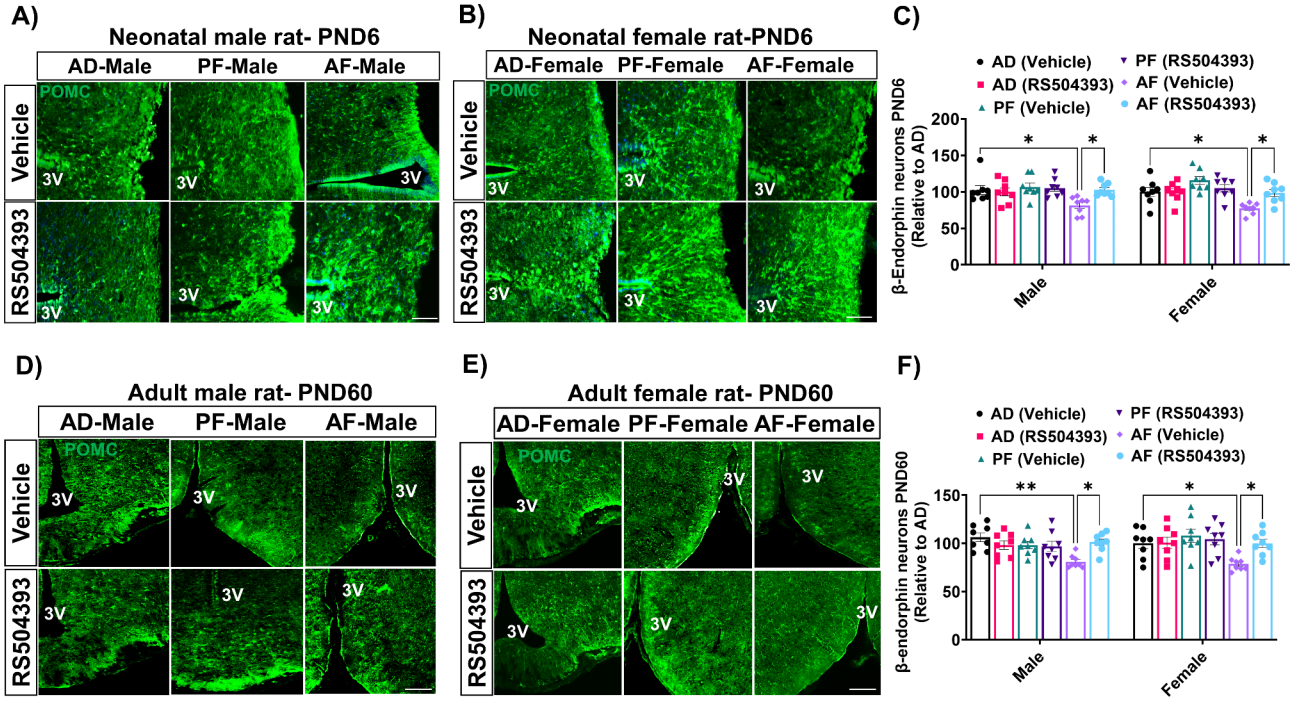

Fig. 4 Protective effects of RS504393 on postnatal ethanol-induced loss of β-endorphin neurons in hypothalamus of rats. Postnatal rats were pretreated with RS504393 (1 mg/kg; 1 h before the first feeding) daily and fed from PND 2–6 with ethanol-containing milk formula (AF), pair-fed isocaloric milk formula (PF), or left undisturbed in the litter with mother (AD). β-Endorphin neurons were processed for immunofluorescence staining and colored green. Representative images of β-endorphin positive neurons in the hypothalamus of different treatment groups are shown in **A**, **B** for neonatal rats (PND6) and adult rats (PND60) in **D**, **E**. Histograms **C**, **F** represent mean ± SEM values of β-endorphin neuronal number (relative to AD using the ImageJ analysis) and are analyzed using two-way ANOVA with Tukey's multiple comparison post hoc test. Significant differences between the groups are marked with *, $p < 0.05$; **, $p < 0.01$

