2019

THE ROLE OF ENDOPLASMIC RETICULUM STRESS IN ETHANOL-INDUCED NEURODEGENERATION

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Digital Object Identifier: https://doi.org/10.13023/etd.2019.450

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THE ROLE OF ENDOPLASMIC RETICULUM STRESS IN ETHANOL-INDUCED NEURODEGENERATION

DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Medicine at the University of Kentucky

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2019

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ABSTRACT OF DISSERTATION

THE ROLE OF ENDOPLASMIC RETICULUM STRESS IN ETHANOL-INDUCED NEURODEGENERATION

Heavy ethanol use causes neurodegeneration manifested by neuronal loss and dysfunction. It is becoming imperative to delineate the underlying mechanism to promote the treatment of ethanol-induced neurodegeneration. Endoplasmic reticulum (ER) stress is a hallmark and an underlying mechanism of many neurodegenerative diseases. This study aims to investigate the role of ER stress in ethanol-induced neurodegeneration. In experimental design, adult mice were exposed to binge ethanol drinking by daily gavage for 1, 5, or 10 days and the response of ER stress was examined. We found the induction of ER stress appeared at 5 days and remained at 10 days. Moreover, the induction of ER stress was accompanied by an increase in neurodegeneration. With cell culture, we demonstrated that ethanol exposure resulted in neuronal apoptosis and that blocking ER stress by sodium phenylbutyrate (4-PBA) abolished ethanol-induced neuronal apoptosis, suggesting that ER stress contributes to ethanol-induced neurodegeneration.

Mesencephalic astrocyte-derived neurotrophic factor (MANF) responds to ER stress and has been identified as a protein upregulated in ethanol-exposed developmental mouse brains. To investigate its implication in ethanol-induced neurodegeneration, we established a central nervous system (CNS)-specific *Manf* knockout mouse model and examined the effects of MANF deficiency on ethanol-induced neuronal apoptosis and ER stress using a third-trimester equivalent mouse model. We found MANF deficiency worsened ethanol-induced neuronal apoptosis and ER stress and that blocking ER stress abrogated the harmful effects of MANF deficiency on ethanol-induced neuronal apoptosis. Moreover, a whole transcriptome RNA sequencing supported the involvement of MANF in ER stress modulation and revealed candidates that may mediate the ER stress-buffering capacity of MANF. Collectively, these data suggest that MANF is neuroprotective against ethanol-induced neurodegeneration via ameliorating ER stress.

Because MANF is a neurotrophic factor, we also examined the effects of MANF deficiency on neurogenesis. We observed that MANF deficiency increased neurogenesis

in the subgranular zone of the hippocampal dentate gyrus and subventricular zone of the lateral ventricles in the mouse brain. Mechanistically, this finding was supported by a decrease of cell cycle inhibitors (p15 and p27), an increase of G2/M marker (phospho-histone H3), and an increase of neural progenitor markers (Sox2 and NeuroD1) in the brain of conditional *Manf* knockout mice. Our in vitro studies demonstrated that the gain-of-function of MANF inhibited cell cycle progression, whereas the loss-of-function of MANF promoted cell cycle progression. Taken together, these data suggest that MANF may affect the process of neurogenesis through altering cell cycle progression.

KEYWORDS: ER Stress, Ethanol-induced Neurodegeneration, Neurogenesis, Mesencephalic Astrocyte-derived Neurotrophic Factor

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THE ROLE OF ENDOPLASMIC RETICULUM STRESS IN ETHANOL-INDUCED NEURODEGENERATION

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DEDICATION

I want to dedicate this to my mother in heaven. She is my hero, and my missing for her is increasing every single day. I want to dedicate this to my wife and my two children for their love and support. I would also like to dedicate this to my previous mentor, Dorothy Tuan, who passed away during my time as a Ph.D. student.

ACKNOWLEDGMENTS

The following dissertation would never turn out this well without the expertise and support of several people. First and foremost, my mentor, Dr. Jia Luo, exemplified the high quality of scholarship and mentorship for which I am so thankful. I also thank Dr. Nancy Webb for taking me as a T32 trainee. Next, I would like to thank the entire dissertation committee and my external examiner, respectively: Dr. Jia Luo, Dr. Porter Nada, Dr. Gang Chen, Dr. Haining Zhang, and Dr. Susan Barron. Each of you provided timely and instructive comments through the years to make this project completed on schedule.

I benefited greatly from many thoughtful individuals who provided valuable comments and supports at every stage of the dissertation process. In particular, I would like to thank: Drs. Chi Wang, Daheng He, and Marco Clementino for helping me with RNA-Seq data analysis; Jacqueline A. Frank and Hui Li for helping me set up my projects; James Wise for helping me proofread and format my fellowship, manuscripts, and dissertation drafts. I wish to thank: Mei Xu, Xin Wang, Fang Yang, Dexiang Liu, Hong Xu, Wen Wen, Huaxun Wu, Guangbin Li, for their help in every aspect of my research. Lucky I am.

I am blessed with having the understanding and support of my family. My father provided unbelievable support, always telling me to put my research and career as my priority. My wife is one of the most unselfish women in the world. While having to pursue her career, she took care of our two kids and never complained to me. Lastly, I am so grateful to my kids, who are so considerate and have brought us so much joy!

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CHAPTER 1. INTRODUCTION

1.1 Concepts and Definitions Related to Ethanol Use

1.1.1 Standard Drink

In the United States, one standard drink contains roughly 14 grams of pure ethanol. This amount of ethanol content is equal to (1) 12 ounces of regular beer, (2) 5 ounces of wine, or (3) 1.5 ounces of distilled spirits [1].

1.1.2 Moderate Ethanol Drinking

According to the " Dietary Guidelines for Americans 2015-2020," U.S. Department of Health and Human Services and U.S. Department of Agriculture, moderate drinking was defined as up to 1 drink per day for women and up to 2 drinks per day for men [2].

1.1.3 Drinking at Low Risk

For women, low-risk drinking is considered as no more than 3 drinks on any single day and no more than 7 drinks per week. For men, it is considered as no more than 4 drinks on any single day and no more than 14 drinks per week [2]. Research from the National Institute on Alcohol Abuse and Alcoholism (NIAAA) suggested that about 2 per 100 people drinking within these limits developed alcohol use disorder [2].

1.1.4 Binge Drinking and Heavy Alcohol Use

According to NIAAA, binge drinking is defined as a drinking pattern that brings BEC levels to 0.08g/dL. It typically occurs after having 4 drinks for women and 5 drinks for men within 2 hours. The Substance Abuse and Mental Health Services Administration (SAMHSA) conducts National Survey on Drug Use and Health annually, and defines binge drinking as 5 or more alcoholic drinks for males or 4 or more alcoholic drinks for females

on the same occasion, i.e., within a couple of hours of each other, on at least one day in the past month [2].

SAMHSA defines heavy alcohol use as a drinking pattern of having binge drinking for no less than 5 days in the past month [2].

1.1.5 Alcohol Use Disorder (AUD)

AUD, also called alcoholism, is a brain disease, manifested by compulsive ethanol use and loss of control of ethanol intake despite the knowledge of adverse social, occupational, and health consequences. Prior to the fifth edition of the *Diagnostic and Statistical Manual* (DSM-V), ethanol abuse and ethanol dependence were regarded as two ethanol-related disorders. They were integrated into a single disease called alcohol use disorder or AUD in DSV-V. To be diagnosed as AUD, patients must meet at least two of the 11 criteria that are outlined in the DSM-V. AUD can range from mild to severe, based on the number of features present [3].

1.1.6 Fetal Alcohol Spectrum Disorders (FASDs)

FASDs is an umbrella term that includes fetal alcohol syndrome (FAS), partial fetal alcohol syndrome (pFAS), alcohol-related neurodevelopmental disorder (ARND), and alcohol-related birth defects (ARBD). FASDs are characterized by damage to the central nervous system; the damaged CNS is coupled with cognitive dysfunction and behavioral changes [4].

1.1.7 Wernicke-Korsakoff Syndrome (WKS)

WKS is a type of brain disorder, manifested by the combined presence of Wernicke encephalopathy and alcoholic Korsakoff syndrome, due to thiamine (vitamin B1) deficiency. Symptoms of WKS may include confusion, unsteady stance, and impaired

memory and cognition. Chronic ethanol misuse and malnutrition can cause thiamine deficiency and are the most common causes of WKS [4].

1.1.8 Disability-Adjusted Life Years (DALYs)

DALYs are expressed as the number of years lost due to unhealthy conditions. The sum of these DALYs in the population is a measure of overall disease burden in society, reflecting the gap between current health status and an ideal health situation, namely, free of disease and disability [4].

1.2 Overview of Ethanol Consumption

People drink to socialize, celebrate, or relax. In many of today's societies, alcoholic beverages are a routine part of the social landscape. A global status report on ethanol and health, published by the World Health Organization (WHO) in 2018, outlined ethanol consumption worldwide and the drinking patterns and trends since 2000 [4]. First, the prevalence of drinking and total ethanol per capita consumption has declined in some WHO regions, e.g., European regions, but has increased in other WHO regions, e.g., Western Pacific Region since 2000 [4]. Thus, the prevalence of drinking and total ethanol per capita worldwide, in general, remained stable since 2000. Secondly, binge drinking, defined as having more than 4 drinks for women or 5 drinks for men on one occasion at least once per month, was prevalent among drinkers and was particularly popular among young people and college students [4]. Thirdly, in all WHO regions, females drank less and were less often current drinkers than males. However, the absolute number of current women drinkers has increased in the world [4]. Lastly, the economic wealth of a region correlates with higher ethanol consumption and the prevalence of current drinkers, and the incidence of drinking and total ethanol per capita was projected to increase in the future [4].

Ethanol consumption has effects on people, and these effects vary from person to person depending on a variety of factors, namely: (1) how much you drink; (2) how often you drink; (3) how early you are exposed to ethanol drinking; (4) health status; and (5) family history or genetic variety. In general, drinking at a mild or moderate level is not necessarily a problem [5]; however, drinking too much can cause a range of severe consequences [4]. In 2016, the harmful use of ethanol caused 3 million death, amounting to 5.3% of all death worldwide and 132.6 million disability-adjusted life years (DALYs), accounting for 5.1% of all DALYs in that year [4]. The mortality due to ethanol use was higher than that caused by diseases such as tuberculosis, HIV/AIDS, and diabetes [4]. Of all the deaths due to ethanol use, 28.7% were due to injuries; 21.3% were due to digestive diseases; 19% were due to cardiovascular diseases; 12.9% were due to infectious diseases; 12.6% were due to cancers [4]. About 49% of ethanol-attributable DALYs were related to non-communicable and mental conditions, and about 40% were due to injuries [4]. An estimate of 237 million men and 46 million women had AUD, with the highest prevalence of AUD among men and women in the European Region [4]. Besides, long-term ethanol use may cause a deficiency of thiamine in people, resulting in WKS, a brain disease due to a lack of thiamine [4].

The harms due to ethanol use affected not only the drinkers, but also those people around him or her, e.g., others in the family, relatives, and friends, or strangers encountered on the street. For instance, the drinker may cause injury, or anxiety or depression to family members or pass infectious diseases to sexual partners. Beyond health consequences, the harm resulted from drinking may also be social, e.g., assault, community nuisance, or economic, e.g., damage to property or money for family necessities [4].

1.3 Ethanol Use in the United States

A National Survey on Drug Use and Health (NSDUH) was conducted annually in the United States. NSDUH asked respondents aged 12 and older about their ethanol use in the lifetime, in the past year or the past month. The data were classified by age groups and further sub-classified into different groups according to the drinking pattern or the quantities consumed, e.g., binge drinking or heavy alcohol use. The legal drinking age, in the United States, is 21. Drinking at an age below 21 is viewed as underage drinking.

1.3.1 Ethanol Use in Adult

According to the survey in 2015, about 209.8 million people aged 18 and older, representing 86.4% of this age group, reported that they drank ethanol at some point in their lifetime; About 170.2 million people aged 18 and older, representing 70.1% of this age group, said that they drank at some point in the past year; About 136.0 million people aged 18 and older, representing 56% of this age group, said that they drank at some point in the past month [6]. About 65.2 million people aged 18 and older, representing 26.9% of this age group, reported that they were engaged in binge drinking at least once in the past month; About 17.1 million people aged 18 and older, representing 7.0% of this age group, reported that they were involved in heavy drinking in the past month [7].

1.3.2 Underage Ethanol Use

According to the 2015 NSDUH, 33.1% of 15-year-old teenagers reported that they had at least one drink in their lives. About 7.7 million people aged between 12 and 20, representing 20.3% of this age group, reported that they drank ethanol at some point in the past month. That included 19.8% of males and 20.8% of females [8-10]. About 5.1 million people, representing 13.4% of people aged between 12 and 20, said that they had binge

drinking at least once in the past month. That included 13.4% of males and 13.3% of females [9, 10]. Approximately 1.3 million people, representing 3.3% of teenagers aged between 12 and 20, reported that they are involved in heavy alcohol use in the past month. That included 3.6% of males and 3.0% of females [9, 10].

1.3.3 Ethanol Use in College Students

According to the 2015 NSDUH, 58.0% of full-time college students aged between 18 and 22 said they drank ethanol at least once in the past month compared to 48.2% of other persons of the same age [11]. 37.9% of college students aged between 18 and 22 reported that they were engaged in binge drinking in the past month compared to 32.6% of other persons of the same age [11]. 12.5% of college students aged between 18 and 22 reported that they were involved in heavy alcohol use compared to 8.5% of other persons of the same age [11].

1.4 Health Consequences due to Ethanol Use

Excessive ethanol consumption affects the functionality of multiple organs such as the brain, heart, pancreas, and liver, causing severe health consequences. Around 88,000 people, including 62,000 men, and 26,000 women, died from ethanol-related diseases annually, making ethanol the third leading preventable cause of death in the United States after tobacco and poor diet and physical inactivity [12]. About 19,500 deaths, representing 3.5% of all cancer deaths, were attributed to ethanol consumption in 2009. The National Toxicology Program of the US Department of Health and Human Services even listed ethanol as a human carcinogen [13]. Recently, ethanol use has also been shown to increase the risk of HIV infection and transmission. The significant impacts that ethanol consumption has on health have been depicted and summarized in this section [13].

1.4.1 Infectious Diseases

HIV/AIDS

Ethanol use has been recently demonstrated to increase the risk of HIV/AIDS [14]. It is associated both with an increase of risk of acquiring HIV infection and with the negative results in terms of treatment, morbidity, and mortality of patients with HIV [14]. The harmful use of ethanol has an impact on HIV infection and transmission mainly in three ways. First, ethanol consumption increases the risk of HIV transmission, notably through engaging in risky sexual behaviors such as multiple sexual partnerships or inconsistent condom use [15]. Secondly, ethanol intake has adverse effects on HIV treatment due to ethanol-and-drug interaction, an increase of resistance to antiretroviral medications, or failure in following treatment plans [16]. Lastly, ethanol drinking can weaken the immune system, making it susceptible to infection and disease progression [17].

In 2015, the U.S. Centers for Disease Control and Prevention (CDC) reported that in the United States, 39,513 people were diagnosed with HIV infection. More than 1.2 million people were HIV carriers in the United States, and 1 in 8 of them was unaware of that [18]. The data from epidemiological research showed that ethanol misuse contributed to the spread of HIV and hurt the treatment of infected patients.

Tuberculosis (TB)

Ethanol use, especially heavy ethanol intake, is associated with an increase in the risk of tuberculosis infection. On the one hand, ethanol consumption can suppress the immune system, making it amenable to active tuberculosis [19]; on the other hand, ethanol consumption can impact the absorption and metabolism of tuberculosis drugs, causing

hepatotoxicity-a common side effect of tuberculosis medications [20]. People with alcohol use disorders are at higher risk of poor treatment adherence and treatment failure of tuberculosis infection.

In 2017, a total of 9,105 TB cases were reported, and the national incidence rate was 2.8 cases in 100,000 persons in the United States. With patient samples spanning from 1997 to 2012, a meta-analysis study showed that excessive ethanol use was associated with TB transmission and high mortality [21].

1.4.2 Non-communicable Diseases

Cardiovascular Diseases

There is increasing evidence showing that ethanol consumption is associated with the occurrence of cardiovascular diseases [22]. For instance, a high amount of ethanol consumption has been shown to increase the risk of high blood pressure [23], cardiomyopathy [24], arrhythmias [25], and strokes [26]. Scientists found the onset of ischemic heart diseases and strokes was associated with a high volume of ethanol intake and irregular drinking patterns; people with low-to-moderate ethanol consumption had a significantly lower disease risk [22, 27].

Cancers

It has been well established that ethanol consumption is not only linked with the occurrence of cancer but also associated with the progression of cancer. Types of cancer that are associated with ethanol drinking include oropharynx, larynx, esophagus, liver, colon, rectum, and breast cancer [28]. People who drink 50 or more grams of ethanol have a higher risk of developing head and neck cancer by two or three times. Even moderate ethanol intake, e.g., 25 grams of pure ethanol, has been shown to increase the risk of

developing breast cancer [28]. More importantly, tobacco use has been shown to increase the risks of ethanol-associated cancer.

Several mechanisms are believed to be responsible for the occurrence and progression of cancer resulted from ethanol consumption. Ethanol has been shown to cause DNA strands damage, and impair DNA repair processes primarily through the intermediate metabolite–acetaldehyde. Ethanol use is also linked with the defect of one-carbon metabolism, resulting in nutritional deficiencies and defects of DNA replication [29]. Ethanol has also been shown to modulate estrogen pathways, thereby increasing the risk of developing breast cancer in females [30].

Liver Diseases

Heavy drinking causes a variety of liver diseases such as steatosis, alcoholic hepatitis, fibrosis, and cirrhosis. Acute alcoholic hepatitis and liver cirrhosis are associated with high mortality; the median survival time of patients with advanced liver cirrhosis or acute alcoholic hepatitis can be as low as 1–2 years [31]. The causal relationship between ethanol consumption and liver diseases has been well established; ethanol consumption can cause hepatocellular damage due to malnutrition or mechanisms related to ethanol metabolism [32]. Of all cirrhosis deaths in 2013, 47.9% of those were attributable to ethanol consumption. Almost 1 in 3 liver transplants in the United States in 2009 were due to ethanol-related liver diseases [3].

1.4.3 Ethanol and Mental Health

Intoxication

Ethanol is a psychoactive substance, generating motivation and desire for rewarding stimuli or pleasurable feelings through reinforcing dopamine and

norepinephrine neurotransmitter system. Meanwhile, ethanol, by facilitating GABAergic transmission and inhibiting glutamatergic transmission, is also a central nervous system depressant, which is accountable for ethanol intoxication. The effects of ethanol intoxication are dependent on BEC and are shown by various psychological and behavioral manifestations. In general, a low level of BEC causes poor coordination and loss of attention that is associated with driving accidents; higher level of BEC results in ataxia, poor judgment, and disinhibition of behavioral control that are associated with aggression and crime violence; extreme level of BEC is fatal, causing an inability to breathe, coma and death [33, 34].

In the United States, acute intoxication results in 2,200 deaths per year directly, and more than 30,000 deaths per year indirectly. In 2014, ethanol-related reckless driving caused 9,967 deaths, amounting to 31% of overall driving fatalities in the United States. 1,825 college students died from ethanol-related unintentional injuries [35]. 696,000 students aged between 18 and 24 were assaulted by another student who had been drinking [36]. 97,000 students aged between 18 and 24 reported ethanol-related sexual assault and date rape [36].

AUD

AUD is characterized by ethanol abuse and ethanol dependence despite the knowledge of negative consequences. The prefrontal cortex, temporal cortex, and cerebellum are the most affected brain regions, manifested by volume loss of cortical and subcortical structure and shrinkage of both white matter and gray matter [37, 38]. This structural change is coupled with cognition deficits, visual and spatial recognition and learning inability, and impaired movement coordination [37, 39]. It has been proposed that

chronic ethanol abuse can cause an alteration of brain structure and thereby impair behavioral control that in turn, can further promote ethanol abuse [37].

According to the 2015 NSDUH, 15.1 million adults aged 18 and older, approximately 6.2% of this age group were diagnosed with AUD. They included 9.8 million men, about 8.4% of men in this age group and 5.3 million women, about 4.2% of women in this age group. An estimate of 623,000 adolescents aged 12-17, about 2.5% of this age group had AUD. They included 298,000 males, about 2.3% of males in this age group and 325,000 females, about 2.7% of females in this age group. Roughly 20% of college students aged between 18 and 22 met the criteria for AUD [40-42].

FASDs

FASDs refer to a spectrum of fetal disorders due to women drinking in pregnancy. The phenotypes associated with FASDs include a small head, retarded growth, abnormal appearance, and behavior problems. FAS is the most severe form of FASDs, which could be attributed to the damage to CNS at the structural, neurological, and functional level. The occurrence of FAS birth and FASDs is about 0.1% and 1% respectively; however, drinking large quantities, e.g., 4-6 drinks in a short time, gives a risk of 50% to a FAS birth [43].

The prevalence of FAS in the United States was estimated by the Institute of Medicine in 1996 to be between 0.5 and 3.0 cases per 1,000. More recent reports from specific U.S. sites reported the prevalence of FAS to be 2 to 7 cases per 1,000, and the prevalence of FASDs to be as high as 20 to 50 cases per 1,000 [44, 45].

1.5 Ethanol Consumption and Neurodegeneration

1.5.1 Ethanol-induced Neurodegeneration

One of the most devastating features in FASDs and AUD is the damage to the central nervous system that manifests as neuronal loss or degeneration [46-51]. This structural damage is accompanied by neurological and functional impairments [37, 52]. Postmortem analysis and *in vivo* imaging of brain structure of alcoholics found significant volume loss of cortical and subcortical structure and shrinkage of white matter and gray matter [37, 38]. The frontal cortex, especially the prefrontal cortex, was the most affected region, showing a significant neuronal loss [37, 53]. The frontal cortex regulates complex cognitive function, e.g., thinking, judgment, planning, attention and memory formation in conjunction with the hippocampus; the damage of the frontal cortex causes a range of neurological and behavioral abnormalities [37, 39]. It has been suggested that the alteration of brain structure due to ethanol consumption may reinforce the loss of behavioral control on ethanol abuse that can further deteriorate ethanol abuse and brain damage [37, 54].

