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Ethanol-induced Damage to the Developing Spinal Cord: The Involvement of CCR2 Signaling

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Running title: Developmental ethanol exposure causes spinal cord damage

Abbreviation

ATF6: Activating transcription factor 6
CCR2: chemokine (C–C motif) receptor 2
CHOP: C/EBP Homologous Protein
CNS: Central nervous system
DNP: Dinitrophenol
eIF2 α : Eukaryotic Initiation Factor 2 α
ER: Endoplasmic reticulum
FASDs: Fetal alcohol spectrum disorders
HNE: 4-Hydroxynonenal
GFAP: Glial fibrillary acidic protein
GSK3 β : Glycogen synthase kinase 3 β
JNK: c-Jun NH (2)-terminal kinase
MANF: Mesencephalic astrocyte derived neurotrophic factor
MAPK: mitogen-activated protein kinase
MCP-1: Chemokines monocyte chemoattractant protein-1
MCPIP1: Monocyte chemotactic protein-1-induced protein-1
PARP: Poly (ADP-ribose) polymerase
PERK: Protein kinase R-like endoplasmic reticulum kinase
PD: Postnatal day
XBP1: X-box binding protein-1
UPR: Unfolded protein response

ABSTRACT

Ethanol exposure during development causes fetal alcohol spectrum disorders (FASD). A large body of evidence shows that ethanol produces multiple abnormalities in the developing central nervous system (CNS), such as smaller brain size, reduced volume of cerebral white matter, permanent loss of neurons, and alterations in synaptogenesis and myelinogenesis. The effects of ethanol on the developing spinal cord, however, receive little attention and remain unclear. We used a third trimester equivalent mouse model to investigate the effect of ethanol on the developing spinal cord. Ethanol caused apoptosis and neurodegeneration in the dorsal horn neurons of mice of early postnatal days, which was accompanied by glial activation, macrophage infiltration, and increased expression of CCR2, a receptor for monocyte chemoattractant protein 1 (MCP-1). Ethanol-induced neuronal death during development resulted in permanent loss of spinal cord neurons in adult mice. Ethanol stimulated endoplasmic reticulum (ER) stress and oxidative stress, and activated glycogen synthase kinase 3 β (GSK3 β) and c-Jun N-terminal kinase (JNK) pathways. Knocking out MCP-1 or CCR2 made mice resistant to ethanol-induced apoptosis, ER stress, glial activation, and activation of GSK3 β and JNK. CCR2 knock out offered much better protection against ethanol-induced damage to the spinal cord. Thus, developmental ethanol exposure caused permanent loss of spinal cord neurons and CCR2 signaling played an important role in ethanol neurotoxicity.

INTRODUCTION

Fetal alcohol-spectrum disorders (FASD) are a collection of physical and neurobehavioral disabilities caused by prenatal exposure to ethanol. The developing nervous system is particularly vulnerable to ethanol toxicity [1-3]. Ethanol causes significant structural and functional abnormalities in the developing central nervous system (CNS), such as microcephaly, abnormal cortical thickness, reduced cerebral white matter volume, ventriculomegaly, and cerebellar hypoplasia [4-10]. These CNS abnormalities may underlie the neurobehavioral deficits observed in FASD [6, 7, 11, 12].

Ethanol-induced damages to the developing CNS have been extensively investigated in humans and animals. The studies using experimental models which include animals and *in vitro* models have clearly demonstrated that ethanol can cause neuroinflammation and neurodegeneration in the developing brain [13-15]. However, the effects of ethanol on the developing spinal cord receive little attention. It is unclear whether ethanol causes damage to the developing spinal cord and if the damage persists to adulthood. We hypothesized that developmental exposure to ethanol causes neurodegeneration, resulting in long lasting deficits in the spinal cord. The current study used a well-established mouse model of third trimester equivalent ethanol exposure to test this hypothesis. The model was previously used to examine ethanol-induced neuron death in the developing brain and behavioral deficits [16-21]. Using this model, we showed ethanol exposure during the synaptogenesis period (early postnatal days) produced robust apoptosis and neurodegeneration in the developing brain [19-23]. In

the current study, we demonstrated that ethanol exposure during postnatal day (PD) 4 to PD7 induced neuronal death in the form of apoptosis which resulted in permanent loss of spinal cord neurons in adult mice.

MCP-1, also known as C-C chemokine ligand 2 (CCL2), is a major chemoattractant for monocytes and memory T cells. MCP-1 binds to a specific cell-surface receptor, CC-chemokine receptor-2 (CCR2), and plays an important role in various inflammatory diseases [24, 25]. MCP-1 is mainly produced by glial cells in the CNS while CCR2 is expressed in both neurons and glial cells [26]. MCP-1/CCR2 signaling is a key regulator of neuroinflammation following CNS injuries and has been implicated in many neurodegenerative disorders [26-29]. We hypothesized that MCP-1/CCR2 signaling played an important role in ethanol-induced damage to the developing spinal cord and explored the potential underlying cellular/molecular mechanisms.

MATERIALS AND METHODS

Materials

The information for primary antibodies used for immunohistochemistry/immunofluorescent staining and immunoblotting was provided in Table 1 and Table 2, respectively. Anti- β -actin antibody and DAB staining kit were obtained from Sigma-Aldrich (St. Louis, MO). HRP-conjugated anti-rabbit, anti-mouse, anti-goat and anti-rat secondary antibodies were purchased from GE Healthcare Life Sciences (Piscataway, NJ). Mounting media with DAPI were obtained from Vector Laboratories (Burlingame, CA). Alexa-488 conjugated anti-rabbit, anti-mouse and Alexa-594 conjugated anti-rabbit, anti-mouse antibodies were obtained from Life Technologies (Grand Island, NY). OxiSelect™ Protein Carbonyl ELISA Kit and OxiSelect™ HNE Adduct Competitive ELISA Kit were obtained from Cell Biolabs (San Diego, CA). Ketamine/xylazine was obtained from Butler Schein Animal Health (Dublin, OH).

Animals and ethanol exposure

C57BL/6 mice, MCP-1^{-/-} (B6.129S4-Ccl2^{tm1Roi}/J) and CCR2^{-/-} (B6.129S4-Ccr2^{tm1lf}/J) mice were obtained from Jackson Laboratories (Bar Harbor, Maine) and maintained in the Division of Laboratory Animal Resources of the University of Kentucky Medical Center. All procedures were performed in accordance with the guidelines set by the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Kentucky (Account #: 2008-0401). Animals were

maintained in a 12/12 hours light/dark cycle with a temperature of $22\pm 1^{\circ}\text{C}$ and relative humidity of $60\pm 5\%$, and received standard chow and water ad libitum. The weights of C57BL/6 and MCP-1^{-/-} and CCR2^{-/-} mice were around 3, 5 and 10 g for PD4, PD7, and PD12 respectively. The pups with abnormal weight and activity were removed from the study. A well-established mouse model of third trimester ethanol exposure was used, in which mice are exposed to ethanol during early postnatal days (PD) [21, 22]. To eliminate litter effects, different litters were pooled and each pup was randomly assigned to either control or ethanol-treated group. Ethanol was administered on either PD4, PD7 or PD12. The pups received two subcutaneous injections of ethanol which were two hours apart. Each injection contained ethanol (2.5 g/kg, 20% solution in saline). Total volume for the injection for each pup was approximately 30 μl . Control pups received an equal volume injection of saline. We have previously determined that the blood alcohol concentration (BAC) was 338 mg/dl 8 hours after the first injection [19]. Eight hours after the first injection, mice were euthanized, and the spinal cords were dissected and processed for further analysis. In some experiments, mice that received ethanol injection on PD7 were kept to adulthood to analyze the prolonged impact of ethanol exposure.

Tissue preparation and immunoblotting

Animals were anesthetized with intraperitoneal injection of ketamine/xylazine (100 mg/kg/10 mg/kg), and the entire spinal cord was dissected from the vertebrae and immediately frozen in dry ice and then stored at -80°C . The spinal cord was homogenized and the protein was extracted and subjected to immunoblotting analysis

as previously described [30]. Briefly, the spinal cord was homogenized in an ice cold lysis buffer containing 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM EGTA, 0.5% NP-40, 0.25% SDS, 1 mM PMSF, 5 µg/ml leupeptin, and 5 µg/ml aprotinin. Homogenates were centrifuged at 20,000 g for 30 min at 4°C and the supernatant fraction was collected. After determining protein concentration, aliquots of the protein samples (30 µg) were separated on a SDS-polyacrylamide gel by electrophoresis. The separated proteins were transferred to nitrocellulose membranes. The membranes were blocked with 5% BSA in 0.01 M PBS (pH 7.4) and 0.05% Tween-20 (TPBS) at room temperature for 1 hour. Subsequently, the membranes were probed with primary antibodies overnight at 4°C. After three washes (5 min each) in TPBS, the membranes were incubated with a secondary antibody conjugated to horseradish peroxidase. The immune complexes were detected by the enhanced chemiluminescence substrate (GE Healthcare, Chalfont, Buckinghamshire, UK). The density of immunoblotting was quantified with the software of Image lab 5.2 (Bio-Rad Laboratories, Hercules, CA).

Measurement of dinitrophenol (DNP) and 4-hydroxynonenal (HNE)

Oxidative stress was determined by protein oxidation and lipid peroxidation by measuring the protein carbonyl content dinitrophenol (DNP) and lipid peroxidation byproduct 4-hydroxynonenal (4-HNE), respectively. Eight hours after the first ethanol exposure, animals were euthanized, and the spinal cord was dissected. The tissues were homogenized in an ice cold lysis buffer. The supernatant fraction was collected after centrifuging at 20,000 g for 30 min at 4°C. An enzymatic immunoassay for rapid detection and quantitation of DNP was performed using the OxiSelect™ Protein

Carbonyl ELISA Kit from Cell BioLabs (San Diego, CA) in accordance with the manufacturer's instructions. Samples were diluted into 10 µg/mL, and 100 µl of sample was assayed. The amount of DNP was presented as mmol/mg. An enzymatic immunoassay for rapid detection and quantitation of HNE protein adducts was performed using the OxiSelect™ HNE Adduct Competitive ELISA Kit Cell BioLabs (San Diego, CA) in accordance with the manufacturer's instructions. 50 µL of sample was added to the wells of the HNE conjugate coated plate, and the amount of HNE protein adducts was presented as µg/ml.

Fluoro-Jade C

Fluoro-Jade C is a sensitive fluorescent marker for degenerating neurons [31]. The procedure for Fluoro-Jade C staining has been previously described with some modifications [31]. Briefly, frozen slides of spinal cord were prepared at a thickness of 10 µm, and first immersed in a basic alcohol solution consisting of 1% sodium hydroxide in 80% ethanol for 5 min. They were then rinsed for 2 min in 70% ethanol, for 2 min in distilled water, and then incubated in 0.06% potassium permanganate solution for 10 min. Slides were then transferred for 10 min to a 0.0001% solution of Fluoro-Jade C (Chemicon, Temecula, CA) dissolved in 0.1% acetic acid vehicle. The proper dilution was accomplished by first making a 0.01% stock solution of the dye in distilled water and then adding 1 ml of the stock solution to 99 ml of 0.1% acetic acid vehicle. The working solution was used within 2 hours of preparation. The slides were then rinsed through three changes of distilled water for 1 min per change. Excess water was drained onto a paper towel, and the slides were then air dried on a slide warmer at 50°C

for at least 5 min. The air dried slides were then cleared in xylene for at least 1 min and then coverslipped with DPX (Sigma) nonfluorescent mounting media. The slides were examined and recorded with a fluorescent microscope (IX81, Olympus).

Immunohistochemistry

The procedure for immunohistochemistry (IHC) has been previously described with some modifications [30]. Briefly, animals were deeply anesthetized with intraperitoneal injection of ketamine/xylazine and then intracardially perfused with PBS followed by 4% paraformaldehyde in PBS (pH7.4). The spinal cord tissues were removed, post fixed in 4% paraformaldehyde for an additional 24 hours and then transferred to sucrose solution (concentration from 10% to 30%) until the tissues sunk to the bottom. The tissues were frozen in OCT compound and sectioned at 10 μ m in a sagittal plane using a Cryostat Microtome (Thermo Scientific). The sections were incubated in 0.3% H₂O₂/30% methanol in PBS for 10 min. After washing with PBS, sections were mounted on slides and dried. The slides were then blocked with 5% goat serum and 0.5% Triton X-100 in PBS for 1 hour at room temperature. After blocking, the slides were treated with a rabbit anti- cleaved caspase-3 (1:4,000), rat anti-CD68 (1:800), and mouse anti-NeuN (1:800) antibodies overnight at 4°C. After washing with PBS, slides were incubated with biotin-conjugated goat anti-rabbit secondary antibody (1: 800) for 1 hour at room temperature and then washed with PBS. Avidin–biotin–peroxidase complex was prepared according to the manufacturer's instructions. The slides were developed in 0.05% 3,3-diaminobenzidine (DAB) (Sigma-Aldrich, Inc.) containing 0.003% H₂O₂ in PBS. The sections were then dehydrated through graded

alcohol, and cleared with xylene and mounted with synthetic resin. The images were recorded using an Olympus BX51 microscope. Negative controls were performed by omitting the primary antibody. For NeuN staining, the positive cells were counted at 40X magnification, and the positive cell number and average cell size in the dorsal horn of spinal cord were counted in twenty randomly selected sections using the software of Image lab 5.2 (Bio-Rad Laboratories, Hercules, CA). Four-five animals were analyzed for each group.