studies we found that RS504393 was effective in preventing alcohol neurotoxic effect on β-endorphin neurons, therefore we assessed if RS504393 is effective in preventing the hyperstress response and behavioral abnormalities often observed in postnatal alcohol exposed rats. Adult rats given postnatal alcohol or control diet and pretreated with RS504393, or vehicle were employed to determine whether MCP1 antagonist would suppress the peripheral levels of corticosterone in response to an restraint stress. At baseline, none of the treatment group displayed any changes in peripheral blood levels of corticosterone (Fig. 6A, B). As expected, AF group compared to control groups (AD and PF) shows increased peripheral blood levels of corticosterone in response to 60 min of restraint stress, which persisted following restraint. Pretreatment with RS504393 prevented the increased response to restraint in AF group, suggesting that MCP1-CCR2 regulates postnatal alcohol effects on stress response. The corticosterone-responses to stress between the treatment groups was higher in females than those in males [Supplemental Fig. 1; F (30, 210) = 1.682, $P = 0.0191$].

To examine the impact of alcohol on anxiety-like behaviors, open field (OF) and elevated plus maze (EPM) tests were conducted in adult male and female rats. EPM data showed that alcohol fed (AF) male and female rats showed a significant decrease of time spent in open arms when compared to AD and PF rat groups (Fig. 6C, E), while a significant increase was found in the amount of time spent in closed arms in only female rats (Fig. 6F). In male rats, the mean level of time spent in closed arm was also increased but was not significantly different [Supplemental Fig. 2; EPM-Open arm, F (1, 5) = 23.58, $p = 0.0046$; EPM-closed arm, F (1, 5) = 3.225, $p = 0.1325$]. Treatment with RS504393 significantly elevated the amount of time spent in open arms and reduced the time spent in closed arms in AF + RS504393 group when compared with AF group. Time spent in the center and corner zones of open field test (OF) in alcohol fed adult male and female rats was also analyzed. We found that AF male and female rats had significantly decreased time in the center zone of the open field test and increased time in the corner zone when compared to AD and PF group rats (Fig. 6G-J). MCP1 antagonist treatment significantly increased the time spent in center zone in AF + RS504393 group when compared with AF group in male rats, while the antagonist reduced the amount of time spent in the corner zone in both male and female rats when compared to AF rats (Supplementary Fig. 3; OF-corner time, F(1,30) = 0.3946, $p = 0.5347$; OF-center time, F(1,30) = 30.94, $p = 0.001$).