It is noteworthy that abstinence from ethanol may be associated with a recovery of brain damage [37, 55]. It was reported that short-term abstention, e.g., one month was linked with an increase of gray matter and long-term abstinence, e.g., one year was associated with shrinkage of the third ventricle [55]. Magnetic resonance spectroscopy showed that there was a significant improvement in neuronal integrity during abstinence [55]. Likewise, cognitive deficits such as learning, spatial recognition, attention, and motor function, improved with abstinence as well [56]. The potential of this recovery and the extent of regeneration varied across brain regions and were dependent on factors such as genetic variation, drinking pattern, and age [37].

1.5.2 Mechanisms that Underlie Ethanol-induced Neurodegeneration

Ethanol intoxication has been suggested to inhibit *N*-methyl-D-aspartate (NMDA) receptors and increase the density of voltage-gated calcium channels in the brain [57, 58]. Excessive excitability of glutamate receptor and calcium ion channel during ethanol withdrawal was regarded as the mechanism for ethanol-induced neurodegeneration. However, the employment of inhibitors or antagonists for glutamate receptor or calcium ion channels was not able to block ethanol-induced brain damage [59, 60]. Moreover, mounting evidence showed that alcoholic neurodegeneration occurred mainly during intoxication rather than during ethanol removal [39, 61]. In agreement with this finding, the occurrence and frequency of intoxication were linked with the extent of alcoholic brain damage [37]. Currently, multiple mechanisms are appreciated with oxidative stress and neuroinflammation regarded as the main mechanisms for ethanol-induced neurodegeneration [37, 62-64].

Ethanol can cross the blood-brain barrier (BBB) and activate nuclear factor kappa-light-chain-enhancer of activated B cells **(**NF-κβ) mediated inflammatory response [65]. As a consequence, inflammatory cytokines or substances such as high mobility group box 1 (HMGB1) and damage-associated molecular patterns (DAMPs) are released from microglial cells to activate toll-like receptors (TLRs) and receptor for advanced glycation end products (RAGE) that in turn, further promote NF-κβ mediated inflammatory response [65]. On the other hand, ethanol can induce inflammation in the liver and cause a systemic inflammation; the induced inflammatory cytokines, e.g., tumor necrosis factor-alpha (TNFα), can cross BBB through receptor-mediated transportation and contribute to the existing inflammation in the brain [65, 66]. Collectively, the induced inflammation arising

from local inflammation and reinforcement from systemic inflammation constitutes one of the mechanisms for ethanol-induced neurodegeneration, manifested by neuronal death, axonal degeneration and synaptic dysfunction [65] (Figure 1.1).

The second mechanism for ethanol-induced neurodegeneration is oxidative stress [64, 67, 68]. Several mechanisms are accountable for the genesis of oxidative stress. First, ethanol metabolism in cells is coupled with the conversion of nicotinamide adenine dinucleotide (NAD^+) to NADH [69]. The backward reaction from NADH to NAD^+ increases the formation of reactive oxygen species (ROS) [69]. Meanwhile, ethanol metabolism in microsomes catalyzed by cytochrome P450 2E1 (CYP2E1) can increase ROS formation directly [69]. Alternatively, ethanol mediates the activation of NADPH oxidase (NOX), thereby increasing ROS formation [69, 70]. Oxidative stress in cells is devastating. On the one hand, oxidative stress causes neuronal death directly; on the other hand, it can crosstalk with the inflammatory pathway and potentiate the harmful effects of neuroinflammation [71, 72]. Besides, it has been reported that oxidative stress can inhibit neurogenesis and therefore can indirectly contribute to a neuronal loss [65] (Figure 1.1).

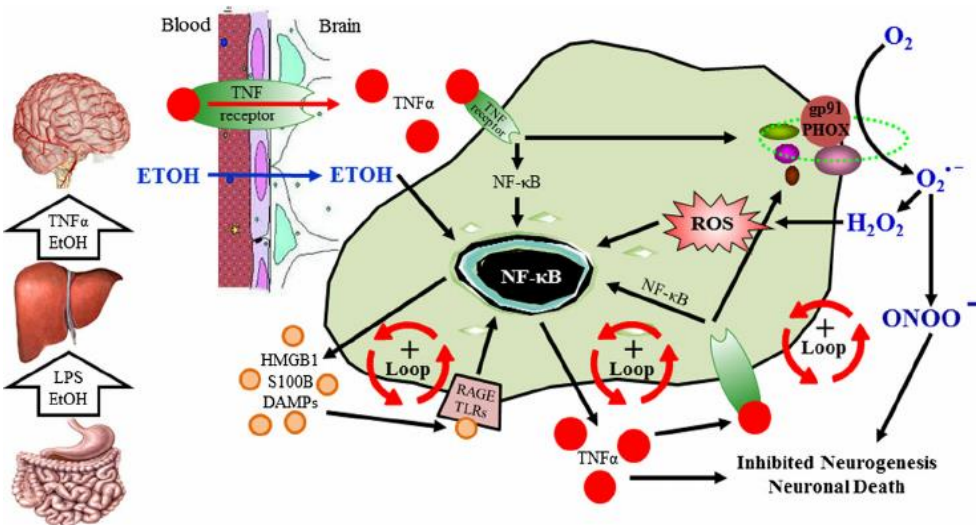


Figure 1.1. Mechanisms that Underlie Ethanol-induced Brain Damage.
Fulton T Crews, et al., 2016. See text for detail**.**

1.5.3 ER Stress and Neurodegenerative Diseases

In eukaryotic cells, ER regulates protein folding and primes the degradation of misfolded proteins through proteasome, lysosome, and autophagy pathways [73]. Protein folding in the ER is tightly regulated, and only properly folded proteins can pass through quality control surveillance and shuttle to the Golgi compartment. Misfolded proteins are retained in ER through interactions with chaperones and eventually targeted for degradation either by the ubiquitin-proteasome system (UPS) or by the autophagy-lysosome system. A variety of conditions, such as pathogen infection, nutrient deprivation, inflammation, alterations in ER luminal Ca^{2+} or redox status, and toxic chemicals can disrupt protein folding in the ER and cause the accumulation of unfolded proteins, which

is referred to as ER stress [74]. ER stress activates an unfolded protein response (UPR) to eliminate unfolded or misfolded proteins in the ER. The UPR is regulated through three ER-localized transmembrane proteins, Inositol-requiring kinase (IRE1), protein kinase RNA-like endoplasmic reticulum kinase (PERK), and activating transcription factor 6 (ATF6) [75]. When ER stress is excessively activated, and the cellular function cannot be restored, it leads to cell death [76].

Accumulation of misfolded protein is a sharing characteristic of many neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease, Huntington's disease, and Amyotrophic Lateral Sclerosis (ALS) [76, 77]. ER stress has been implicated as one of the underlying mechanisms for these neurodegenerative diseases. With animal and preclinical models, targeting UPR through pharmacological and genetic approaches in these diseases generated profound effects on the outcomes of these diseases [76]. For example, *Perk* haploinsufficiency exacerbated ALS in transgenic mice induced with a *SOD1* mutation [78]; Employment of salubrinal, an eIF2α phosphatase inhibitor, blocked protein translation and ameliorated ER stress, thereby generating positive effects in improving neuronal survival and motor performance [76, 79, 80]. It is noteworthy that inhibition of ER stress is not certainly associated with the improvement of the diseases. Dependent on the disease models and the stages of the disease, ER stress inhibition that has proved to be effective in one disease model may be ineffective or operate oppositely in the other disease models. For instance, salubrinal improved the outcome of ALS and Parkinson disease by reducing ER stress but exacerbated Prion-related disease in Scrapie prion-infected mice [81]. Ethanol-induced ER stress is linked with multiple organ injuries.

ER stress was found in acute pancreatitis [82-84] and implicated in Ethanol-induced liver disease (ALD) and in the myocardium of Ethanol-fed mice [85-90].

1.5.4 MANF and its Implication in ER Stress

MANF, also recognized as arginine-rich mutated in early stages of tumor (ARMET) was identified in a ventral mesencephalic astrocyte cell line 1 in search of astrocyte-derived neurotrophic factor [91]. MANF is widely expressed in the cerebral cortex, hippocampus, and cerebellum and can also be detected in the liver, kidney, and testes [92]. The crystal structure of mammalian MANF has revealed that MANF contains several motifs: a saposin-like domain in the amino-terminal, an SAP-like domain in the carboxy-terminal and a C-X-X-C motif. The saposin-like domain can interact with lipid and membrane, thereby linking MANF to the ER membrane. The SAP-like domain shares high homology with the SAP domain in Ku70 that has been reported to interact with proapoptotic proteins such as Bad and Bax and inhibit their function. The C-X-X-C motif is commonly found in protein disulfide isomerase and mediates the formation of disulfide bonds [93, 94].

MANF is involved in neural development, such as neurite outgrowth and neuronal migration [95]. MANF has been found to have trophic effects on embryonic dopaminergic neurons both *in vitro* and *in vivo* [91, 96, 97]. Neves *et al.* showed MANF was induced in innate immune cells during retina injury; the induced MANF was involved in the alternative activation of microglial cells, which was essential for retinal repair and regeneration [98]. It has also been shown that MANF can cause insulin resistance in the neurons of the hypothalamus, thereby increasing food intake and causing hyperphagia phenotype in mice [99]. Meantime, MANF has been found to respond to ER stress and was

identified as a protein up-regulated by UPR [100]. It was upregulated in myocardial infarction and brain diseases such as cerebral epilepsy and ischemia wherein ER stress was a sharing characteristic [92, 101] (Figure 1.2).

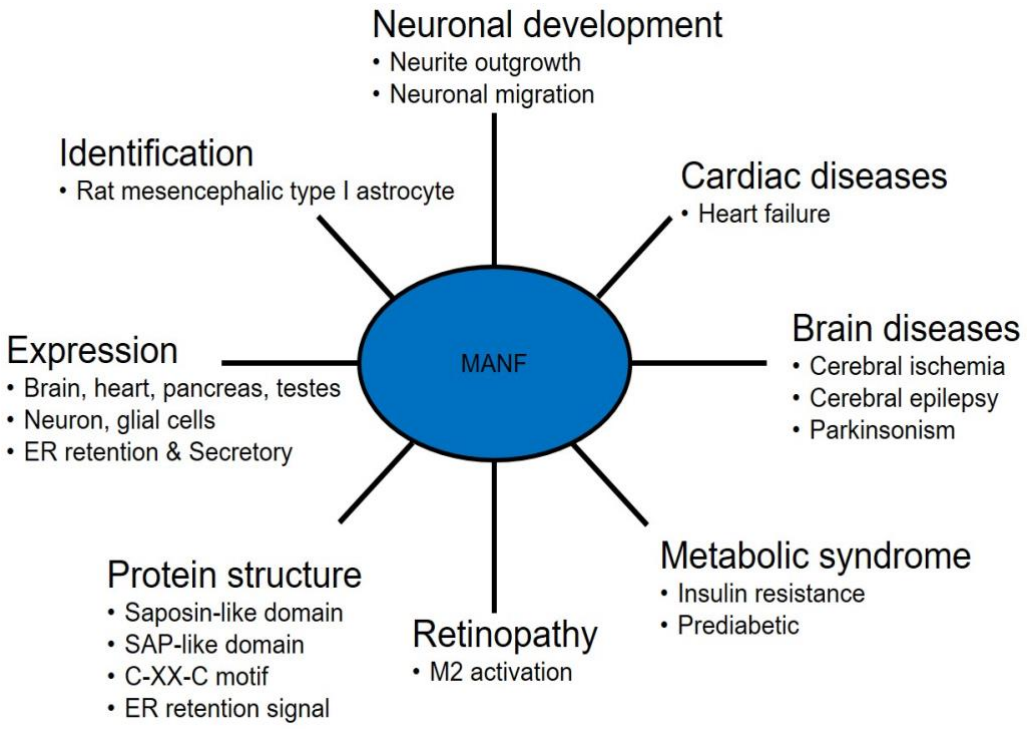


Figure 1.2. Background on MANF. See text for detail.

1.6 Ethanol Consumption and Neurogenesis Dysregulation

Neurogenesis is the process in which neural stem cells (NSCs) give rise to various types of neurons of the organism [102, 103]. In humans and rodents, neurogenesis is most active during the embryonic stage [104]. Whether neurogenesis occurs in the adult brain is debatable for decades. The first discovery of neurogenesis in the adult rodent brain was

made in the hippocampal dentate gyrus in the 1960s [105]. Subsequent studies confirmed this finding and well established the existence of neurogenesis throughout life at least in two brain regions: (1) in the subgranular zone (SGZ) of the hippocampal dentate gyrus, a brain region critical for learning and memory formation, and (2) in the subventricular zone (SVZ) of the anterior lateral ventricles, the origin of olfactory bulb neurons [104, 106, 107]. Neurons born in the adult SVZ can migrate through the rostral migratory system (RMS) and eventually evolve into granule neurons and periglomerular neurons in the olfactory bulb [102]. Neurons born in the adult SGZ can migrate to the granule layer of the dentate gyrus and differentiate into granule cells [102]. These newly formed neurons need to integrate into local circuitry and receive input from other neurons to become functional. Neurogenesis in adulthood is the most robust form of neuroplasticity in the adult mouse brain, which is associated with various brain functions such as mood, learning, cognition, and memory formation [102, 103].

Neurogenesis is heavily regulated by genetic and environmental factors at multiple levels, including NSCs proliferation, differentiation, migration, and the survival and maturation of newly born neurons [102, 103]. Ethanol exposure may affect this process at any one of these individual phases or through a combination of the effects at multiple stages [108]. Depending on the species and Ethanol exposure paradigm, Ethanol may promote or inhibit neurogenesis [108]. For example, it was shown that a single dose of 5 g/kg of ethanol reduced NSCs proliferation by 40% in rats [109]. The neurogenesis inhibitory effects of ethanol were confirmed with another ethanol exposure paradigm; rats fed with an ethanol-containing liquid diet that rendered a lower BEC (86.4 mg/dl) also exhibited a 40% decrease in neuronal proliferation [110]. However, the repetition of similar ethanol

exposure on mice presented an opposite finding; mice fed with an ethanol-containing diet for 14 days showed a 2-fold increase in the number of proliferating neurons in the SGZ of the dentate gyrus [111, 112]. These conflict findings may result from a difference in term of ethanol metabolism rate and consequently, a timing shift of ethanol-induced neurogenesis in mice when compared to rats [108]. Besides, a few studies showed no effect or an increase of neurogenesis upon chronic ethanol exposure in rats and argued the decrease of neurogenesis was an acute effect or a transient effect resulting from ethanol exposure [108, 113]. These controversial findings may result from a range of variances such as ethanol exposure patterns that leads to different BECs at the time of analysis and the method or markers that are used to label proliferative cells [108]. Overall, the vast majority of *in vivo* findings supported that ethanol-induced intoxication can cause a reduction in neurogenesis due to the inhibitory effects of ethanol on cell proliferation.

1.7 Study Overview and Objectives

Drinking too much on a single occasion or overtime is harmful, resulting in a range of health problems. It may affect the proper function of the brain, heart, liver, and pancreas and trigger tumorigenesis or promote the progression of existing tumors. Neuronal loss or degeneration is one of the most devastating features in FASDs and AUD [46-49]. Postmortem and *in vivo* imaging of alcoholic brains revealed a significant alternation of brain structure and neuronal loss [37]. The brain structure alternation and neuronal loss were coupled with neurological dysfunction, manifested by the impairment of visual-spatial learning, memory formation, motor performance, and cognitive deficits [37, 52].

Currently, multiple mechanisms are believed to be responsible for ethanol-induced neurodegeneration [37, 62-64]. Misfolded proteins are accumulated in many

neurodegenerative diseases, and ER stress is believed to be an underlying mechanism for their pathogenesis [76]. We previously showed that ethanol induces ER stress in cultured neuronal cells and the developing brain [114, 115]. However, whether ER stress is involved in ethanol-induced neurodegeneration is unclear. *In my first project, the role of ER stress in ethanol-induced neurodegeneration was investigated with a binge drinking animal model and cell culture.*

MANF was identified as a neurotrophic factor in an astrocyte cell line and has been proven to be neuroprotective, especially to dopaminergic neurons both *in vivo* and *in vitro* [91, 96, 97]. Several lines of studies found MANF was an ER stress-responsive protein and up-regulated in many pathophysiological conditions wherein ER stress was an underlying characteristic [92, 101]. We recently showed that ethanol exposure increased MANF level in the developing brain [115, 116]. Therefore, we postulated that the induced MANF may be associated with ER stress regulation. *Hence, in my second project, the role of MANF in ER stress regulation and its association with ethanol-induced neurodegeneration were examined.*

Currently, scientists believe that, under normal conditions, adult neurogenesis exists at least in two brain regions: the SVZ of the lateral ventricles and the SGZ of the dentate gyrus in the hippocampus [102, 103]. Adult neurogenesis is regulated at a multitude of levels, including the proliferation of NSCs, fate determined differentiation of progenitor cells, and the survival and maturation of newly born neurons [102, 103]. Various signaling pathways, growth factors, cytokines, neurotransmitters, and neurotrophic factors are involved in this complex regulation of neurogenesis. Because MANF is a neurotrophic factor, we speculated that MANF may participate in or affect the process of adult

neurogenesis. *In my third project, the effects of MANF deficiency on neurogenesis in the SVZ of the anterior lateral ventricles and SGZ of the hippocampal dentate gyrus in the mouse brain were investigated.*

CHAPTER 2. MATERIALS AND METHODS

2.1 Regents and Antibodies

Reagents were purchased for blood ethanol levels from Analox Instruments (London, UK). 4-Hydroxynonenal (HNE) adduct assay was from Cell Biolabs, Inc. (San Diego, CA). Rapamycin was from EMD Millipore (Burlington, MA). ATF6 was from Life Span Biosciences (Seattle, WA). Mammalian target of rapamycin (mTOR), p-mTOR, 4EBP1, p-4EBP1, p70S6K, p-p70S6K, eukaryotic initiation factor 2α (eIF2α), p-eIF2α, Ki-67, p-PERK, cyclinE1, cyclin A2, Sox2, NeuroD1, HA, phospho-histone H3 (pHH3), Calbindin, PCNA, p15, p27, cleaved caspase-3, and cleaved caspase-12 antibodies, were from Cell Signaling Technology (Danvers, MA). GRP78 antibody was from Novus Biologicals (Littleton, CO). X-box binding protein-1s (XBP1s) antibody was from BioLegend (San Diego, CA). Doublecortin (DCX), BrdU and MANF antibodies were from Abcam (Cambridge, MA). CCAAT/enhancer-binding protein homologous protein (CHOP) antibody was from Thermo Fisher Scientific (Rockford, IL). HRP-conjugated anti-rabbit and anti-mouse secondary antibodies were from GE Healthcare Life Sciences (Piscataway, NJ). Mounting media containing 4′, 6-diamidino-2-phenylindole (DAPI) was from Vector Laboratories (Burlingame, CA). Alexa-488 conjugated anti-rabbit and Alexa-594 conjugated anti-mouse antibodies were from Life Technologies (Grand Island, NY). Ketamine/xylazine was from Butler Schein Animal Health (Dublin, OH). Other chemicals and reagents were purchased either from Sigma-Aldrich or Life Technologies (Frederick, MD).

2.2 Immunoblotting

Proteins were extracted from the cerebellum, cortex, subcortical area, heart, liver,

or cultured cells. Around 30–50μg of extracted protein was used in immunoblots to examine the levels of ER stress markers (GRP78, p-eIF2α, CHOP, XBP1s, and ATF6), mTOR signaling (p-mTOR, p-4EBP1, and p-p70S6K), MANF, HA, cell cycle inhibitors (p15 and p27), cell cycle markers (PCNA, pHH3, cyclin A2 and cyclin E1), neural progenitor markers (Sox2 and NeuroD1) and apoptotic markers (cleaved caspase-3 and caspase-12). The nitrocellulose membranes were first probed with specific primary antibodies overnight at 4°C. After washing with phosphate-buffered saline (PBS) containing 0.05% Tween-20 three times, the membranes were incubated with anti-rabbit or anti-mouse secondary antibodies (horseradish peroxidase-conjugated) for 1h at room temperature. Protein-specific signals were then detected with enhanced chemiluminescence substrate (GE Healthcare, Chalfont, Buckinghamshire, UK) using a Chemi™Doc imaging system (Bio-Rad 215 Laboratories, Hercules, CA) and then quantified with the software of Image lab 5.2 (Bio-Rad Laboratories, Hercules, CA).

2.3 Fluoro-Jade C Staining

Fluoro-Jade C is fluorescein-derived fluorochrome. Fluoro-Jade C staining is a commonly used method to label degenerating neurons due to its great signal-to-background ratio [117]. Brain tissues were fixed with 4% paraformaldehyde (PFA) overnight and then transferred to 30% sucrose to dehydrate. The brain tissues were sectioned sagittally at a thickness of 15 μm and then mounted onto superfrost/plus slides. After immunostained with NeuN (a marker for mature neurons) antibody, these sections were rehydrated in distilled water for 2 minutes and then transferred to a 0.06% potassium permanganate solution and incubated for 10 minutes. After rinsing in distilled water for 2 minutes, the sections were covered with Fluoro-Jade C working solution (0.0001% Fluoro-Jade C in

0.1% acetic acid) for 10 minutes. Next, the sections were rinsed in distilled water, dehydrated and covered with mounting and imaged with an inverted fluorescent microscope (IX81, Olympus).