Immunofluorescent staining

The procedure for immunofluorescent staining has been previously described with some modifications [23]. Briefly, the cross sections of the spinal cord were prepared at a thickness of 10 μ m. After blocking with 1% BSA and 0.5% TritonX-100 in PBS for 1 hour at room temperature, the slides were incubated with a rabbit anti-cleaved caspase 3 (1:800), anti-Iba-1 (1:800); anti-MANF (1:1,000), mouse anti-GFAP (1:400) or anti-NeuN (1:400) overnight at 4°C. After rinsing in PBS, the sections were incubated with Alexa Fluor 488-conjugated anti-rabbit or anti-mouse and/or Alexa Fluor 594-conjugated anti-rabbit or anti-mouse IgG in the dark at room temperature for 1 hour. After rinsing, the slides were covered in mounting media with DAPI and examined/recorded with a fluorescent microscope (IX81, Olympus). Negative controls were performed by omitting the primary antibody.

Statistics

Quantitative data were presented as the means \pm SEM. Differences between two groups were analyzed using unpaired t test. Differences between multiple groups were analyzed using ANOVA followed by Dunnett's test. Differences in which p was less than 0.05 were considered statistically significant.

RESULTS

Ethanol causes neuroapoptosis and neurodegeneration in the developing spinal cord

We have previously demonstrated that cleaved caspase-3 is a reliable indicator for neuroapoptosis in the developing mouse brain [19, 30]. Ethanol increased the expression of cleaved caspase-3 in the spinal cord of PD4 and PD7 mice but not PD12 mice (Fig. 1). The cleaved-caspase-3 positive cells were located in the lamina II-III of the dorsal horn. Ethanol-induced cleavage of caspase-3 was observed 4 hours after ethanol injection and increased drastically after 8 hours; then the effect diminished after 24 hours (Fig. 1B). On PD7, ethanol-induced expression of cleaved caspase-3 occurred in all segments (cervical, thoracic and lumbar) after 8 hours of ethanol exposure (Fig. 1C). The cells positive for cleaved caspase-3 were also NeuN-positive, indicating their neuronal identity (Fig. 1D). Immunoblotting results confirmed that ethanol increased the expression of cleaved caspase-3, caspase 8, caspase 9, and PARP in the spinal cord (Fig. 2). Ethanol increased Fluoro-Jade C-positive cells in a similar area of the spinal cord, indicative of neurodegeneration (Fig. 1E).

Developmental ethanol exposure causes permanent neuron loss in the adult spinal cord

To determine whether ethanol exposure during development has a permanent effect, we examined the number of neurons in the spinal cord of 8-week-old C57BL6 mice which received ethanol or saline injection on PD7. The neurons were identified by

NeuN IHC (Fig. 3A). Quantitative analysis showed that both the number and size of neurons in the dorsal horn were significantly decreased in the ethanol-exposed group (Fig. 3B). Ethanol exposure also reduced the number of neurons in the ventral horn but not their size (Fig. 3C).

Ethanol stimulates glial activation and macrophage infiltration in the developing spinal cord

Ethanol causes neuroimmune activation in the developing brain, which is manifested by the glial activation, macrophage infiltration, and production of proinflammatory molecules [14, 32]. Neuroinflammation is one of the potential mechanisms for ethanol neurotoxicity in the developing brain. We first examined the expression of Iba-1 and GFAP in the spinal cord by immunofluorescence staining and immunoblotting. Ethanol increased the expression of Iba-1 and GFAP in the spinal cord (Figs. 4A-C). CD68, a macrophage marker, was also significantly increased by ethanol exposure (Figs. 4D and 4E). P2X7 is a regulator of inflammatory responses, and involved in microglial activation and cytotoxicity [33]. Ethanol increased the expression of P2X7 (Fig. 4F). Ethanol also increased the expression of MHC-II (Fig. 10), another marker for microglial activation and neuroinflammation [34].

Ethanol stimulates CCR2 signaling in the developing spinal cord

MCP-1 and its receptor CCR2 play an important role in the recruitment and activation of monocytes/macrophages [35]. To determine whether CCR2 signaling was involved in ethanol-induced damage of the spinal cord, we examined the expression of

MCP-1 and CCR2 by immunoblotting (Fig. 5). Ethanol did not affect MCP-1 but significantly increased the expression of CCR2 in the spinal cord (Fig. 5B). Monocyte chemoattractant protein-1-induced protein-1 (MCP-1IP1), a transcriptional activator, is downstream of CCR2 and regulates the expression of IL-1 β , MCP-1 and TNF α [36]. Ethanol significantly increased the protein level of MCP-1IP1 in the spinal cord (Fig. 5C).

Ethanol causes ER stress and oxidative stress in the developing spinal cord

Both ER stress and oxidative stress have been proposed to play an important role in ethanol-induced brain damage [13, 37-40]. To determine whether ethanol exposure causes ER stress in the developing spinal cord, we examined the expression of a spectrum of proteins involved in the unfolded protein response (UPR) (Fig. 6). Ethanol significantly increased the expression of activating transcription factor 6 (ATF6), phosphorylated PERK (p-PERK), phosphorylated eIF2 α (p-eIF2 α), GRP94, X-box binding protein-1 (XBP-1), cleaved caspase-12, mesencephalic astrocyte-derived neurotrophic factor (MANF), and C/EBP Homologous Protein (CHOP).

We then determined the effect of ethanol on oxidative stress. We examined the protein oxidation and lipid peroxidation by assaying the protein carbonyl content with immunoblotting using an anti-dinitrophenol (DNP) antibody and lipid peroxidation byproduct using an anti-4-hydroxynonenal (4-HNE) antibody, respectively. As shown in Fig. 7A, ethanol significantly increased DNP and 4-HNE. The finding was confirmed by ELISA (Fig. 7B).

Effect of ethanol on intracellular signaling

We examined the effect of ethanol on the activity of several key signaling proteins that regulate neuroinflammation and neurodegeneration. GSK3 β plays an important role in neurogenesis, neuronal migration, cell adhesion, synapse formation, neuronal survival, and cell polarity/neurite outgrowth in the developing CNS [41, 42]. The activity of GSK3 β is inhibited by the phosphorylation at Ser9 but stimulated by the phosphorylation at Tyr216 [43]. Ethanol significantly decreased the phosphorylation of GSK3 β at Ser9 (Figs. 8A and B), without affecting phosphorylation at Tyr216 (data not shown), indicating the activation of GSK3 β . c-Jun N-terminal kinases (JNKs) are a family of protein kinases that play a central role in stress signaling pathways and regulate neuronal death and neuroinflammation [44]. Ethanol significantly increased the phosphorylation of JNK (Thr183/Tyr185) (Fig. 8A and C). Phosphorylated c-Jun, a substrate of JNK, was consistently upregulated by ethanol (Figs. 8A and D). The data suggested that ethanol induced the activation of JNK and p-c-Jun. However, ethanol had little effect on p38 MAPK (data not shown).

MCP-1^{-/-} and CCR2^{-/-} mice are resistant to ethanol-induced apoptosis in the developing spinal cord

Since MCP-1/CCR-2 signaling plays an important role in neuroinflammation and neurodegeneration in the nervous system, we hypothesized that this signaling pathway may mediate ethanol-induced damage to the developing spinal cord. We compared ethanol-induced apoptosis in the developing spinal cord among wild-type (WT), MCP-1^{-/-} and CCR2^{-/-} mice. As shown in Fig. 9A, ethanol drastically increased the immunoreactivity of cleaved caspase-3 in WT mice; ethanol also increased the

immunoreactivity of cleaved caspase-3 in MCP-1^{-/-} and CCR2^{-/-} mice, but to a much lesser extent. It appeared that CCR2^{-/-} mice were much more resistant to ethanol-induced caspase 3 activation. The results from immunoblotting analysis confirmed that MCP-1^{-/-} and CCR2^{-/-} mice were less affected by ethanol-induced caspase 3 activation and PARP cleavage (Fig. 9B). In MCP-1^{-/-} mice, ethanol-induced activation of caspase-8, caspase-9 and caspase-12 was completely blocked (Fig. 9C). Interestingly in CCR2^{-/-} mice, ethanol actually inhibited the activation of caspase-8, caspase-9 and caspase-12. These results suggested that MCP-1^{-/-} and CCR2^{-/-} mice were resistant to ethanol-induced apoptosis in the developing spinal cord, and CCR2 knock out was more effective.

MCP-1^{-/-} and CCR2^{-/-} mice are resistant to ethanol-induced glial activation and macrophage infiltration

We compared ethanol-induced glial activation and macrophage infiltration among WT, MCP-1^{-/-} and CCR2^{-/-} mice (Fig. 10). Ethanol induced glial activation and macrophage infiltration in the developing spinal cord of WT mice (Figs. 4 and 10). Ethanol induced an increase of GFAP, CD68, P2X7 and MHC-II, but not Iba-1. In CCR2^{-/-} mice, ethanol failed to increase the expression of Iba-1 and MHC-II. Interestingly, ethanol actually inhibited the expression of GFAP, CD68 and P2X7 in CCR2^{-/-} mice (Figs. 10A, 10C and 10D). These results suggested that MCP-1^{-/-} and CCR2^{-/-} mice were resistant to ethanol-induced glial activation and macrophage infiltration in the developing spinal cord, and CCR2^{-/-} knock out was more effective.

MCP-1^{-/-} and CCR2^{-/-} mice are resistant to ethanol-induced ER stress

We compared ethanol-induced oxidative stress and ER stress among WT, MCP-1^{-/-} and CCR2^{-/-} mice. There was little difference in ethanol-induced oxidative stress among these mice (data not shown). However, MCP-1^{-/-} and CCR2^{-/-} mice were resistant to ethanol-induced ER stress. Ethanol caused ER stress as demonstrated by the increase in the expression of various UPR proteins in WT mice (Figs. 6 and 11). In MCP-1^{-/-} mice, we did not observe ethanol-induced expression of ATF6, p-eIF2 α , GRP94, XBP-1, and MANF; but ethanol still upregulated the expression of p-PERK and GRP78 (Fig. 11). In CCR2^{-/-} mice, ethanol failed to induce all UPR proteins investigated. Interestingly, ethanol actually inhibited the expression of GRP94 (Fig. 11A). These results suggested that MCP-1^{-/-} and CCR2^{-/-} mice were resistant to ethanol-induced ER stress in the developing spinal cord, and CCR2^{-/-} knock out was more effective.

Effect of ethanol on intracellular signaling in WT, MCP-1^{-/-} and CCR2^{-/-} mice

We further compared the effect of ethanol on the activation of GSK3 β and JNKs among WT, MCP-1^{-/-} and CCR2^{-/-} mice. In MCP-1^{-/-} mice, although ethanol still activated GSK3 β and JNKs, the activation was significantly reduced, compared to WT mice (Fig. 12). In CCR2^{-/-} mice, ethanol failed to activate both GSK3 β and JNKs. The results suggested that MCP-1/CCR2 signaling played an important role in ethanol-induced activation of pro-inflammatory and pro-apoptotic pathways, such as GSK3 β and JNKs in the developing spinal cord.

DISCUSSION

Temporal and regional susceptibility to ethanol

The developing nervous system is sensitive to ethanol exposure and there is a temporal and regional susceptibility. It appears that the period of synaptogenesis is particularly vulnerable to ethanol-induced neurodegeneration [16]. In humans, this period spans the last 3 months of pregnancy and extends into the first several postnatal years; whereas in rodents it begins a day or two before the birth and terminates at approximately 14 days after birth [45]. The third trimester equivalent model for ethanol exposure causes wide-spread neuron death in the CNS of rodents [16]. We have previously shown that the BAC was 338 mg/dl in these pups [19]. High BACs are not uncommon in human alcoholics [46-52]. Some alcoholics with a BAC greater than 400 mg/dl were alert and oriented [47, 50]. BACs greater than 500 mg/dl have been reported in heavy drinkers [48, 49, 52].

We show here that ethanol activates caspase-3 in the developing spinal cord of PD4 and PD7 mice, but not PD12 mice (Fig. 1A). This is consistent with our previous study showing that ethanol caused caspase-3 activation in the developing brain of PD4 but not PD12 mice [21]. Therefore, the susceptibility to ethanol diminishes as the spinal cord matures. Ethanol triggers apoptotic death in the spinal cord which is evident by the activation of caspase-3, caspase-8 caspase-9, and PARP (Fig. 2). Ethanol-induced apoptosis occurs rapidly; it occurs at 4 hours after ethanol exposure, persists to 12 hours and then declines thereafter (Fig. 1B). The co-localization of cleaved caspase-3 and NeuN indicates that the apoptosis occurs in neurons (Fig. 1D). The activation of

caspase-3 and Fluoro-Jade C staining are observed in similar regions of the spinal cord, indicating the occurrence of neurodegeneration (Fig. 1E). It appears that all spinal segments are affected by ethanol exposure since similar results are observed in cervical, thoracic and lumbar samples (Fig. 1C).