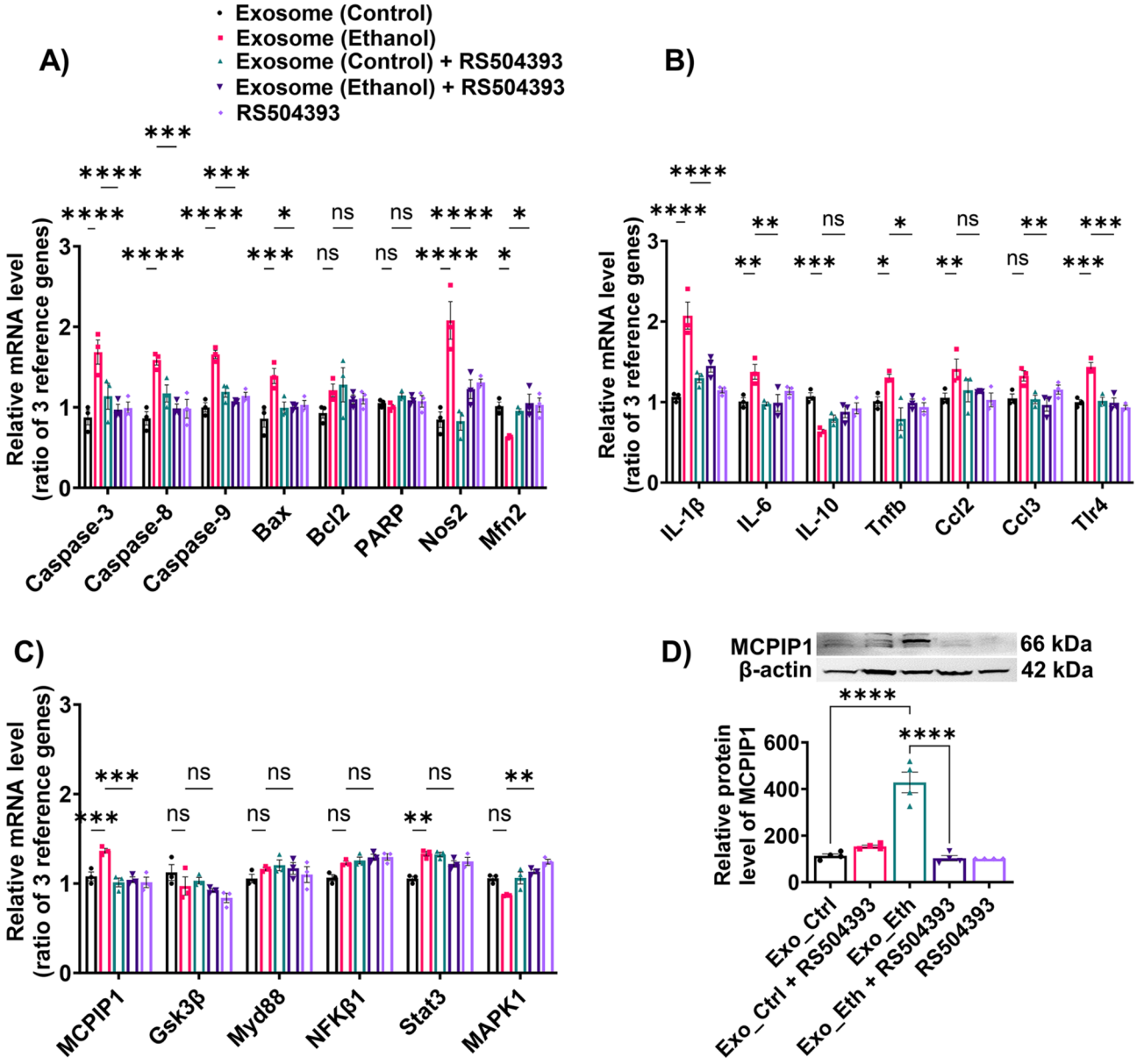

Fig. 5 Gene transcripts change in β-endorphin neurons following treatment with ethanol-activated exosomes with or without RS504393. β-endorphin neurons treated with microglia derived ethanol activated exosomes (Exosome-Ethanol) or vehicle treated exosomes (Exosome-Control). RS504393 (10μM/ml) was treated 3 h prior addition of ethanol activated exosomes. Changes in gene transcripts levels related to apoptosis, inflammation and transcriptional regulation are measured using qPCR and shown in panels **A**, **B**, and **C**, respectively, Changes in MCPIP1, a transcriptional factor downstream to MCP-1-CCL2 receptor CCR2 signaling were detected both at the gene transcript level **(C)** and the protein level using western blot measurements **(D)**. Values are represented as mean ± SEM ($n = 3$) for qPCR and ($n = 4$) for western blot. Data are analyzed using one-way ANOVA followed by Newman-Keuls post hoc test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, ****, $p < 0.0001$

Discussion

In this study, we showed that ethanol exposure causes microglia to release elevated levels of exosomes containing MCP1. This ethanol-activated microglial exosome when added to the *β*-endorphin neuronal cells in primary cultures increases the levels of MCPIP1, which is a key downstream transcriptional factor in MCP1-CCR2 signaling, apoptotic genes and inflammatory cytokines in association with elevated cellular apoptosis in these cells. A pharmacological blocker of MCP1-CCR2 signaling, RS504393, prevented the death of *β*-endorphin neurons induced by ethanol-activated microglial exosomes. RS504393 was also able to reduce the postnatal alcohol exposure-induced loss of *β*-endorphin neurons during postnatal and adult periods, as well as corticosterone hyper-response to stress, and increased anxiety-like behaviors in adult period. These data suggest that MCP1 participates in the ethanol-activated microglial