2.4 In vivo Labeling with BrdU

BrdU (Bromodeoxyuridine/5-Bromo-2'-deoxyuridine) is an analog of the nucleoside thymidine and can be incorporated into DNA during replication *in vivo* and *in vitro* [118, 119]. In our study, BrdU was constituted in PBS to make a sterile solution of 10 mg/ml. Based on the literature, BrdU solution was administered to postnatal day 6 (PD6) pups (CTL and KO) through intraperitoneal injection at a dose of 100 mg/kg [120]. At 4 hours after BrdU treatment, the animals were sacrificed and the brains were removed, dissected, fixed, paraffin-embedded, sectioned, and then subjected to analysis with antibodies against BrdU and Calbindin according to standard immunofluorescence protocol (IF).

2.5 Immunohistochemistry and Immunofluorescence

Mouse brains were dissected and processed for immunofluorescence (IF) or immunohistochemistry (IHC) staining as previously described [115, 121]. In brief, brain tissues were fixed with 4% paraformaldehyde (PFA) overnight and then transferred to 30% sucrose to dehydrate. Next, the brain tissues were sectioned sagittally at a thickness of 15 μm and then mounted onto superfrost/plus slides. The slides mounted with brain sections were incubated with 0.3% H_2O_2 in methanol, permeabilized with 1% Triton X-100 and blocked with 1% BSA at room temperature. After that, these slides were immunostained with the following individual or combined primary antibodies: DCX (1:500), Ki-67 (1:500), MANF (1:200), cleaved caspase 3 (1:200), CHOP (1:50), or NeuN (1:200). The

slides were then incubated with anti-rabbit or anti-mouse secondary antibody conjugated to Alexa Fluor 488 or biotin. The immunolabelings were visualized using nickel-enhanced 3,3'-Diaminobenzidine (DAB) or Alexa Fluor 488 (green) and imaged with an inverted fluorescent microscope (IX81, Olympus).

2.6 Protein Carbonyl Assay and HNE Assay

Protein carbonyl assay is a colorimetric assay for the measurement of oxidized proteins, indicative of oxidative stress. Cerebellum, cortex or liver were homogenized in 1 ml of cold buffer containing 50 mM of phosphate, pH 6.7 and 1 mM EDTA with the final protein concentration at a range of 1-10 ug/ul. The homogenate was centrifuged, and the supernatant was collected for the assay. Briefly, 200 ul of the supernatant was transferred to two 2 ml tubes; one tube was the sample tube, and the other one was the control tube. 800 µl of 2,4-*dinitrophenylhydrazine* (DNPH) was added to the sample tube, and 800 ul of 2.5 M HCl was added to the control tube. Both tubes were vortexed every 15 minutes and remained at room temperature for one hour. Next, 1 ml of 20% trichloroacetic acid (TCA) was added to the sample tube and the control tube, and the mixtures remained on the ice for five minutes. Proteins were precipitated by centrifuging, and the pellets were washed using ethanol/ethyl acetate mixture twice. The protein pellets were resuspended in 500 ul of guanidine hydrochloride. Then, 150 ul of protein was transferred from the sample tube and control tube to three wells in a 96-well plate, and the absorbance was measured at a wavelength of 360-385 nm using a plate reader. The corrected absorbance (CA) was calculated by subtracting the average absorbance of the control tube from the average absorbance of the sample tube. The final carbonyl content was determined using the following equation: Carbonyl content (mmol/mg)=[CA/(*11 mM^{-1})]*(500 µl/ 200 µl)

/(protein mg/ml).

HNE adduct assay is for the measurement of lipid peroxidation, another index for oxidative stress. Protein lysates were prepared from samples (cerebellum, cortex, or liver). 50 µl of protein lysates or HNE-BSA standards were added to wells pre-coated with HNE conjugates. After a brief incubation, 50 µl of the diluted HNE antibody was added to the wells and incubated for 1 hour, followed by the addition of an HRP conjugated secondary antibody. The wells were washed and then incubated with substrates for 20 minutes. The absorbance was read on a microplate reader using 450nm, and the content of HNE protein adducts was calculated by comparison with a predetermined HNE-BSA standard curve.

2.7 Cell Culture and Drug Treatments

N2a cells are mouse neuroblastoma cell line and were maintained in DMEM medium supplemented with 10% fetal bovine serum (FBS) and 1% streptomycin/penicillin. In ethanol treatment, N2a cells were serum-starved overnight and then incubated with complete medium containing 0.2%, 0.4% (volume/volume) ethanol for 12 hours. Tunicamycin (TM) is a conventional ER stress inducer by inhibiting N-linked glycosylation. We dissolved TM in dimethyl sulfoxide (DMSO) to a concentration of 10 mg/ml. In TM treatment, N2a cells were serum-starved overnight and then treated with TM at a working concentration of 0.5 or 1 µg/ml in complete medium for 12 hours. 4-PBA is a chemical chaperone that is widely used to block ER stress induction. We dissolved 4-PBA in water at a concentration of 20 mg/ml. To block ER stress induction, N2a cells were serum-starved overnight and then pretreated with 4-PBA at a working concentration of 10 mM in complete medium for two hours prior to ethanol or TM treatment. Rapamycin is an mTOR inhibitor and was dissolved in DMSO at a concentration of 1 mM. In rapamycin

treatment, N2a cells were serum-starved overnight and then treated with rapamycin at a working concentration of 0.5, 1, 2, 5 or 10 μM in complete medium for 12 hours. An equal volume of DMSO or water was used as the corresponding control in these drug treatments of N2a cells.

2.8 RNA Isolation and Real-time RT-PCR

Total RNA from the brain or liver was extracted using Trizol Reagent (Life Technologies) and treated with RNA-free DNAase I to remove remnant DNA as described previously [122]. cDNA used for gene detection was synthesized as described previously using SuperScript III reverse transcriptase and random primers [122]. The primers used for this study were purchased from Fisher Scientific as below: *UBXN6*, Mm01272178; *HSPA5*, Mm00517691; *UBE2J2*, Mm01263784; *PDIA6*, Mm01270904; *ERLEC1*, Mm00458735; *HYOU1*, Mm00491279; *Manf*, Mm00512511; *Tnf-α*, Mm099999068; *IL1β*, Mm00434228; *IL-6*, Mm00446190; *MCP-1*, Mm00441242; *18s rRNA*, Mm03928990. The relative expression was normalized to internal control (*18s rRNA*) using cycle time (Ct). The relative difference between the control and treatment group was expressed and calculated as relative increases using $2^{-\Delta\Delta Ct}$ and setting control as 1.

2.9 MANF Adenovirus and Transduction

MANF adenoviruses (AD-MANF) were prepared in Applied Biological Materials (ABM). In brief, a DNA sequence encoding MANF was inserted into pAdenoG-HA vector and then packaged into adenoviruses (Cat No. 210179A). An empty vector virus, AD-vector (Cat. No. 000047A) was used as the control virus. Both adenoviruses were prepared at titer more than $1x10^6$pfu/ml and amplified in our lab by transducing HEK293 cells. To obtain the overexpression of MANF in N2A cells, we infected N2A cells with AD-MANF

or AD-vector for 48 hours following the protocol from manufacture (ABM). In brief, N2A cells were seeded in 6-well plates or 6-well chambers at 50% confluency one day before transduction. In transduction, N2A cells were covered with viral culture (AD-MANF or AD-vector) for 2 hours and then incubated with fresh media. After transduction for 48 hours, N2A cells were collected for the subsequent immunochemical or flow cytometric analysis.

2.10 Generation of *Manf* Knockout Cell Lines

Several stable *Manf*-knockout cell lines were established in our lab using the CRISPR/Cas9 gene-editing method. In brief, N2A cells were transfected with *Manf* CRISPR/Cas9 KO plasmid (Cat No. sc-428989-NIC) or the control plasmid (Cat No. sc-418922) according to the protocol from manufacture (Santa Cruz Biotechnology). Colonies with successful transfection of the KO plasmids or control plasmids were visually confirmed by the detection of the green fluorescent protein (GFP) and immunoblot of MANF. Three colonies with complete *Manf* knockout (verified by immunoblot of MANF) were selected for the following immunochemical or flow cytometric analysis with colonies transfected with control plasmids as the control.

2.11 Cell Cycle Analysis

To determine the effects of MANF on cell cycle progression, N2A cells with MANF overexpression (AD-MANF) or *Manf* knockout (KO) and their corresponding control cell lines were stained with propidium iodide (PI) and then subjected to cell cycle analysis by flow cytometry. In brief, samples were trypsinized and fixed in cold 70% ethanol for 30 minutes. Next, these samples were treated with ribonuclease I (5ug/ml), incubated with PI for at least 30 minutes and then sent to Flow Cytometry and Immune

Monitoring Core for flow cytometric analysis of PI content. ModFit LT V3.3.11 (software) was used to quantitate the percentage of cells in each cell cycle phase.

2.12 TUNEL Assay

Brain samples (sections) were prepared and mounted to slides as described in the section of "Immunohistochemistry and Immunofluorescence" and then subjected to TUNEL analysis according to the protocol from manufacture (Roche, Cat No. 11684817910). In brief, the brain sections/slides were incubated with a blocking solution (3% H_2O_2 in methanol) for 10 minutes and then incubated with permeabilization solution (0.1% Triton X-100 and 0.1% sodium citrate) for 2 minutes. Next, these sections/slides were incubated with TUNEL reaction mixture at 37 °C in a humidified atmosphere in the dark for 60 minutes. A brain section incubated with labeling solution was used as the negative control and a brain section digested with DNase I and incubated with TUNEL reaction mixture as the positive control. The slides were then mounted with DAPI-containing medium and imaged with an inverted fluorescent microscope (IX81, Olympus).

2.13 Cell Viability and Proliferation Assay

Cell viability and proliferation assay were performed using MTT assay according to the protocol from manufacture (Roche, Cat No. 11465007001). In brief, N2A cells in 96-well plates were transduced with adenoviruses (AD-CTL or AD-MANF) as described above for 72 hours. 10 ul of MTT labeling reagent was then added into each well and incubated for 4 hours in a humidified atmosphere. Next, 100 ul of the solubilized solution was added to each well and incubated overnight in the incubator. The relative cell numbers were assessed by measuring the spectrophotometric absorbance of the samples using a microplate reader at the wavelength of 595 nm.

2.14 Animal Models and Behavioral Tests

2.14.1 Animal Models

Binge Ethanol Drinking Mice

All study procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Kentucky and performed following regulations for the Care and Use of Laboratory Animals set forth by the National Institutes of Health (NIH) Guide. Eight-week male C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, Maine). Twenty four male mice were used for each time course and randomly assigned to two groups: water control group (n=12) and the ethanol group (n=12). The mice received water or ethanol (5 g/kg, i.g., 25% ethanol w/v) intragastrically once daily for 1, 5 or 10 days with volume matched. One hour after the last gavage, blood was extracted from the mouse tail vein, spun down, and the supernatant (serum) was aspirated for the measurement of blood ethanol concentration using an Analox AM 1 analyzer (Lunenburg, MA). Six hours after the last gavage, the mice were sacrificed and the brains were dissected for biochemical or histological analyses. For behavioral tests, twenty male mice were gavaged with water (n=10) or ethanol (n=10) at the same dose for 10 consecutive days and then subjected to behavioral tests as described below.

Nervous System (NS)-Specific *Manf* Knockout

Embryonic stem cell clone EPD0162_3_D06 (*Manf* $^{tm1a(KOMP)Wtsi}$) as illustrated in Figure 2.1 was generated by the Wellcome Sanger Institute and injected into blastocyst by the Knockout Mouse Project (KOMP) Repository and the Mouse Biology Program at the University of California (UC) Davis. The generated chimeric mice were then bred with

C57BL/6N mates. Then via UC Davis Mouse Biology Program, MANF mice were crossed with an FLP recombinase line for *in vivo* recombination to convert to the conditional (floxed) allele. The FLP recombinase line was obtained from the MMRRC: C57BL/6-Tg(CAG-Flpo)1Afst/Mmucd, 032247-UCD. Two generations of breeding were performed to ensure excision in the germ cells and remove the FLP transgene. They also bred to homozygosity at our request. Nestin-Cre$^{+/}$ transgenic mice (B6.Cg-Tg(Nes-Cre)1Kln/J) were obtained from Jackson Laboratory. *Manf* floxed$^{+/+}$ mice were first crossed with Nestin-Cre$^{+/}$ mice to generate *Manf* floxed$^{+/-}$ Nestin-Cre$^{+/}$ mice. Then the offspring was crossed with *Manf* floxed$^{+/+}$ mice to generate *Manf* floxed$^{-/-}$ Nestin-Cre$^{+/}$ mice in which *Manf* was specifically knocked out in the central nervous system and peripheral nervous system. For genotyping, mice tail DNA was extracted using Fast Tissue/Tail PCR Genotyping Kit (EZ BioResearch) according to the manufacturer's instructions. Primers used for genotyping are as follows: *Manf-f2*, GAGATGGCGCAACGCAATTAATG; *Manf-r2*, CCATGGTGATGCTGTAACTGTCACG; *Manf-r1*, TGCTCAGCTGCAGAGTTAGAGTTCC; *Manf-f1*, TGAAGCAAGAGGCAAAGAGAATCGG. *Cre*-F, GGTTCGCAAGAACCTGATGG; *Cre*-R, GCCTTCTCTACACCTGCGG. Touch-Down PCR protocol was used for the reactions. For the first 10 cycles which annealed at 65°C decreasing in temperature by 1°C per cycle and an additional 30 cycles annealed at 55 °C. The amplifications were performed in 12 µl volume, and the PCR cycles were 94°C for 5 min, 94°C for 15 sec, annealing for 30 sec, 72°C for 40 sec and final extension 72°C for 5 min. The length of amplified bands for *Manf*$^{+/+}$, *Manf* $^{flox/flox}$ (CTL), and conditional *Manf* $^{-/-}$ (cKO) targeted locus are listed as below (Figure 2.1).

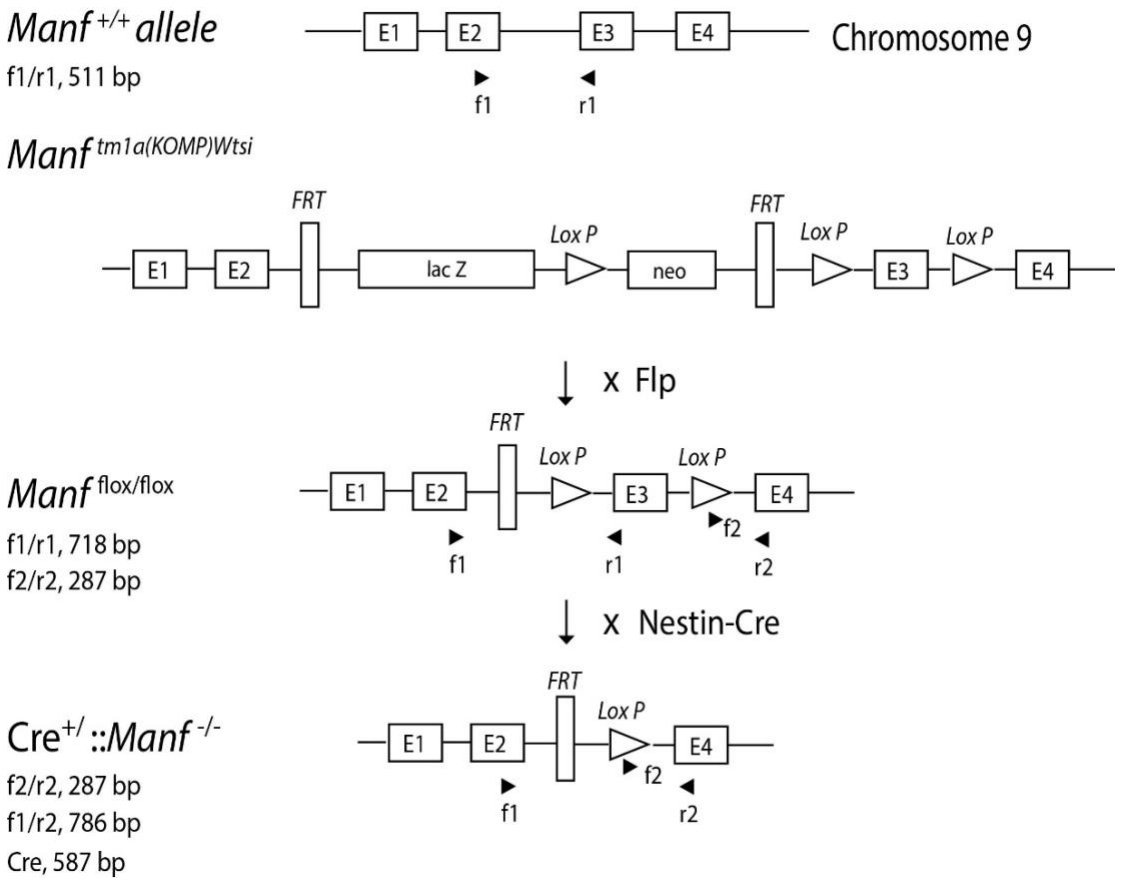


Figure 2. Schematic Illustration of NS-Specific *Manf* Knockout. Schematic representation of *Manf*$^{+/+}$ wild-type, *Manf*$^{flox/flox}$, and Cre::*Manf*$^{-/-}$ targeted locus. E: exon. Arrowheads indicate priming sites used in genotyping by PCR. See text for detail.

Ethanol- and TM-induced Neurodegenerative Mice

Two animal models were used in this study to investigate the effects of MANF on ER stress-induced neuronal damage: ethanol-induced neurodegeneration and TM-induced neurodegeneration. In ethanol-induced neurodegeneration, PD7 pups (CTL and cKO) were injected with PBS or ethanol (1.5 g/kg, 20% solution in PBS) subcutaneously twice at a two-hour interval. Neuronal apoptosis and ER stress were assessed 8 hours after the first injection. In TM-induced neurodegeneration, PD7 pups (CTL and cKO) were injected with 150 mM dextrose (Mock treatment) or TM (3 mg/kg, 30% solution in dextrose) subcutaneously once. Neuronal apoptosis and ER stress were assessed 24 hours after the injection. For pre-inhibition of ER stress with 4-PBA, PD6 pups (CTL and cKO) were injected with 4-PBA or PBS subcutaneously twice at a dose of 100 mg/kg 24 hours and 2 hours in advance and then subjected to ethanol or TM treatment following the same procedure as above. Neuronal apoptosis and ER stress in the brain from the CTL group and cKO mice were evaluated with immunoblotting and immunochemistry of ER stress markers and apoptotic markers as well as TUNEL assay.

2.14.2 Behavioral Tests

Open Field Activity and Elevated Plus Maze are experimental tests used to assess general levels of locomotor activity and anxiety in rodents. Morris Water Maze is a behavioral procedure to assess spatial learning and memory in rodents. They have been widely used to study the effect of ethanol exposure on animal behavioral changes [123, 124]. Behavioral tests were started on the third day after the last gavage when the animals

were recovered from intoxication at the University of Kentucky Rodent Behavior Core.

Open Field Activity (OFA)

All animals were brought from their housing room to the test room in clean holding cages for a ten-minute habituation period before testing. The open-field test was conducted in a square chamber (50 x 50 x 40 cm) with opaque white walls and floors. Animals were placed in the chamber and tested for 15 minutes individually using a computer-operated EthoVision XT system (Noldus, Wageningen, The Netherlands). The total distance traveled and time spent in the center area was calculated by tracking the animal's midpoint relative to predetermined boundaries on the video images of each apparatus. The center was defined as a centered, square zone with a length and width half the length and width of the open field.

Elevated Plus Maze (OPM)

Following the OFA, the test animals were given a two-hour rest period before beginning the EPM test. EPM is made up of two open arms (50 x 10 cm) and two closed arms (50 x 10 x 40 cm) above the floor. Each animal was placed in the intersection between open arms and closed arms and tested for a five-minute session using a computer-linked EthoVision XT system. The total distance traveled, and total entries to the open arms or closed arms were recorded and calculated by tracking the animal's midpoint relative to predetermined boundaries on the video images of each apparatus.

Morris Water Maze (MWM)

All animals were rested for one day and then transferred to the test room for a ten-minute habituation period before MWM testing. The MWM is a round plastic tub, about 107 cm in diameter, made opaque using white non-toxic water-based paint and then filled

with water (25-26°C). We placed a hidden escape platform (15 x 15 cm) 1 cm under the surface of the water in a fixed location. Four visible extra-maze cues were posted on the walls around the maze. Each mouse was placed in the pool from different drop locations between trials and allowed to swim until they found the submerged platform. If the animals could not find the platform within 60 seconds, they were gently guided to the platform and remained on the platform for 10 seconds before being removed for a five-minute intertrial interval. Each animal had four trials for five consecutive days, and the latency to reach the platform and distance traveled for each trial was recorded with EthoVision XT.

2.15 RNA Sequencing and Data Analysis

Total RNA was isolated from brain tissues of two groups of PD7 mice with each group having three samples: CTL group and cKO group. Therefore, six RNA samples were submitted to the University of Kentucky Genomics Core Laboratory for the whole transcriptome sequencing. Libraries were assembled using the KAPA RNA HyperPrep Kits with RiboErase (HMR) for Illumina Platforms and indexed with KAPA Single-Indexed Adapter Kit (KAPA Biosystems). Indexed libraries were pooled in equimolar concentrations and the denatured pool diluted to a final concentration of 10 pM. This pool was spiked with 5% PhiX Control library. The combined pool was clustered on board using a HiSeq SR Rapid Cluster Kit v2 (Illumina) and sequenced on an Illumina HiSeq 2500 Sequencing system using a HiSeq Rapid SBS Kit v2 (Illumina).

The transcriptome of each sample was analyzed with ensemble GRCm38 Mouse Transcriptome as reference. Transcript abundance was calculated with RNA-Seq by Expectation-Maximization (RSEM) analysis [125] and differentially expressed (DE) genes were calculated using EBSeq [126]. A false detection rate (FDR) analysis with a 0.05

threshold was performed. The lists of DE genes were submitted to Enrichr for gene ontology enrichment (GOEA) analysis [127].

