Upregulation of Mesencephalic Astrocyte-derived Neurotrophic Factor (MANF) Expression Offers Protection Against Alcohol Neurotoxicity

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Abstract

Alcohol exposure has detrimental effects on both the developing and mature brain. Endoplasmic reticulum (ER) stress is one of the mechanisms that contributes to alcohol-induced neuronal damages. Mesencephalic astrocyte-derived neurotrophic factor (MANF) is an ER stress responsive protein and is neuroprotective in multiple neuronal injury and neurodegenerative disease models. MANF deficiency has been shown to exacerbate alcohol-induced ER stress and neurodegeneration. However, it is unknown whether MANF supplement is sufficient to protect against alcohol neurotoxicity. Alcohol alters MANF expression in the brain, but the mechanisms underlying alcohol modulation of MANF expression remain unclear. This study was designed to determine how alcohol alters MANF expression in neuronal cells and whether exogenous MANF can alleviate alcohol neurotoxicity. We showed that alcohol increased MANF transcription and secretion without affecting MANF mRNA stability and protein degradation. ER stress was necessary for alcohol-induced MANF upregulation, as pharmacological inhibition of ER stress by 4-PBA diminished alcohol-induced MANF expression. In addition, the presence of ER stress response element II (ERSE-II) was required for alcohol-stimulated MANF transcription. Mutations or deletion of this sequence abolished alcohol-regulated transcriptional activity. We generated MANF knockout (KO) neuronal cells using CRISPR/Cas9. MANF KO cells exhibited increased unfolded protein response (UPR) and were more susceptible to alcohol-induced cell death. On the other hand, MANF upregulation by addition of recombinant MANF protein or adenovirus gene transduction, protected neuronal cells against alcohol-induced cell death. Further studies using early postnatal mouse pups demonstrated that enhanced MANF expression in the brain by intracerebroventricular (ICV) injection of MANF adeno-associated viruses ameliorated alcohol-induced cell death. Thus, alcohol increased MANF expression through inducing ER stress,
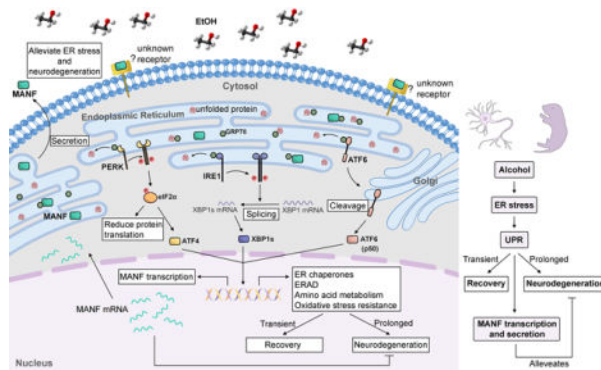
which could be a protective response. Exogenous MANF was able to protect against alcohol-induced neurodegeneration.

Graphical abstract

(By Figdraw)

Alcohol triggers endoplasmic reticulum (ER) stress and neurodegeneration. Mesencephalic astrocyte-derived neurotrophic factor (MANF) is an ER stress responsive protein that is neuroprotective in various neurodegenerative disease models. In this study, we demonstrated that alcohol upregulated MANF expression through ER stress and the activation of ER stress response element II (ERSE-II). MANF deficient neuronal cells exhibited increased unfolded protein response (UPR) and were more susceptible to alcohol-induced neuronal damage. Exogenous MANF supplementation protected neurons against alcohol neurotoxicity in both neuronal cell culture and postnatal mouse brains. These results suggest that alcohol-induced MANF upregulation could be a protective response against alcohol neurotoxicity.

Keywords

MANF; alcohol neurotoxicity; neurodegeneration; endoplasmic reticulum stress

Introduction

Alcohol is a commonly consumed substance that can pose detrimental effects on the structure, physiology, and function of the central nervous system (CNS) [1, 2]. Immature neurons are especially vulnerable to alcohol neurotoxicity. The exposure of developing fetus to alcohol during pregnancy can result in a condition known as fetal alcohol spectrum disorder (FASD). FASD manifests a wide variety of alcohol-related physiological and cognitive impairments in children [3]. FASD is a highly prevalent, yet preventable cause of mental disabilities worldwide. Numerous clinical and animal studies have shown that even acute and moderate alcohol exposure during development can induce profound damages to the developing brain with long-term effects that can impair neuronal function later in life [4–6]. The neurobehavioral impairments in individuals with FASD arise from the structural and functional changes in the CNS, with neurodegeneration being the most profound characteristic [7, 8]. Chronic heavy alcohol intake in adult also results in structural and

functional alterations in the brain [9, 10]. Brain imaging revealed smaller frontal cortical and white matter regions with larger ventricles in the alcoholics brain, which was associated with functional deficits in human alcoholics [11].

Alcohol-induced neurodegeneration may be mediated by multiple cellular and molecular mechanisms, including neuroinflammation [12], oxidative stress [13], autophagy [14], disruption of neurotrophic factor functions [15], and dysregulation of microRNA [16]. Recently, the involvement of endoplasmic reticulum (ER) stress has been suggested as a contributing factor in the process of alcohol-induced neurodegeneration [17, 18]. The ER is an organelle regulating protein synthesis and folding, posttranslational modification and secretion, and lipid metabolism. ER stress can be induced by various pathological insults that affect proper protein folding and disturb the homeostasis of the ER, including glucose deprivation, abnormal ER Ca^{2+} levels, inflammation, and toxic chemical exposure. ER stress can be resolved by the activation of a highly conserved adaptive response called unfolded protein response (UPR). UPR is regulated by three ER-transmembrane proteins: protein kinase R-like ER kinase (PERK), inositol-requiring enzyme 1α (IRE1α), and activating transcription factor 6 (ATF6). It alleviates ER stress by suppressing the rate of protein synthesis, increasing the ER protein folding capacity, and eliminating aberrant proteins through ER-associated protein degradation (ERAD) [19]. If the UPR is insufficient to overcome ER stress, prolonged UPR activation can ultimately result in apoptosis [20, 21]. In neurons, the maintenance of ER homeostasis is essential for normal neuronal functions as the dysregulation of UPR often lead to neurodegeneration. The aggregation of misfolded/unfolded proteins is a common pathogenesis observed in many neurodegenerative diseases, including Parkinson's disease [22], Alzheimer's disease [23], and stroke [24]. We and other investigators have demonstrated that alcohol exposure can cause ER stress and cell death in the developing, mature, and aged CNS [25–31]. Pharmacologically inhibiting ER stress has the potential to protect alcohol-sensitive immature neurons from the detrimental effects of alcohol-induced neurodegeneration [32].

Mesencephalic astrocyte-derived neurotrophic factor (MANF) is an ER-stress inducible protein that was originally identified as a secreted trophic factor to promote dopaminergic neuron survival in cell cultures [33]. It is widely expressed in the developing and mature brain [34, 35]. MANF is involved in multiple neurodevelopment processes, including neurogenesis [36], neurite outgrowth [37, 38], and neuron migration [39]. Increasing evidence suggest that MANF not only regulates normal neurodevelopment and survival [40, 41], but also protects neurons from pathological conditions such as brain ischemia, Alzheimer's disease, Parkinson's disease, traumatic brain injury, and retinal degeneration [33, 40–48].

Previously, we have demonstrated that deficiency of neuronal MANF exacerbated alcohol-induced neurodegeneration [49], and alcohol exposure caused ER stress and increased MANF expression in the developing brain [26, 32, 49]. In this study, we investigated the mechanisms underlying alcohol-induced upregulation of MANF expression and sought to determine whether exogenous MANF supplementation is sufficient to protect neurons against alcohol neurotoxicity.

Materials and Methods

Reagents and antibodies

The following reagents and materials were used: MEM (11095–080), high glucose DMEM (10569–010), and antibiotic-antimycotic (15240112) were from Life Technologies (Carlsbad, CA). FBS (35–010-CV) was from Corning (Glendale, AZ). MTT (M5655), DAPI (D9542), tunicamycin (T7765), and fibronectin (F3667) were from Sigma-Aldrich (St. Louis, MO). Ethanol 200 Proof (64-17-5) was from Decon Labs (King of Prussia, PA). Paraformaldehyde (PFA) (15714) was from Electron Microscopy Sciences (Hatfield, PA). Recombinant human MANF (3748-MN-050) was from R&D systems (Minneapolis, MN). Recombinant human MANF HA-tagged MANF adenovirus (mouse) (279970540200) and CMV Null control adenovirus (000047A) were from Applied Biological Materials (Richmond, BC, Canada). VECTASHIELD mounting medium (H-1400) was from Vector Laboratories (Burlingame, CA). Actinomycin D (GR300–0005) was purchased from Enzo Lifesciences (Farmingdale, NY). Cycloheximide (10187) was purchased from VWR (Radnor, PA). Trizol Reagent (15596026) and Lipofectamine 2000 transfection reagent (11668027) were purchased from Invitrogen (Waltham, MA). DNase I recombinant, RNase-free (04716728001) was from Roche (South San Francisco CA). RNase inhibitor (N8080119), High-Capacity cDNA Reverse Transcription Kit (4368814), and TaqMan™ fast universal PCR master mix (4352042) were from Applied Biosystems (Waltham, MA). Taqman gene expression assay for Manf (Mm00512511) and 18 s rRNA (Mm03928990) were purchased from Thermo Fisher Scientific (Rockford, IL). *DC* protein assay kit (5000112) was from Bio-Rad Laboratories (Hercules, CA). Sodium phenylbutyrate (4-PBA) (SML0309) was from MilliporeSigma (Burlington, MA). Cytiva Amersham™ ECL™ Prime Western Blotting Detection Reagent (45-002-401) was purchased from GE Healthcare Life Sciences (Piscataway, NJ). Luciferase Assay System (E1500) was purchased from Promega (Madison, WI). Control CRISPR/Cas9 plasmid (sc-418922), mouse ARP double nickase plasmid (sc-428989-NIC), UltraCruz transfection reagent (sc-395739), and plasmid transfection medium (sc-108062) were purchased from Santa Cruz Biotechnology (Dallas, TX). Fisherbrand™ Superfrost™ Plus Microscope Slides (12-550-15) and Cytiva Vivaspin™ 6 Sample Concentrators (45-001-571) were purchased from Fisher Scientific (Hampton, NH).