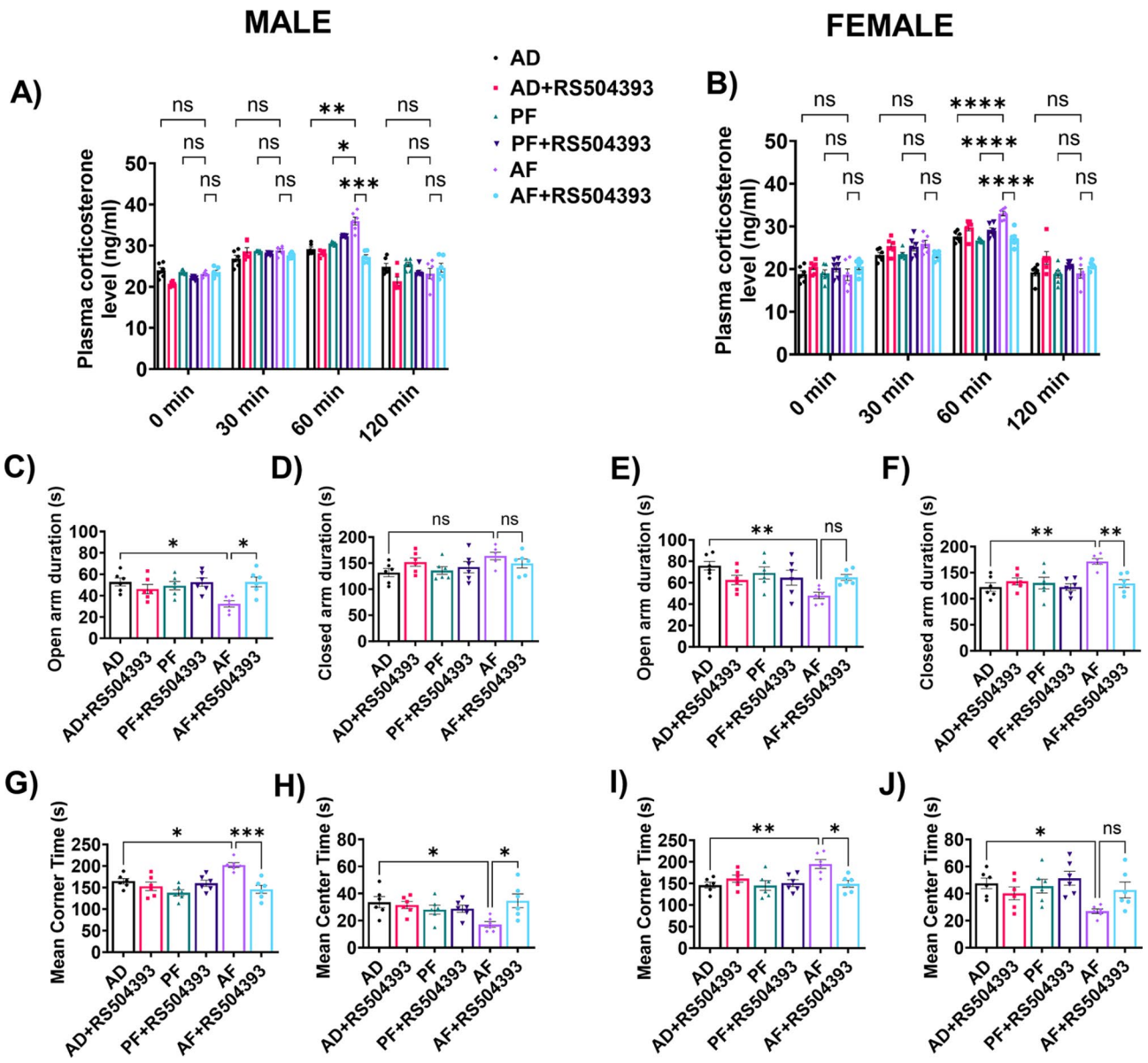

Fig. 6 RS504393 prevents postnatal ethanol-induced stress hyper-response and anxiety-like behavior in adult male and female rats. Time-dependent changes in plasma corticosterone levels following restrain in AD, AD + RS504393, PF, PF + RS504393, AF, AF + RS504393 male and female offspring are shown in panel **A** and **B**, respectively. Data are shown as mean ± SEM ($n = 5–8$/group) and were analyzed using two-way analysis of variance with Tukey's multiple comparison post hoc test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, ****, $p < 0.0001$. Effect of prenatal ethanol exposure on anxiety-like behaviors was evaluated by the elevated plus maze (EPM) and open field (OF) in male and female offsprings at PND 60. For EPM analysis, time (sec) in closed and open arms (**C**) while for OF analysis, time (sec) in corner and center (**D**) was analyzed in male and female rat offspring. Data are shown as mean ± SEM, $n = 5–8$ in each group. The data were analyzed using one-way ANOVA followed by the Student Newman-Keuls post hoc test. Significant differences are indicated for AD vs. AF and AF vs. AF + RS504393 groups with *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$

exosome-mediated killing of β-endorphin neurons and in resultant abnormalities in hormonal and behavioral responses to stress.