2.16 Measurement of Blood-Ethanol Concentration (BEC)

One hour after ethanol gavage or ethanol injection, blood samples were collected from the tail vein. The blood was centrifuged, and 10 ul of the plasma was used to measure BEC using an Analox AM 1 analyzer (Lunenburg, MA) as previously described [128].

2.17 Statistics

The data was expressed as mean ± SEM, and statistical significance was determined using Student Unpaired t-test, one-way ANOVA, or two-way ANOVA followed by Bonferroni Multiple Comparison test (GraphPad Prism version 7). A p-value of less than 0.5 was considered statistically significant.

CHAPTER 3. ER STRESS CONTRIBUTES TO ETHANOL-INDUCED NEURODEGENERATION

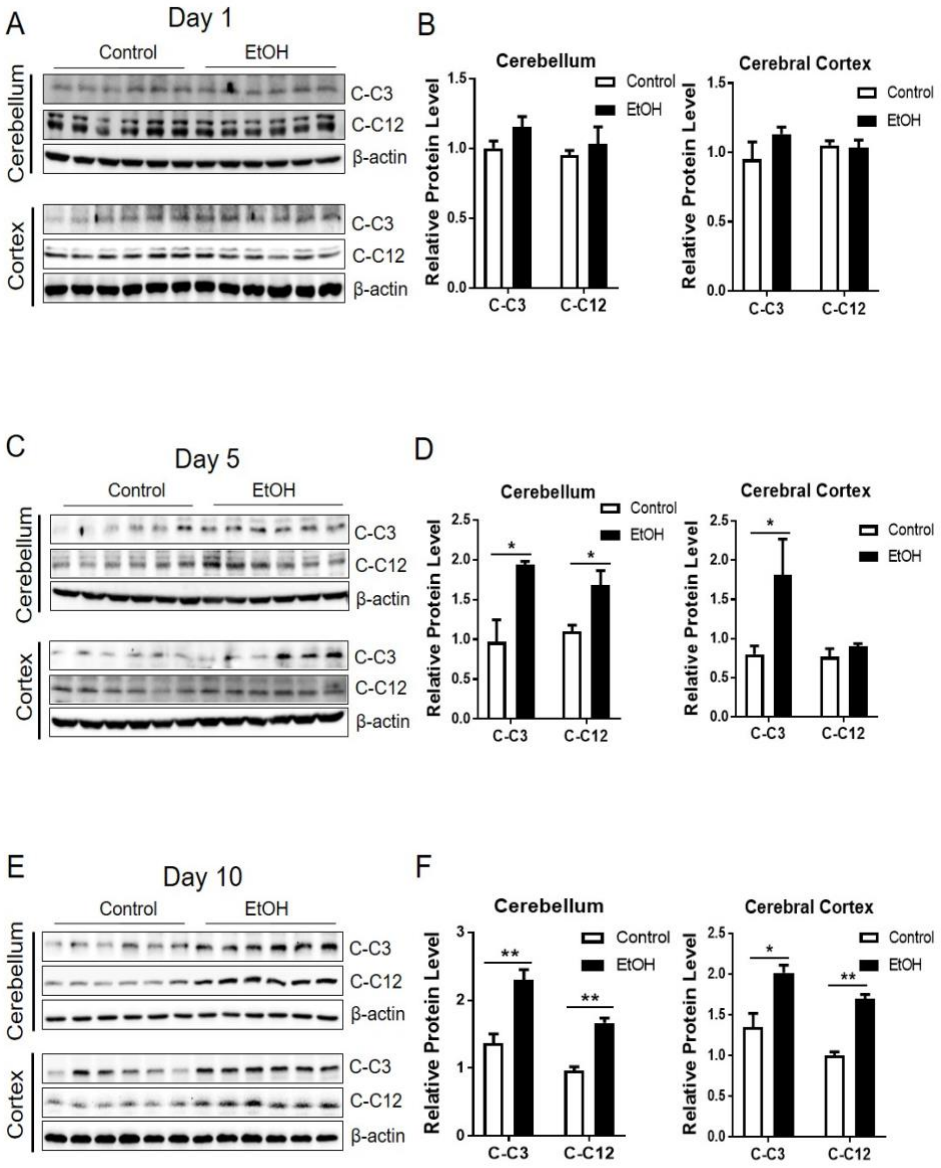
3.1 Overview

Heavy alcohol use causes brain damage that manifests as neuronal loss and dysfunction as well as mental and behavioral abnormalities. The aggregation of misfolded proteins is a sharing trait among several neurodegenerative diseases, and ER stress has been proposed as the underlying mechanism for their pathogenesis. We previously showed that ethanol exposure induces ER stress in cultured neuronal cells and the developing mouse brain [114, 115]. However, whether ER stress contributes to ethanol-induced neurodegeneration is unclear. In this section, I investigated the role of ER stress in ethanol-induced neurodegeneration with a binging drinking animal model and cell culture.

3.2 Binge Ethanol Exposure Induces Neurodegeneration and Neuroinflammation in the Adult Male Mouse Brain

BEC was measured on days 1, 5, and 10 on these male mice gavaged with water or ethanol. The BEC is 351 ± 29 mg/dl on day 1, 374 ± 21 mg/dl on day 5, and 463 ± 25 mg/dl on day 10 in the ethanol-treated group as compared to 1.7 ± 0.5 mg/dl on day 1, 6.4 ± 0.6 mg/dl on day 5, and 7.6 ± 0.5 mg/dl on day 10 in the water-treated group. We also measured the body weights of these mice and found there was no significant difference in body weight between the water-gavaged group and the ethanol-gavaged group (data not shown). Next, we determined the effects of binge ethanol exposure on neurodegeneration. As shown in Figure 3.1A-F, binge ethanol exposure for one day failed to induce the expression of cleaved caspase-3 and caspase-12. However, binge ethanol exposure for 5 or 10 days significantly increased the levels of cleaved caspase-3 and caspase-12 in the cerebellum

and cerebral cortex. Cleaved caspase-3 is indicative of apoptosis, and cleaved caspase-12 suggests that the induced apoptosis originates from ER perturbation [129]. We confirmed this ethanol-induced neurodegeneration with Fluoro-Jade C labeling, a method commonly used to label degenerative neurons. As shown in Figure 3.1G, ethanol exposure for 10 days increased the number of Fluoro-Jade C-positive cells in the cerebellum and cortex of ethanol-exposed mice. The Fluoro-Jade C-labeled cells colocalized with NeuN, a marker of mature neurons, which indicated the occurrence of neurodegeneration. Consistent with the findings in the brain, ethanol also induced apoptosis in the liver, the primary organ for ethanol metabolism (Figure 3.3A-B). Ethanol also increased the expression of some proinflammatory cytokines and chemokines; binge ethanol exposure up-regulated IL-6 in the cerebellum and MCP-1 and IL1β in the cerebral cortex following 10 days of ethanol exposure (Figure 3.1H).


A
Day 1
Control
EtOH
Cerebellum
C-C3
C-C12
β-actin
Cortex
C-C3
C-C12
β-actin
B
Cerebellum
Control
EtOH
Relative Protein Level
1.5
1.0
0.5
0.0
C-C3
C-C12
Cerebral Cortex
Control
EtOH
Relative Protein Level
1.5
1.0
0.5
0.0
C-C3
C-C12
C
Day 5
Control
EtOH
Cerebellum
C-C3
C-C12
β-actin
Cortex
C-C3
C-C12
β-actin
D
Cerebellum
Control
EtOH
Relative Protein Level
2.5
2.0
1.5
1.0
0.5
0.0
*
*
C-C3
C-C12
Cerebral Cortex
Control
EtOH
Relative Protein Level
2.5
2.0
1.5
1.0
0.5
0.0
*
C-C3
C-C12
E
Day 10
Control
EtOH
Cerebellum
C-C3
C-C12
β-actin
Cortex
C-C3
C-C12
β-actin
F
Cerebellum
Control
EtOH
Relative Protein Level
3
2
1
0
**
**
C-C3
C-C12
Cerebral Cortex
Control
EtOH
Relative Protein Level
2.5
2.0
1.5
1.0
0.5
0.0
*
**
C-C3
C-C12

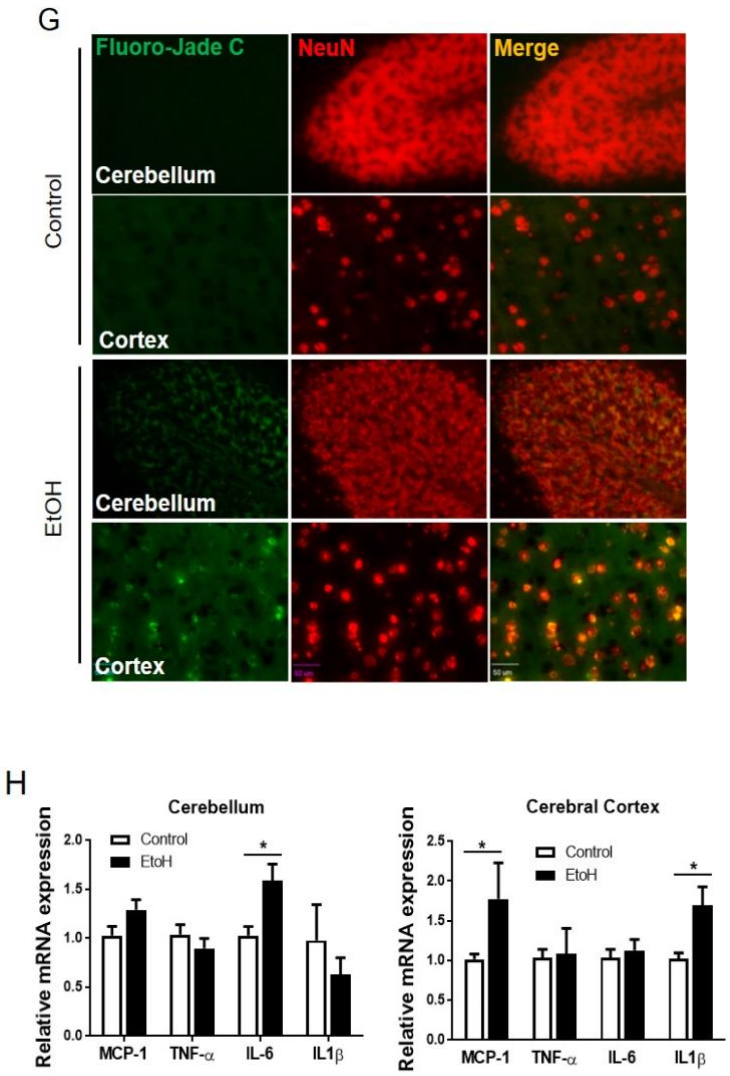
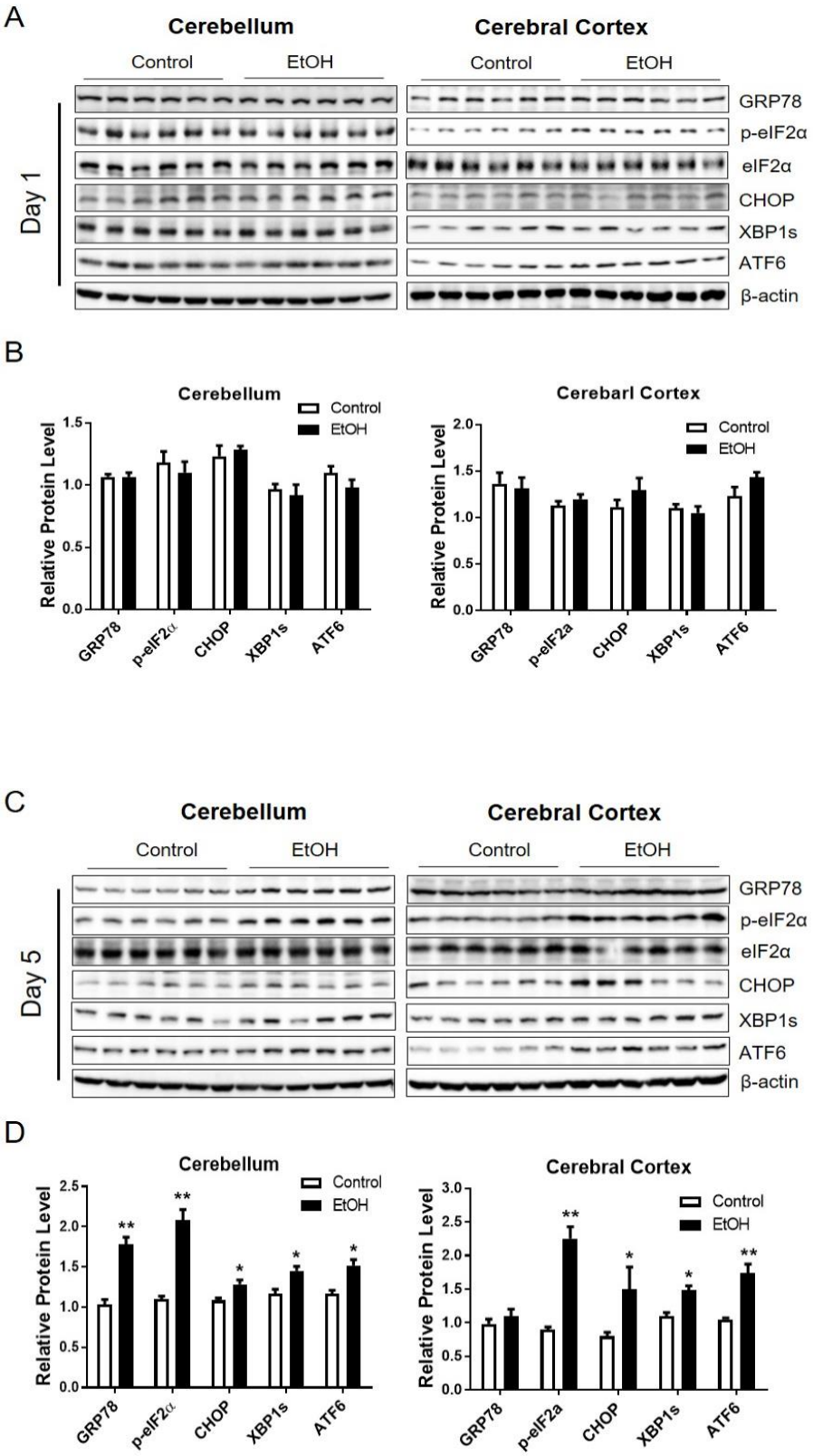


Figure 3.1. Binge Ethanol Exposure Induces Neurodegeneration in the Adult Male Mouse Brain. A, C, E. C57BL/6 male mice received water (control) or ethanol intragastrically for 1, 5, or 10 days and brain tissues were dissected to assess the level of cleaved caspase-3 (C-C3) and cleaved caspase-12 (C-C12) by immunoblotting. **B, D, F.** The protein levels of C-C3 and C-C12 were normalized to β-actin and expressed relative to control. Six animals (n=6) were used in each group to calculate the mean ± SEM. *P<0.05 or **P<0.01 vs. control. **G.** Representative images showing Fluoro-Jade C- (green) and NeuN- (red) positive cells in the brain of either water- or ethanol-treated mice for 10 days. **H.** Total RNA was prepared from the cerebellum or cerebral cortex of water- (control) or ethanol-treated mice for 10 days to assess the level of proinflammatory cytokines (MCP-1, TNF-α, IL-6, and IL1β) by RT-qPCR. The mRNA level of these cytokines was normalized to 18s rRNA and expressed relative to control. Three to six samples (n = 3-6) were used in each group to calculate the mean ± SEM. *P<0.05 vs. control. Scale bar: 20 um.

3.3 Binge Ethanol Exposure Induces ER Stress in the Adult Male Mouse Brain

With the same ethanol exposure paradigm, we next sought to determine whether binge ethanol exposure induces ER stress. Consistent with the timeline of neurodegeneration, binge ethanol exposure for 1 day did not cause a change to the levels of ER stress markers (Figure 3.2A and B). However, ethanol exposure for 5 and 10 days significantly increased the levels of ER stress markers including GRP78, p-eIF2α, CHOP, XBP1s, and ATF6 both in the cerebellum and cerebral cortex (Figure 3.2C-F). As expected, ethanol also increased the levels of ER stress markers in the liver (Figure 3.3A-B) drastically. Oxidative stress has been suggested as a potential mechanism for ethanol-induced neurodegeneration [62, 130]. Therefore, we examined the level of oxidative stress following binge ethanol exposure. While ethanol caused oxidative stress in the liver, manifested by an increase of protein carbonyl and HNE adducts, it had little effect on that in the male mouse brain (Figure 3.4A-B).


A
Cerebellum
Cerebral Cortex
Control
EtOH
Control
EtOH
Day 1
GRP78
p-eIF2α
eIF2α
CHOP
XBP1s
ATF6
β-actin
B
Cerebellum
Control
EtOH
Relative Protein Level
1.5
1.0
0.5
0.0
GRP78
p-eIF2α
CHOP
XBP1s
ATF6
Cerebarl Cortex
Control
EtOH
Relative Protein Level
2.0
1.5
1.0
0.5
0.0
GRP78
p-eIF2a
CHOP
XBP1s
ATF6
C
Cerebellum
Cerebral Cortex
Control
EtOH
Control
EtOH
Day 5
GRP78
p-eIF2α
eIF2α
CHOP
XBP1s
ATF6
β-actin
D
Cerebellum
Control
EtOH
Relative Protein Level
2.5
2.0
1.5
1.0
0.5
0.0
**
**
*
*
*
GRP78
p-eIF2α
CHOP
XBP1s
ATF6
Cerebral Cortex
Control
EtOH
Relative Protein Level
3.0
2.5
2.0
1.5
1.0
0.5
0.0
**
*
*
**
GRP78
p-eIF2a
CHOP
XBP1s
ATF6

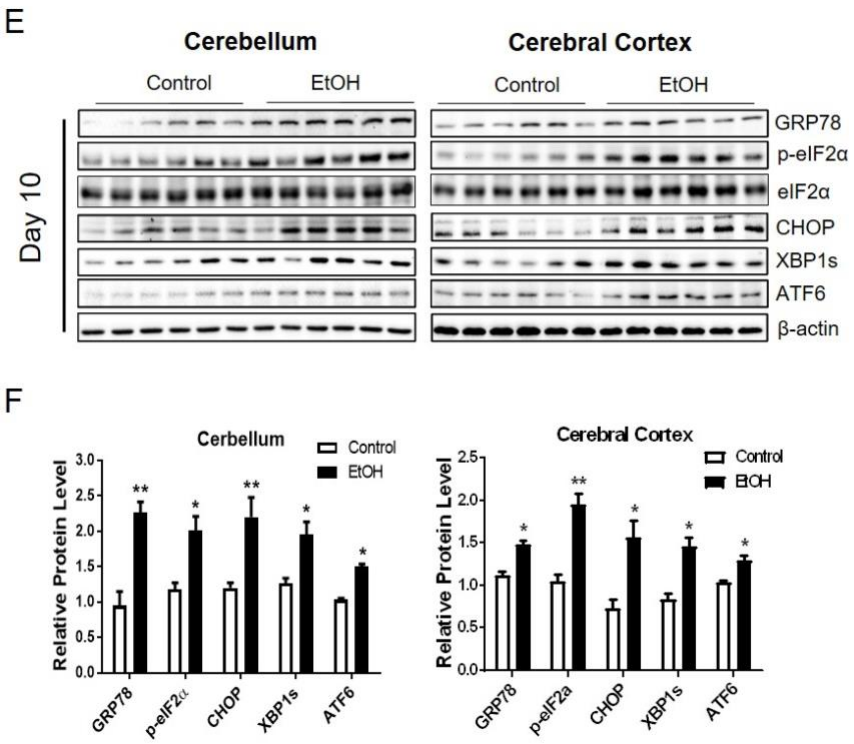


Figure 3.2. Binge Ethanol Exposure Induces ER Stress in the Adult Male Mouse Brain. A. C57BL/6 male mice received water (control) or ethanol intragastrically for 1 day and brains tissues were harvested to assess the level of ER stress markers by immunoblots. **B.** The protein levels of ER stress markers were normalized to β-actin and expressed relative to control. Six animals (n = 6) were used in each group to calculate the mean ± SEM. *P<0.05 or **P<0.01 vs. control. **C.** C57BL/6 male mice received water (control) or ethanol intragastrically for 5 days and brains tissues were harvested to assess the level of ER stress markers by immunoblots. **D.** The protein levels of ER stress markers were normalized to β-actin and expressed relative to control. Six animals (n = 6) were used in each group to calculate the mean ± SEM. *P<0.05 or **P<0.01 vs. control. **E.** C57BL/6 male mice received water (control) or ethanol intragastrically for 10 days and brains tissues were harvested to assess the level of ER stress markers by immunoblots. **F.** The protein levels of ER stress markers were normalized to β-actin and expressed relative to control. Six animals (n = 6) were used in each group to calculate the mean ± SEM. *P<0.05 or **P<0.01 vs. control.