With this ethanol exposure paradigm, ethanol-induced cleaved caspase 3 and Fluoro-Jade C staining is consistently localized in the lamina II-III of the dorsal horn, indicating a region-specific neurodegeneration. In the lamina I-IV of the dorsal horn, more than 90% of the neurons are local interneurons, which serve as the first relay station for sensory and nociceptive signals reaching the CNS from the periphery [53, 54]. These interneurons, including excitatory glutamatergic neurons and inhibitory neurons settle in the dorsal horn of the spinal cord in the embryo [55, 56]. It appears that the loss of these neurons is permanent (Fig. 3). Dysfunctions of dorsal horn interneurons may contribute to symptoms of chronic pain such as the increased sensitivity to noxious stimuli (hyperalgesia) [57]. Chronic ethanol exposure can induce hyperalgesia in adult rats, which may be mediated by the activation of microglia and mGlu5 receptors in the spinal cord [58, 59]. Children with FASD display sensory-processing impairments, such as tactile, visual/auditory, and taste/smell sensitivity [60-62]. They also show inefficient integration of visual, vestibular, and somatosensory information, which may contribute to poor balance and diminished postural control [63, 64]. Although the underlying mechanisms for sensory deficits are unclear, our findings of ethanol-induced loss of dorsal horn interneurons in the spinal cord provide an important insight into the mechanisms of behavioral deficits observed in FASD.

Although neuroapoptosis and degeneration are not obvious in the ventral horn of developing spinal cord, a reduction of ventral horn neurons is also observed in adult spinal cord (Fig. 3C). It has been reported that prenatal ethanol exposure throughout gestation selectively decreases the number and size of motoneurons in the ventral horn of the spinal cord of rats [65, 66]. The loss of motor neurons may contribute to motor deficits in children with FASD [67, 68].

Ethanol-induced glial activation and macrophage infiltration

It is believed that neuroinflammation contributes to the neuropathologic and behavioral consequences in FASD [14, 69]. In the developing CNS, ethanol-induced apoptotic neurodegeneration is usually accompanied with the activation of glial cells and neuroinflammation [70-73]. Ethanol increases the expression of GFAP and Iba-1 in the developing spinal cord (Figs. 4A-C), suggesting the activation of astrocytes and microglia, respectively. Ethanol also increases the expression of MHC-II, a marker for microglial activation and neuroinflammation [34]. Ethanol-induced CD68 expression suggests macrophage infiltration (Figs. 4D and 4E). Pathological activation of microglia leads to neuroinflammation and neurodegeneration [74-76]. It is believed that ethanol-induced microglial activation in the developing brain may contribute to neuronal damage [69]. Recent studies indicate that the expression of P2X7 is also a good indication of microglial activation [77]. Microglial P2X7 activation causes neuroinflammation, which in turn induces neurodegeneration by releasing pro-inflammatory cytokines [33, 77, 78]. We show that ethanol increases the expression of P2X7, suggesting the involvement of microglial activation and neuroinflammation in ethanol-induced spinal cord damage.

Ethanol-induced activation of pro-apoptotic and pro-inflammatory signaling

Three key signaling proteins regulating neuroinflammation, namely glycogen GSK3 β , JNKs, and p38 MAPK, were examined. GSK3 β , a multifunctional serine/threonine kinase, responds to various cellular stresses, and regulates diverse developmental events in the immature brain, such as neurogenesis and neuronal differentiation, migration, and survival [41, 42, 79]. It also regulates microglial activation and neuroinflammation [80]. GSK3 β plays an important role in ethanol-induced neurodegeneration and inhibition of differentiation in the developing brain and cultured neuronal cells [22, 41, 81]. Ethanol activates GSK3 β in the developing spinal cord by inhibiting Ser9 phosphorylation (Fig. 8). This finding is consistent with the previous observation that ethanol causes Ser9 dephosphorylation in the developing brain [22, 81].

JNKs are stress responsive proteins and regulate gene expression, neuronal plasticity, neurodegeneration, and neuroinflammation [44]. JNKs are also involved in microglial activation in the CNS [82, 83]. Previous studies show that ethanol exposure activates JNKs which may contribute to ethanol-induced neuronal death in the developing brain of PD7 rat pups [84, 85]. We show that ethanol increases the phosphorylation of JNK and c-Jun, indicating the activation of the JNK pathway in the developing spinal cord.

Endoplasmic reticulum (ER) stress and oxidative stress

We have previously shown that ethanol caused ER stress in the brain of early postnatal mouse pups [19]. The current study demonstrates that ethanol significantly

up-regulates the expression of a number of UPR proteins, such as, ATF6, PERK, p-EIF2 α , GRP94, XBP-1, CHOP, cleaved caspase-12, and MANF in the developing spinal cord, indicating ER stress. We also show that ethanol increases DNP and 4-HNE, indicating oxidative stress. There is considerable interaction between oxidative stress and ER stress [13]. Therefore, it is possible that the interaction of ER stress and oxidative stress contributes to ethanol-induced spinal cord damage.

Role of MCP-1 and CCR2 in ethanol-induced damage to the developing spinal cord

MCP-1/CCR2 signaling regulates neuroinflammation following CNS injuries and has been implicated in many neurodegenerative disorders [26-29]. Knocking out MCP-1 and CCR2 offers protection against CNS damage associated with neuroinflammation. For example, MCP-1^{-/-} and CCR2^{-/-} mice show a decreased expression of pro-inflammatory cytokines and macrophage infiltration, lessened brain injury/neurodegeneration, and improved hippocampal based learning/ memory after LPS injection or traumatic brain injury [86-92].

In this study, we show that MCP-1^{-/-} and CCR2^{-/-} mice are less susceptible to ethanol-induced apoptotic cell death, glial activation, macrophage infiltration, and ER stress in the developing spinal cord (Figs. 9-12). The protection provided by MCP-1 and CCR2 knock out is likely mediated by a reduction of pathologic neuroninflammation. In experimental autoimmune encephalomyelitis (EAE), we have previously demonstrated that MCP-1/CCR2 signaling plays an important role in microglial activation and macrophage infiltration in adult spinal cord, and inhibition of MCP-

1/CCR2 signaling offers significant protection [93]. Although our findings suggest that CCR2-mediated neuroinflammation was involved, we could not rule out the possibility of ethanol directly killing neurons. It is possible that ethanol initially targets neurons which causes neuronal damage, triggering CCR2-mediated neuroinflammation, thereby exacerbating neuroapoptosis.

MCP-1/CCR2 signaling regulates GSK3 β and JNKs as MCP-1^{-/-} and CCR2^{-/-} mice show attenuated or blocked activation of GSK3 β and JNKs in response to ethanol exposure (Fig. 12). However, the mechanisms underlying the connection between MCP-1/CCR2 signaling and GSK3 β /JNK activation are currently unclear. Both GSK3 β and JNKs are important regulators of microglial activation. Therefore, the activation of MCP-1/CCR2, GSK3 β and JNKs pathway could result in microglial activation which is evident by increased P2X7 and Iba-1 expression.

MCP-1/CCR2 signaling may play an important role in ethanol-induced ER stress because ethanol-induced upregulation of UPR molecules is significantly reduced or blocked in MCP-1^{-/-} and CCR2^{-/-} mice (Fig. 11). The role of MCP-1/CCR2 signaling in ER stress has been previously demonstrated. For example, Kim et al [94] show that CCR2 inhibitor attenuates ER stress and decreases the expression of inflammatory cytokines in the liver of type 2 diabetic mice. The activation of MCP-1/CCR2 signaling may cause ER stress through the upregulation of MCP-1 in cardiomyocytes and osteoclasts [95]. We show that ethanol increased the expression of MCP-1 which is associated with enhanced ER stress, confirming the involvement of MCP-1/CCR2 signaling in ethanol-induced ER stress. It appears that MCP-1/CCR2 signaling has little involvement in oxidative stress in the developing spinal cord as MCP-1^{-/-} and CCR2^{-/-}

mice are similarly affected by ethanol-induced oxidative stress compared to WT mice (data not shown).

Ethanol increases the expression of CCR2 but not MCP-1 (Fig. 5), and CCR2^{-/-} mice are much more resistant than MCP-1^{-/-} mice to ethanol-induced damage to the spinal cord (Figs. 9-12). CCR2 has multiple ligands. MCP-1 binds and activates CCR2, but other chemokines, such as CCL7, 8, 12, 13 and 16 can also interact with CCR2 and stimulate CCR2-mediated signaling [96]. Therefore, the levels of CCR2 are a more critical determinant for the action of MCP-1 and other chemokines [96, 97]. As a result, CCR2 knock out is more effective. It remains to be tested whether other chemokines, besides MCP-1, are involved in ethanol-induced damage in the developing spinal cord. It is noted that ethanol increases GFAP and Iba-1 expression in MCP-1^{-/-} mice (Fig. 10). This suggests that ethanol may activate glial cells independent of MCP-1; however, the underlying mechanisms are unclear.

In summary, the developing spinal cord is vulnerable to ethanol. In the animal model, the developmental exposure to ethanol may produce permanent deficits associated with spinal cord function. However, the clinical relevance needs to be further validated in FASD. Furthermore, the current study indicates that ethanol-induced apoptotic death of spinal cord neurons may be caused by the combined action of microglial activation, neuroinflammation, ER stress, and oxidative stress. Although the underlying mechanisms are not clear, MCP-1/CCR2 signaling is involved these pathologic processes and play an important role in ethanol-induced damages to the developing spinal cord.

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Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

References

- [1] O.A. Parsons, Neurocognitive deficits in alcoholics and social drinkers: a continuum?, *Alcoholism, clinical and experimental research*, 22 (1998) 954-961.
- [2] C. Harper, The neuropathology of alcohol-specific brain damage, or does alcohol damage the brain?, *Journal of neuropathology and experimental neurology*, 57 (1998) 101-110.
- [3] J. Luo, Effects of Ethanol on the Cerebellum: Advances and Prospects, *Cerebellum*, 14 (2015) 383-385.
- [4] C.R. Goodlett, K.H. Horn, Mechanisms of alcohol-induced damage to the developing nervous system, *Alcohol research & health : the journal of the National Institute on Alcohol Abuse and Alcoholism*, 25 (2001) 175-184.
- [5] J. Luo, Autophagy and ethanol neurotoxicity, *Autophagy*, 10 (2014) 2099-2108.
- [6] S.K. Clarren, E.C. Alvord, Jr., S.M. Sumi, A.P. Streissguth, D.W. Smith, Brain malformations related to prenatal exposure to ethanol, *The Journal of pediatrics*, 92 (1978) 64-67.
- [7] V.W. Swayze, 2nd, V.P. Johnson, J.W. Hanson, J. Piven, Y. Sato, J.N. Giedd, D. Mosnik, N.C. Andreasen, Magnetic resonance imaging of brain anomalies in fetal alcohol syndrome, *Pediatrics*, 99 (1997) 232-240.
- [8] S.L. Archibald, C. Fennema-Notestine, A. Gamst, E.P. Riley, S.N. Mattson, T.L. Jernigan, Brain dysmorphology in individuals with severe prenatal alcohol exposure, *Developmental medicine and child neurology*, 43 (2001) 148-154.

- [9] F.L. Bookstein, A.P. Streissguth, P.D. Connor, P.D. Sampson, Damage to the human cerebellum from prenatal alcohol exposure: the anatomy of a simple biometrical explanation, *Anatomical record. Part B, New anatomist*, 289 (2006) 195-209.
- [10] E.R. Sowell, S.N. Mattson, E. Kan, P.M. Thompson, E.P. Riley, A.W. Toga, Abnormal cortical thickness and brain-behavior correlation patterns in individuals with heavy prenatal alcohol exposure, *Cerebral cortex*, 18 (2008) 136-144.
- [11] K.L. Jones, D.W. Smith, Recognition of the fetal alcohol syndrome in early infancy, *Lancet*, 302 (1973) 999-1001.
- [12] C. Guerri, A. Bazinet, E.P. Riley, Foetal Alcohol Spectrum Disorders and alterations in brain and behaviour, *Alcohol and alcoholism*, 44 (2009) 108-114.
- [13] F. Yang, J. Luo, Endoplasmic Reticulum Stress and Ethanol Neurotoxicity, *Biomolecules*, 5 (2015) 2538-2553.
- [14] C.J. Kane, P.D. Drew, Inflammatory responses to alcohol in the CNS: nuclear receptors as potential therapeutics for alcohol-induced neuropathologies, *Journal of leukocyte biology*, 100 (2016) 951-959.
- [15] P.D. Drew, C.J. Kane, Peroxisome Proliferator-Activated Receptor-gamma Agonists: Potential Therapeutics for Neuropathology Associated with Fetal Alcohol Spectrum Disorders, *Journal of clinical & cellular immunology*, 7 (2016).
- [16] J.W. Olney, Fetal alcohol syndrome at the cellular level, *Addiction biology*, 9 (2004) 137-149; discussion 151.
- [17] J.W. Olney, T. Tenkova, K. Dikranian, Y.Q. Qin, J. Labruyere, C. Ikonomidou, Ethanol-induced apoptotic neurodegeneration in the developing C57BL/6 mouse brain, *Brain research. Developmental brain research*, 133 (2002) 115-126.