Exosomes play an essential role in cellular communication via transporting protein, RNA and bioactive metabolites between the cells [8, 32]. The role of exosomes in relevance to the neurotoxic effects of prenatal ethanol is not well understood. We have previously shown that following activation by alcohol, microglia secrete exosomes containing various cytokines (e.g., IL-2, TNF-α) and proteins (e.g., C1q) which might be involved in various functions including neuroinflammation and neurotoxicity [8, 18]. Our previous study identified [18], complement protein C1q is elevated within ethanol-activated microglia which participated in β-endorphin neuronal apoptosis involving C1q-C3-C4-membrane attack complex. This study provides evidence that ethanol activated microglia releases exosomes containing elevated level of

chemokine, MCP1, which participates to induce apoptosis in *β*-endorphin neurons. MCP1-CCR2 signaling may also involve C1q which leads to apoptotic death. Both studies provide and connect underlying apoptotic mechanism for β-endorphin neuronal cell death.

MCP1 within the brain is known to be produced by the astrocytes and microglia [10]. CCR2, a receptor for the chemokine has been found to be expressed by the adult rat brain neurons [13, 33]. MCP1-CCR2 signaling has been implicated in various neuroinflammatory and neurodegenerative diseases that involve neuronal degeneration such as stroke, multiple sclerosis and Alzheimer's [33]. Chronic ethanol exposure have been reported to cause increased expression of MCP1 in adult mice brain [34, 35]. Ethanol stimulated activation of MCP1/CCR2 signaling have been found to induce apoptosis of spinal cord neurons of early postnatal mice while genetic deletion of MCP1 or CCR2 protect mice from ethanol induced damage to developing spinal cord [36]. In the present study, we have provided evidence that ethanol induced increased level of MCP1 within microglial exosomes enhance the MCP1-CCR2 signaling mediated death of stress-regulatory β-endorphin neurons in developing brain. Based on the findings of the present study, we propose that ethanol exposure elevates MCP1 in hypothalamic microglia-derived exosomes. These exosomes when transported to hypothalamic β-endorphin neurons lead to activation CCR2 signaling with MCPIP1 as downstream effector and transcriptional factor that induces apoptotic pathway and aggravating neuronal cell death [37] (Fig. 7). CCR2 signaling mediated upregulation of MCPIP1 has also been reported to cause endoplasmic reticulum (ER) stress in cardiomyocytes and osteoclast [38]. CCR2 inhibitor RS102895 have been found to attenuate ER stress and decrease inflammatory cytokine expression in liver of type 2 diabetic mice [39, 40]. ER stress has also been implicated in ethanol induced damage to developing brain [41]. Ethanol mediated ER stress in developing spinal cord neurons was significantly reduced in MCP1 and CCR2 knockout mice [36]. Thus, there may be possibility of involvement of ER stress in