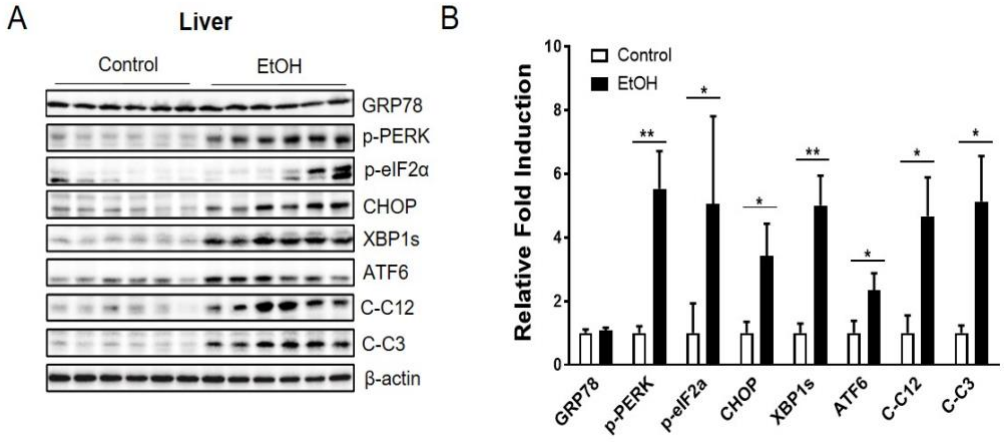


Figure 3.3. Binge Ethanol Exposure Induces ER Stress and Hepatocyte Death in the Adult Male Mouse Liver. A. C57BL/6 male mice received water (control) or ethanol intragastrically for 10 days, and the liver was dissected to assess the level of ER stress markers, cleaved caspase-3 (C-C3) and caspase-12 (C-C12). **B.** The protein levels of ER stress markers, C-C3 and C-C12, were normalized to β-actin and expressed relative to control. Six animals (n = 6) were used in each group to calculate the mean ± SEM. *P<0.05 or **P<0.01 vs. control.

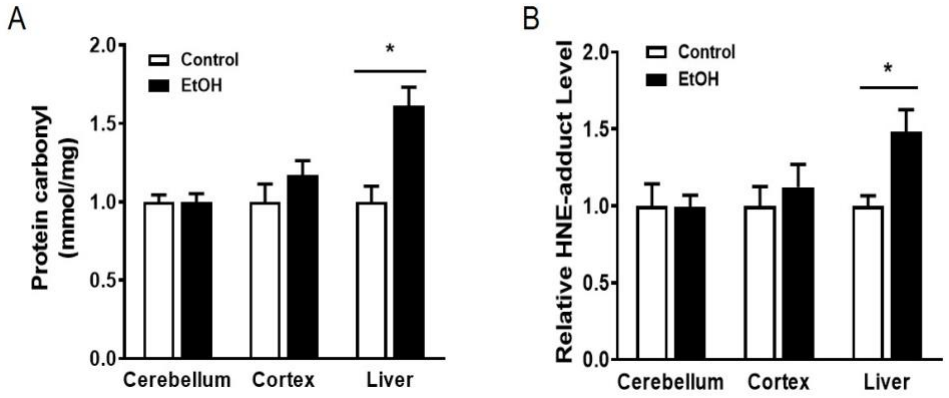
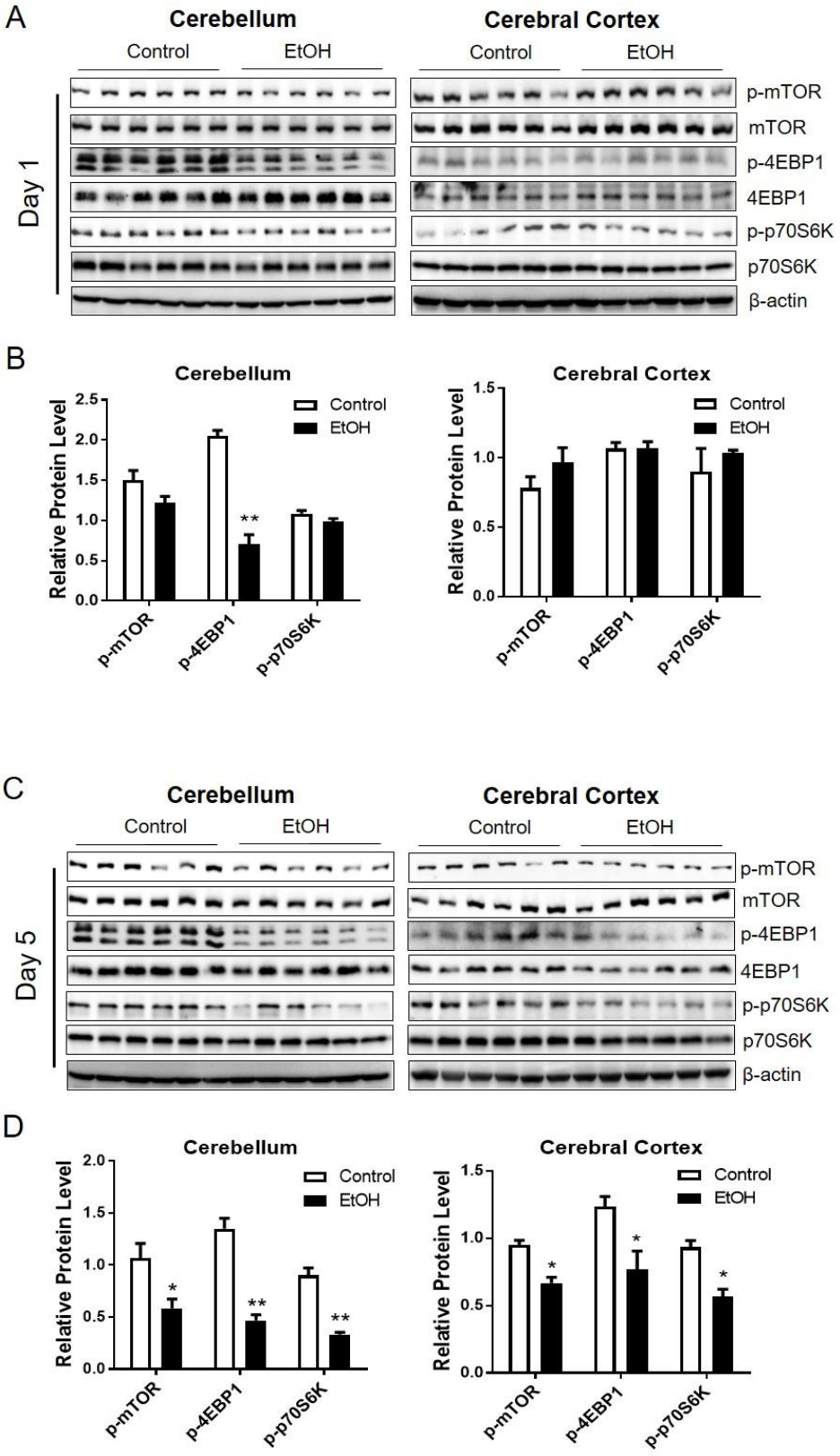


Figure 3.4. The Effect of Binge Ethanol Exposure on the Induction of Oxidative Stress. C57BL/6 male mice received water (control) or ethanol intragastrically for 10 days as described in Fig. 1. The brain (cerebellum and cerebral cortex), and the liver tissues were extracted and assayed for the level of oxidized protein **(A)** and lipid peroxidation **(B)** as described in the Materials and Methods. The levels of protein carbonyl or HNE-BSA were normalized to total lysates and expressed relative to control. Three to six samples (n = 3-6) were used in each group to calculate the mean ± SEM. *P<0.05 vs. control.

3.4 Binge Ethanol Exposure Downregulates mTOR signaling in the Adult Male Mouse Brain

Since there was considerable evidence of interaction between mTOR signaling and ER stress [131], we assessed the effects of binge ethanol exposure on mTOR signaling in the mouse brain. We found that binge ethanol exposure downregulated mTOR activity and its downstream signaling in the mouse brain manifested by a decrease of p-mTOR, and its target effectors, p-4EBP1, and p-p70S6K (Figure 3.5C-F). Interestingly, ethanol-induced downregulation of mTOR signaling parallelled the induction of neurodegeneration and ER stress; that is, ethanol-induced downregulation of mTOR was only evident after 5 days of ethanol exposure.


A
Cerebellum
Cerebral Cortex
Control
EtOH
Control
EtOH
Day 1
p-mTOR
mTOR
p-4EBP1
4EBP1
p-p70S6K
p70S6K
β-actin
B
Cerebellum
Cerebral Cortex
Relative Protein Level
Control
EtOH
**
p-mTOR
p-4EBP1
p-p70S6K
C
Cerebellum
Cerebral Cortex
Control
EtOH
Control
EtOH
Day 5
p-mTOR
mTOR
p-4EBP1
4EBP1
p-p70S6K
p70S6K
β-actin
D
Cerebellum
Cerebral Cortex
Relative Protein Level
Control
EtOH
*
**
p-mTOR
p-4EBP1
p-p70S6K

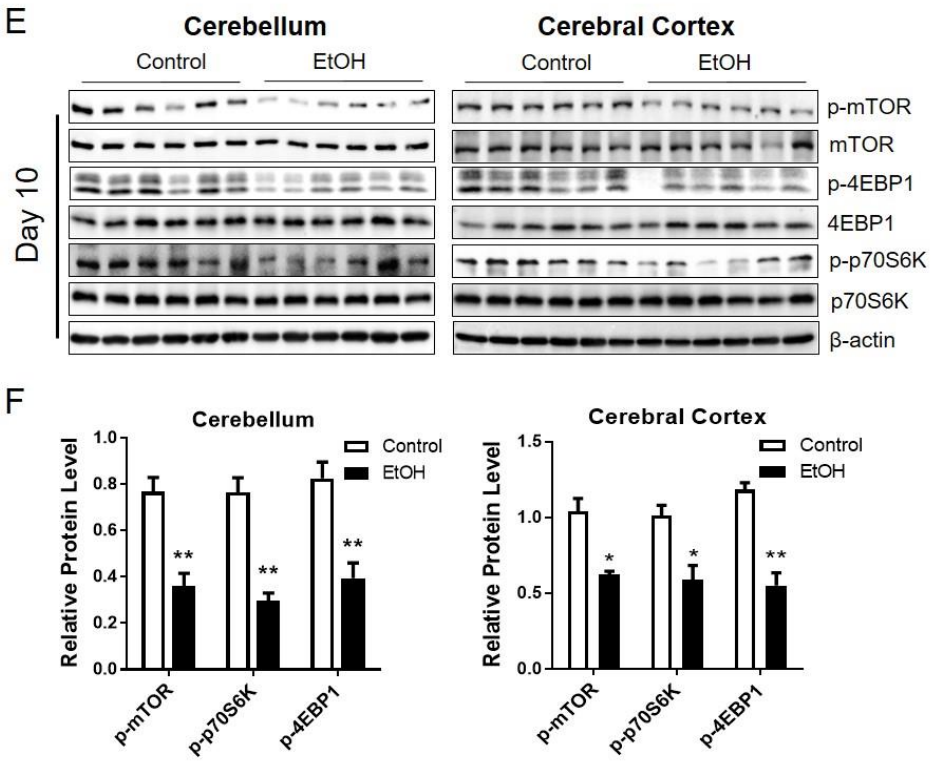


Figure 3.5. Binge Ethanol Exposure Downregulates mTOR Signaling in the Adult Male Mouse Brain. A. C57BL/6 mice received water (control) or ethanol intragastrically for 1 day and brains tissues were harvested to assess the activity of mTOR signaling by immunoblots. **B.** The protein levels of p-mTOR and its downstream effectors were normalized to β-actin and expressed relative to control. Six animals (n = 6) were used in each group to calculate the mean ± SEM. *P<0.05 or **P<0.01 vs. control. **C.** C57BL/6 mice received water (control) or ethanol intragastrically for 5 days and brains tissues were harvested to assess the activity of mTOR signaling by immunoblots. **D.** The protein levels of p-mTOR and its downstream effectors were normalized to β-actin and expressed relative to control. Six animals (n = 6) were used in each group to calculate the mean ± SEM. *P<0.05 or **P<0.01 vs. control. **E.** C57BL/6 mice received water (control) or ethanol intragastrically for 10 days and brains tissues were harvested to assess the activity of mTOR signaling by immunoblots. **F.** The protein levels of p-mTOR and its downstream effectors were normalized to β-actin and expressed relative to control. Six animals (n = 6) were used in each group to calculate the mean ± SEM. *P<0.05 or **P<0.01 vs. control.

3.5 Inhibition of ER Stress Ameliorates Ethanol-induced Neurodegeneration

To determine whether ER stress contributes to ethanol-induced neurodegeneration, we performed *in vitro* experiments with a mouse neuroblastoma cell line (N2a). First, we found that ethanol at a concentration of 0.2% or 0.4% induced ER stress and neuronal apoptosis manifested by an increase of ER stress markers (GRP78, XBP1s, and ATF6) and apoptotic marker (cleaved caspase-3) (Figure 3.6A, B, C, D, and F). More importantly, cotreatment with 4-PBA, a chemical chaperone used to inhibit ER stress, abolished ethanol-induced ER stress and neuronal apoptosis, suggesting that ER stress contributes to ethanol-induced neurodegeneration (Figure 3.6A, B, C, D, and F). In addition, consistent with the findings *in vivo*, ethanol also downregulated mTOR activity in N2a cells (Figure 3.6A and E).

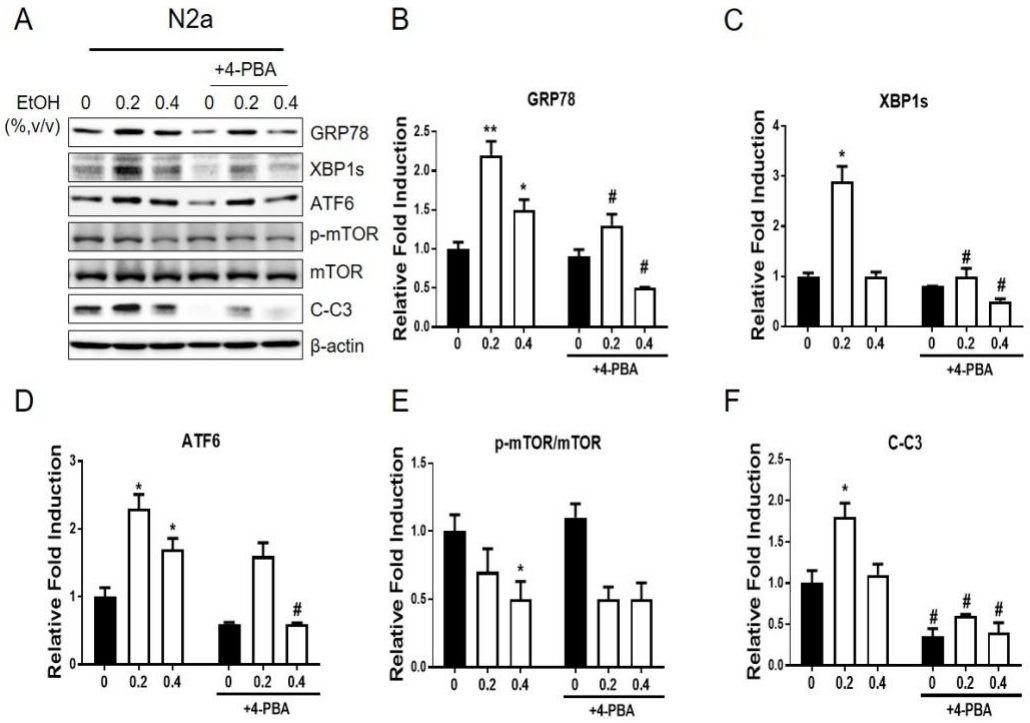


Figure 3.6. Inhibition of ER Stress Reduces Ethanol-induced Neuronal Death. A. N2a cells were serum-starved overnight and followed by pretreatment with or without 4-PBA (10 mM) for 2 hours before ethanol treatment (0.2% or 0.4%, v/v) for 12 hours. Cell lysates were collected to assess the levels of ER stress markers and cleaved caspase-3 (C-C3) by immunoblots. **B-F.** The protein levels of ER stress markers and C-C3 were normalized to β-actin and expressed relative to control (without treatment by ethanol and 4-PBA). Three independent experiments (n = 3) were performed to calculate the mean ± SEM. *P<0.05 or **P<0.01 vs. control. #P<0.05 vs. the corresponding points without 4-PBA treatment.

3.6 Inhibition of mTOR signaling Contributes to the Induction of ER Stress

To delineate the crosstalk between mTOR signaling and ethanol-induced ER stress, we employed pharmacological approaches to intervene in mTOR signaling and ER stress in N2a cells. TM is an ER stress inducer by inhibiting N-linked glycosylation in the endoplasmic reticulum. TM treatment of N2A cells induced ER stress, and the induction of ER stress was blocked by cotreatment with 4-PBA. However, both TM and 4-PBA exerted no significant effects on mTOR signaling (Figure 3.7A-E). Likewise, although 4-PBA alleviated ethanol-induced ER stress, it can not reverse ethanol-mediated downregulation of mTOR signaling in N2a cells (Figure 3.7A and E). However, treatment of N2A cells with rapamycin–an mTOR kinase inhibitor–inhibited mTOR signaling and meanwhile induced ER stress (Figure 3.7F-J). Collectively, these results suggest that mTOR signaling operates upstream of ethanol-induced ER stress and is involved in the regulation of ER stress in response to binge ethanol exposure.

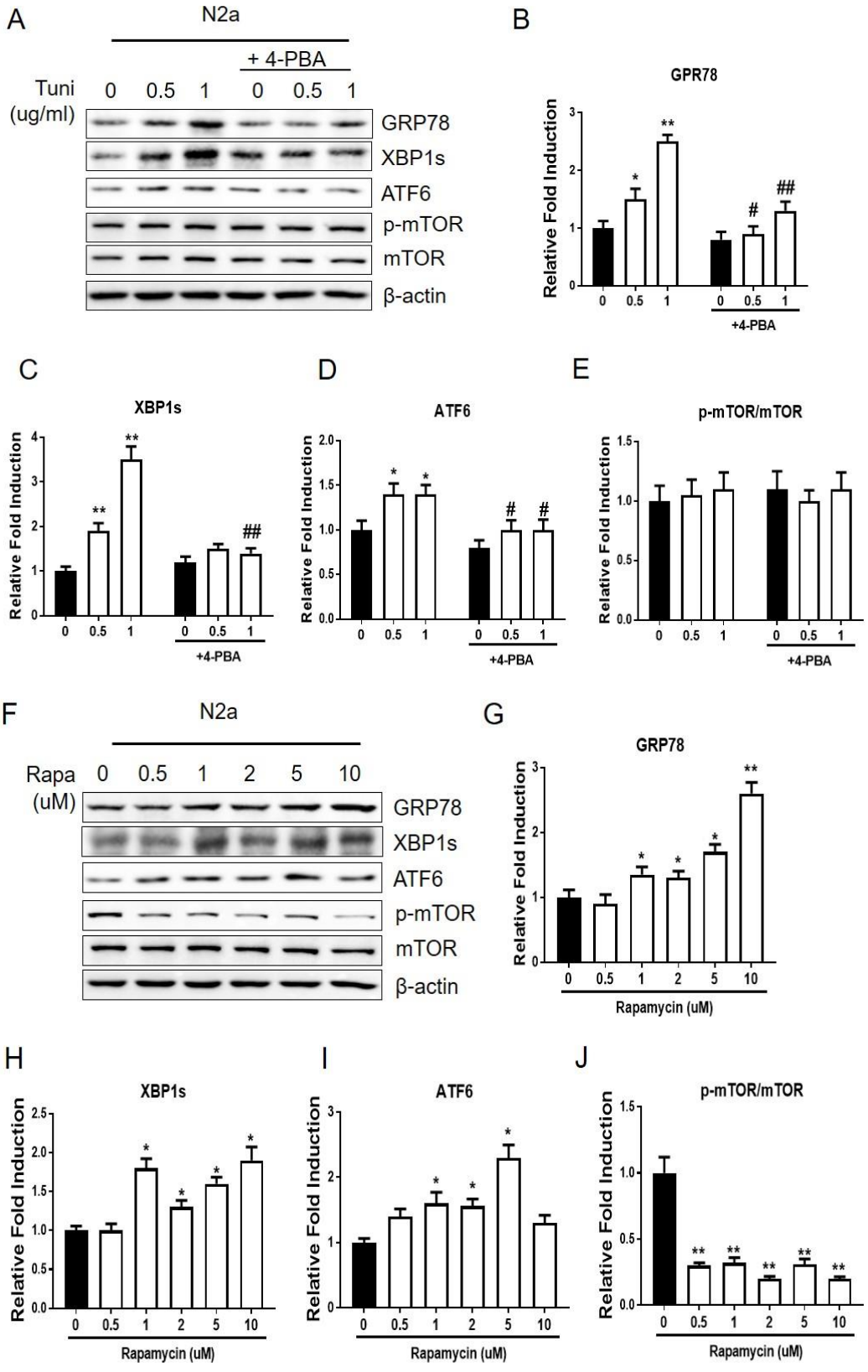

A
N2a
+ 4-PBA
Tuni (ug/ml)
0 0.5 1 0 0.5 1
GRP78
XBP1s
ATF6
p-mTOR
mTOR
β-actin
B
GPR78
Relative Fold Induction
+4-PBA
C
XBP1s
D
ATF6
E
p-mTOR/mTOR
F
N2a
Rapa (uM)
0 0.5 1 2 5 10
G
GRP78
Rapamycin (uM)
H
XBP1s
I
ATF6
J
p-mTOR/mTOR

Figure 3.7. Inhibition of mTOR Signaling Induces ER Stress. A. N2a cells were serum-starved overnight and followed by pretreatment with or without 4-PBA (10 mM) for 2 hours before TM treatment (0.5 or 1 ug/ml) for 12 hours. Cell lysates were collected to assess the levels of ER stress markers and mTOR signaling. **B-E.** The protein levels of ER stress markers and p-mTOR/mTOR were normalized to β-actin and expressed relative to control (without treatment by TM and 4-PBA). Three independent experiments (n = 3) were performed to calculate the mean ± SEM. *P<0.05 or **P<0.01 vs. control. #P<0.05 vs. the corresponding points without 4-PBA treatment. **F.** N2a cells were serum-starved overnight followed by treatments with rapamycin (0, 0.5, 1, 2, 5 or 10 uM) for 12 hours to assess the level of ER stress markers and mTOR signaling. **G-J.** Quantification was normalized to β-actin and expressed relative to control (without rapamycin treatment) and calculated as mean ± SEM for three independent experiments (n = 3). *P<0.05 or **P<0.01 vs. control.