The following antibodies were used: ATF6 antibody (LS-B2516, 1:1000) was purchased from Life Span Biosciences (Seattle, WA). Cleaved caspase-3 (9661, 1:1000), phosphorylated-eIF2α (3398, 1:1000), eIF2α (9722, 1:1000), ATF4 (11815, 1:1000), IRE1α (3294, 1:1000), phosphorylated-PERK (3179, 1:1000), PERK (3192, 1:1000), HA-Tag (3724, 1:1000), and β-Actin (3700, 1:10000) antibodies were from Cell Signaling Technology (Danvers, MA). GRP78 antibody (NBP1–06274, 1:2000) was from Novus Biologicals (Littleton, CO). XBP1s antibody (658802, 1:1000) was from BioLegend (San Diego, CA). Phosphorylated-IRE1α (ab48187, 1:1000), PDIA1 (ab2792, 1:100) and MANF (ab67271 and ab67203, 1:1000) antibodies were from Abcam (Cambridge, MA). CHOP antibody (MA1–250, 1:1000) was from Thermo Fisher Scientific (Rockford, IL). Horseradish peroxidase (HRP)-conjugated anti-rabbit (NA934V, 1:200) and anti-mouse (NA931V, 1:200) secondary antibodies were from GE Healthcare Life Sciences (Piscataway,

NJ). Alexa-594 conjugated anti-rabbit antibody (A-11012, 1:200) was purchased from Invitrogen (Waltham, MA).

Cell culture and treatments

Mouse neuronal cell line N2a (ATCC®CCL-131, RRID:CVCL_0470) and human neuronal cell line SH-SY5Y (ATCC®CRL-2266, RRID:CVCL_0019) were purchased from ATCC (Manassas, VA). These cell lines are not listed as a commonly misidentified cell line by the International Cell Line Authentication Committee. N2a and SH-SY5Y cells were maintained in MEM and DMEM, respectively, supplemented with 10% FBS and 1% antibiotic-antimycotic at 37°C in 5% CO_2 in a humidified incubator. The culture medium was changed every 2 days, and cells were subcultured after reaching 90% confluence. A maximum of 5 cell passages were used. No further authentication was performed in the laboratory. For alcohol treatment, N2a and SH-SY5Y cells were starved in serum free medium overnight and then incubated with complete medium containing 0.4% weight/volume (w/v) ethanol, unless noted otherwise. Cells were put into a sealed container with 5% CO_2 to maintain proper ethanol concentrations in the medium. To decide the appropriate concentration of recombinant human MANF (rhMANF) treatment, rhMANF was added into the medium at various concentrations of 5, 10, 20, and 50 ng/ml two hours prior to alcohol treatment. 20 ng/ml rhMANF were chose for subsequent experiments as it generated the best outcome. For ER stress induction, cells were incubated with 5 μg/ml tunicamycin or 1.5 μM thapsigargin for 24 hours.

RNA extraction and cDNA synthesis

Cells were dissociated from the plates by trypsin. After PBS wash, total RNA was extracted from cells using Trizol reagent. One μg of total RNA was reverse transcribed into cDNA using SuperScriptTM III reverse transcriptase and random primers according to the manufacturer's instruction (Invitrogen).

Quantitative real-time PCR (qPCR)

qPCR was performed on the QuantStudio 3 Real-Time PCR Systems using the TaqMan™ fast universal PCR master mix. Taqman gene expression assay for Manf (Mm00512511) and 18 s rRNA (Mm03928990) were purchased from Thermo Fisher Scientific. All reactions were performed in triplicates. The relative *Manf* expression was normalized to the housekeeping 18 s rRNA using cycle time (Ct) values. The relative differences among different treatments were calculated as relative fold changes normalized to control using $2^{-\Delta Ct}$ method.

Protein extraction and immunoblotting

Protein was extracted from cells or postnatal mice brains as previously described [36]. Briefly, samples were lysed on ice for 15 minutes in RIPA buffer (150 mM NaCl, 1 mM EGTA, 50 mM Tris-HCl pH 7.5, 0.5% NP-40, 0.25% SDS, and freshly added protease inhibitors). Protein concentration was determined using the DC protein assay according to the manufacture's instruction. To enrich MANF protein from the culture medium, 3 ml of

conditioned medium from each treatment was centrifuged at $4,000 \times g$ for 90 minutes at 25 °C using the Cytiva Vivaspin™ 6 Sample Concentrators (45-001-571).

For immunoblotting, 20–30 μg protein samples from cell lysates or 20 μl enriched culture medium were separated on 12% or 15% polyacrylamide gels by electrophoresis and transferred to nitrocellulose membranes. Membranes were blocked in 5% BSA and incubated with primary antibodies at 4°C overnight. Membranes were then washed with TBST and incubated with horseradish peroxidase conjugated secondary antibodies. Blots were developed using the Cytiva Amersham™ ECL™ Prime Western Blotting Detection Reagent on Chemi™Doc imaging system (Bio- Rad Laboratories, Hercules, CA). Band intensity was quantified using Image Lab software (Bio-Rad Laboratories). β-actin was used as housekeeping control for proteins from cell lysates and ponceau staining image was used as loading control for proteins from culture media.

Immunocytochemistry and immunofluorescence

Cells were seeded in 24-well plates onto sterile coverslips coated with 10μg/ml fibronectin. Following treatment, the cells were fixed using 4% PFA and made permeable using 0.25% Triton X-100 in PBS. Subsequently, the cells were blocked in PBS with 1% BSA and 2% goat serum for 30 minutes and incubated with primary antibodies for 1 hour at room temperature and Alexa fluor dyes-conjugated secondary antibodies for 30 minutes in the dark. DAPI was used to counterstain the nuclei.

For immunofluorescence labeling, postnatal mouse brains were removed, post-fixed in 4% PFA for 48 hours at 4 C°, cryopreserved in 30% sucrose in PBS, frozen and sectioned sagittally at a thickness of 10 μm using a cryostat. Brain sections were mounted onto superfrost/plus slides. Slides were incubated with primary antibodies at 4 C° overnight and Alexa fluor-conjugated secondary antibodies for 1 hour at room temperature in the dark. DAPI was used to counterstain the nuclei.

Images were obtained using the IX83 inverted fluorescent microscope (Olympus). Fluorescent intensity and the number of positive cells were analyzed using the Olympus cellSens Dimention software.

mRNA stability assay

To determine MANF mRNA stability, N2a cells were cultured in 6-well plates and starved in serum free medium overnight. Cells were then treated for 0, 0.5, 1, 3, 6, and 8 hours with 10 μg/ml actinomycin D with or without 0.4% (w/v) alcohol in complete media. Total RNA was extracted, and reverse transcribed into cDNA for subsequent qPCR analysis on MANF transcripts.

Cycloheximide (CHX)-chase assay

N2a cells were cultured in 6-well plates and starved in serum free medium overnight. Cells were then treated for 0, 0.5, 1, 3, and 6 hours with 100 μg/ml CHX with or without 0.4% (w/v) alcohol in complete media. Protein was obtained from the cell lysate and subsequently

utilized for immunoblotting analysis. The level of MANF was normalized to actin and the ratio of each time points to 0 hour was calculated.

Pharmacological inhibition of ER stress by 4-PBA

Sodium phenylbutyrate (4-PBA) inhibits ER stress by acting as a chemical chaperone that facilitate protein folding, thus prevents their aggregation. N2a cells were preincubated with 10 mM of 4-PBA (SML0309, MilliporeSigma) for 2 hours then treated with alcohol as described above.

Luciferase activity assay

Luciferase reporter constructs with wild type or mutant mouse MANF promoter regions were described previously [50] and generously provided by Dr. Oh-Hashi from Gifu University, Japan. N2a cells were cultured in 6-well plates and transfected with 1 μg/well reporter constructs using Lipofectamine 2000. Forty-eight hours after the transfection, cells were treated with 0.4% (w/v) ethanol or 5 μg/ml tunicamycin for 6 hours. Luciferase activities were detected using the Luciferase Assay System (Promega) according to the manufacture's instruction. Relative MANF promoter activities were normalized to cells transfected with pGL3-Basic empty vector.

Generation of MANF knockout (KO) N2a cells using CRISPR/Cas9

MANF KO N2a cells were generated and described previously [37]. Briefly, N2a cells were seeded onto 6-well plate and transfected with 1 μg of either control CRISPR/Cas9 or mouse ARP double nickase plasmids (Santa Cruz Biotechnology). Forty-eight hours after the transfection, cells were selected for GFP by flow cytometry and selected by 1 μg/ml puromycin for 5 days. Single cells were dispensed into 96-well plates by cell sorting using BD FACSAria II Cell Sorter (BD Biosciences, Franklin Lakes, NJ), and allowed to expand in complete media for 2 weeks. Protein was extracted from each single cell colony and immunoblotting for MANF was performed to confirm MANF knock out. We generated 16 MANF KO colonies in N2a cells and 2–3 colonies were used for experiments in this study to confirm data reproducibility.

Cell viability assay

MTT assay was used to determine cell viability. Cells were seeded in 96-well plates and starved in serum free medium overnight and then incubated with complete media with or without alcohol. Five-hundred μg/ml MTT was added to each well and incubated at 37°C for 2 hours. After the incubation, culture medium in each well was replaced with 100 μl DMSO. Results were detected using the SpectraMax iD3 Multi-Mode Microplate Reader (Molecular Devices, San Jose,CA) at the wavelength of 570 nm. The percentage of cell survival after alcohol exposure was calculated by normalizing alcohol- treated groups to no alcohol control groups at each time points.