- [18] D.F. Wozniak, R.E. Hartman, M.P. Boyle, S.K. Vogt, A.R. Brooks, T. Tenkova, C. Young, J.W. Olney, L.J. Muglia, Apoptotic neurodegeneration induced by ethanol in neonatal mice is associated with profound learning/memory deficits in juveniles followed by progressive functional recovery in adults, *Neurobiology of disease*, 17 (2004) 403-414.
- [19] Z. Ke, X. Wang, Y. Liu, Z. Fan, G. Chen, M. Xu, K.A. Bower, J.A. Frank, M. Li, S. Fang, X. Shi, J. Luo, Ethanol induces endoplasmic reticulum stress in the developing brain, *Alcoholism, clinical and experimental research*, 35 (2011) 1574-1583.
- [20] Z. Ke, Y. Liu, X. Wang, Z. Fan, G. Chen, M. Xu, K.A. Bower, J.A. Frank, X. Ou, X. Shi, J. Luo, Cyanidin-3-glucoside ameliorates ethanol neurotoxicity in the developing brain, *Journal of neuroscience research*, 89 (2011) 1676-1684.
- [21] A. Alimov, H. Wang, M. Liu, J.A. Frank, M. Xu, X. Ou, J. Luo, Expression of autophagy and UPR genes in the developing brain during ethanol-sensitive and resistant periods, *Metabolic brain disease*, 28 (2013) 667-676.
- [22] Y. Liu, G. Chen, C. Ma, K.A. Bower, M. Xu, Z. Fan, X. Shi, Z.J. Ke, J. Luo, Overexpression of glycogen synthase kinase 3 β sensitizes neuronal cells to ethanol toxicity, *Journal of neuroscience research*, 87 (2009) 2793-2802.
- [23] G. Chen, Z. Ke, M. Xu, M. Liao, X. Wang, Y. Qi, T. Zhang, J.A. Frank, K.A. Bower, X. Shi, J. Luo, Autophagy is a protective response to ethanol neurotoxicity, *Autophagy*, 8 (2012) 1577-1589.
- [24] Y.S. Kang, J.J. Cha, Y.Y. Hyun, D.R. Cha, Novel C-C chemokine receptor 2 antagonists in metabolic disease: a review of recent developments, *Expert opinion on investigational drugs*, 20 (2011) 745-756.

- [25] M. Xia, Z. Sui, Recent developments in CCR2 antagonists, Expert opinion on therapeutic patents, 19 (2009) 295-303.
- [26] S. Bose, J. Cho, Role of chemokine CCL2 and its receptor CCR2 in neurodegenerative diseases, Archives of pharmacal research, 36 (2013) 1039-1050.
- [27] L. Gao, H. Tang, K. Nie, L. Wang, J. Zhao, R. Gan, J. Huang, S. Feng, R. Zhu, Z. Duan, Y. Zhang, X. Zhao, Y. Zhang, L. Wang, MCP-1 and CCR2 gene polymorphisms in Parkinson's disease in a Han Chinese cohort, Neurological sciences : official journal of the Italian Neurological Society and of the Italian Society of Clinical Neurophysiology, 36 (2015) 571-576.
- [28] B. Moser, M. Wolf, A. Walz, P. Loetscher, Chemokines: multiple levels of leukocyte migration control, Trends in immunology, 25 (2004) 75-84.
- [29] M.M. Acharya, N.H. Patel, B.M. Craver, K.K. Tran, E. Giedzinski, B.P. Tseng, V.K. Parihar, C.L. Limoli, Consequences of low dose ionizing radiation exposure on the hippocampal microenvironment, PloS one, 10 (2015) e0128316.
- [30] H. Wang, X. Wang, Z.J. Ke, A.L. Comer, M. Xu, J.A. Frank, Z. Zhang, X. Shi, J. Luo, Tunicamycin-induced unfolded protein response in the developing mouse brain, Toxicology and applied pharmacology, 283 (2015) 157-167.
- [31] L.C. Schmued, C.C. Stowers, A.C. Scallet, L. Xu, Fluoro-Jade C results in ultra high resolution and contrast labeling of degenerating neurons, Brain research, 1035 (2005) 24-31.
- [32] P.D. Drew, C.J. Kane, Fetal alcohol spectrum disorders and neuroimmune changes, International review of neurobiology, 118 (2014) 41-80.

- [33] C. Volonte, S. Apolloni, S.D. Skaper, G. Burnstock, P2X7 receptors: channels, pores and more, *CNS & neurological disorders drug targets*, 11 (2012) 705-721.
- [34] M. Gupta, G. Kaur, Aqueous extract from the *Withania somnifera* leaves as a potential anti-neuroinflammatory agent: a mechanistic study, *Journal of neuroinflammation*, 13 (2016) 193.
- [35] T.Y. Na, Y.H. Han, N.L. Ka, H.S. Park, Y.P. Kang, S.W. Kwon, B.H. Lee, M.O. Lee, 22-S-Hydroxycholesterol protects against ethanol-induced liver injury by blocking the auto/paracrine activation of MCP-1 mediated by LXRA, *The Journal of pathology*, 235 (2015) 710-720.
- [36] J. Jura, L. Skalniak, A. Koj, Monocyte chemotactic protein-1-induced protein-1 (MCP-1) is a novel multifunctional modulator of inflammatory reactions, *Biochimica et biophysica acta*, 1823 (2012) 1905-1913.
- [37] G. Chen, C. Ma, K.A. Bower, X. Shi, Z. Ke, J. Luo, Ethanol promotes endoplasmic reticulum stress-induced neuronal death: involvement of oxidative stress, *Journal of neuroscience research*, 86 (2008) 937-946.
- [38] M. Comporti, C. Signorini, S. Leoncini, C. Gardi, L. Ciccoli, A. Giardini, D. Vecchio, B. Arezzini, Ethanol-induced oxidative stress: basic knowledge, *Genes & nutrition*, 5 (2010) 101-109.
- [39] C. Ji, Mechanisms of alcohol-induced endoplasmic reticulum stress and organ injuries, *Biochemistry research international*, 2012 (2012) 216450.
- [40] F.U. Amin, S.A. Shah, M.O. Kim, Glycine inhibits ethanol-induced oxidative stress, neuroinflammation and apoptotic neurodegeneration in postnatal rat brain, *Neurochemistry international*, 96 (2016) 1-12.

- [41] J. Luo, GSK3beta in ethanol neurotoxicity, *Molecular neurobiology*, 40 (2009) 108-121.
- [42] J. Luo, The role of GSK3beta in the development of the central nervous system, *Frontiers in biology*, 7 (2012) 212-220.
- [43] A.K. Srivastava, S.K. Pandey, Potential mechanism(s) involved in the regulation of glycogen synthesis by insulin, *Molecular and cellular biochemistry*, 182 (1998) 135-141.
- [44] R. Yarza, S. Vela, M. Solas, M.J. Ramirez, c-Jun N-terminal Kinase (JNK) Signaling as a Therapeutic Target for Alzheimer's Disease, *Frontiers in pharmacology*, 6 (2015) 321.
- [45] J. Dobbing, J. Sands, Comparative aspects of the brain growth spurt, *Early human development*, 3 (1979) 79-83.
- [46] T. Urso, J.S. Gavalier, D.H. Van Thiel, Blood ethanol levels in sober alcohol users seen in an emergency room, *Life sciences*, 28 (1981) 1053-1056.
- [47] D. Cartlidge, A.D. Redmond, Alcohol and conscious level, *Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie*, 44 (1990) 205-208.
- [48] A.W. Jones, The drunkenest drinking driver in Sweden: blood alcohol concentration 0.545% w/v, *Journal of studies on alcohol*, 60 (1999) 400-406.
- [49] J.J. van Hoof, N. Van Der Lely, S.H. Bouthoorn, W.E. Van Dalen, R.R. Pereira, Adolescent alcohol intoxication in the Dutch hospital departments of pediatrics: a 2-year comparison study, *The Journal of adolescent health : official publication of the Society for Adolescent Medicine*, 48 (2011) 212-214.
- [50] A.W. Jones, P. Harding, Driving under the influence with blood alcohol concentrations over 0.4 g%, *Forensic science international*, 231 (2013) 349-353.

- [51] E. Van Zanten, T. Van der Ploeg, J.J. Van Hoof, N. Van der Lely, Gender, age, and educational level attribute to blood alcohol concentration in hospitalized intoxicated adolescents; a cohort study, *Alcoholism, clinical and experimental research*, 37 (2013) 1188-1194.
- [52] K. Malejko, H. Graf, M. Gahr, Survival of Very High Blood Alcohol Concentration Without Consequential Damage in a Patient Without a Previous Substance Use Disorder, *Journal of forensic sciences*, 61 (2016) 1155-1157.
- [53] P. Punnakal, C. von Schoultz, K. Haenraets, H. Wildner, H.U. Zeilhofer, Morphological, biophysical and synaptic properties of glutamatergic neurons of the mouse spinal dorsal horn, *The Journal of physiology*, 592 (2014) 759-776.
- [54] A.J. Todd, Neuronal circuitry for pain processing in the dorsal horn, *Nature reviews. Neuroscience*, 11 (2010) 823-836.
- [55] R. Mizuguchi, S. Kriks, R. Cordes, A. Gossler, Q. Ma, M. Goulding, *Ascl1* and *Gsh1/2* control inhibitory and excitatory cell fate in spinal sensory interneurons, *Nature neuroscience*, 9 (2006) 770-778.
- [56] A. Pillai, A. Mansouri, R. Behringer, H. Westphal, M. Goulding, *Lhx1* and *Lhx5* maintain the inhibitory-neurotransmitter status of interneurons in the dorsal spinal cord, *Development*, 134 (2007) 357-366.
- [57] H.U. Zeilhofer, D. Benke, G.E. Yevenes, Chronic pain states: pharmacological strategies to restore diminished inhibitory spinal pain control, *Annual review of pharmacology and toxicology*, 52 (2012) 111-133.

- [58] K. Miyoshi, M. Narita, M. Takatsu, T. Suzuki, mGlu5 receptor and protein kinase C implicated in the development and induction of neuropathic pain following chronic ethanol consumption, *European journal of pharmacology*, 562 (2007) 208-211.
- [59] M. Narita, K. Miyoshi, M. Narita, T. Suzuki, Involvement of microglia in the ethanol-induced neuropathic pain-like state in the rat, *Neuroscience letters*, 414 (2007) 21-25.
- [60] L. Franklin, J. Deitz, T. Jirikowic, S. Astley, Children with fetal alcohol spectrum disorders: problem behaviors and sensory processing, *The American journal of occupational therapy : official publication of the American Occupational Therapy Association*, 62 (2008) 265-273.
- [61] T. Jirikowic, H.C. Olson, D. Kartin, Sensory processing, school performance, and adaptive behavior of young school-age children with fetal alcohol spectrum disorders, *Physical & occupational therapy in pediatrics*, 28 (2008) 117-136.
- [62] L. Williams, C.P. Jackson, N. Choe, L. Pelland, S.H. Scott, J.N. Reynolds, Sensory-motor deficits in children with fetal alcohol spectrum disorder assessed using a robotic virtual reality platform, *Alcoholism, clinical and experimental research*, 38 (2014) 116-125.
- [63] T.L. Jirikowic, S.W. McCoy, A. Lubetzky-Vilnai, R. Price, M.A. Ciol, D. Kartin, L.Y. Hsu, B. Gendler, S.J. Astley, Sensory control of balance: a comparison of children with fetal alcohol spectrum disorders to children with typical development, *Journal of population therapeutics and clinical pharmacology = Journal de la therapeutique des populations et de la pharamcologie clinique*, 20 (2013) e212-228.

- [64] K.D. Hansen, T. Jirikowic, A comparison of the sensory profile and sensory processing measure home form for children with fetal alcohol spectrum disorders, *Physical & occupational therapy in pediatrics*, 33 (2013) 440-452.
- [65] P. David, K. Subramaniam, The effects of prenatal alcohol exposure on the morphological characteristics of spinal motoneurons, *Birth defects research. Part A, Clinical and molecular teratology*, 85 (2009) 791-799.
- [66] M.B. Barrow Heaton, K. Kidd, D. Bradley, M. Paiva, J. Mitchell, D.W. Walker, Prenatal ethanol exposure reduces spinal cord motoneuron number in the fetal rat but does not affect GDNF target tissue protein, *Developmental neuroscience*, 21 (1999) 444-452.
- [67] R. Doney, B.R. Lucas, R.E. Watkins, T.W. Tsang, K. Sauer, P. Howat, J. Latimer, J.P. Fitzpatrick, J. Oscar, M. Carter, E.J. Elliott, Visual-motor integration, visual perception, and fine motor coordination in a population of children with high levels of Fetal Alcohol Spectrum Disorder, *Research in developmental disabilities*, 55 (2016) 346-357.
- [68] R. Feldmann, N. Girke, Mental and Motor Development of Children with Preterm Birth and Children with Fetal Alcohol Syndrome, *Klinische Padiatrie*, 227 (2015) 259-263.
- [69] L.G. Chastain, D.K. Sarkar, Role of microglia in regulation of ethanol neurotoxic action, *International review of neurobiology*, 118 (2014) 81-103.
- [70] C.J. Kane, S.M. Smith, R.C. Miranda, J. Kable, Proceedings of the 2010 annual meeting of the Fetal Alcohol Spectrum Disorders Study Group, *Alcohol*, 46 (2012) 107-114.