3.7 Effects of Binge Ethanol Exposure on Animal Behaviors

To determine whether the paradigm of binge ethanol exposure affects animal behaviors, we evaluated locomotor activity by OFA, anxiety-like behaviors by EPM, and spatial-based learning and memory by MWM following ethanol exposure. In OFA, although the mice in the ethanol group showed a tendency for less activity, the total distance traveled was not significantly different between the control group and the ethanol group (Figure 3.8A, p=0.0697). Also, during OFA, the mice in the ethanol group spent a similar period in the center compared with the mice in the control group (Figure 3.8B, p=0.4717). In EPM, the ratio of open arm entries versus total entries and the total distance traveled between the ethanol group and the control group also did not significantly differ (Figure 3.8C, p=0.0545; Figure 3.8D, p=0.0757). Lastly, the results from the MWM test showed there was no significant difference in term of latency or distance traveled between the control group and ethanol group (Figure 3.8E, p=0.2816; Figure 3.8F, p=0.3285). Altogether, these results suggest that this paradigm of binge ethanol exposure does not cause a significant change in locomotor activity, anxiety-like behaviors, and spatial memory and learning.

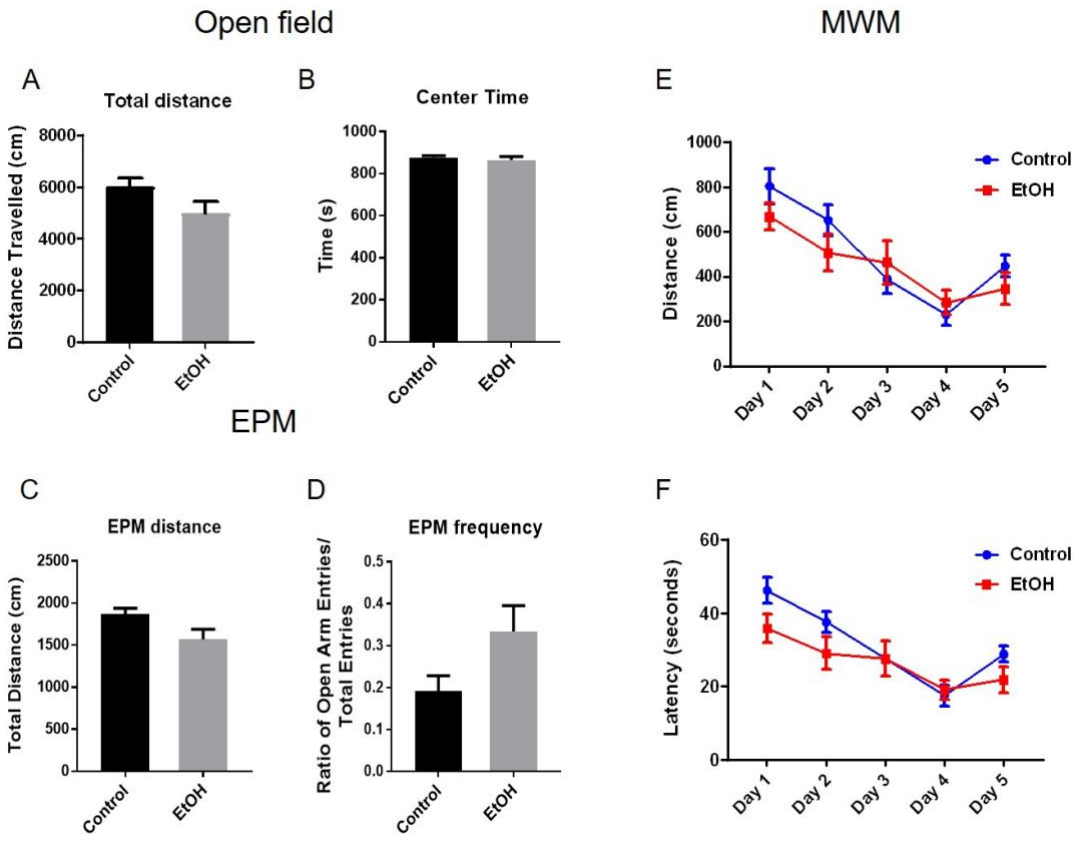


Figure 3.8. The Effects of Ethanol-induced Neurodegeneration on Animal Behaviors. C57BL/6 mice received water (control) or ethanol intragastrically for 10 days. At 3 days after the last gavage, animals were subjected to OFA test **(A-B)** once. After 2 h following OFA, animals were subjected to EPM test once **(C-D).** At 5 days after the last gavage, animals were subjected to MWM test **(E-F)** for 5 consecutive days. Quantification of each parameter was calculated as the mean ± SEM of ten animals (n=10) in each group. Unpaired student t-test was used assess the difference between control group and ethanol group in OFA and EPM and two-way ANOVA was used in MWM to assess the difference between two groups.

CHAPTER 4. MANF IS NEUROPROTECTIVE AGAINST ETHANOL-INDUCED NEURODEGENERATION VIA AMELIORATING ER STRESS

4.1 Overview

MANF was initially identified as a neurotrophic factor in search of astrocyte-derived neurotrophic factors [91]. Meanwhile, MANF is an ER stress-inducible protein and has been identified as a protein upregulated in myocardial infarction, cerebral ischemia, and epilepsy in which ER stress is an underlying characteristic [92, 101, 132]. Moreover, we recently demonstrated that MANF was induced in ethanol-induced neurodegeneration in the developing mouse brain [115, 116]. We speculated that MANF may be associated with ER stress regulation and thereby exert a neuroprotective role in ethanol-induced neurodegenerative diseases. Hence, in this section, I investigated the role of MANF in ER stress regulation and its association with ethanol-induced neurodegeneration using a third-trimester equivalent mouse model.

4.2 Characterization of the CNS-specific *Manf* Knockout Mouse Model

CNS-*Manf*-KO (cKO) mice exhibited a CNS-specific *Manf* knockout (Figure 1B). The expression level of MANF was dramatically decreased across the entire mouse brain compared with that in control (CTL) littermates (*Manf* $^{flox/flox}$ mice) (Figure 4.1A and B). We also measured the body weight and brain weight and calculated the ratio of brain weight to body weight in cKO mice and CTL littermates. In brevity, the cKO mouse brain and body tended to weigh less compared with those in the CTL group (Figure 4.1C and 1D). However, the ratio of brain weight to body weight between these two groups showed no significant difference (Figure 4.1E). It is noteworthy that the bodyweight difference can extend to the adult stage (Figure 4.1F). We think the decrease in body weight and brain weight is likely due to the involvement of MANF in neuronal development and energy

metabolism.

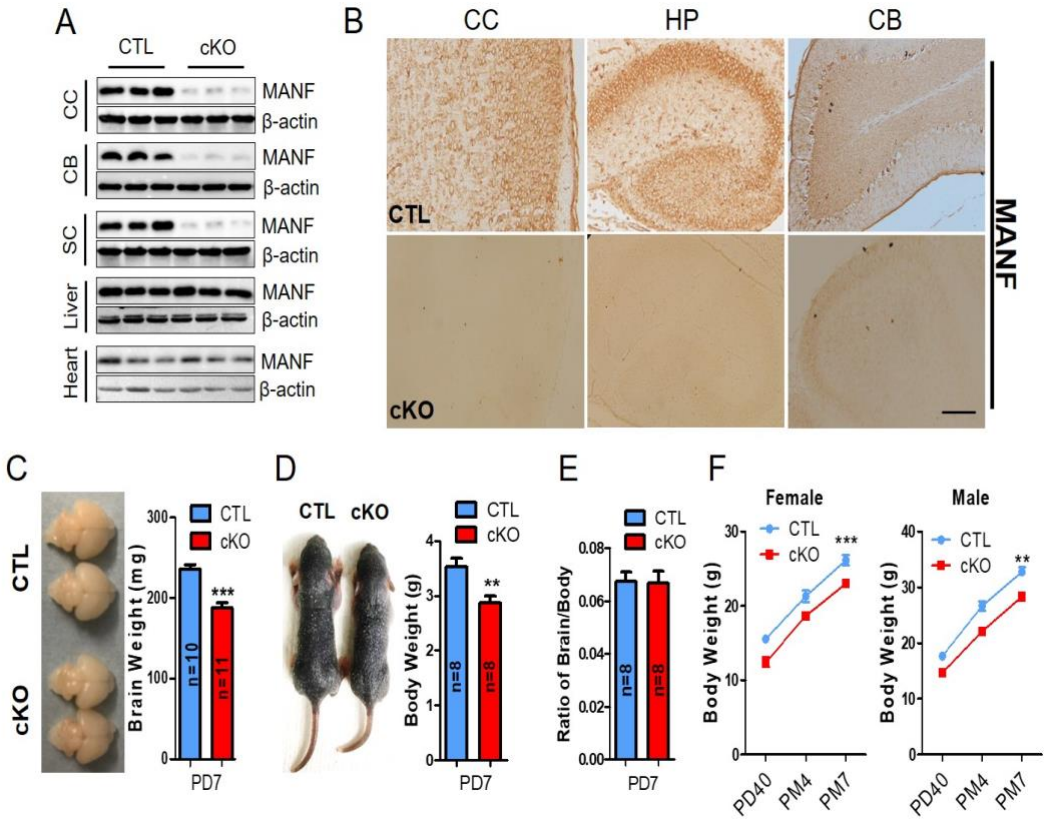
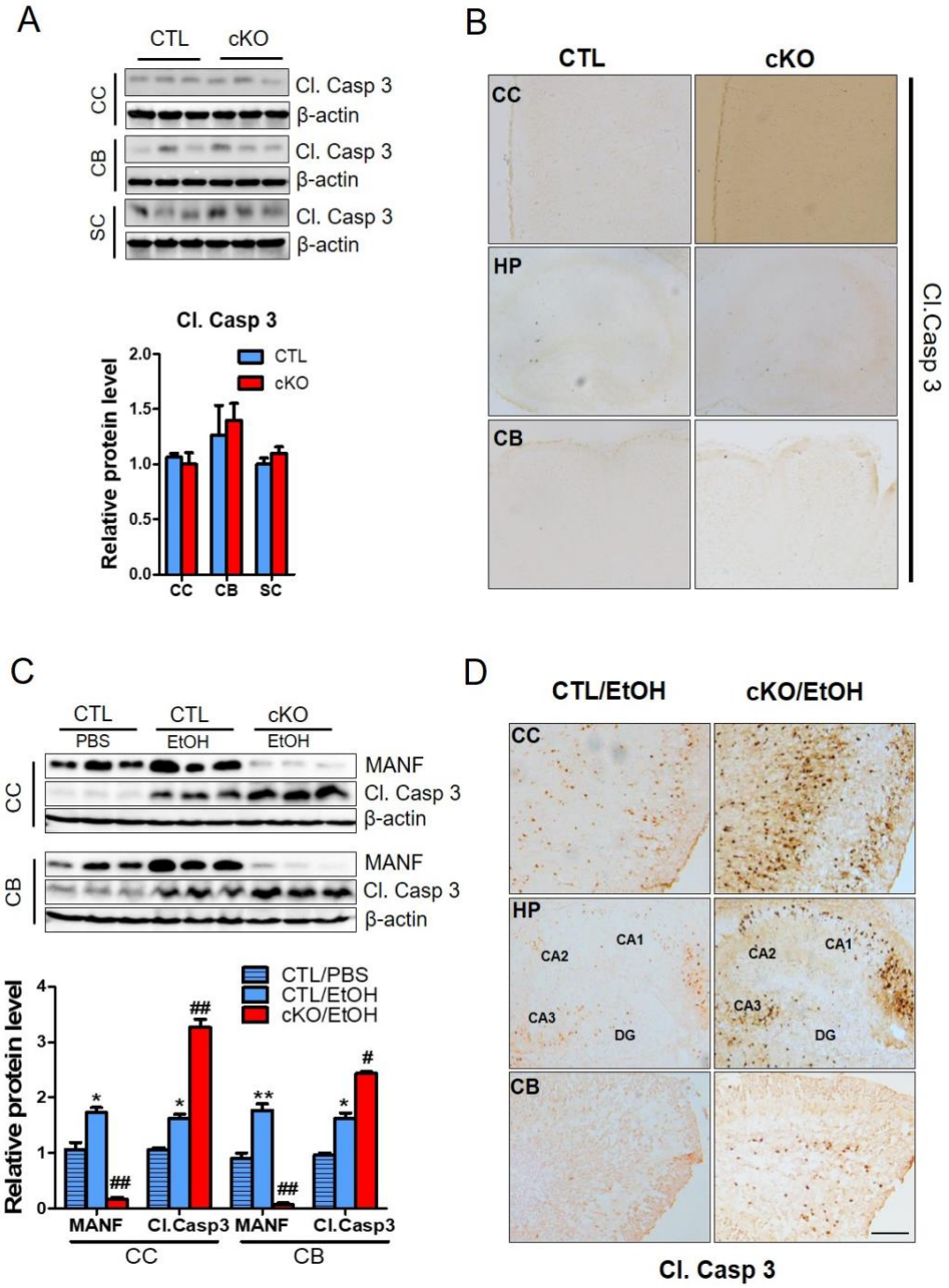


Figure 4.1. Characterization of the CNS-specific *Manf* Knockout Mouse Model. A. Representative immunoblots of MANF in cerebral cortex (CC), cerebellum (CB), subcortical structure (SC), liver and heart in the control (CTL) and *Manf* conditional knockout (cKO) mice. **B.** Representative immunochemical staining of MANF in CC, HP (hippocampus) and CB sections of PD7 pups from the CTL and cKO groups. Scale bar: 20um. **C-F.** Measurement and calculation of brain weight **(C)** and body weight **(D and F)** and the ratio of brain weight to body weight **(E)** in the CTL and cKO mice. PD: postnatal day. PM: postnatal month. Mean ± SEM. n=8-11 per group, **p<0.01 or ***p<0.001 vs. CTL.

4.3 MANF Deficiency Exacerbates Ethanol- and TM- induced Neuronal Apoptosis in the Developing Mouse Brain

First, we determined the effects of MANF deficiency on neuronal apoptosis and ER stress at basal conditions (without ethanol or TM treatment). On postnatal day (PD) 6, pups were sacrificed, and the brains were dissected to compare the levels of neuronal apoptosis and ER stress between the CTL group and cKO group. There was no significant induction of neuronal apoptosis (Figure 4.2A and B) even though we found a slight induction of ER stress in the brain of cKO mice (data not shown). To investigate its effects on ethanol-induced neuronal apoptosis, a modified neonatal ethanol exposure paradigm was employed [133]. Blood ethanol concentration was first measured to be around 185 ± 10 mg/dl in ethanol-injected group as compared to 7.5 ± 1.4 mg/dl in the PBS-injected group (data not shown). While ethanol exposure alone resulted in neuronal apoptosis to a certain degree in the developing mouse brain, we found that MANF deficiency can dramatically promote ethanol neurotoxicity (Figure 4.2C and D). Co-labelling with TUNEL and NeuN (a neuron marker) revealed that almost all of the apoptotic cells were neurons (Figure 4.2E). The apoptotic neurons, in general, spread across the entire brain, but some regions (e.g., frontal cortex, cerebellum, thalamus, and hippocampus), indeed, showed more vulnerability (Figure 4.2D). To investigate whether the effects of MANF are ubiquitous to all ER stress-characterized neurodegenerative diseases, we treated the mice with a generic ER stress inducer, TM (3mg/kg, 30% solution in dextrose) following the same procedure except that the treatment duration was 24 hours. The results presented a similar pattern showing that MANF deficiency exacerbated TM-induced neuronal apoptosis, especially in the frontal cortex, cerebellum, and hippocampus (Figure 4.2F and G). Taken together, these results

suggest that MANF is neuroprotective against ethanol-induced neuronal apoptosis and that the neuroprotective effects of MANF likely apply to ER stress-characterized neurodegenerative diseases.



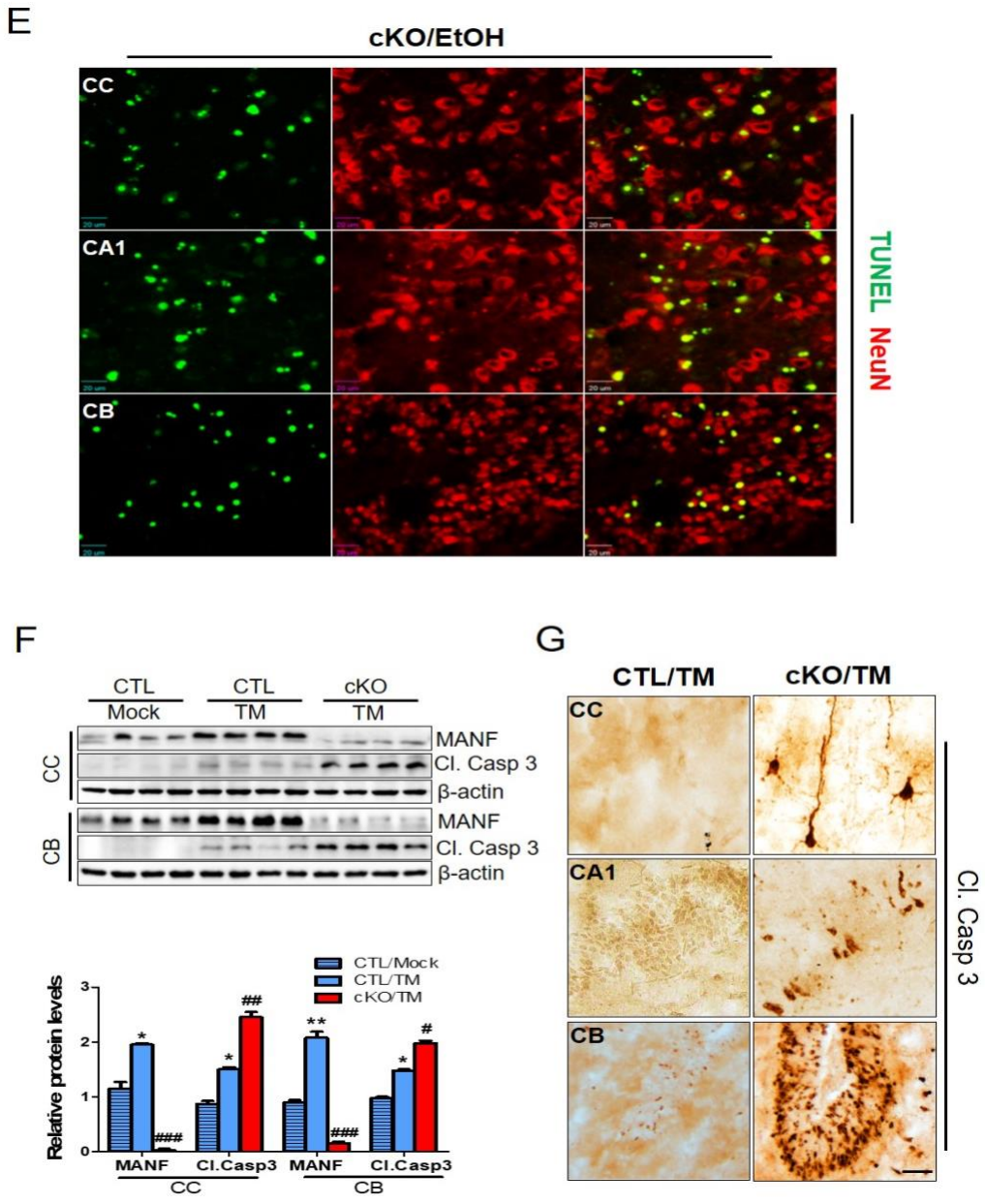

E
cKO/EtOH
CC
CA1
CB
TUNEL NeuN
F
CTL
Mock
CTL
TM
cKO
TM
CC
CB
MANF
Cl. Casp 3
β-actin
MANF
Cl. Casp 3
β-actin
CTL/Mock
CTL/TM
cKO/TM
Relative protein levels
MANF
Cl.Casp3
MANF
Cl.Casp3
CC
CB
G
CTL/TM
cKO/TM
CC
CA1
CB
Cl. Casp 3

Figure 4.2. MANF Deficiency Exacerbates Ethanol- and TM-induced Neuronal Apoptosis. A. Representative immunoblots of cleaved caspase 3 (Cl. Casp 3) in cerebral cortex (CC), cerebellum (CB) and subcortical structure (SC) in the CTL and cKO mice. **B.** Representative immunochemical staining of Cl. Casp 3 in CC, HP (hippocampus) and CB sections of PD7 pups from the CTL and cKO groups. Scale bar: 20um. **C.** Representative immunoblots of MANF and cleaved caspase 3 (Cl.Casp3) in cerebral cortex (CC) and cerebellum (CB) of PD7 pups from the control (CTL/PBS and CTL/EtOH) and cKO (cKO/EtOH) groups. Mean ± SEM. n=3-5, *p<0.05 or **p<0.01 vs. CTL/PBS. #p<0.05 or ##p<0.01 vs. CTL/EtOH. Scale bar: 20um. **D.** Representative immunochemical staining of Cl.Casp3 in CC, HP (hippocampus), and CB sections of PD7 pups from the control (CTL/EtOH) and cKO (cKO/EtOH) groups. **E.** Representative images of co-labeling with TUNEL (green) and NeuN (red) in CC, CA1, and CB sections of PD7 pups from the cKO group. **F.** Representative immunoblots of MANF and Cl. Casp3 in cerebral cortex (CC) and cerebellum (CB) of PD7 pups from the control (CTL/mock and CTL/TM) and cKO (cKO/TM) groups. Mean ± SEM. n=3-5, *p<0.05 or **p<0.01 vs. CTL/mock. #p<0.05, ##p<0.01 or ###p<0.001 vs. CTL/TM. **G.** Representative staining of Cl.Casp3 in CC, CA1, and CB sections of PD7 pups from the control (CTL/TM) and cKO (cKO/TM) groups.