Adenovirus transduction

Crude viral stock of HA-tagged mouse MANF adenovirus (MANF-AD) and CMV-Null control adenovirus (Ctrl-AD) were produced at a titer ranging from 1×10^7- 1×10^8 pfu/ml

by the manufacture (Applied Biological Materials). They were amplified in HEK293 cells as described previously [37]. To obtain MANF overexpression, N2a cells were incubated with 120 μl per well MANF-AD in 6-well plate for 2 hours. Cells underwent subsequent alcohol treatment, MTT analysis, and immunoblot at desired time points as described above. Although Enzyme-linked immunosorbent assay (ELISA) was used in previous reports to determine MANF secretion [51], the current study aimed to detect both endogenous MANF and HA-tagged exogenous MANF and therefore employed immunoblotting approach. To enrich MANF in the culture media, 3 ml of conditioned medium from each treatment was centrifuged at $4,000 \times g$ for 90 minutes at 25 °C using the Cytiva Vivaspin™ 6 Sample Concentrators (45-001-571). The enriched culture media were subject to immunoblot as described in the previous section.

Neonatal intracerebroventricular (ICV) injection of adeno-associated virus (AAV)

All experimental procedures were performed following regulations for the Care and Use of Laboratory Animals set forth by the National Institutes of Health (NIH) Guide and approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Iowa (#3042295). pAAV-CMV>mManf[NM_029103.4](ns):T2A:EGFP:WPRE vector (MANF-AAV) and pAAV-CMV>EGFP:WPRE vector (control-AAV) were generated and packaged into AAV-PHP.eB virus at a minimum titer of 10^{13} viral particles (vp)/ml by VectorBuilder (Chicago, IL). Viruses were diluted with sterile PBS and mixed with 0.04% trypan blue. For free-hand ICV delivery, postnatal day 0 (PD0) neonatal C57BL/6 mice were cryo-anesthetized and injected with control- or MANF-AAV at a dose of 10^{10} vp/cerebral hemisphere for both hemispheres as previously described [52]. Injections were performed using a 10 μl Hamilton 701 Cemented Needle Special (SN) Syringe with a 10 millimeter 33-gauge 30° bevel needle (Hamilton Company, Reno, NV). The skull was pierced at 2/5 distance from the lambda to the eye with 3 mm in depth. After injection, pups were placed on a heating pad to recover body heat. Brains of injected mice were collected and cryosectioned on PD3, 7, and 14 for the assessment of GFP expression. Widespread GFP expression was observed in the postnatal brain at all time points, indicative of successful AAV expression.

Animals and alcohol exposure

All experimental procedures were performed following regulations for the Care and Use of Laboratory Animals set forth by the National Institutes of Health (NIH) Guide and approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Iowa (#3042295). Four pregnant C57BL/6 mouse dams (RRID:MGI:2159769) were purchased from The Jackson Laboratory. Dams were single housed with *ad libitum* access to chow and water on a 14-hour light/10-hour dark cycle. They gave birth to a total of 21 pups. Pups were numbered from 1 to 21 and then divided randomly into 4 groups using the RAND formula in Excel: control-AAV+PBS (n=5), control-AAV+EtOH (n=5), MANF-AAV+PBS (n=5), MANF-AAV+EtOH (n=6). On PD0, pups were injected with control- or MANF-AAV as described above. On PD7, they were subcutaneously injected with either PBS control or ethanol (2.5 g/kg, 20% solution in PBS) twice with a 2-hour interval as previously described [49]. The first and second injection were performed at 9 and 11 am, respectively. After 8 hours following the initial injection, pups were cryo-anesthetized and decapitated

to collect the brain. Neuronal apoptosis was assessed by immunofluorescence labeling and immunoblotting analysis for cleaved-caspase 3. Briefly, freshly dissected postnatal mouse brains were cut in half saggitally with one half frozen on dry ice for protein extraction and immunoblot as described above. The other half was fixed in 4% PFA and used for fluorescence labeling for cleaved-caspase 3 as describe above. Fluorescence images were obtained using the IX83 inverted fluorescent microscope (Olympus). Two sections were examined for each brain. The total number of cleaved-caspase 3 positive cells in the cerebral cortex and cerebellum was counted using the Olympus cellSens Dimention software. The timeline for the experimental procedure is shown in Figure 7A. Investigators were blinded for experimental groups when assessing the immunofluorescence labeling.

Statistical analysis

Three to five independent cell culture preparations were used for all *in vitro* experiments and 5–6 animals per group were used for *in vivo* experiments. Sample size was not determined by statistical method as the number of independent experiments we used was sufficient to detect significant changes as reported previously [36]. No exclusion criteria were pre-determined. Data of all animals in the experiments were included for analysis.

All statistical analyses were performed using GraphPad Prism 9.0 (GraphPad software, La Jolla, CA). Test for outlier were not performed. The data were displayed as mean ± SEM. Data were tested for normal distribution using the Shapiro-Wilk normality test. Two tailed Student's unpaired t-test was used for comparison between two groups that were normally distributed. One-way analysis of variance (ANOVA) was used to compare more than two groups of data with one variable. Two-way ANOVA was used for comparison of more than two groups with two variables. ANOVA tests were followed by Tukey post hoc test. p values of < 0.05 were considered significant.

Results

Alcohol increases MANF transcription and secretion without altering the stability of MANF mRNA and protein

To examine the effect of alcohol exposure on MANF expression and secretion, N2a and SH-SY5Y cells were treated with 0.4% (w/v) ethanol for various length of time, then total RNA and protein were extracted from cell lysates or conditioned culture media. Quantitative real-time PCR analysis revealed an upregulation of MANF transcripts that peaked at 6 hours after alcohol treatment in both N2a and SH-SY5Y cells (Fig. 1A, 1B). A corresponding increase in MANF protein level was observed after 12 hours of alcohol treatment (Fig. 1C, 1D). Increased MANF immunofluorescent signal was also detected in N2a cells 12 hours following alcohol exposure, which was co-localized with the MANF interacting protein PDIA1 in the ER (Fig. 1E) [53]. Although MANF localized in the ER, its secretion can be induced in pathological conditions [54]. Next, we detected the amount of secreted MANF in the conditioned media and found that it was elevated by alcohol treatment in a time dependent manner for both N2a and SH-SY5Y cells (Fig. 1C, 1D).

Alcohol-induced upregulation of MANF could be mediated by either increased transcription/translation or enhanced mRNA/protein stability. We then examined the effect of alcohol exposure on MANF mRNA stability. N2a cells were treated with actinomycin D, which inhibits new RNA synthesis so that the degradation of pre-existing MANF mRNA could be examined. The level of MANF mRNA in N2a cells was measured at various time points by qPCR following alcohol exposure. The result showed that the rate of MANF mRNA degradation was not affected by alcohol (Fig. 2A).

Similarly, we also examined whether alcohol affected the stability of MANF protein. CHX inhibits new protein synthesis by blocking ribosomal protein translation. N2a cells were treated with 100 μg/ml of CHX for various timepoints and then MANF protein half-life was detected by immunoblotting. As shown in Fig. 2B, MANF protein level was degraded to about 75% after 3 hours, and to about 60% after 6 hours. Alcohol did not significantly affect the rate of MANF protein degradation (Fig. 2B).

Alcohol-induced MANF expression is dependent on elevated ER stress

Studies have shown that alcohol can induce ER stress in both the developing and mature mouse brain [25, 49]. We examined the protein levels of unfolded protein response (UPR) genes in alcohol treated N2a cells and confirmed that all UPR genes were upregulated (Fig. 3A and 3B), indicating that alcohol indeed triggered ER stress in N2a cells. Notably, while all the UPR genes were upregulated by 12 hours of alcohol treatment, some UPR genes were elevated as soon as 3 hours post alcohol treatment, such as p-PERK and XBP1s. MANF is known as a protein that responds to ER stress. To determine whether alcohol-induced MANF expression is dependent on ER stress activation, N2a cells were treated with alcohol with or without pre-incubation of an ER stress inhibitor 4-PBA. The level of MANF transcripts were examined after 6 hours of alcohol treatment and MANF protein levels were examined after 12 hours of treatment. While alcohol alone upregulated MANF expression as expected, pre-incubation of 4-PBA significantly diminished alcohol-induced MANF upregulation (Fig. 4A and 4B). MANF transcription is regulated by several transcription factors in the UPR pathways, such as the binding of ATF6 and XBP1s with the MANF promoter region with ER stress response element II (ERSE-II) [50, 55–57]. To further examine if ERSE-II transactivation is required for alcohol-induced MANF transcription, MANF promoter luciferase reporter constructs with wild type or mutant/deleted ERSE-II motifs were transfected into N2a cells. The consensus sequence of the ERSE-II motif and the mutated/deleted sequences were shown in Fig. 4C. Transfected cells were incubated with alcohol for 6 hours. Tunicamycin was used as a positive control as high level of MANF expression was able to be induced by tunicamycin in N2a cells (Fig. S1). Luciferase activity was significantly elevated by both alcohol and tunicamycin treatment in cells expressing wild type MANF promoter (Fig. 4D), and as expected, the luciferase activity was higher in tunicamycin-treated group than in alcohol-treated group. However, mutation or deletion of the ATF6/XBP1s binding site in the ERSE-II region of MANF promoter abolished the luciferase activity in both tunicamycin- and alcohol-treated groups (Fig. 4D). These data indicated that ER stress and ERSE-II were required for alcohol-induced MANF upregulation.

MANF-deficient neuronal cells are more susceptible to alcohol-induced cell death

To investigate the effects of MANF deficiency on neuronal cell death induced by alcohol, MANF knockout (KO) N2a cells were generated using CRISPR/Cas9 (Fig. 5A) [37]. The basal expression level of UPR genes GRP78 and XBP1s were increased in MANF KO cells (Fig. 5B), suggesting a predisposition of ER stress in MANF deficient cells. Control and MANF KO cells were exposed to alcohol and the amount of cleaved-caspase 3 was examined at different time points. While in control cells cleaved-caspase 3 was induced by 48 hours of alcohol exposure, it was detected in MANF KO cells as early as 24 hours post alcohol treatment (Fig. 5C) and its expression level was much higher in MANF KO cells than in control cells. To further assess cell viability, MTT assay was perform. As shown in Fig. 5D, alcohol exposure caused more reduction of cell viability over time in MANF KO cells than control cells, indicating MANF-deficient neuronal cells are more susceptible to alcohol-induced cell death (Fig. 5D). To further examine whether the increased susceptibility to alcohol neurotoxicity was specific to MANF knockout, cells were incubated with 20 ng/ml recombinant human MANF (rhMANF) 2 hours prior to and during alcohol treatment. MTT assay revealed that the addition of rhMANF was able to rescue the decrease in cell survival induced by alcohol in MANF knockout (KO) cells (Fig. 5D).