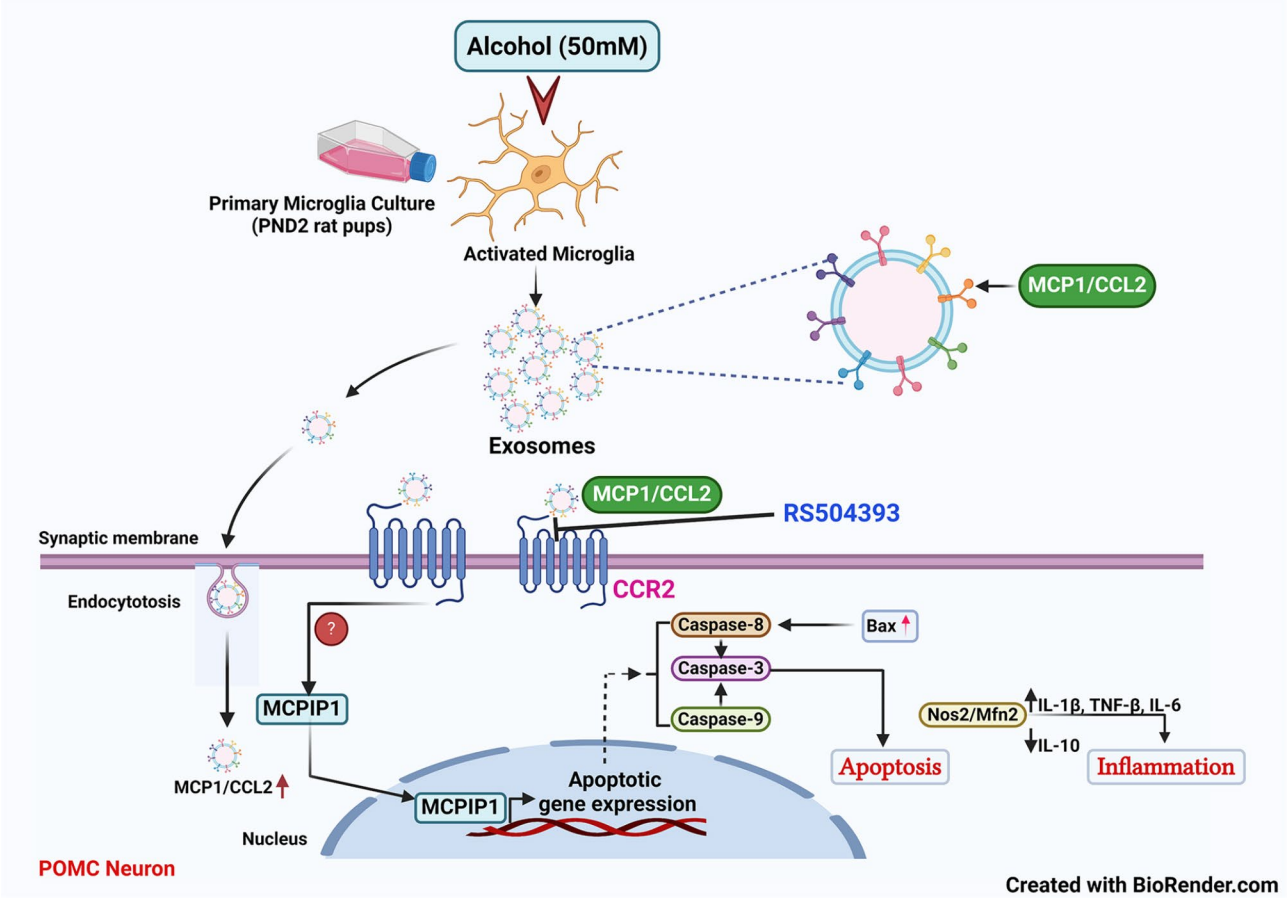

Fig. 7 Schematic representing mechanisms by which microglial extravesicular MCP1 induces postnatal alcohol-activated apoptosis in β-endorphin neurons. Postnatal alcohol exposure (PAE) causes hypothalamic microglia to release exosomes containing MCP1 (also known as CCL2). MCP1 by activating it's receptor CCR2 increases the transcriptional factor MCPIP1 which elevate the levels of apoptotic and inflammatory genes leading to *β*-endorphin neuronal death. CCR2 antagonist RS504393 prevents *β*-endorphin neuronal death and improve stress response and anxiety-like behavior in PAE offspring

MCP1-CCR2-MCPIP mediated death of β-endorphin neurons which needs further investigation.

Conclusions

In conclusion, we revealed the important role of exosomes in neuroinflammatory and neurotoxic actions of microglia. We show here that ethanol exposure during the developmental period increase microglia to release exosome containing chemokine MCP1 which activates CCR2 and MCPIP1 signaling along with increased production of inflammatory cytokines and apoptotic proteins and reactive super-oxygen species in β-endorphin neurons and resulting in demise of these neurons and stress-related behavioral responses. Thus, targeting MCP1-CCR2 signaling may provide new strategies for alleviating fetal alcohol induced brain injury and behavioral abnormalities.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12974-024-03274-6.

Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Supplementary Material 4

Supplementary Material 5

Supplementary Material 6

Author contributions

PT, SC and SM conducted experiments and analyzed data. DS and PT wrote the main manuscript text. PT, SC and DS prepared the figures. All authors reviewed the manuscript.

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Data availability

Data is provided within the manuscript or supplementary information files.

Declarations

Ethics approval

All animal experiments were performed in accordance with institutional guidelines, and protocols were approved by the Rutgers Institutional Animal Care and Facilities Committee (approval number 999900286).

Competing interests

The authors declare no competing interests.

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