- [71] Y.N. Zhao, F. Wang, Y.X. Fan, G.F. Ping, J.Y. Yang, C.F. Wu, Activated microglia are implicated in cognitive deficits, neuronal death, and successful recovery following intermittent ethanol exposure, *Behavioural brain research*, 236 (2013) 270-282.
- [72] K.E. Ahlers, B. Karacay, L. Fuller, D.J. Bonthius, M.E. Dailey, Transient activation of microglia following acute alcohol exposure in developing mouse neocortex is primarily driven by BAX-dependent neurodegeneration, *Glia*, 63 (2015) 1694-1713.
- [73] M. Saito, G. Chakraborty, M. Hui, K. Masiello, M. Saito, Ethanol-Induced Neurodegeneration and Glial Activation in the Developing Brain, *Brain sciences*, 6 (2016).
- [74] G. Kaur, S.J. Han, I. Yang, C. Crane, Microglia and central nervous system immunity, *Neurosurgery clinics of North America*, 21 (2010) 43-51.
- [75] K.M. Lucin, T. Wyss-Coray, Immune activation in brain aging and neurodegeneration: too much or too little?, *Neuron*, 64 (2009) 110-122.
- [76] M.A. Lynch, The multifaceted profile of activated microglia, *Molecular neurobiology*, 40 (2009) 139-156.
- [77] A. Bhattacharya, K. Biber, The microglial ATP-gated ion channel P2X7 as a CNS drug target, *Glia*, 64 (2016) 1772-1787.
- [78] S.D. Skaper, P. Debetto, P. Giusti, The P2X7 purinergic receptor: from physiology to neurological disorders, *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*, 24 (2010) 337-345.
- [79] J. Luo, Lithium-mediated protection against ethanol neurotoxicity, *Frontiers in neuroscience*, 4 (2010) 41.

- [80] D.W. Maixner, H.R. Weng, The Role of Glycogen Synthase Kinase 3 Beta in Neuroinflammation and Pain, *Journal of pharmaceutics & pharmacology*, 1 (2013) 001.
- [81] G. Chen, K.A. Bower, M. Xu, M. Ding, X. Shi, Z.J. Ke, J. Luo, Cyanidin-3-glucoside reverses ethanol-induced inhibition of neurite outgrowth: role of glycogen synthase kinase 3 Beta, *Neurotoxicity research*, 15 (2009) 321-331.
- [82] C. Wang, X. Nie, Y. Zhang, T. Li, J. Mao, X. Liu, Y. Gu, J. Shi, J. Xiao, C. Wan, Q. Wu, Reactive oxygen species mediate nitric oxide production through ERK/JNK MAPK signaling in HAPI microglia after PFOS exposure, *Toxicology and applied pharmacology*, 288 (2015) 143-151.
- [83] H. Zhao, L. Cheng, Y. Liu, W. Zhang, S. Maharjan, Z. Cui, X. Wang, D. Tang, L. Nie, Mechanisms of anti-inflammatory property of conserved dopamine neurotrophic factor: inhibition of JNK signaling in lipopolysaccharide-induced microglia, *Journal of molecular neuroscience : MN*, 52 (2014) 186-192.
- [84] J.Y. Han, E.Y. Jeong, Y.S. Kim, G.S. Roh, H.J. Kim, S.S. Kang, G.J. Cho, W.S. Choi, C-jun N-terminal kinase regulates the interaction between 14-3-3 and Bad in ethanol-induced cell death, *Journal of neuroscience research*, 86 (2008) 3221-3229.
- [85] S.A. Shah, G.H. Yoon, M.O. Kim, Protection of the developing brain with anthocyanins against ethanol-induced oxidative stress and neurodegeneration, *Molecular neurobiology*, 51 (2015) 1278-1291.
- [86] S. Gyoneva, D. Kim, A. Katsumoto, O.N. Kokiko-Cochran, B.T. Lamb, R.M. Ransohoff, Ccr2 deletion dissociates cavity size and tau pathology after mild traumatic brain injury, *Journal of neuroinflammation*, 12 (2015) 228.

- [87] D.R. Huang, J. Wang, P. Kivisakk, B.J. Rollins, R.M. Ransohoff, Absence of monocyte chemoattractant protein 1 in mice leads to decreased local macrophage recruitment and antigen-specific T helper cell type 1 immune response in experimental autoimmune encephalomyelitis, *The Journal of experimental medicine*, 193 (2001) 713-726.
- [88] D.J. Mahad, R.M. Ransohoff, The role of MCP-1 (CCL2) and CCR2 in multiple sclerosis and experimental autoimmune encephalomyelitis (EAE), *Seminars in immunology*, 15 (2003) 23-32.
- [89] W.L. Thompson, W.J. Karpus, L.J. Van Eldik, MCP-1-deficient mice show reduced neuroinflammatory responses and increased peripheral inflammatory responses to peripheral endotoxin insult, *Journal of neuroinflammation*, 5 (2008) 35.
- [90] A.J. Sawyer, W. Tian, J.K. Saucier-Sawyer, P.J. Rizk, W.M. Saltzman, R.V. Bellamkonda, T.R. Kyriakides, The effect of inflammatory cell-derived MCP-1 loss on neuronal survival during chronic neuroinflammation, *Biomaterials*, 35 (2014) 6698-6706.
- [91] A.R. Allen, K. Eilertson, S. Sharma, D. Schneider, J. Baure, B. Allen, S. Rosi, J. Raber, J.R. Fike, Effects of radiation combined injury on hippocampal function are modulated in mice deficient in chemokine receptor 2 (CCR2), *Radiation research*, 180 (2013) 78-88.
- [92] K. Belarbi, T. Jopson, C. Arellano, J.R. Fike, S. Rosi, CCR2 deficiency prevents neuronal dysfunction and cognitive impairments induced by cranial irradiation, *Cancer research*, 73 (2013) 1201-1210.

- [93] Z. Ji, Z. Fan, Y. Zhang, R. Yu, H. Yang, C. Zhou, J. Luo, Z.J. Ke, Thiamine deficiency promotes T cell infiltration in experimental autoimmune encephalomyelitis: the involvement of CCL2, *Journal of immunology*, 193 (2014) 2157-2167.
- [94] H.M. Kim, E.S. Lee, B.R. Lee, D. Yadav, Y.M. Kim, H.J. Ko, K.S. Park, E.Y. Lee, C.H. Chung, C-C chemokine receptor 2 inhibitor ameliorates hepatic steatosis by improving ER stress and inflammation in a type 2 diabetic mouse model, *PloS one*, 10 (2015) e0120711.
- [95] P.E. Kolattukudy, J. Niu, Inflammation, endoplasmic reticulum stress, autophagy, and the monocyte chemoattractant protein-1/CCR2 pathway, *Circulation research*, 110 (2012) 174-189.
- [96] S.L. Deshmane, S. Kremlev, S. Amini, B.E. Sawaya, Monocyte chemoattractant protein-1 (MCP-1): an overview, *Journal of interferon & cytokine research : the official journal of the International Society for Interferon and Cytokine Research*, 29 (2009) 313-326.
- [97] G. Yang, Y. Meng, W. Li, Y. Yong, Z. Fan, H. Ding, Y. Wei, J. Luo, Z.J. Ke, Neuronal MCP-1 mediates microglia recruitment and neurodegeneration induced by the mild impairment of oxidative metabolism, *Brain pathology*, 21 (2011) 279-297.

Figure legends

Figure 1. Ethanol induces neuroapoptosis and neurodegeneration in the developing spinal cord

A: C57BL6 mice of postnatal (PD) 4, PD7 and PD12 were exposed to ethanol as described in the Materials and Methods. Eight hours after ethanol exposure, mice were sacrificed and the spinal cord was processed for IHC of cleaved caspase-3. Bar = 200 μ m. **B:** C57BL6 mice of PD7 were exposed to ethanol as described above. At 4, 8, 12 or 24 hours following ethanol exposure, the lumbar spinal cord was processed for IHC of cleaved caspase-3. Bar = 200 μ m. **C:** C57BL6 mice of PD7 were exposed to ethanol. At 8 hours following ethanol exposure, the spinal cord was dissected and the cervical, thoracic and lumbar segments were processed for IHC of cleaved caspase-3. Bar = 200 μ m. **D:** C57BL6 mice of PD7 were exposed to ethanol for 8 hours. The spinal cord was dissected and processed for immunofluorescence staining of cleaved caspase 3 and NeuN. Bar = 100 μ m at the upper panel and 20 μ m at the lower panel. **E:** The spinal cord samples shown in panel **D** were assayed for neurodegeneration by Fluoro-Jade C staining. Bar = 100 μ m. Four-five animals were analyzed for each group.

Figure 2. Ethanol causes apoptosis in the developing spinal cord

C57BL6 mice of PD7 were exposed to ethanol for 8 hours and the spinal cord tissues were processed for immunoblotting analysis of cleaved caspase-3, caspase-8, caspase-9, and PARP. The expression of these proteins was quantified and normalized to the expression of β -actin. Each data point was the mean \pm SEM of three independent

experiments. * denotes significant difference ($p < 0.05$). ** denotes significant difference ($p < 0.01$).

Figure 3. Developing exposure to ethanol results in reduced neurons in adult spinal cord

C57BL6 mice of PD7 were exposed to ethanol as described in the Materials and Methods. The mice were maintained and sacrificed at the age of 8-weeks-old. **A**: The neurons in the spinal cord were visualized by NeuN IHC. Bar = 200 μ m in upper panels; 100 μ m in lower panel). The images show the lumbar section of the spinal cord. **B and C**: The number and size of NeuN-positive cells in the dorsal and ventral horn of the spinal cord were determined in twenty randomly selected sections using the software of Image lab 5.2. Four-five animals were analyzed for each group. ** denotes significant difference ($p < 0.01$).

Figure 4. Effect of ethanol on glial activation and macrophage infiltration

C57BL6 mice of PD7 were exposed to ethanol for 8 hours. **A and B**: Mice were sacrificed and the spinal cord was processed for immunofluorescent staining of Iba-1 (red), GFAP (green), and DAPI (blue). Bar = 100 μ m in panel **A**; bar = 200 μ m in panel **B**. **C**: The expression of Iba-1 and GFAP was determined by immunoblotting, and quantified and normalized to β -actin. **D**: The infiltration of macrophages in the spinal cord was determined by CD68 IHC. Bar = 200 μ m in left column, Bar = 100 μ m in middle and left columns. **E and F**: The expression of CD68 and P2X7 was determined by immunoblotting, and quantified and normalized to β -actin. Each data point was the

mean \pm SEM of three animals. * denotes statistical difference ($p < 0.05$), and ** denotes significant difference ($p < 0.01$).

Figure 5. Effect of ethanol on MCP-1, CCR2 and MCP-1P expression

C57BL6 mice of PD7 were exposed to ethanol for 8 hours. Mice were sacrificed and the spinal cord was analyzed for the expression of MCP-1 (**A**), CCR2 (**B**) and MCP-1P (**C**) by immunoblotting. The expression was quantified and normalized to the expression of β -actin. Each data point was the mean \pm SEM of three animals. * denotes statistical difference ($p < 0.05$).

Figure 6. Ethanol induces ER stress in the developing spinal cord

C57BL6 mice of PD7 were exposed to ethanol for 8 hours. Mice were sacrificed and the spinal cord tissues were processed for the immunoblotting analysis of a number of ER stress markers (ATF6, p-PERK, p-eIF2 α , GRP94, XBP-1 and CHOP) (**A**), cleaved caspase 12 (**B**) and MANF (**C**). The relative expression of these ER stress markers were quantified and normalized to β -actin. Each data point was the mean \pm SEM of three animals. * denotes statistical difference ($p < 0.05$) and ** denotes significant difference ($p < 0.01$).

Figure 7. Ethanol induces oxidative stress in the developing spinal cord

C57BL6 mice of PD7 were exposed to ethanol for 8 hours. **A**: Mice were sacrificed and the spinal cord tissues were processed for the immunoblotting analysis of protein oxidation marker dinitrophenol (DNP) and lipid peroxidation marker 4-hydroxynonenal

(HNE). The relative expression of DNP and HNE was quantified and normalized to β -actin. **B**: The levels of HNE and DNP in the spinal cord tissues were quantified by ELISA as described in the Materials and Methods. Each data point was the mean \pm SEM of three animals. * denotes statistical difference ($p<0.05$) and ** denotes significant difference ($p<0.01$).