Exogenous MANF protects neuronal cells against alcohol-induced cell death

Overexpression of MANF can alleviate cellular damages induced by ER stress in multiple disease models [46, 58–61]. However, little is known on whether MANF can mitigate alcohol-induced ER stress and cell death in neuronal cells. To determine whether MANF overexpression is protective on alcohol neurotoxicity, N2a cells were transduced with control- or MANF-adenovirus (MANF-AD). MANF-AD transduction resulted in strong intracellular MANF overexpression, which can be further augmented by ER stress inducers tunicamycin and thapsigargin (Fig. 6A and 6B). MTT assay demonstrated that alleviation of alcohol-induced cell death was observed in MANF-AD transduced N2a cells (Fig. 6E). Notably, MANF-AD transduced exogenous MANF can be secreted into culture media and the secretion was enhanced by thapsigargin treatment, which was consistent with previous reports showing MANF was induced by thapsigargin [62] (Fig. 6C and 6D). As a result, the protective effect of MANF against alcohol may be due to the action of both intracellular and extracellular MANF.

To determine if the protective effect of MANF observed in cell cultures can be replicated *in vivo*, we enhanced MANF expression in the developing brain through the intracerebroventricular (ICV) injection of MANF-AAV to the mouse brain. Control- or MANF-AAV were administered to neonatal mouse pups on postnatal day 0 (PD0) via bilateral ICV injection (Fig. 7A). Widespread GFP expression was observed in the postnatal brain on PD3, 7, and 14, indicative of successful AAV expression (Fig. S2 and data not shown). By PD7, GFP expression was detected in multiple brain regions, including the olfactory bulb, cerebral cortex, hippocampus, striatum, thalamus, and pontine nuclei (Fig. S2). Immunoblot of cerebral cortex lysates indicated a ~1.5-fold increase of MANF protein expression when compared to its endogenous level (Fig. 7C). Control- or MANF-AAV injected pups were exposed to alcohol on PD7, and then apoptosis was assessed by immunofluorescence labeling and immunoblotting for cleaved-caspase 3. Alcohol exposure

led to widespread neuronal apoptosis throughout the developing brain with some areas, such as the cortex, superior and inferior colliculus, and cerebellum showing more susceptibility (data not shown). Alcohol exposure caused increased numbers of cleaved-caspase 3^{+} cells in control-AAV injected cerebral cortex (Fig. 7B). Consistently, immunoblotting also showed an upregulation of cleaved-caspase 3 expression (Fig. 7C). Applaudably, MANF overexpression significantly alleviated alcohol-induced neuronal apoptosis in the cerebral cortex as indicative by reduced cleaved-caspase 3 expression (Fig. 7B and 7C). Notably, alcohol exposure induced a similar elevation in the number of cleaved-caspase 3+ cells in the cerebellum of both control- and MANF-AAV injected animals, where exogenous MANF/GFP expression was not detected (Fig. S2H and H', Fig. S3). Taken together, these results suggested that MANF was neuroprotective and exogenous MANF was sufficient to ameliorate alcohol-induced neuronal apoptosis.

Discussion

In this study, we provided evidence that alcohol induces the expression of MANF in neuronal cells. MANF expression was reported to be induced by ER stress inducers in different types of tissues and cells [44, 57, 63]. Animal studies also indicated that MANF expression was induced in pathological conditions where ER stress was activated. For example, MANF expression was induced by ischemia-related ER stress in neurons, glial cells, and cardiac tissues [64–67]. MANF upregulation was implicated to attenuate ER stress-related cellular damages by modulating the stress via unknown mechanisms, probably through facilitating protein folding and regulating immune response [45, 68–70].

Alcohol exposure has been reported to induce MANF expression in multiple tissues including postnatal mouse brain, adult mouse liver and mouse pancreatic acinar cells [26, 32, 49, 71, 72]. However, the molecular mechanism of how alcohol stimulates MANF expression and whether enhanced MANF expression alleviates alcohol neurotoxicity are unclear. Numerous studies have shown that alcohol interferes with protein homeostasis in the ER, causing ER stress and triggering unfolded protein response (UPR) in the pancreas, liver, heart, and brain (reviewed in [73–75]). Our results support that alcohol-induced MANF expression in neuronal cells is mediated by ER stress, because pharmacological inhibition of UPR activation by 4-PBA diminished alcohol-induced MANF expression.

We demonstrated that alcohol increased MANF transcription without affecting MANF mRNA stability and protein degradation. The expression of ER molecular chaperones, such as GRP78 and GRP94, are often regulated by the ERSE located in the promoter region [76]. Under ER stress conditions, ATF6 and/or XBP1s recognize and bind to CCACG and form complex with the general transcription factor NF-Y that binds to the CCAAT region, resulting in the transcriptional upregulation of ER chaperones. ERSE-II with the consensus sequence of ATTGG-N-CCACG is another ERSE-like *cis*-acting element first identified in the human homocysteine-induced endoplasmic reticulum protein (*HERP*) gene [55]. It also possesses the ATF6/XBP1s binding sequence of CCACG and the NF-Y binding sequence of CCAAT, although they are located at the opposite strands [56]. ERSE-II is present in both human and mouse MANF promoter region, while an additional canonical ERSE was identified in the human MANF promoter [57, 77]. Mizobuchi et al. demonstrated

that MANF expression in mouse tissues and cell cultures were induced by ER stress via ERSE-II [57]. Oh-Hashi *et al.* further showed that ATF6α, but not ATF6β nor XBP1s, strongly induced MANF promoter activity via ERSE-II in N2a mouse neuronal cells [50]. XBP1s was reported to mainly bind to the canonical ERSE promoter region and promote MANF transcription in human tissues [77]. We showed that the presence of ERSE-II was necessary for alcohol-induced MANF transcription in mouse neuronal cells. ATF6α and XBP1s were induced by alcohol in neuronal cells. Mutations or deletion of the ATF6/XBP1s binding sequence in the ERSE-II abolished alcohol-induced MANF luciferase reporter activity. Taken together, these results suggest alcohol may enhance MANF transcription through promoting ATF6/ XBP1s binding to ERSE-II. This conclusion is supported by the evidence that cells lacking ATF6 or XBP1s exhibited a decrease in MANF expression [78]. In addition, a mouse model of spinocerebellar ataxia demonstrated a decline in MANF expression, which was attributed to reduced association of XBP1s with the MANF promoter [79].

We also detected increased MANF secretion after alcohol exposure. Although MANF is located in the ER, its extracellular secretion has been reported to be protective against many ER stress-associated diseases, including kidney diseases, skeletal diseases, diabetes, cardiac ischemia, stroke, and Parkinson's disease in rodent models and human patients [42, 54, 64, 67, 80–82]. The mechanism of MANF secretion is not fully understood. Based on the available information, MANF secretion is regulated in conjugation by interaction of MANF with KDEL receptors and calcium-dependent binding of MANF to GRP78 [62, 83, 84]. ER retention is largely relied on Lys-Asp-Glu-Leu (KDEL), a canonical C-terminal ER-retention sequence that are possessed by many soluble ER resident proteins such as GRP78, GRP94, and protein disulfide isomerases (PDIs) [85]. MANF possessed a C-terminal Arg-Thr-Asp-Leu (RTDL) sequence that resembles the function of the canonical KDEL but with a weaker affinity to the KDEL receptor, which renders its secretion under stress conditions [67, 84, 86, 87]. Increased MANF secretion was observed with deletion of the RTDL sequence [83]. In addition, it was reported that the secretion of MANF was partially regulated by the calcium-dependent interaction of MANF with the ER molecular chaperone GRP78 [62]. GRP78 is a major ER chaperone molecule critical for protein folding and the activation of the UPR sensors. In unstressed condition, GRP78 anchors to the luminal domain of the ER stress sensors, thereby preserving their inactive state. Upon ER stress, GRP78 dissociates with the sensors and governs the activation of UPR [19]. Similar to the binding of GRP78 to UPR sensors, direct physical interaction of MANF and GRP78 was detected in a manner that is dependent on calcium levels [62]. Depletion of ER calcium by chemical calcium depletion compounds triggered the dissociation of the MANF-GRP78 complex, facilitating the subsequent secretion of MANF [62]. Alcohol has long been known to disrupt neuron calcium homeostasis and calcium responses by increasing cytoplasmic free calcium level, altering voltage- and ligand-gated calcium channels, and depleting intracellular calcium pool in the ER and mitochondrial [88–90]. Whether the increased MANF secretion in response to alcohol is due to the calcium-dependent MANF-GRP78 interaction requires further investigations.

The most devastating outcome of alcohol exposure is neurodegeneration observed in individuals with excessive alcohol consumption or prenatal alcohol exposure [6, 9]. It

is usually associated with long-lasting cognitive and behavioral abnormalities throughout an individual's life in alcoholics and children with FASD. Alcohol triggered widespread apoptotic neuronal death in a third trimester equivalent alcohol exposure mouse model [7]. Evidence have suggested that ER stress is one of the contributing factors for alcohol-induced neurodegeneration. A study using cultured SH-SY5Y cells and primary cerebellar granule neurons showed that alcohol significantly potentiated tunicamycin- and thapsigargin-induced expression of UPR genes, indicating that alcohol was synergistic with chemical ER inducers in enhancing ER stress [91]. Activation of the UPR was observed in the developing mouse brain using a third-trimester equivalent alcohol exposure model [26]. When alcohol induced UPR activation was blocked pharmacologically, neuronal apoptosis was significantly reduced [32]. MANF has been implicated to attenuate ER stress-related cellular damages in multiple diseases. We have previously shown that MANF deficiency rendered neurons more susceptible to alcohol induced ER stress and neuronal apoptosis in the postnatal mouse brain in a neuron specific MANF knockout model [49].