4.4 MANF Deficiency Exacerbates Ethanol- and TM- induced ER Stress in the Developing Mouse Brain

To determine whether the resulting increase of neuronal apoptosis is attributed to ER stress over-activation, we examined ER stress levels in the entire mouse brain and compared that level between CTL pups and cKO pups following the same treatment and procedure. Consistent with our previous results, we found a moderate increase in ER stress due to ethanol or TM exposure alone (Figure 4.3A and D). However, we found MANF deficiency potentiated ethanol- or TM-induced ER stress significantly, i.e., causing a further dramatic enhancement of ER stress in the cerebral cortex, cerebellum, and hippocampus of the mouse brain. This data suggest that MANF may function as an ER stress-buffering agent and the deficiency of MANF exacerbates ethanol- or TM-induced ER stress (Figure 4.3A-E).

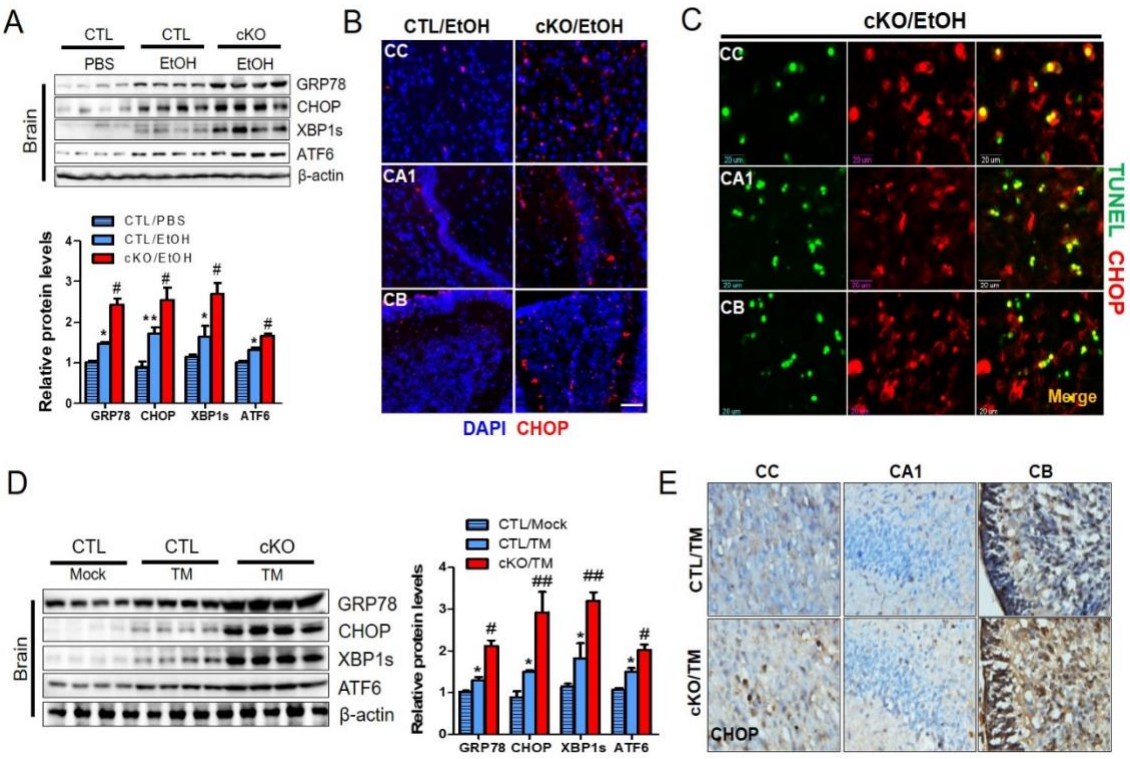


Figure 4.3. MANF Deficiency Exacerbates Ethanol- and TM-induced ER Stress. **A.** Representative immunoblots of ER stress markers (GRP78, CHOP, XBP1s and ATF6) in the entire brains of PD7 pups from control (CTL/PBS and CTL/EtOH) and cKO (cKO/EtOH) groups. Mean ± SEM. n=3-5, *p<0.05 or **p<0.01 vs. CTL/PBS. #p<0.05 vs. CTL/EtOH. **B.** Representative immunochemical staining of CHOP (red) and DAPI (blue) in CC, CA1, and CB sections of PD7 pups from control (CTL/EtOH) and cKO (KO/EtOH) groups. **C.** Representative images of co-labeling with TUNEL (green) and CHOP (red) in CC, CA1, and CB sections of PD7 pups from the cKO/EtOH group. **D.** Representative immunoblots of ER stress markers (GRP78, CHOP, XBP1s and ATF6) in the entire brains of PD7 pups from control (CTL/mock and CTL/TM) and cKO (cKO/TM) groups. Mean ± SEM. n=3-5, *p<0.05 vs. CTL/mock. #p<0.05 or ##p<0.01 vs. CTL/TM. **E.** Representative staining of CHOP (brown) and Hematoxylin (blue) in CC, CA1, and CB sections of PD7 pups from control CTL/TM and cKO/TM groups.

4.5 Blocking ER Stress Abrogates the Deleterious Effects of MANF Deficiency on Ethanol- and TM-induced Neuronal Apoptosis

We hypothesized that the increase of neuronal apoptosis due to MANF deficiency was attributed to the exacerbation of ER stress. First, co-labeling with TUNEL and CHOP (an ER stress marker) revealed that most apoptotic labeling co-localized with ER stress labeling, indicating that ER stress may be correlated with neuronal apoptosis (Figure 4.3C). This hypothesis was also supported by the employment of the ER stress blocker–4-PBA. 4-PBA, as an ER stress blocker, is a chemical chaperone that has been widely used to prevent ER stress in the liver, adipose tissue, and brain. In our animal model, pretreatment with 4-PBA can block ethanol- and TM- induced ER stress both in the CTL group and cKO mice (Figure 4.4A and C). Meanwhile, ethanol- and TM- induced neuronal apoptosis was also abolished by the employment of 4-PBA (Figure 4.4 A, B, C, and D). Collectively, this data suggests that the overactivation of ER stress is accountable for the exacerbation of neuronal apoptosis. It is also noteworthy that, in this study, we intentionally reduced the dose of ethanol or TM which was used in our previous study so that ethanol- or TM-induced neuronal apoptosis and ER stress was not saturated and the effects of MANF deficiency can be detected.

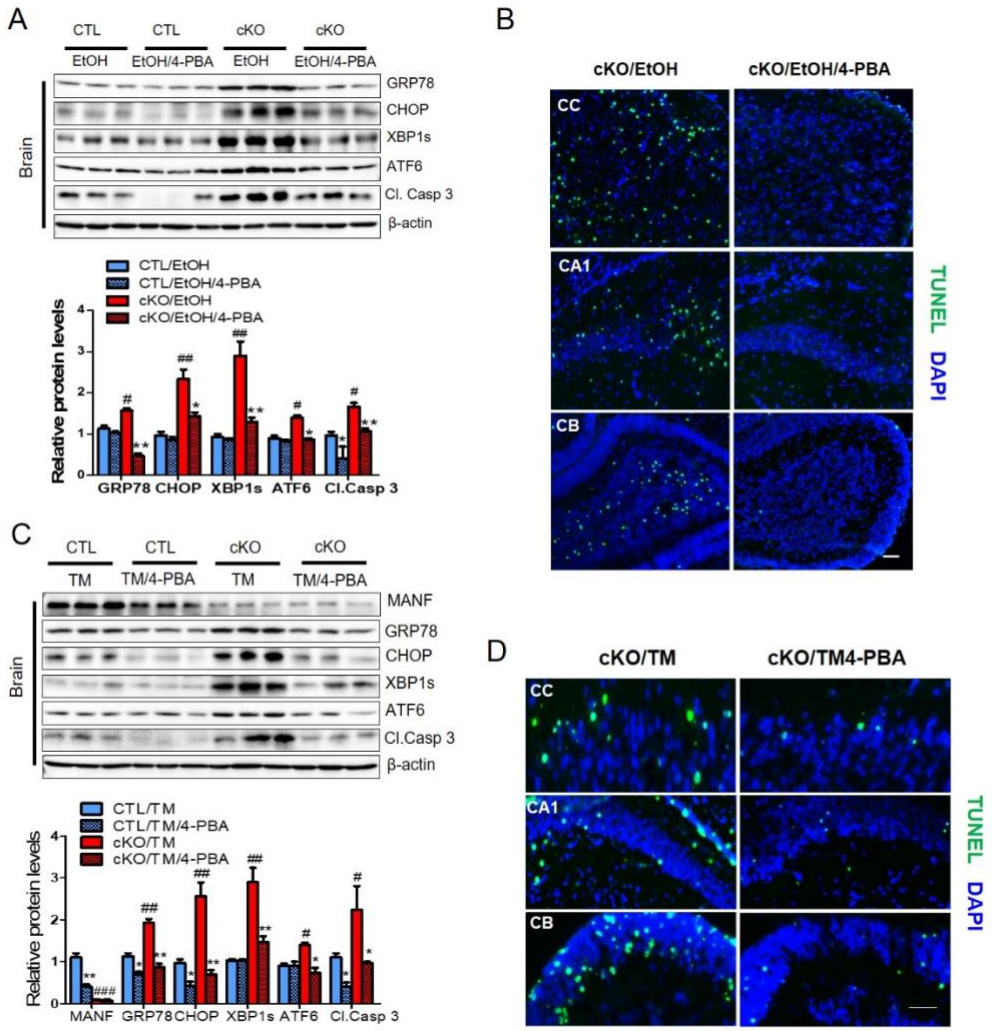


Figure 4.4. Blocking ER Stress Abrogates the Deleterious Effects of MANF Deficiency on Ethanol- and TM-induced Neuronal Apoptosis. A. Representative immunoblots of ER stress markers and Cl. Casp 3 in the entire brains of PD7 pups from the control (CTL/EtOH and CTL/EtOH/4-PBA) and cKO (cKO/EtOH and cKO/EtOH/4-PBA) groups. Mean ± SEM. n=3-5, *p<0.05 or **p<0.01 vs. corresponding treatment control (CTL/EtOH or cKO/EtOH) groups. #p<0.05 or ##p<0.01 vs. CTL/EtOH. **B.** Representative images of TUNEL (green) and DAPI (blue) labeling in CC, CA1, and CB sections of PD7 pups from cKO/EtOH and cKO/EtOH/4-PBA groups. Scale bar: 20um. **C.** Representative immunoblots of ER stress markers and Cl.Casp3 in the entire brains of PD7 pups from control (CTL/TM and CTL/TM/4-PBA) and cKO (KO/TM and KO/TM/4-PBA) groups. Mean ± SEM. n=3-5, *p<0.05 or **p<0.01 vs. corresponding treatment control (CTL/TM or KO/TM) groups. #p<0.05 or ##p<0.01 vs. CTL/TM. **D.** Representative images of TUNEL (green) and DAPI (blue) labeling in CC, CA1, and CB sections of PD7 pups from cKO/TM and cKO/TM/4-PBA groups. Scale bar: 20um.

4.6 Potential Targets of MANF that Mediate the Regulation of ER Stress

As described in the section of the method, we first ran RSEM analysis to obtain the reads that can be mapped out. The abundance of these transcripts was expressed as transcripts per million (TPM). As shown in Table 4.1, more than 98 percent of reads were mapped, and more than 90 percent of reads were uniquely mapped; only a few portions (less than five percent) were mapped to multiple loci. From the RSEM results, we ran an EBseq analysis that gave us a list of genes differentially expressed (DE) between the CTL group and the cKO group. A total of 2212 DE genes were detected (not shown here); however, only 933 had identified IDs for them (not shown here). From the list of DE genes, we performed a gene ontology enrichment analysis (GOEA) that assigned DE genes to their associated signaling pathways. The 933 genes are associated with many signaling pathways with protein processing in endoplasmic reticulum being one of them. A total of 18 DE genes were identified to be associated with protein assessing in the endoplasmic reticulum (Table 4.2 and Figure 4.5). Among these 18 genes, most of them were upregulated by *Manf* cKO; three genes (EDEM3, UBE2J2, and UBXN6) were downregulated in the brain of *Manf* cKO mice (Table 4.2 and Figure 4.5A). In term of gene function, eight genes (PDIA3, HSPA5, PDIA6, DDOST, PDIA4, HSP90B1, DNAJC3, and HYOU1) are associated with protein chaperoning function or protein folding, seven genes (EDEM3, UBE2J2, ERLEC1, SELENOS, UGGT1, UBXN6, and ATF4) are associated with protein quality control or ER-associated degradation, whereas three genes (RRBP1, SEC61A1, and SEC24D) are mainly involved in protein trafficking or secretory pathways. Next, we verified those DE genes that showed more than 1.5 folds change between the CTL group and the cKO group and confirmed *Manf* cKO in the cKO group by RT-qPCR

(Figure 4.5B-H). As expected, *Manf* mRNA level is significantly reduced in the brain of *Manf* cKO mice (Figure 4.5B). Overall, the results are consistent with our RNA-Seq data reflecting the same change of those targets in the *Manf* cKO group (Figure 4.5C-H). However, we did find that the expression level of HSPA5 and ERLEC1 does not differ significantly between the control group and *Manf* cKO group (Figure 4.5E and F). In summary, the RNA-Seq results further demonstrated that MANF is likely an ER stress-buffering agent to ameliorate ER stress and that MANF deficiency causes a deterioration of ER stress.

Table 4.1. RNA-Seq Read Mapping Summary

Samples	Input Reads	Uniquely Mapped Reads (%)	Reads Mapped to Multiple Loci (%)	Reads Unmapped (%)	Total (%)
CTL1	24426184	92.89%	4.43%	1.58%	98.90%
CTL2	32792469	93.23%	3.96%	1.69%	98.88%
CTL3	24967970	92.70%	4.46%	1.73%	98.89%
cKO1	26898333	93.52%	4.06%	1.25%	98.83%
cKO2	28616373	91.83%	4.53%	2.52%	98.88%
cKO3	24953320	93.36%	3.94%	1.57%	98.87%

Table 4.2. DE Genes that Are Involved in Protein Processing in the ER

Gene Symbols	TPM CTL			TPM cKO			Fold Change*
	CTL1	CTL2	CTL3	cKO1	cKO2	cKO3	
EDEM3	10.59	0	0	0	0.03	0	-353
PDIA3	178.25	194.95	198.1	236.47	229.3	245.79	1.25
HSPA5	190.45	210.63	200.87	325.24	321.53	373.94	1.70
RRBP1	0	0	0	5.73	0	0	3549
UBE2J2	3.62	2.7	0.56	0	0	0	-2870
PDIA6	106.21	125.87	111.92	168.65	177.94	175.76	1.52
DDOST	74.59	78.86	81.03	100.56	109.77	114.31	1.38
PDIA4	64.16	67.02	72.67	88.98	85.02	91.26	1.30
HSP90B1	281.12	335.28	308.45	463.05	428.79	472.81	1.48
DNAJC3	27.04	32.01	28.88	45.9	34.96	42.65	1.40
SEC61A1	77.17	81.16	81.32	96.02	94.07	105.29	1.23
ERLEC1	6.58	11.19	10.87	14.79	14.02	16.97	1.60
SELENOS	32.21	39.69	36.44	54.88	50.98	45.98	1.40
HYOU1	0	0	0	10.12	8.6	12.97	17439
SEC24D	10.82	15.53	15.35	19.93	19.17	19.79	1.41
UGGT1	14.51	16.23	16.71	20.55	19.63	24.6	1.37
UBXN6	8.03	6.79	4.52	0	0	0	-3004
ATF4	105.87	134.57	107.86	157	149.97	156.83	1.33

*, - and + indicate down- and up-regulation, respectively.

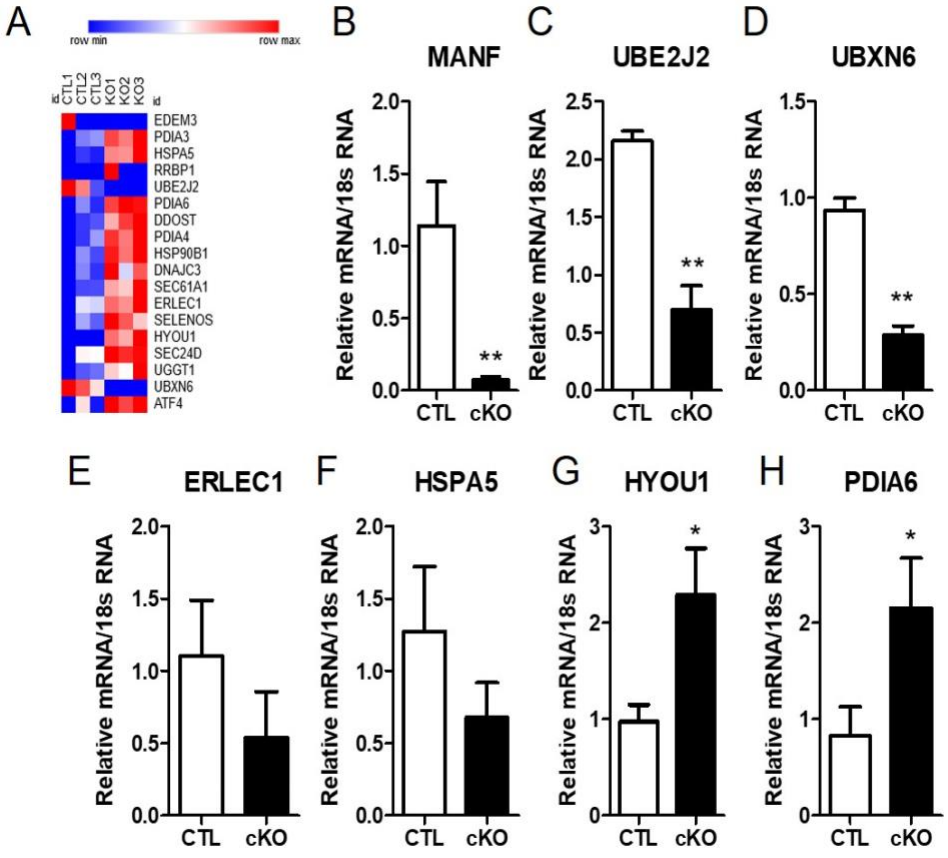


Figure 4.5. Verification of DE Genes that Are Associated with Protein Processing in ER. A. Heat map representing color-coded DE genes that are associated with protein processing in ER. **B-H.** Verification of DE genes that are associated with protein processin in ER by RT-qPCR. Mean ± SEM. n=3, *P<0.05 or **P<0.01 vs. CTL.

CHAPTER 5. MANF IS INVOLVED IN NEUROGENESIS

5.1 Overview

Neurogenesis is regulated at multiple levels, including NSC proliferation, differentiation, and the survival and maturation of newly born neurons [102]. Many signaling pathways, growth factors, cytokines, neurotransmitters, and neurotrophic factors are involved in this complex regulation of neurogenesis [102]. Ethanol exposure can inhibit or promote neurogenesis depending on animal models and ethanol exposure paradigm, e.g., acute ethanol exposure or chronic ethanol exposure [108]. Because MANF responds to ethanol exposure and has been reported to improve the survival of embryonic midbrain dopaminergic neurons both *in vivo* and *in vitro* [91, 96, 115], we speculated that MANF may participate in or affect the process of neurogenesis. Therefore, in this section, I explored the role of MANF in neurogenesis in mouse brains.

5.2 MANF Deficiency Enhances Neurogenesis in the SGZ of the Hippocampal Dentate Gyrus

Neurons born in the SGZ can migrate to the granule cell layer and eventually differentiate into granule cells in the hippocampal dentate gyrus [102]. We evaluated the neurogenesis/proliferation level in SGZ by immunostaining of DCX and BrdU incorporation. DCX is a microtubule-associated protein expressed in neuronal precursor cells and immature neurons [134]. BrdU is an analog of the nucleoside thymidine that can be incorporated into the genome during DNA replication; BrdU labeling is a classical method used for labeling dividing neurons [135]. We first assessed the neurogenesis level in SGZ at the postnatal stage and compared the level between the CTL group and the cKO group. As shown in Figure 5.1A and 5.1C, there was a significant increase in neurogenesis

in the SGZ of cKO mice when compared to their CTL littermates. Interestingly, this enhancement of neurogenesis in the SGZ of cKO mice extended to the adult stage, e.g., in three month- or six-month-old mice (Figure 5.1A). However, we did not observe a significant change in neurogenesis in the SGZ of ten-month-old cKO mice when compared to the neurogenesis level in CTL group mice. The effects of MANF on neurogenesis may reflect the expression pattern of MANF that presents a robust expression at an early age during brain development and declines afterward.