Figure 8. Effect of ethanol on the phosphorylation of JNKs, c-Jun, and GSK3 β

C57BL6 mice of PD7 were exposed to ethanol for 8 hours. **A**: Mice were euthanized, and the spinal cord tissues were evaluated for the expression/phosphorylation of GSK3 β , JNK and c-Jun. **B-D**: The phosphorylation levels of GSK3 β , JNK, and c-Jun were quantified and normalized to β -actin. Each data point was the mean \pm SEM of three animals. * denotes statistical difference ($p<0.05$) and ** denotes significant difference ($p<0.01$).

Figure 9. MCP-1 and CCR2 knockout mice are resistant to ethanol-induced apoptosis

Wild type (WT), MCP-1^{-/-} and CCR2^{-/-} mice of PD7 were exposed to ethanol for 8 hours. **A**: Mice were sacrificed, and the lumbar spinal cord was processed for IHC of cleaved caspase-3. Bar = 200 μ m. **B**: The expression of cleaved caspase 3 and PARP was determined by immunoblotting, and quantified by normalizing to β -actin. **C**: expression of cleaved caspase 8, 9 and 12 determined by immunoblotting, and quantified by normalizing to β -actin. Each data point was the mean \pm SEM of three animals. * ($p<0.05$) or ** ($p<0.01$) indicate difference between control and ethanol-treated groups; # ($p<0.05$)

or ## ($p<0.01$) indicate the difference between ethanol-treated groups of WT and MCP-1^{-/-} or CCR2^{-/-} mice.

Figure 10. MCP-1 and CCR2 knockout mice are resistant to ethanol-induced glial activation and macrophage infiltration

WT, MCP-1^{-/-} and CCR2^{-/-} mice of PD7 were exposed to ethanol for 8 hours. Mice were sacrificed, and the spinal cord was processed for immunoblotting analysis of GFAP (A), Iba-1 (B), CD68 (C), P2X7 (D), and MHC-II (E). The relative expression of these markers was quantified and normalized to β -actin. Each data point was the mean \pm SEM of three animals. * ($p<0.05$) or ** ($p<0.01$) indicate the difference between control and ethanol-treated groups; # ($p<0.05$) or ## ($p<0.01$) indicate the difference between ethanol-treated groups of WT and MCP-1^{-/-} or CCR2^{-/-} mice.

Figure 11 MCP-1 and CCR2 knockout mice are resistant to ethanol-induced ER stress

WT, MCP-1^{-/-} and CCR2^{-/-} mice of PD7 were exposed to ethanol for 8 hours. The expression of ER stress markers (ATF6, p-eIF2 α , p-PERK, GRP94, GRP78, and XBP-1) (A) and MANF (B) in the spinal cord was analyzed by immunoblotting. The relative expression of these markers was quantified and normalized to β -actin. Each data point was the mean \pm SEM of three animals. * ($p<0.05$) or ** ($p<0.01$) indicate the difference between control and ethanol-treated groups; # ($p<0.05$) or ## ($p<0.01$) indicate the difference between ethanol-treated groups of WT and MCP-1^{-/-} or CCR2^{-/-} mice.

Figure 12 Effect of ethanol on p-JNK, p-c-Jun, and p-GSK3 β in wild type (WT), MCP-1^{-/-} and CCR2^{-/-} mice

WT, MCP-1^{-/-} and CCR2^{-/-} mice of PD7 were exposed to ethanol for 8 hours. The expression/phosphorylation of GSK3 β (B), JNK (C), and c-Jun (D) was determined by immunoblotting. The relative expression of these proteins was quantified and normalized to β -actin. Each data point was the mean \pm SEM of three animals. * ($p < 0.05$) or ** ($p < 0.01$) indicate the difference between control and ethanol-treated groups; ## ($p < 0.01$) indicate the difference between ethanol-treated groups of WT and MCP-1^{-/-} or CCR2^{-/-} mice.