We showed that exogenous MANF was able to protect neuronal cells against alcohol neurotoxicity, which was indicated by *in vitro* studies showing that the treatment of rhMANF or MANF overexpression by adenovirus offered protection to alcohol-induced reduction of viability in N2a cells. Similar protection was observed *in vivo* studies showing that enhanced MANF by ICV-injection of MANF-AAV significantly decreased the number of alcohol-induced apoptotic neurons in the developing cortex. These observations were in line with previous studies that MANF overexpression protect neurons from ER stress-induced cell death in various disease models. For example, MANF overexpression reduced cerebral ischemia-induced neurodegeneration and promoted functional recovery after stroke [64, 92]. In Parkinson's disease models, intrastriatal injection of MANF protected and reinstated the functionality of dopaminergic neurons [42, 93]. Addition of MANF also facilitated retinal neurons recovery in various eye injury models [45, 46, 48, 94]. The function of MANF in ER stress modulation is still unclear and it is unknown whether intracellular and extracellular MANF acts via similar or different molecular mechanisms. Intracellular overexpression of MANF reduced UPR gene p-eIF2α, ATF4, CHOP, XBP1s, GRP78 and ATF6α in a 6-hydroxydopamine (6-OHDA)-induced PD model in SH-SY5Y cells [95]. However, the same study showed that extracellular addition of MANF failed to alter any of the UPR genes, but instead it may protect cells against 6-OHDA-induced toxicity via the PI3K/AKT/mTOR pathway [95]. Furthermore, results were also controversial for the effect of MANF on UPR genes expression. For example, extracellular addition of MANF was reported to attenuated cerebral ischemia-induced ER stress by inhibiting the expression of GRP78, p-IRE1, and XBP1s [96]. However, in an earlier study, extracellular MANF was shown to elevate GRP78 expression in 6-OHDA-treated SH-SY5Y cells [97]. As a result, further investigations are needed to understand the mechanisms by which MANF modulates ER stress and UPR.

In summary, we demonstrated that alcohol increased MANF transcription and secretion in neuronal cells. The elevation of UPR and the presence of ERSE-II were necessary for alcohol-induced MANF expression. MANF deficient neuronal cells exhibited increased GRP78 and XBP1s and were more vulnerable to alcohol-induced cell death. Enhanced MANF expression alleviated alcohol-induced cell death in cultured neuronal cells and in the

postnatal mouse brain. Taken together, MANF may be beneficial for the therapy/treatment of alcohol neurotoxicity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements:

Abbreviations

AAV	adeno-associated virus
AKT	protein kinase B
ATF4	activating transcription factor 4
ATF6	activating transcription factor 6
CHX	cycloheximide
CHOP	CCAAT-enhancer-binding proteins homologous protein
CNS	central nervous system
CRISPR	clustered regularly interspaced short palindromic repeats
eIF2α	eukaryotic translation initiation factor 2α
ER	endoplasmic reticulum
ERAD	ER-associated protein degradation
ERSE-II	ER stress response element II
FASD	fetal alcohol spectrum disorder
GRP78	glucose-regulating protein 78
GRP94	glucose-regulated protein 94
HERP	homocysteine-induced endoplasmic reticulum protein
HRP	horseradish peroxidase
ICV	intracerebroventricular
IRE1α	inositol-requiring enzyme 1α
KO	knockout

KDEL	lysine-aspartic acid-glutamic acid-leucine
mTOR	mammalian target of rapamycin
MANF	mesencephalic astrocyte-derived neurotrophic factor
rhMANF	recombinant human mesencephalic astrocyte-derived neurotrophic factor
PD	postnatal day
PDIs	protein disulfide isomerases
PERK	protein kinase R-like ER kinase
PI3K	phosphoinositide 3-kinases
RRID	Research Resource Identifier
RTDL	arginine-threonine-aspartic acid-leucine
TG	thapsigargin
Tun	tunicamycin
UPR	unfolded protein response
vp	viral particles
WT	wild type
XBP1s	spliced form of X-box binding protein 1
4-PBA	sodium phenylbutyrate
6-OHDA	6-hydroxydopamine

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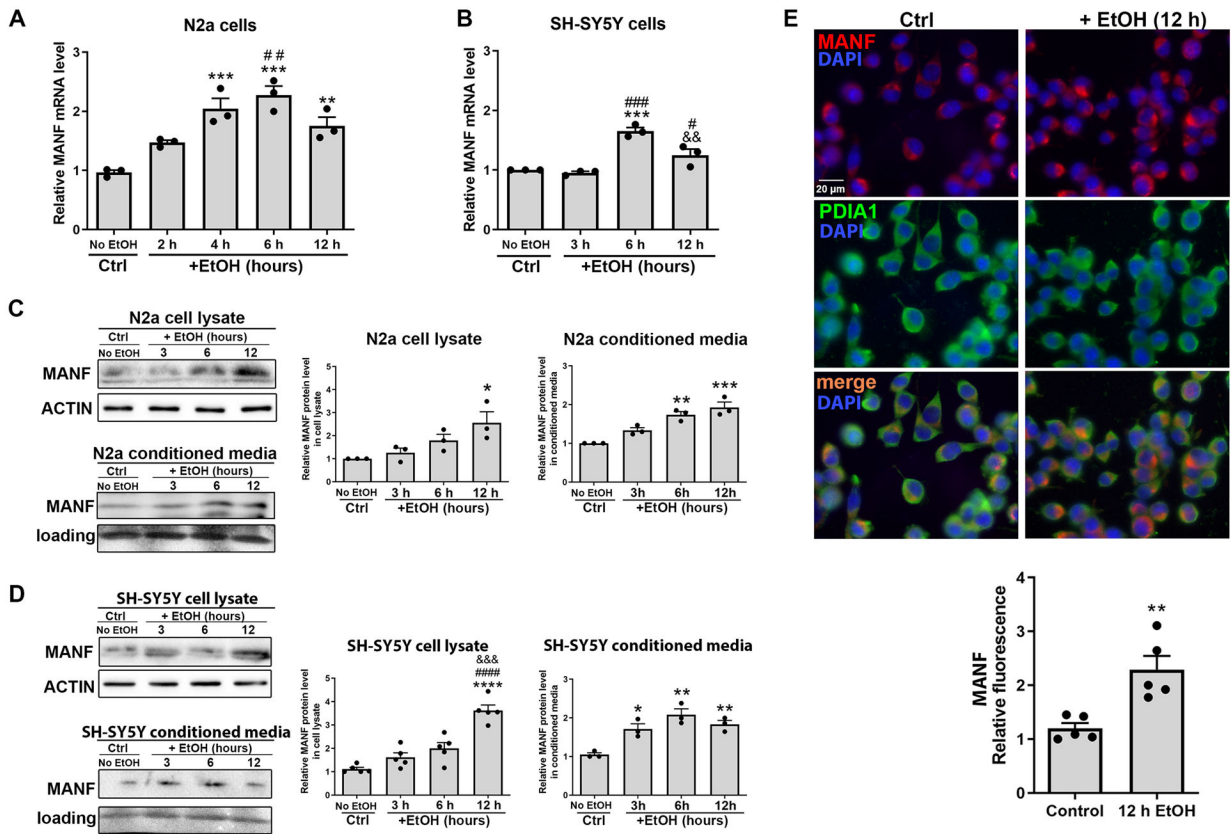
Figure 1. Effects of alcohol on MANF expression and secretion in neuronal cells.
A-B: The levels of MANF mRNA in control and alcohol-treated (0.4% w/v) N2a and
SH-SY5Y cells were examined by real-time PCR. The data was expressed as mean ± SEM
from 3 independent cell culture preparations. One-way ANOVA followed by Tukey post hoc
test, N2a cells: $F_{(4, 10)} = 16.97$, $p = 0.0002$, SH-SY5Y cells: $F_{(3, 8)} = 29.18$, $p = 0.0001$.
** $p < 0.01$ or *** $p < 0.001$ when compared to control; # $p < 0.05$ or ## $p < 0.01$ or ###
$p < 0.001$ when compared to 2 h alcohol-treated group (N2a cells) or 3 h alcohol-treated
group (SH-SY5Y cells); && $p < 0.01$ when compared to 6 h alcohol-treated groups. **C-D:**
Representative immunoblots showing MANF protein levels in cell lysates and conditioned
media in control and alcohol-treated cells. The data was expressed as mean ± SEM from 3–5
independent cell culture preparations. One-way ANOVA followed by Tukey post hoc test,
N2a cell lysate: $F_{(3, 8)} = 5.636$, $p = 0.0226$, SH-SY5Y cell lysate: $F_{(3, 16)} = 29.69$, $p <
0.0001$, N2a conditioned media: $F_{(3, 8)} = 22.37$, $p = 0.0003$, SH-SY5Y conditioned media: F
$_{(3, 8)} = 14.33$, $p = 0.0014$. * $p < 0.05$ or ** $p < 0.01$ or *** $p < 0.001$ or **** $p < 0.0001$ when
compared to control; #### $p < 0.0001$ when compared to 3 h alcohol-treated group; &&& $p
< 0.001$ when compared to 6 h alcohol-treated group. **E:** Representative fluorescent images
of MANF (red) and PDIA1 (green) in N2a cells after 12 hours of alcohol treatment. The data
was expressed as mean ± SEM from 5 independent cell culture preparations. t-test, t = 4.017,
df = 8, $p = 0.0039$. ** $p < 0.01$.

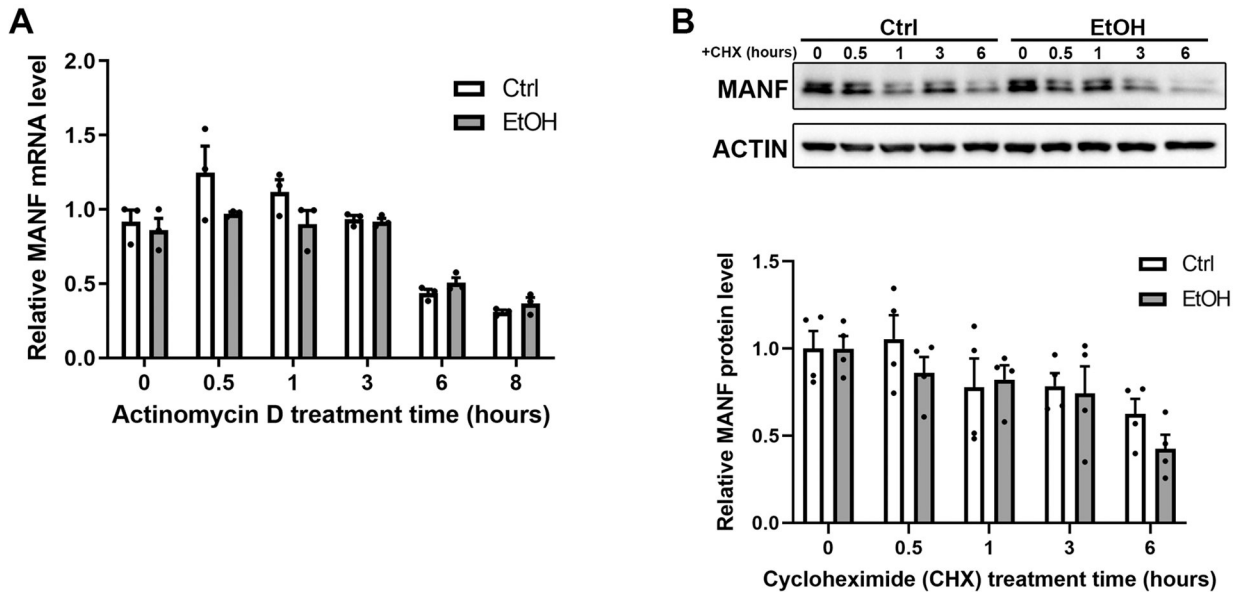**Figure 2. Effects of alcohol on the stability of MANF mRNA and protein.**
A: The levels of MANF mRNA in control and alcohol-treated (0.4% w/v) N2a cells were examined by real-time PCR after new RNA synthesize was inhibited by actinomycin D (10 μg/ml). The data was expressed as mean ± SEM from 3 independent cell culture preparations. Two-way ANOVA followed by Tukey post hoc test, Time: $F_{(1.595, 6.380)} = 33.38$, $p = 0.0005$, EtOH: $F_{(1, 4)} = 4.997$, $p = 0.0891$, Interaction: $F_{(5, 20)} = 1.750$, $p = 0.1693$. No statistical significance was detected between control and EtOH groups. **B:** Representative immunoblots showing MANF protein levels in control and alcohol-treated (0.4% w/v) N2a cells after new protein synthesis was blocked by CHX (100 μg/ml). The data was expressed as mean ± SEM from 4 independent cell culture preparations. Two-way ANOVA followed by Tukey post hoc test, Time: $F_{(4, 30)} = 5.883$, $p = 0.0013$, EtOH: $F_{(1, 30)} = 1.268$, $p = 0.2691$, Interaction: $F_{(4, 30)} = 0.5218$, $p = 0.7204$. No statistical significance was detected between control and EtOH groups.
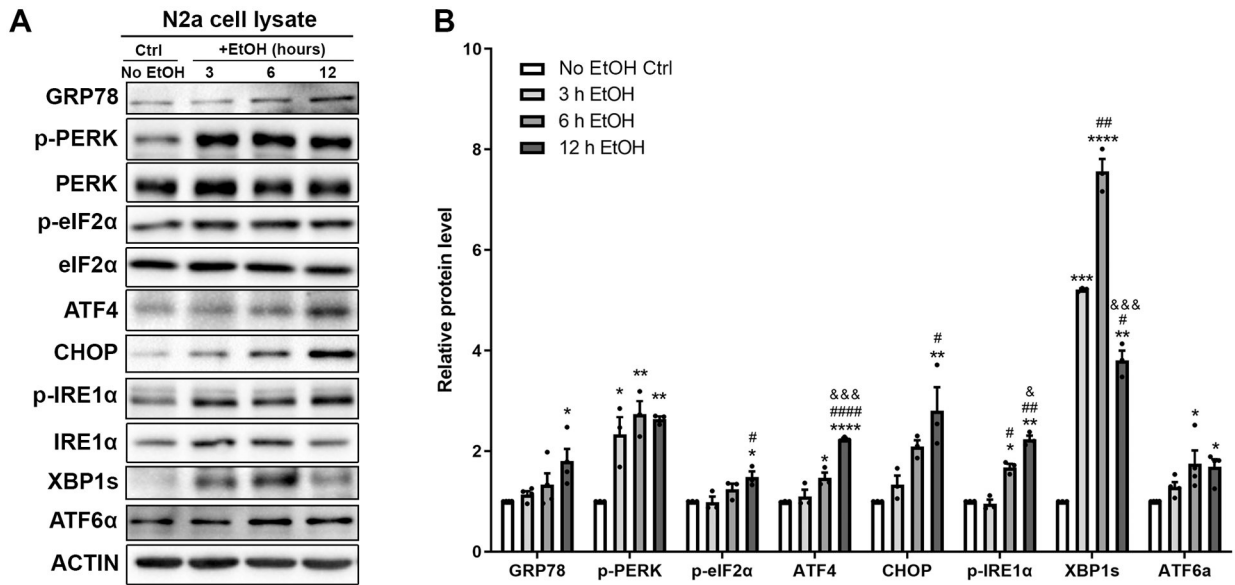
Figure 3. Effects of alcohol on ER stress.

A-B: Representative immunoblots showing the expression of UPR proteins in control and alcohol-treated (0.4% w/v) N2a cell lysates. The data was expressed as mean ± SEM from 3–4 independent cell culture preparations. One-way ANOVA followed by Tukey post hoc test, GRP78: $F_{(3, 12)} = 4.254$, $p = 0.0290$, p-PERK: $F_{(3, 8)} = 13.75$, $p = 0.0016$, p-eIF2α: $F_{(3, 8)} = 11.63$, $p = 0.0027$, ATF4: $F_{(3, 8)} = 106.5$, $p < 0.0001$, CHOP: $F_{(3, 8)} = 9.668$, $p = 0.0049$, p-IRE1α: $F_{(3, 8)} = 84.94$, $p < 0.0001$, XBP1s: $F_{(3, 8)} = 312.0$, $p < 0.0001$, ATF6α: $F_{(3, 12)} = 7.390$, $p = 0.0046$. * $p < 0.05$ or ** $p < 0.01$ or *** $p < 0.001$ or **** $p < 0.0001$ when compared to control; # $p < 0.05$ or ## $p < 0.01$ or #### $p < 0.0001$ when compared to 3 h alcohol-treated group; & $p < 0.05$ or &&& $p < 0.001$ when compared to 6 h alcohol-treated group.
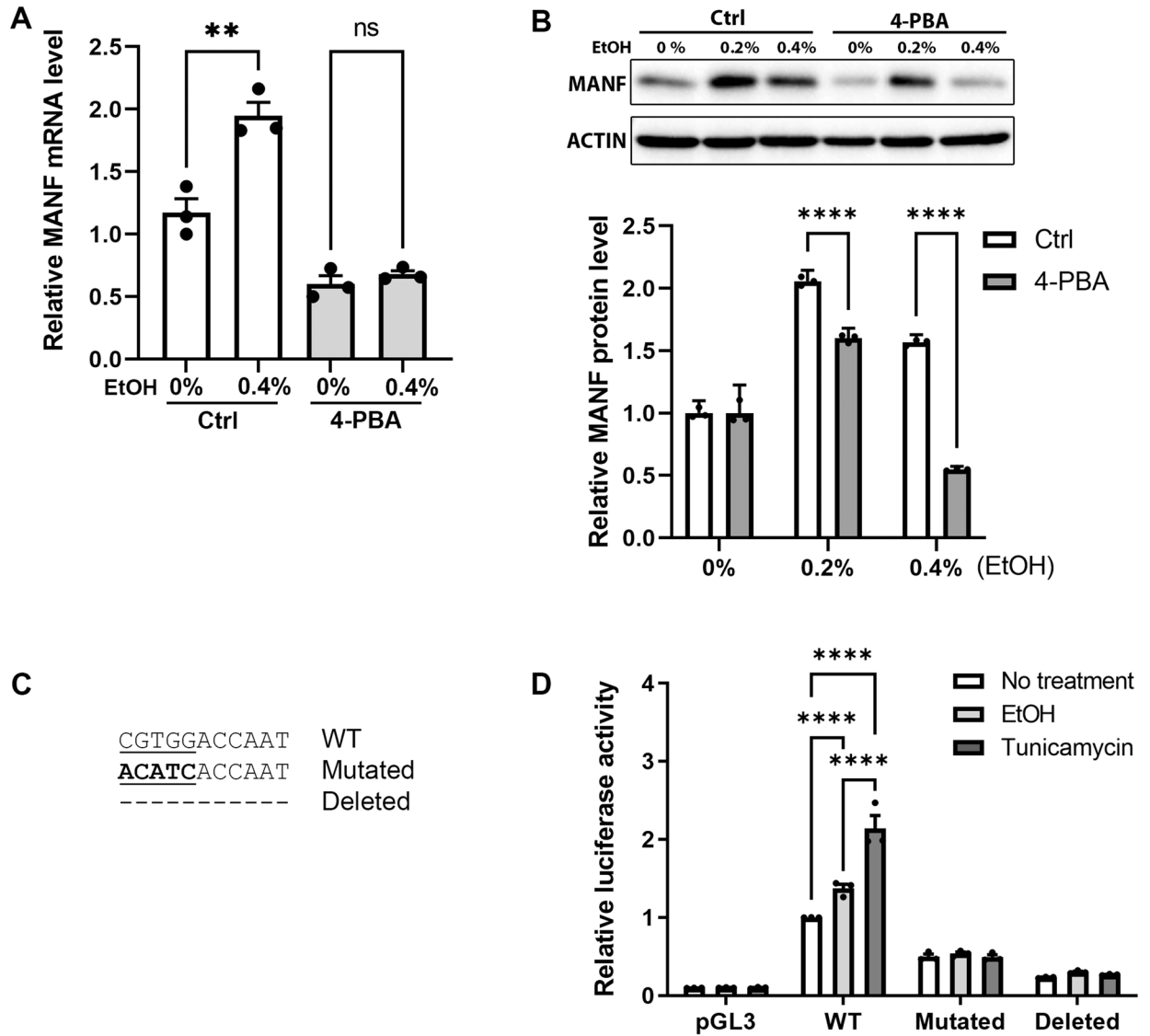
Figure 4. Effects of alcohol and 4-PBA on MANF expression and transcription activity.
A: Real-time PCR result showing the levels of MANF mRNA in control and alcohol-treated
(0.4% w/v) N2a cells with or without the pretreatment of ER stress inhibitor 4-PBA. Cells
were incubated with 0.4% (w/v) ethanol for 6 hours with or without 2 hours pretreatment
of 10 mM 4-PBA. The data was expressed as mean ± SEM from 3 independent cell
culture preparations. One-way ANOVA followed by Tukey post hoc test, $F_{(3, 8)} = 52.15$,
$p < 0.0001$. ** $p < 0.01$, ns not statistically significant. **B:** Representative immunoblots of
MANF expression in control and alcohol-treated N2a cells with or without the pretreatment
of ER stress inhibitor 4-PBA. Cells were incubated with 0.2% or 0.4% (w/v) ethanol for 12
hours with or without 2 hours pretreatment of 10 mM 4-PBA. The data was expressed as
mean ± SEM from 3 independent cell culture preparations. t-test, 0%: t = 4.444e-006, df = 4,
$p = 0.999997$, 0.2%: t = 16.48, df = 4, $p = 0.000079$, 0.4%: t = 64.48, df = 4, $p < 0.000001$.
**** $p < 0.0001$. **C:** The consensus sequence of ERSE-II in MANF promoter is shown and
ATF6/XBP1s binding site is underlined. Mutated sequence is bolded. Dashed line represents