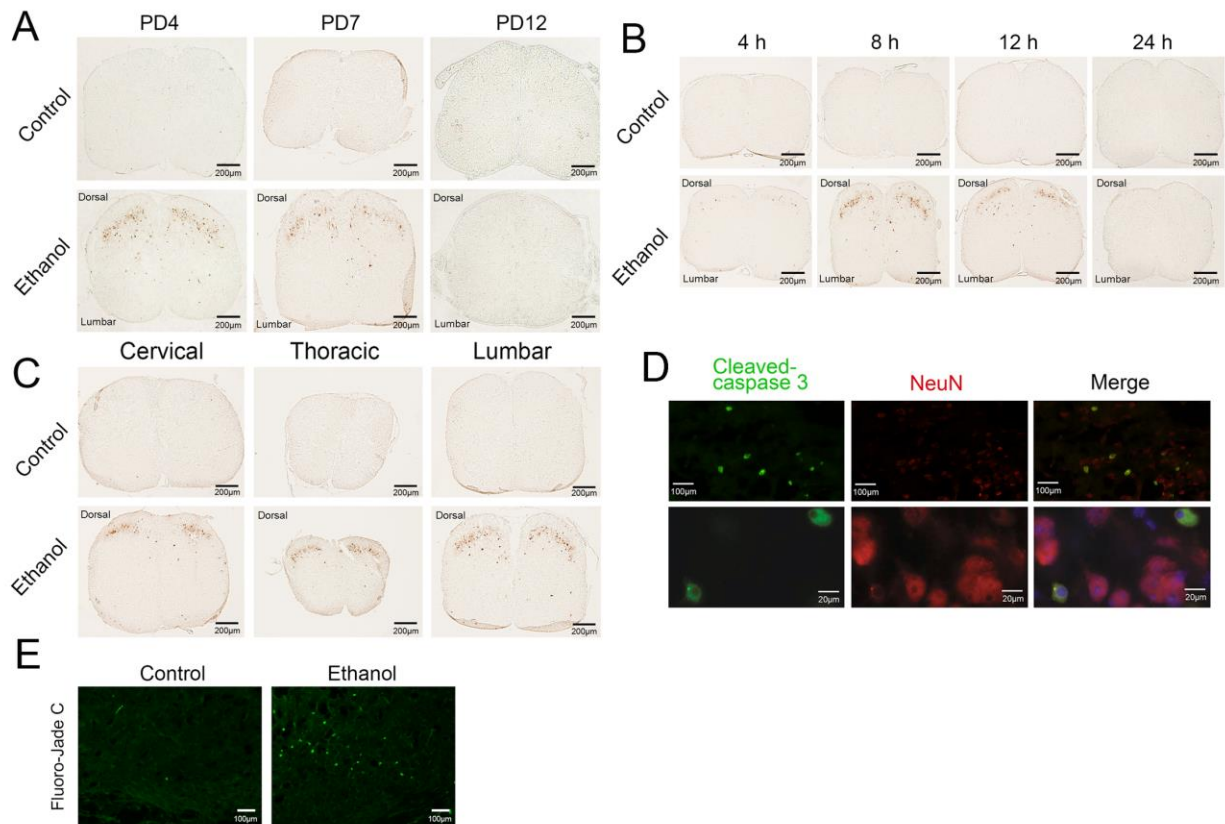


Fig. 1

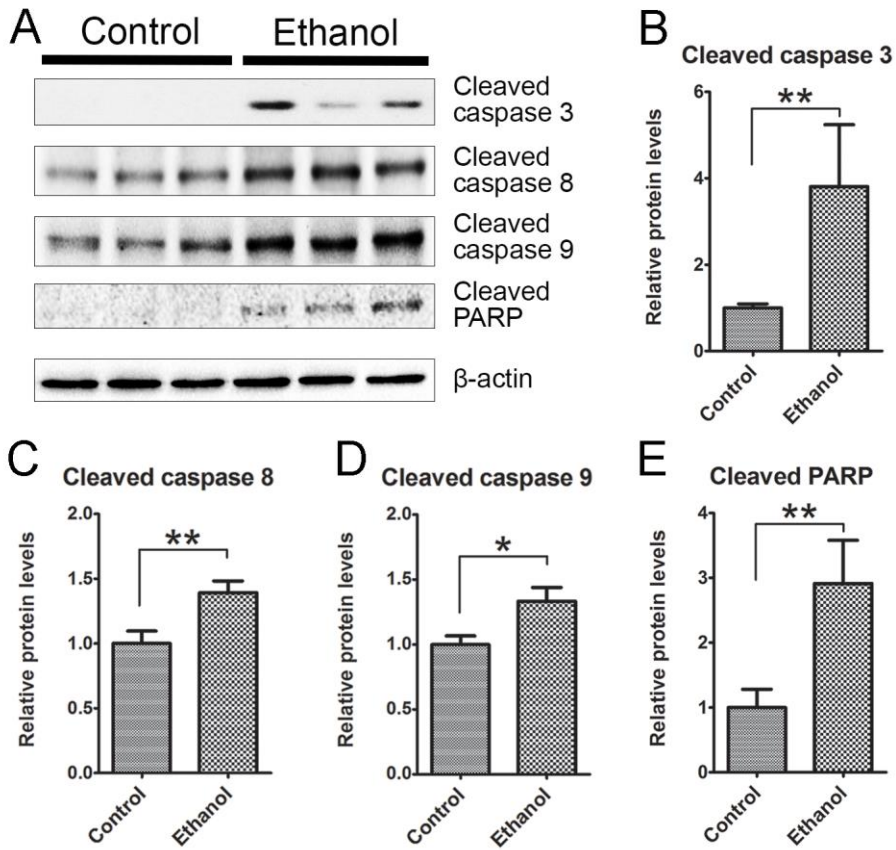


Fig. 2

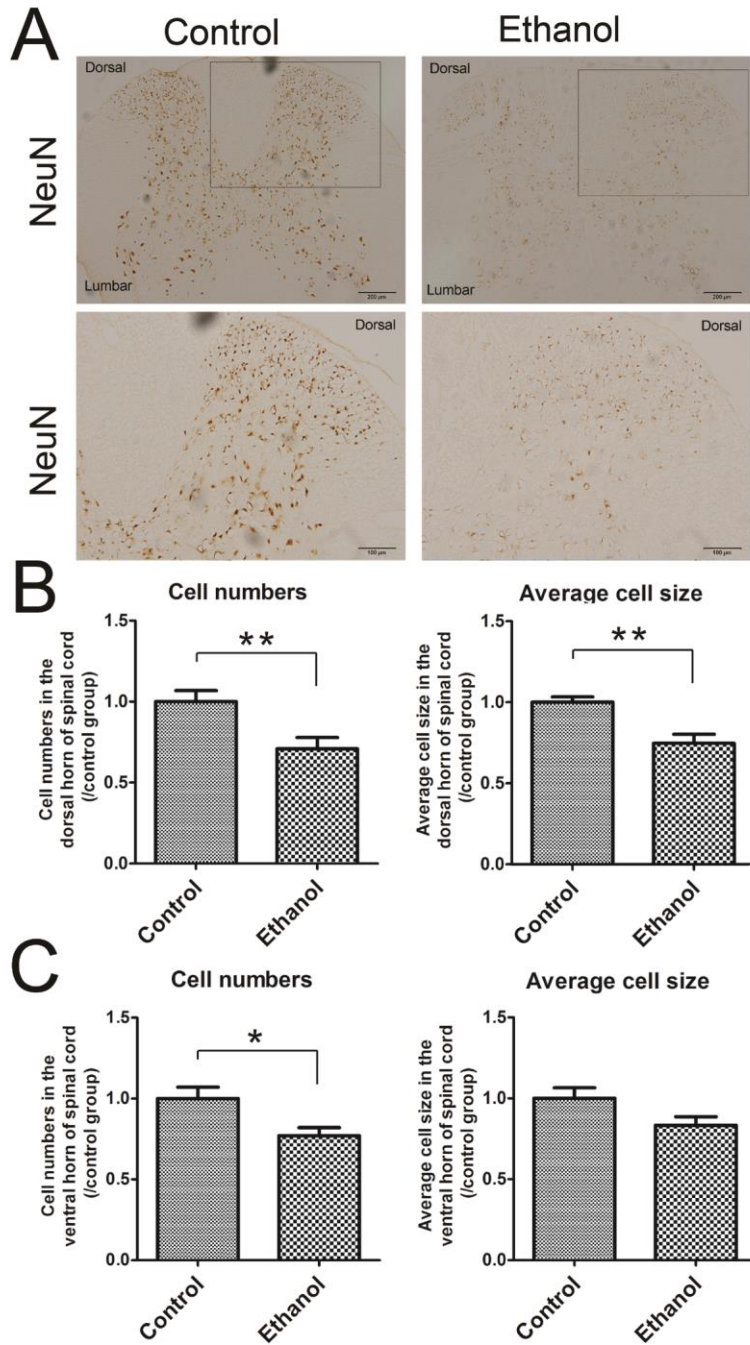


Fig. 3

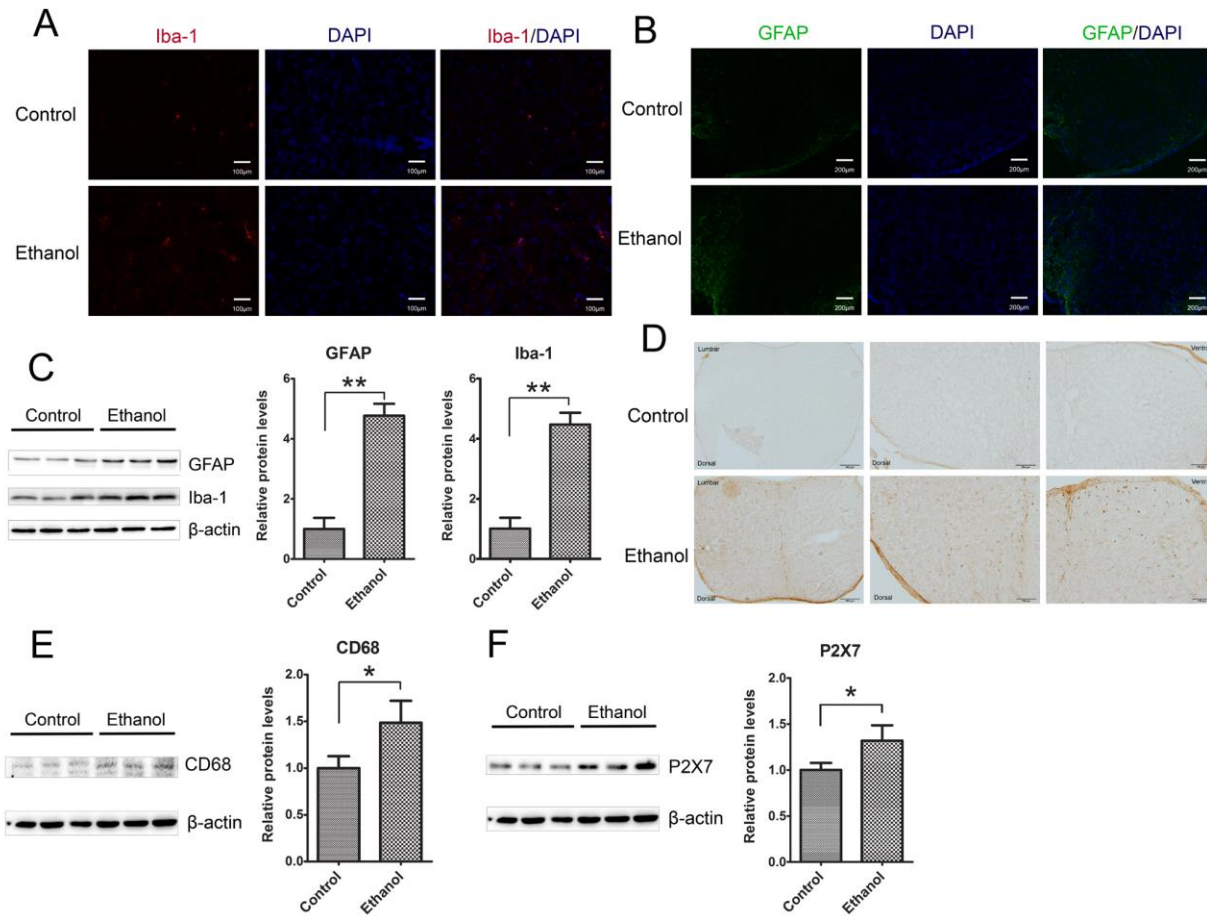


Fig. 4

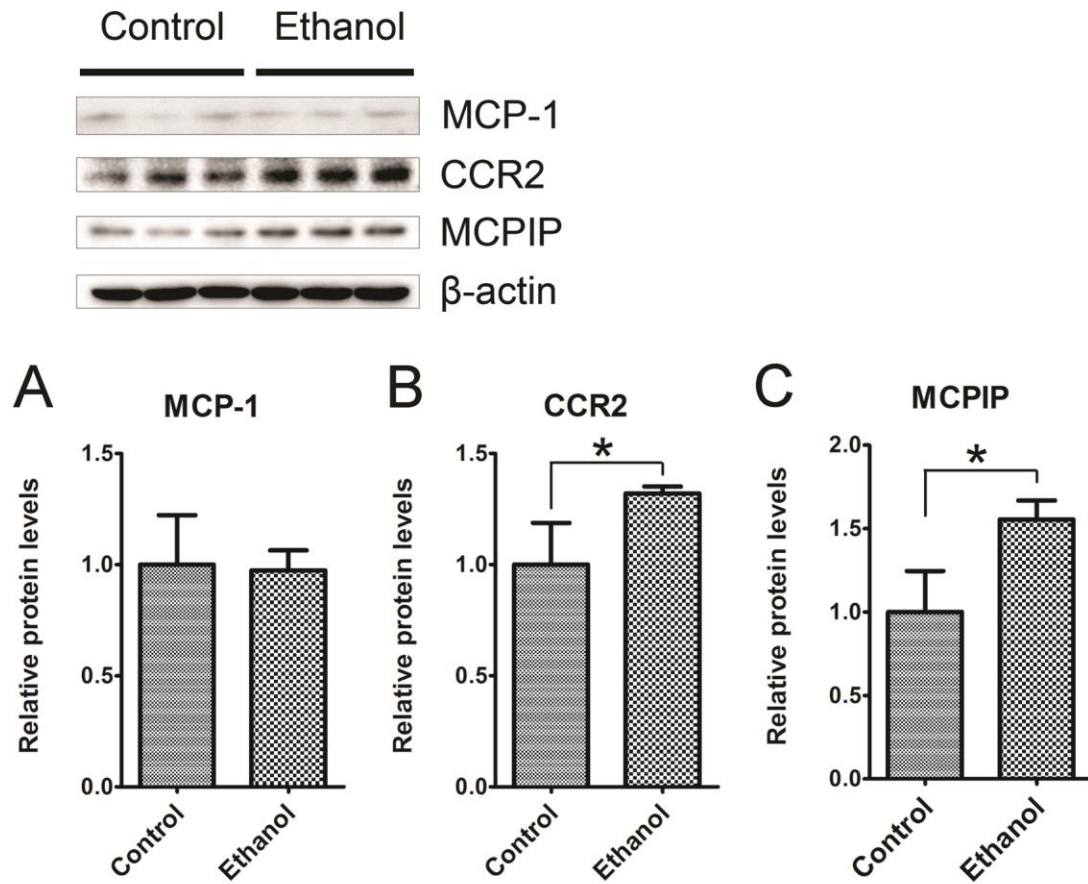


Fig. 5

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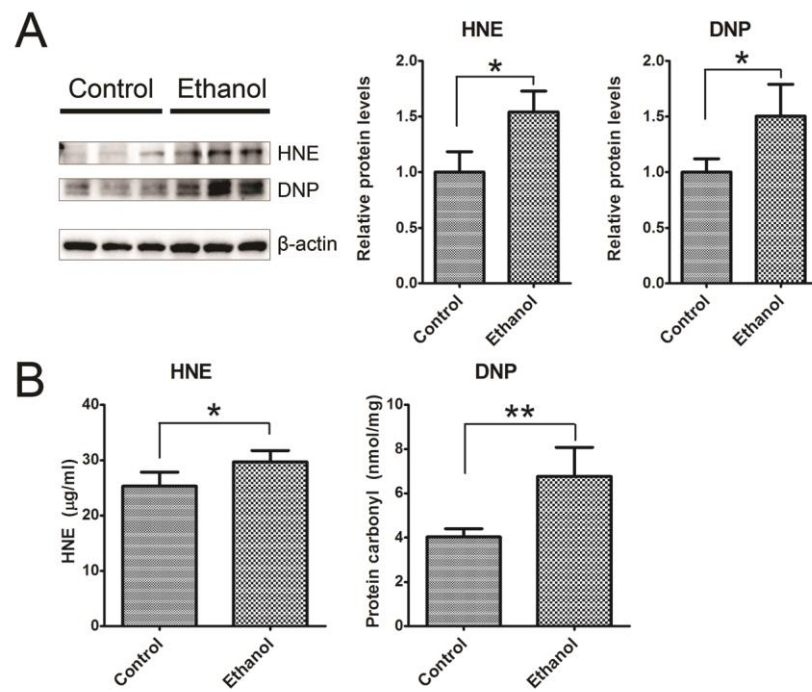