the deletion of the entire ERSE-II sequence. **D:** N2a cells were transfected with luciferase reporter constructs with MANF promotor regions containing wild type (WT), mutated, or deleted ERSE-II sequence. After the transfection, cells were treated with ethanol (0.4% w/v) or tunicamycin (5 μg/ml) for 6 hours. Luciferase activity was measured and normalized to cells that were transfected with WT construct and with no treatment. The data was expressed as mean ± SEM from 3 independent cell culture preparations. Two-way ANOVA followed by Tukey post hoc test, Mutations: $F_{(3, 24)} = 445.6$, $p < 0.0001$, Treatment: $F_{(2, 24)} = 32.67$, $p < 0.0001$, Interaction: $F_{(6, 24)} = 31.84$, $p < 0.0001$. **** $p < 0.0001$.
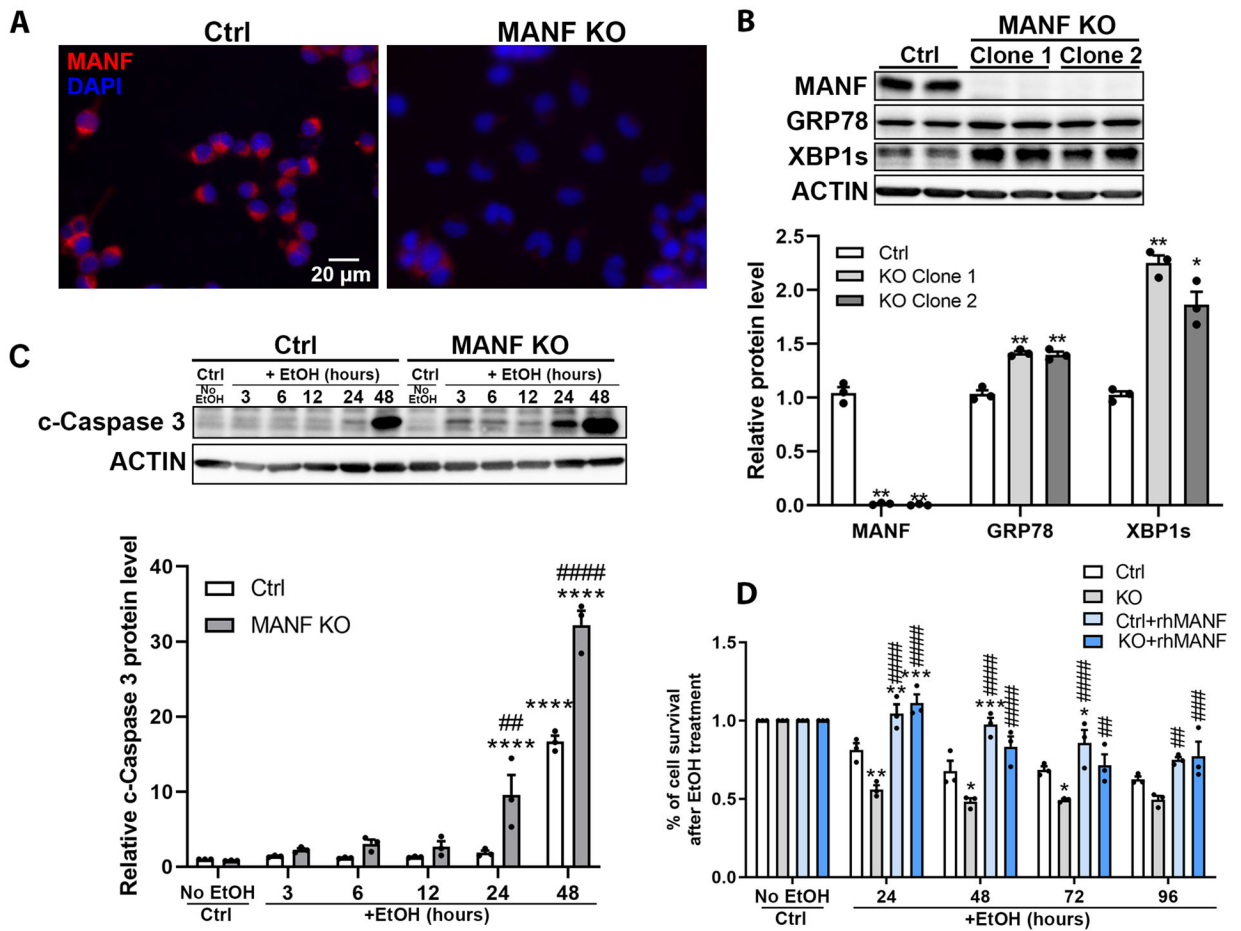
Figure 5. Effects of MANF deficiency on alcohol-induced cell death.

A: Representative fluorescent images confirming MANF knockout (KO) in N2a cells. **B:** Representative immunoblots of MANF, GRP78, and XBP1s expression in control and MANF KO N2a cells. The data was expressed as mean ± SEM from 3 independent cell culture preparations. One-way ANOVA followed by Tukey post hoc test, MANF: $F_{(2, 6)} = 361.0$, $p < 0.0001$, GRP78: $F_{(2, 6)} = 60.17$, $p = 0.0001$, XBP1s: $F_{(2, 6)} = 58.41$, $p = 0.0001$. * $p < 0.05$ or ** $p < 0.01$ when compared to control cells. **C:** Representative immunoblots of cleaved-caspase 3 expression in control and alcohol-treated (0.4% w/v) control and MANF KO N2a cells. The data was expressed as mean ± SEM from 3 independent cell culture preparations. Two-way ANOVA followed by Tukey post hoc test, Time: $F_{(5, 24)} = 161.4$, $p < 0.0001$, KO: $F_{(1, 24)} = 59.67$, $p < 0.0001$, Interaction: $F_{(1, 24)} = 59.67$, $p < 0.0001$. **** $p < 0.0001$ when compared to all earlier time points. ## $p < 0.01$ or #### $p < 0.0001$ when compared between control and MANF KO cells within each time points. **D:** N2a cells were incubated with or without 20 ng/ml rhMANF. Cell viability was tested by MTT assay at various time points after alcohol treatment (0.4% w/v). Percentage of cell survival was calculated by normalizing the alcohol treated group by no alcohol control group at each time points. The data was expressed as mean ± SEM from 3 independent cell culture preparations. Two-way ANOVA followed by Tukey post hoc test, Time: $F_{(4, 40)} = 38.64$, $p < 0.0001$, KO: $F_{(3, 40)} = 48.86$, $p < 0.0001$, Interaction: $F_{(12, 40)} = 4.994$, $p < 0.0001$. * $p <$

0.05, ** $p < 0.01$, *** $p < 0.001$ when compared to control; ## $p < 0.01$, ### $p < 0.01$, #### $p < 0.0001$ when compared to KO at each time points.
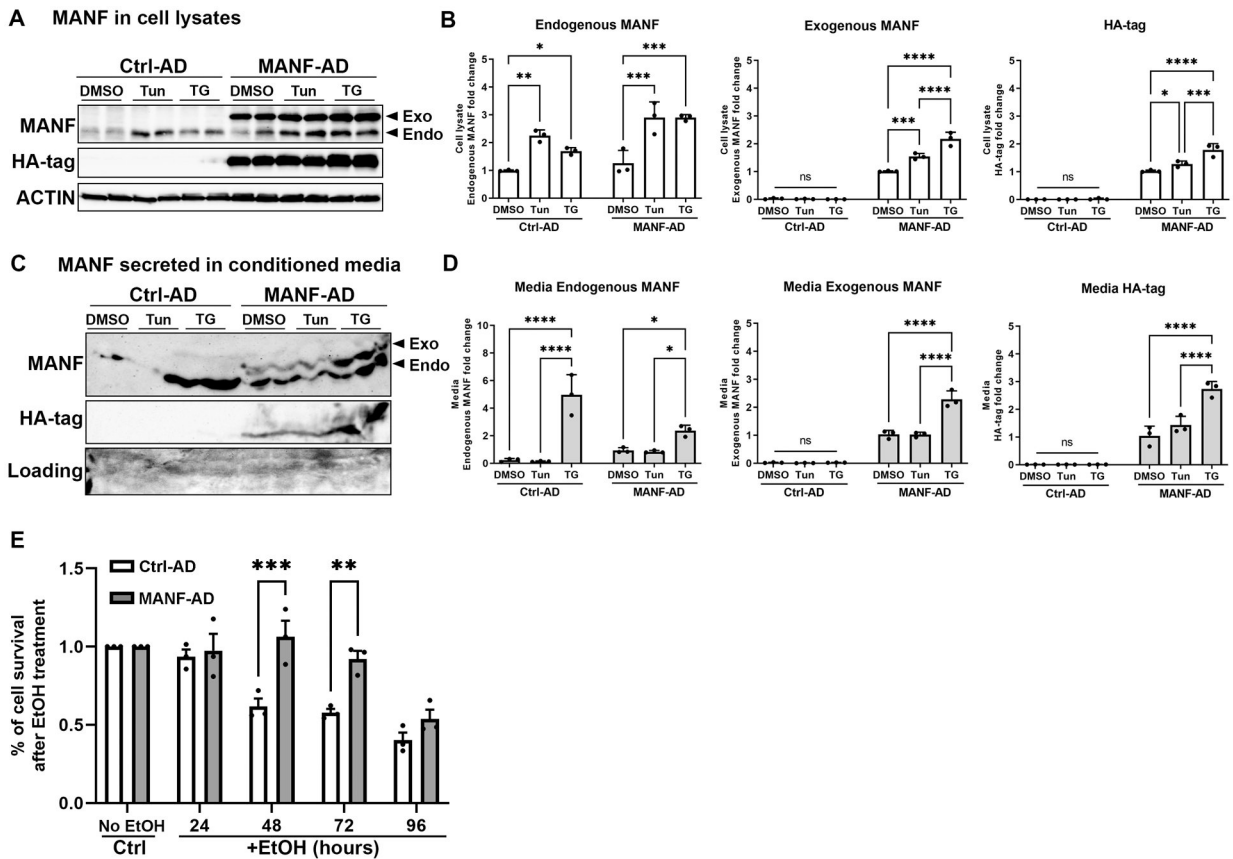
Figure 6. Effects of exogenous MANF on alcohol-induced cell death.

A: N2a Cells were transduced with Ctrl-AD or MANF-AD for 4 hours, then treated with DMSO or tunicamycin (5 μg/ml) or thapsigargin (1.5 μM) for 24 hours. Representative immunoblots of MANF and HA-tag expression in N2a cell lysates. Exogenous (exo) and endogenous (endo) MANF were distinguished by the shift of migration on SDS-PAGE gel electrophoresis. Exogenous MANF migrated slower than endogenous MANF because of the HA tag. Tun: tunicamycin; TG: thapsigargin. **B:** Quantification of the cell lysates immunoblots. The data was expressed as mean ± SEM from 3 independent cell culture preparations. Two-way ANOVA followed by Tukey post hoc test. Endogenous MANF: Treatment $F_{(2, 12)} = 35.41$, $p < 0.0001$, Ctrl- vs MANF-AD $F_{(2, 12)} = 22.67$, $p = 0.0005$, Interaction $F_{(2, 12)} = 3.33$, $p = 0.0707$. Exogenous MANF: Treatment $F_{(2, 12)} = 41.68$, $p < 0.0001$, Ctrl- vs MANF-AD $F_{(2, 12)} = 933.4$, $p < 0.0001$, Interaction $F_{(2, 12)} = 45.01$, $p < 0.0001$. Ha-tag: Treatment $F_{(2, 12)} = 22.21$, $p < 0.0001$, Ctrl- vs MANF-AD $F_{(2, 12)} = 769.4$, $p < 0.0001$, Interaction $F_{(2, 12)} = 20.1$, $p = 0.0001$. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, ns not statistically significant. **C:** Representative immunoblots of MANF and HA-tag expression in conditioned culture media. **D:** Quantification of the conditioned media immunoblots. The data was expressed as mean ± SEM from 3 independent cell culture preparations. Two-way ANOVA followed by Tukey post hoc test. Endogenous MANF: Treatment $F_{(2, 12)} = 49.6$, $p < 0.0001$, Ctrl- vs MANF-AD $F_{(2, 12)} = 1.788$, $p = 0.206$, Interaction $F_{(2, 12)} = 13.82$, $p = 0.00008$. Exogenous MANF: Treatment $F_{(2, 12)} = 39.97$, $p < 0.0001$, Ctrl- vs MANF-AD $F_{(2, 12)} = 459.5$, $p < 0.0001$, Interaction $F_{(2, 12)}$

= 38.93, $p < 0.0001$. Ha-tag: Treatment $F_{(2, 12)} = 23.82$, $p < 0.0001$, Ctrl- vs MANF-AD $F_{(2, 12)} = 272.1$, $p < 0.0001$, Interaction $F_{(2, 12)} = 23.66$, $p = 0.0001$. * $p < 0.05$, **** $p < 0.0001$, ns not statistically significant. **E:** The viability of cells transduced with Ctrl-AD and MANF-AD was tested by MTT assay at various time points following alcohol exposure. Percentage of cell survival was calculated by normalizing the alcohol treated group by no alcohol control group at each time points. The data was expressed as mean ± SEM from 3 independent cell culture preparations. Two-way ANOVA followed by Tukey post hoc test, Time: $F_{(4, 20)} = 24.70$, $p < 0.0001$, MANF: $F_{(1, 20)} = 25.93$, $p < 0.0001$, Interaction: $F_{(4, 20)} = 5.276$, $p = 0.0046$. ** $p < 0.01$, *** $p < 0.001$.
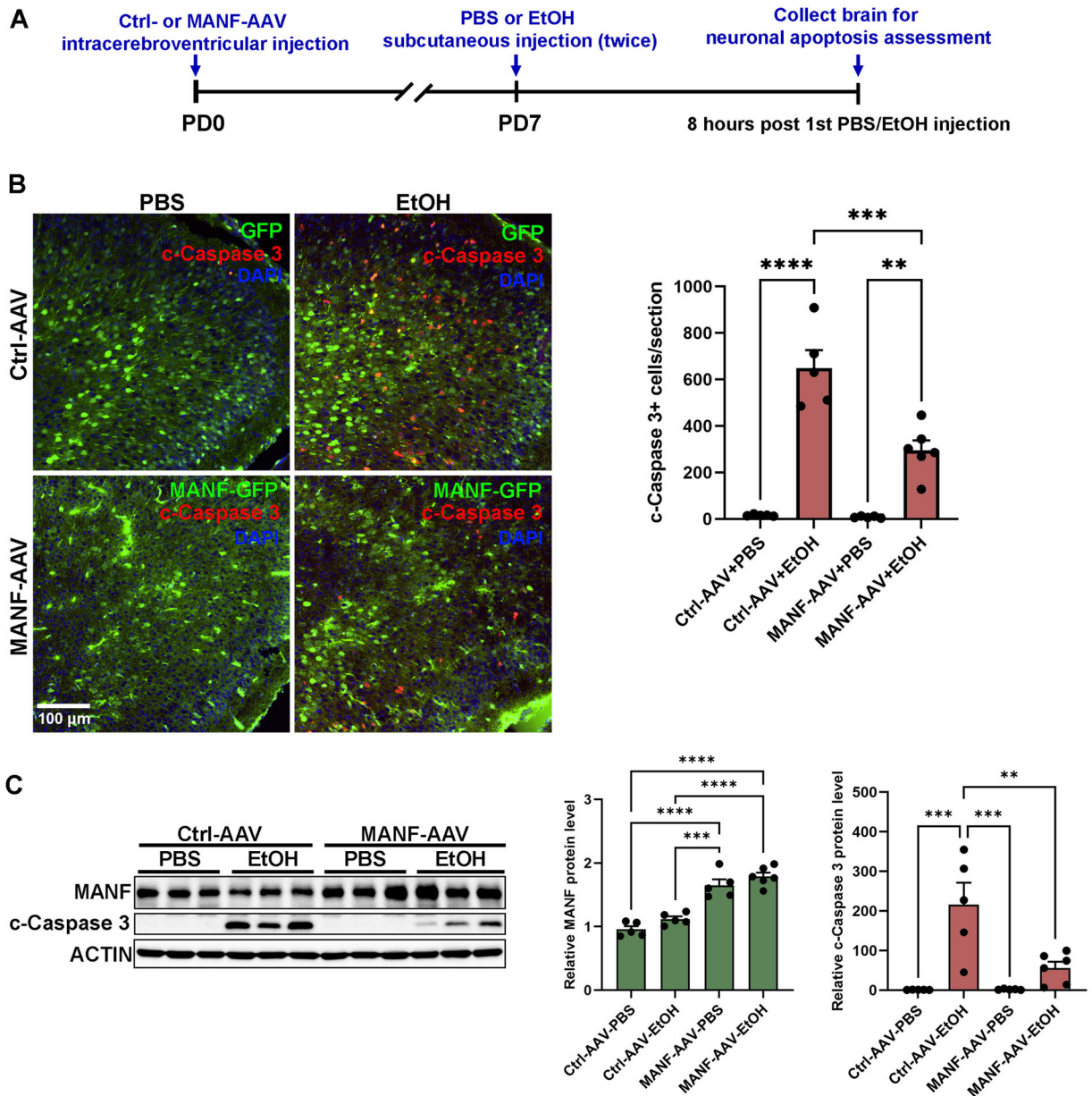
Figure 7. Effects of MANF on alcohol-induced cell death in the developing mouse cerebral cortex.

A: Time-line diagram showing the experimental procedure. Newborn pups were ICV-injected with either control- or MANF-AAV. The pups were then injected with PBS control or alcohol subcutaneously at PD7. Eight hours after alcohol treatment, the pups were sacrificed, and the cerebral cortices were dissected. **B:** Representative fluorescent images of GFP (green) and cleaved-caspase 3 (red) in the PD7 cerebral cortex. The number of cleaved-caspase 3 positive cells in the cerebral cortex was quantified. The data was expressed as mean ± SEM. n = 5–6 animals per group. One-way ANOVA followed by Tukey post hoc test, $F_{(3, 17)} = 45.37$, $p < 0.0001$. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. **C:** Representative immunoblots of MANF and cleaved-caspase 3 in the PD7 cerebral cortex. The data was expressed as mean ± SEM. n = 5–6 animals per group. One-way ANOVA

followed by Tukey post hoc test, MANF: $F_{(3, 17)} = 40.23$, $p < 0.0001$, cleaved-caspase 3: $F_{(3, 17)} = 12.69$, $p = 0.0001$. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.