Fig. 7

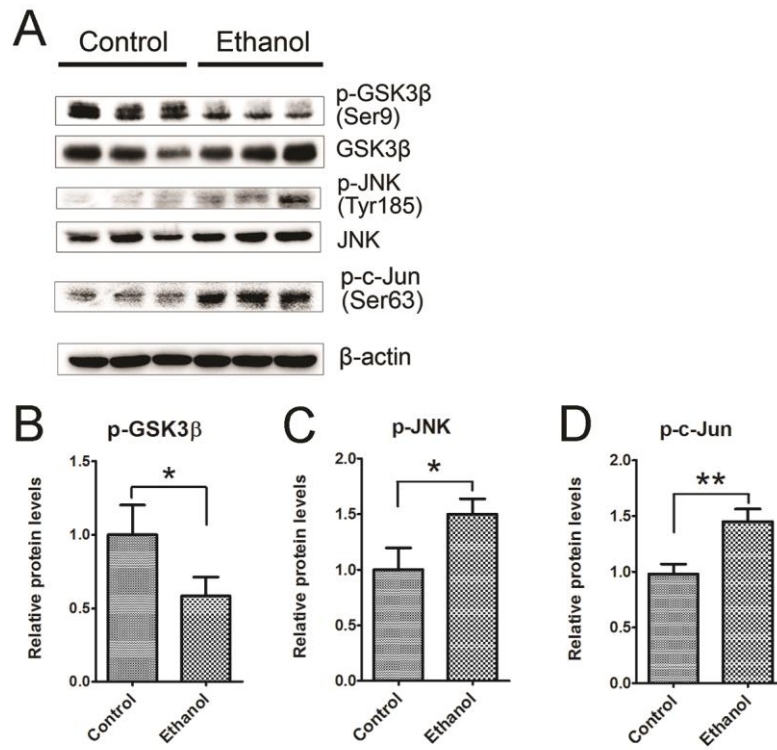


Fig. 8

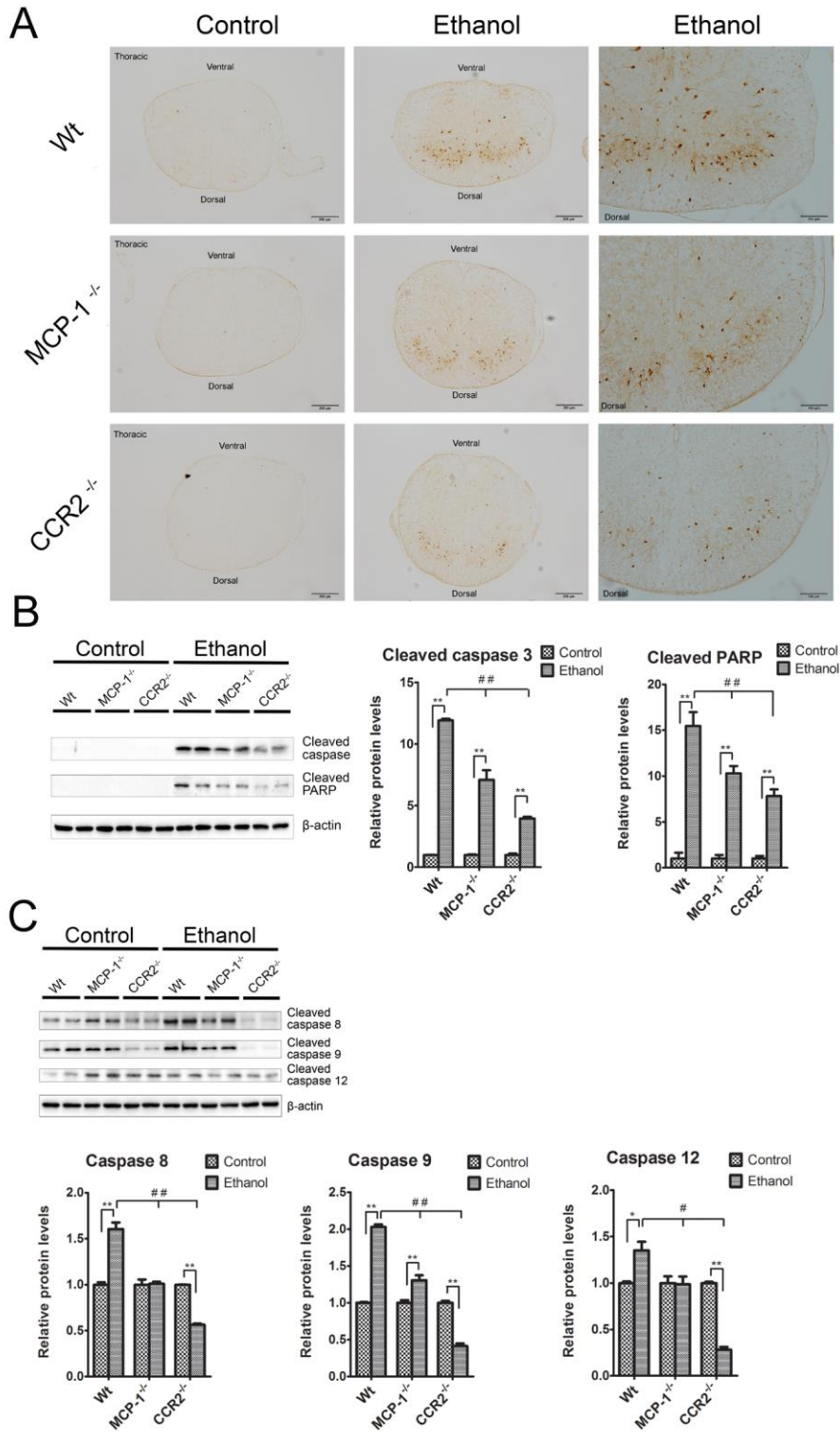


Fig. 9

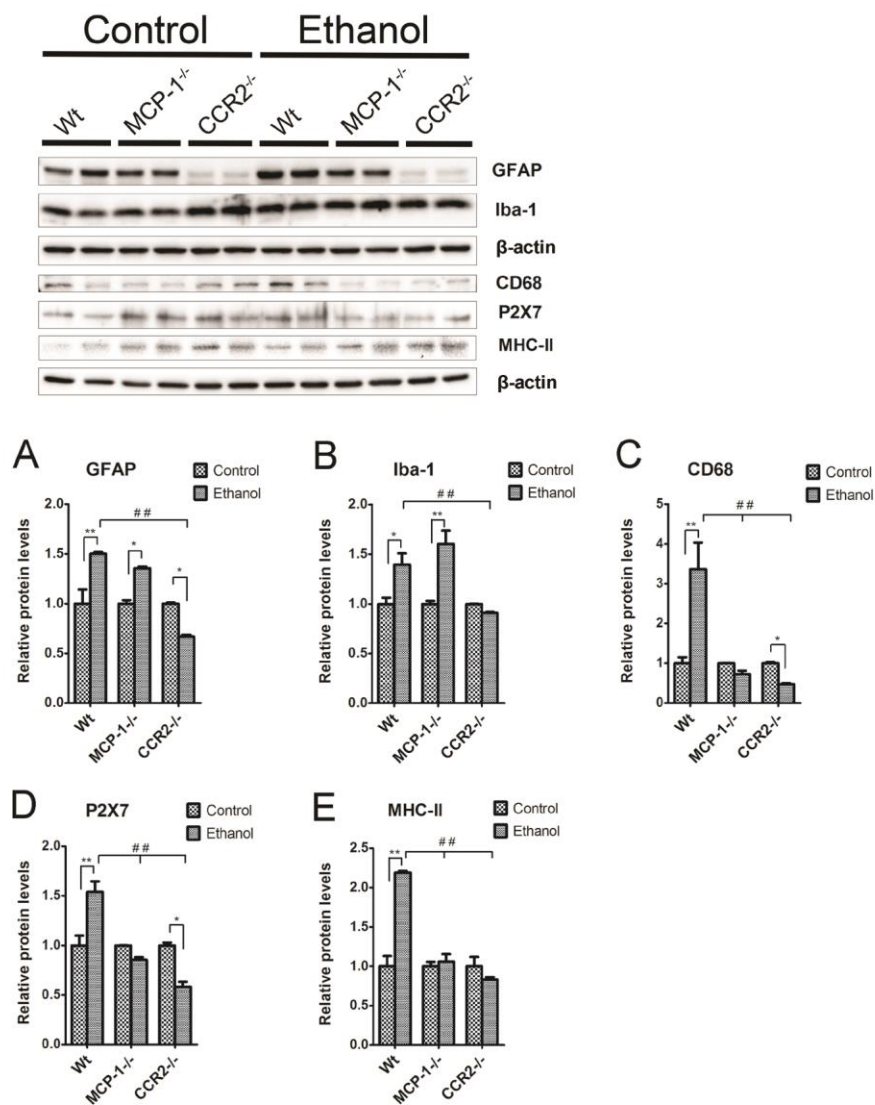


Fig. 10

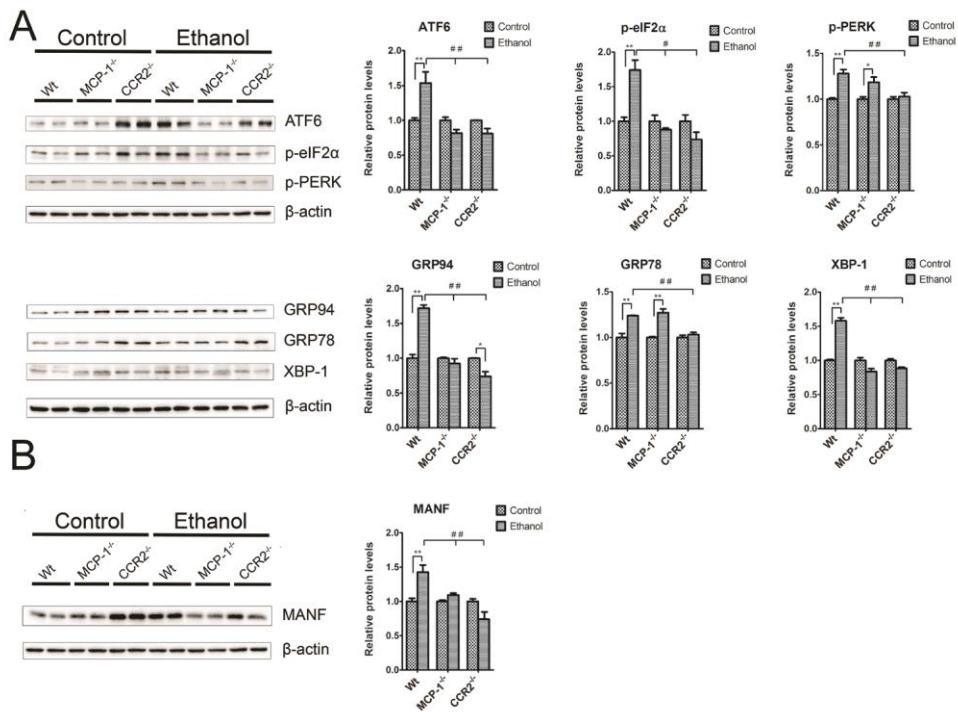


Fig. 11

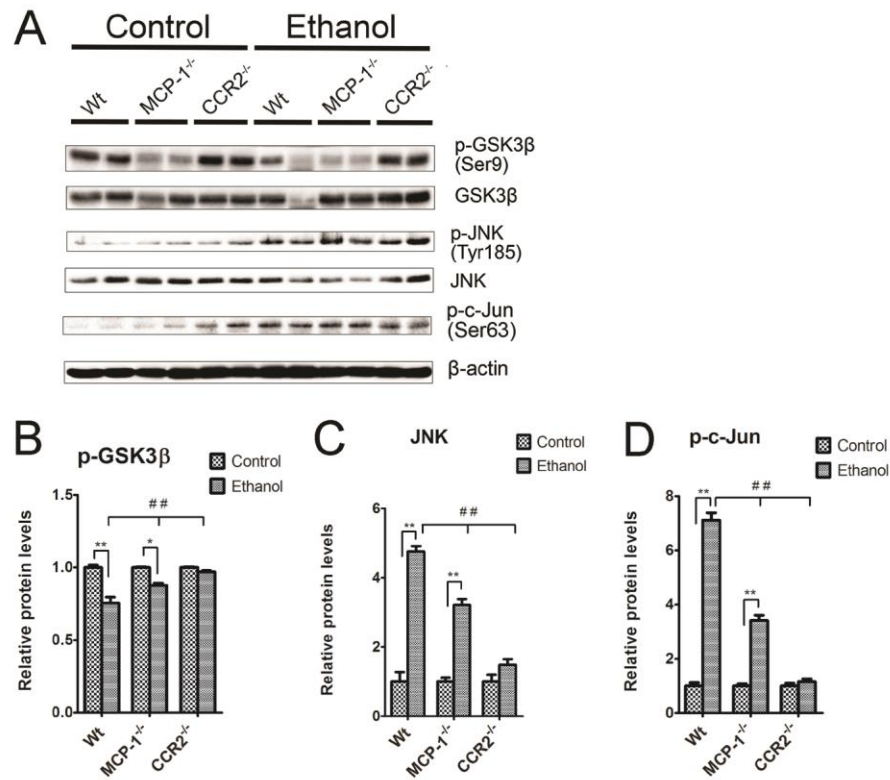


Fig. 12

Table 1. primary antibodies used for Immunohistochemistry and Immunofluorescent staining

Name of Antibody	Target Antigen	Manufacturer	Catalog Number	Species Raised	Dilution Used	Related publications
cleaved caspase-3	Asp175	Cell Signaling	CS9661S	Rabbit, Polyclonal	1:3000	PMID: 22632376
NeuN	clone A60	EMD Millipore	MAB377	Mouse, monoclonal	1:800	PMID: 26708003
Iba-1	C-terminus	Wako Chemicals	019-19741	Rabbit, Polyclonal	1:1000	PMID: 22956822
GFAP	GA5	EMD Millipore	MAB360	Mouse, monoclonal	1:1000	PMID: 27000654
CD68	FA-11	AbD Serotec	MCA1957	Rat, monoclonal	1:500	PMID: 12480978
Fluoro-Jade C		Chemicon	AG325		0.0001%	PMID: 24614080

Table 2. primary antibodies used for Immunoblotting

Name of Antibody	Target Antigen	Manufacturer	Catalog Number	Species Raised	Dilution Used	Related publications
cleaved caspase-3	Asp175	Cell Signaling	CS9661	Rabbit, Polyclonal	1:1000	PMID: 23319806
caspase 8	1C12	Cell Signaling	CS9746	Mouse, monoclonal	1:1000	PMID: 28439094
caspase 9	C9	Cell Signaling	CS9508	Mouse, monoclonal	1:1000	PMID: 28386133
Cleaved PARP	Asp214	Cell Signaling	CS9548	Mouse, monoclonal	1:1000	PMID: 24516177
Iba-1	C-terminus	Wako Chemicals	019-19741	Rabbit, Polyclonal	1:1000	PMID: 22504638
GFAP	GA5	EMD Millipore	MAB360	Mouse, monoclonal	1:2000	PMID: 27083773
CD68	FA-11	AbD Serotec	MCA1957	Rat, monoclonal	1:1000	PMID: 27829437
P2X7	H-265	Santa Cruz	sc-25698	Rabbit, Polyclonal	1:1000	PMID: 24286344
MHC-II	MRC OX-6	AbCam	ab23990	Mouse, monoclonal	1:1000	PMID: 9834080
MCP-1	PMP35	AbD Serotec	AAM43	Rabbit,	1:3000	PMID:

				Polyclonal		9884336
CCR2	amino acid 353	BioVision	3415R	Rabbit, Polyclonal	1:1000	PMID: 22319587
MCPIP	P-12	Santa Cruz	sc-136750	Goat, Polyclonal	1:1000	PMID: 22036805
ATF6	C-terminus	LifeSpan BioSciences	LS-B2516	Rabbit, Polyclonal	1:1000	PMID: 23850081
p-PERK(Thr980)	16F8	Cell Signaling	CS3179	Rabbit, monoclonal	1:1000	PMID: 28366931
p-eIF2 α (Ser51)	D9G8	Cell Signaling	CS3398	Rabbit, monoclonal	1:1000	PMID: 24057571
GRP78	HSPA5	Novus Biologicals	NBP1-06274	Rabbit, Polyclonal	1:2000	PMID: 27527870
GRP94	9G10	Enzo Life Sciences	ADI-SPA-850	Rat, monoclonal	1:3000	PMID: 23671277
XBP-1	Poly6195	BioLegend	619502	Rabbit, Polyclonal	1:1000	PMID: 25086361
CHOP	9C8	Thermo Fisher Scientific	MA1-250	Mouse, monoclonal	1:1000	PMID: 25859045
Caspase 12	Q6UXS9	AbCam	ab18766	Rabbit, Polyclonal	1:1000	PMID: 23209735
MANF	C-terminus	AbCam	ab67271	Rabbit, Polyclonal	1:1000	PMID: 27669143

4-Hydroxynonenal (HNE)		LifeSpan BioSciences	LS-C56815	Goat, Polyclonal	1:3000	PMID:27527870
Dinitrophenol (DNP)	D1D6	Cell Signaling	CS14681	Rabbit, monoclonal	1:1000	PMID: 24872412
p-GSK3 β (Ser9)	5B3	Cell Signaling	CS9323	Rabbit, monoclonal	1:1000	PMID: 27901475
GSK3 β	27C10	Cell Signaling	CS9315	Rabbit, monoclonal	1:1000	PMID: 22796213
p-JNK(Thr183/Tyr185)	98F2	Cell Signaling	CS4671	Rabbit, monoclonal	1:1000	PMID: 27882354
JNK		Cell Signaling	CS4672	Rabbit, Polyclonal	1:1000	PMID: 27313785
p-c-Jun (Ser63)		Cell Signaling	CS9261	Rabbit, Polyclonal	1:1000	PMID: 18187551

Highlights

- Ethanol causes apoptosis and neurodegeneration in the developing spinal cord.
- Ethanol induces neuroinflammation, oxidative stress and ER stress.
- MCP-1/CCR2 signaling may mediate ethanol-induced damage to the spinal cord.