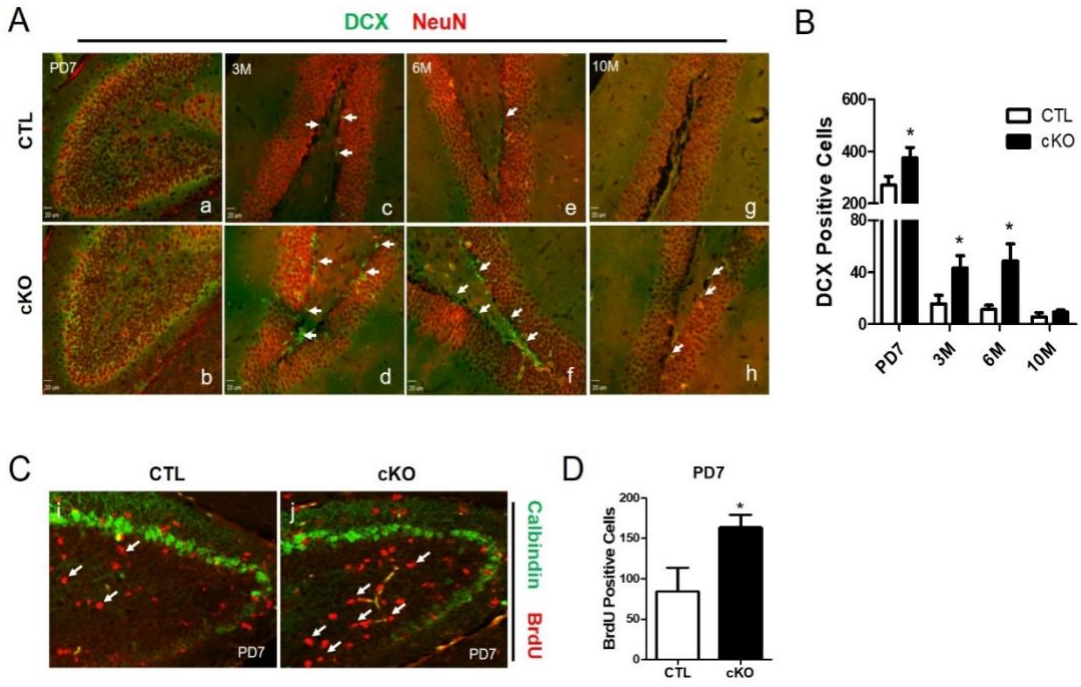


Figure 5.1. MANF Deficiency Enhances Neurogenesis in the SGZ of the Hippocampal Dentate Gyrus. A. Representative co-immunostaining of DCX (green) and NeuN (red) in the hippocampal dentate gyrus sections from the CTL (a, c, e and g) and cKO (b, d, f and h) mice. PD: postnatal day. M: postnatal month. **B.** Quantification of DCX positive cells in the SGZ of CTL and cKO mice. **C.** Representative images of co-labeling with calbindin (green) and BrdU (red) in the hippocampal dentate gyrus sections of PD7 pups from CTL (i) and cKO (j) groups. **D.** Quantification of BrdU positive cells in the SGZ of PD7 pups from CTL and cKO mice. Scale bar: 20um. Mean ± SEM. n=3-5, *P<0.05 vs. CTL.

5.3 MANF Deficiency Enhances Neurogenesis in the SVZ of the Anterior Lateral Ventricles

Neurons born in the SVZ can migrate through the RMS and eventually evolve into granule neurons and periglomerular neurons in the olfactory bulb [102]. We evaluated neurogenesis/proliferation level in the SVZ by immunostaining of Ki-67 and BrdU incorporation. Ki-67 is a general cell proliferating marker and has been widely used as a proliferating marker in cancer biology and neuroscience [135]. Consistent with the uptrend of neurogenesis in the SGZ of the hippocampal dentate gyrus of cKO mice, neurogenesis was also increased in the SVZ of the anterior lateral ventricles of cKO mice at the postnatal stage (Figure 5.2A and 5.2C). Similarly, this increase of neurogenesis in SVZ remained at the early adult stage, e.g., in three-month or six-month-old mice, but disappeared in the late adult stage, e.g., in ten-month-old mice (Figure 5.2A).

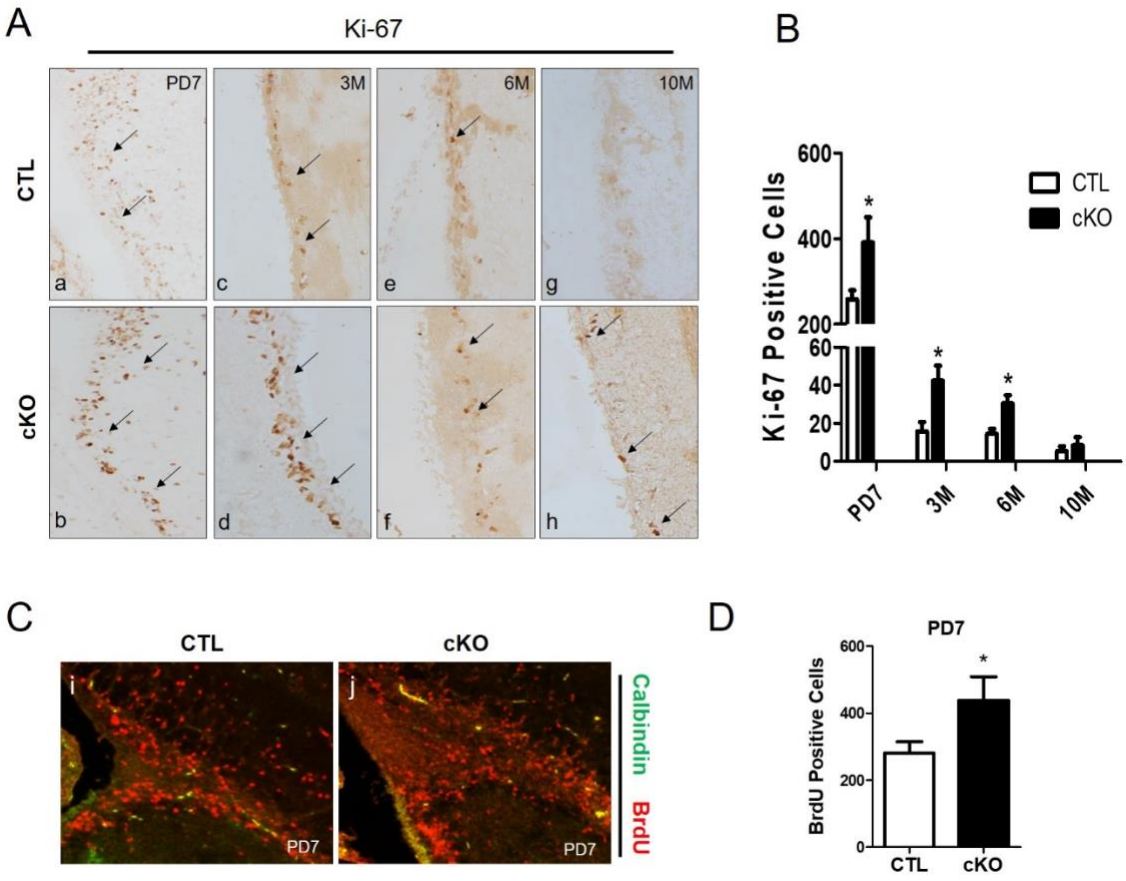


Figure 5.2. MANF Deficiency Enhances Neurogenesis in the SVZ of the Anterior Lateral Ventricles. A. Representative immunochemical staining of Ki-67 in the lateral ventricle sections from the CTL (a, c, e and g) and cKO (b, d, f and h) mice. PD: postnatal day. M: postnatal month. **B.** Quantification of Ki-67 positive cells in the SVZ of CTL and cKO mice. **C.** Representative images of co-labeling with calbindin (green) and BrdU (red) in lateral ventricles sections of PD7 pups from the CTL (i) and cKO (j) groups. **D.** Quantification of BrdU positive cells in the SVZ of PD7 pups from CTL and cKO mice. Scale bar: 20um. Mean ± SEM. n=3-5, *P<0.05 vs. CTL.

5.4 MANF Deficiency Promotes NeuroD1 and Sox2 Expression and Cell Cycle Progression

Over the past decades, many proteins have been identified to express at specific stages during the process of neurogenesis and lineage commitment. Of which, most proteins are transcriptional factors that control lineage progression at the transcriptional level in neurogenesis [103]. Among them, the SRY-related high-mobility group box (Sox) family member Sox2 and NeuroD1 appear to be the most predominant transcriptional factors that maintain the multipotency and proliferative capacities of NSCs [103]. We have examined the protein levels of NeuroD1 and Sox2 in the *Manf* cKO mouse brain and compared with that in the CTL mice. Our results showed that MANF deficiency increased the expression of NeuroD1 and Sox2, suggesting that neurogenesis is increased in *Manf* cKO mice (Figure 5.3). Sox2 controls the expression of several target genes; among them, the nuclear orphan receptor, Tlx, is positively regulated by Sox2. Tlx can promote proliferation and self-renewal of NSCs either by activating canonical Wnt pathway or by inhibiting pathways that maintain cell quiescence. We have examined a number of cell-cycle markers and cell-cycle inhibitors that are involved in cell quiescence. As shown in Figure 5.3, cell-cycle inhibitor (p15 and p27) were downregulated in the brain of *Manf* cKO mice. Consistent with these findings, mitotic markers present in the G2/M phase (cyclin A2 and pHH3) were increased, whereas the quiescent phase marker (cyclin E1) was decreased. Collectively, these findings are consistent with the results from immunochemical staining of DCX and Ki-67, suggesting that neurogenesis is elevated in MANF deficient mice and the increase of neurogenesis may be partly attributed to the acceleration of cell cycle progression.

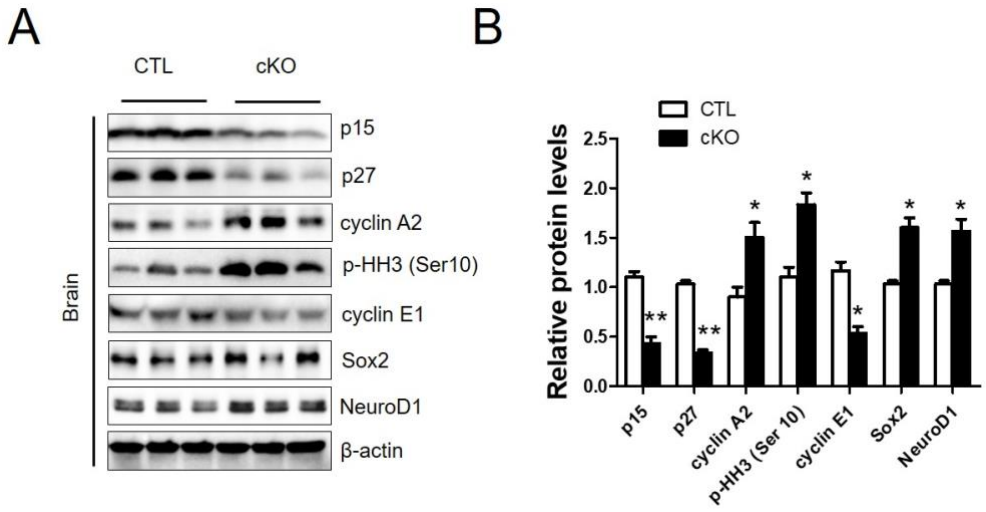


Figure 5.3. MANF Deficiency Promotes NeuroD1 and Sox2 Expression and Cell Cycle Progression. Representative immunoblots **(A)** and quantification **(B)** of NeuroD1, Sox2 and cell cycle inhibitors as well as markers in the brains of the CTL and *Manf* cKO mice. Mean ± SEM. n=3-5, *P<0.05 or **P<0.01 vs. CTL.

5.5 Gain-of-function of MANF Retards Cell Cycle Progression *in vitro*

To consolidate our findings *in vivo*, we have examined the effects of the gain-of-function of MANF on cell proliferation *in vitro*. N2A cells were infected with AD-MANF to obtain MANF overexpression in comparison with cells infected with AD-CTL (AD-vector). First, we confirmed the overexpression of MANF in these cells by immunoblots and immunochemical staining of MANF and HA 48 hours after viral infection (Figure 5.4A and 5.4B). Cell cycle analysis by flow cytometric measurements of propidium iodide (PI) incorporation in DNA revealed a smaller percentage of proliferative cells (the summation of S phase and G2/M phase) in AD-MANF infected cells when compared to that in AD-CTL infected cells (Figure 5.4C). The results were supported by a decrease of S phase or G2/M phase marker (cyclin A2, PCNA, and pHH3) and an increase of cell cycle inhibitors (p15 and p27) in AD-MANF infected cells (Figure 5.4D). MTT assay is a commonly used method to evaluate cell viability or proliferation by measuring cell metabolic activity. The results by MTT assay suggest that the gain-of-function of MANF decreases cell viability or proliferation (Figure 5.4E).

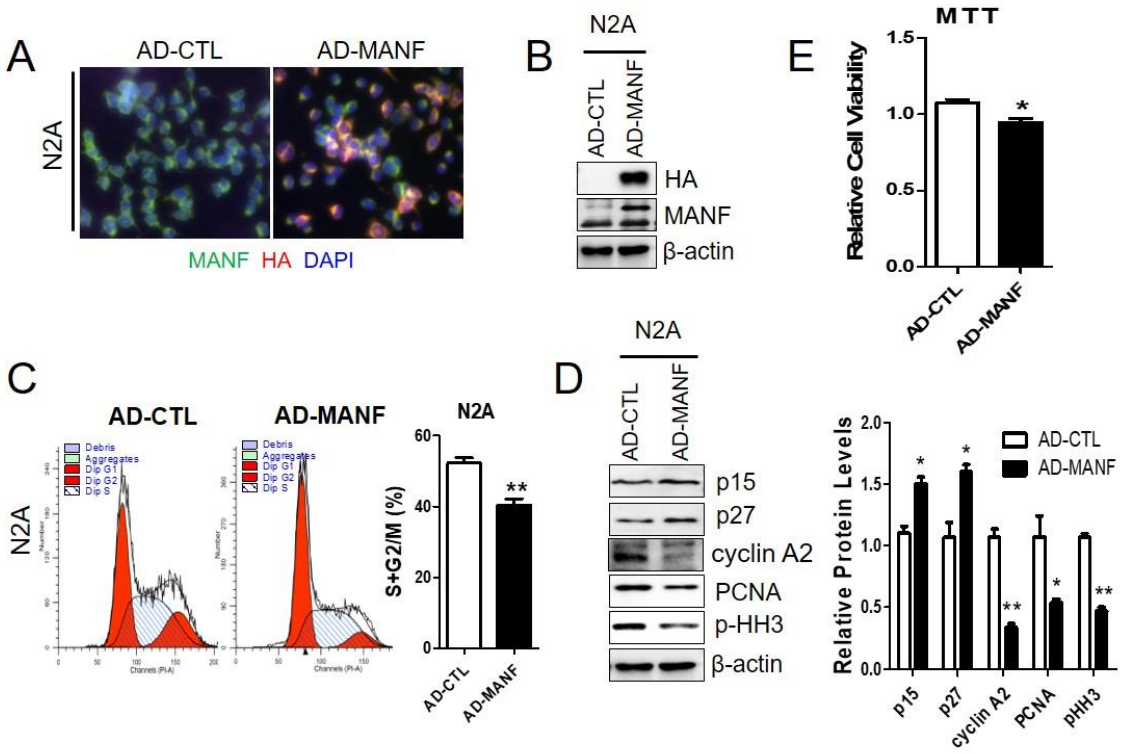


Figure 5.4. Gain-of-function of MANF Retards Cell Cycle Progression in N2A Cells. N2A cells were infected with AD-MANF or AD-CTL viral particles as described in "materials and methods". **A-B.** 48 hours after infection, MANF overexpression was confirmed by co-labeling of MANF (green) and HA (red) as well as DAPI (blue) **(A)** and immunoblotting of MANF and HA **(B)** in these two AD-infected cell lines. **C.** Flow cytometric analysis of PI content was performed for cell cycle analysis and the proliferative phases (S+G2/M) was quantitated in these two infected cell lines. **D.** Representative immunoblots and quantification of cell cycle markers (PCNA, p-HH3 andcyclin A2) and cell cycle inhibitors (p15 and p27) in these two AD-infected cell lines. **E.** Cell viability measured by MTT assay was quantificated and expressed relative to control (AD-CTL). Three independent experiments (n = 3) were performed to calculate the mean ± SEM. *P<0.05 or **P<0.01 vs. AD-CTL.

5.6 Loss-of-function of MANF Promotes Cell Cycle Progression *in vitro*

To investigate the effects of the loss-of-function of MANF on cell proliferation *in vitro*, we have developed several colonies in which *Manf* was knocked out in N2A cells using the CRISPR/Cas9 method. First, we confirmed that MANF was knocked out with a representative colony by immunoblots and immunochemical staining of MANF (Figure 5.5A and 5.5B). Consistent with the finding in N2A cells with MANF overexpression, *Manf* deletion in N2A increased the percentage of proliferative cells (the summation of S phase and G2/M phase) (Figure 5.5C). Likewise, this increase of proliferation was supported by an increase of S phase or G2/M phase marker (cyclin A2, PCNA, and p-HH3) and a decrease of cell cycle inhibitors (p15 and p27) in *Manf* knockout N2a cells (Figure 5.5D). Taken together, the results indicate that the gain-of-function of MANF retards cell cycle progression, whereas the loss-of-function of MANF promotes cell proliferation. The inhibition of cell cycle progression could be the mechanism that underlies the increase of neurogenesis in the *Manf* cKO mice.

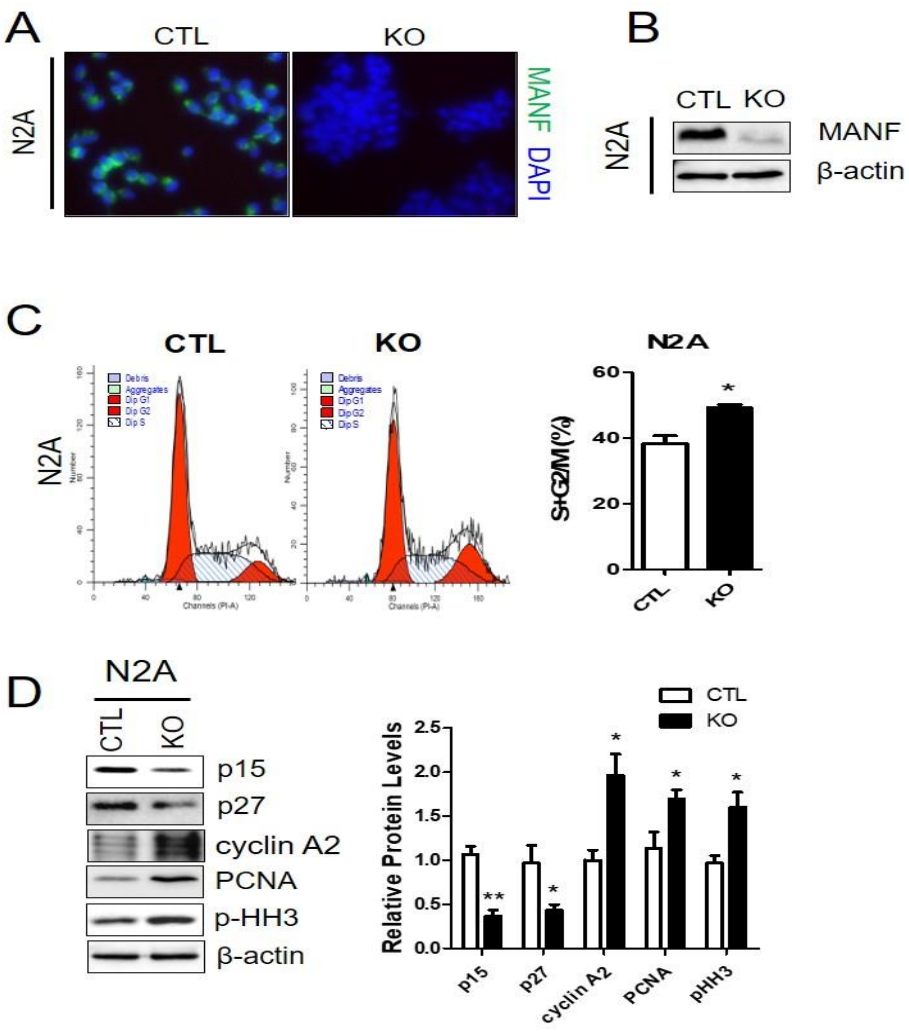


Figure 5.5. Loss-of-function of MANF Promotes Cell Cycle Progression in N2A Cells. Several colonies in which *Manf* is knocked out using CRISPR/Cas9 method were developed as described in "materials and methods". **A-B.** Representative images of co-labeling of MANF (green) and DAPI (blue) **(A)** and immnunoblots of MANF in a representative colony **(B)** in comparison with that in a colony transfected with the control plasmid. **C.** Flow cytometric analysis of PI content was performed for cell cycle analysis and the proliferative phases (S+G2/M) was quantificated in these cells. **D.** Representative immunoblots and quantification of cell cycle markers (PCNA, p-HH3 and cyclin A2) and cell cycle inhibitors (p15 and p27) in these cells. Three independent experiments (n = 3) were performed to calculate the mean ± SEM. *P<0.05 or **P<0.01 vs. CTL.

CHAPTER 6. DISCUSSION

6.1 ER Stress and Ethanol-induced Neurodegeneration

According to the survey on ethanol use worldwide in 2018, men tend to drink more and are more often current drinkers than women [4]. In my first project, the effects of binge ethanol drinking on neurodegeneration and ER stress were evaluated in adult male mice. We demonstrated that binge ethanol exposure caused duration-dependent neurodegeneration, neuroinflammation, and ER stress in the cerebellum and cortex of the adult mouse brain. However, this paradigm of ethanol exposure did not cause significant alterations in behavioral outcomes which were tested by OFA, EPM, and MWM. *In vitro*, our studies suggest that mTOR signaling mediates the induction of ER stress that contributes to ethanol-induced neuronal cell death.

This paradigm of binge ethanol exposure results in high BECs (300-400 mg/dl). Although NIAAA defines binge drinking as a pattern of drinking that leads to a BEC of 80 mg/dL, the actual amount of ethanol consumed in human alcoholics is much higher. BECs greater than 300 mg/dl are not uncommon in human alcoholics [136-139]. Some alcoholics with a BEC larger than 400 mg/dL are coherent and able to drive and BECs greater than 500 mg/dL were reported in heavy drinkers [136, 139]. The paradigms of binge ethanol exposure similar to ours have been used to investigate ethanol-induced neurodegeneration, glial activation, and neuroinflammation in the brain of adult and adolescent mice [63, 64, 140].

Multiple mechanisms have been proposed for ethanol-induced neurodegeneration, including neuronal excitotoxicity, oxidative stress, and proinflammatory response [141, 142]. In our binge ethanol drinking mice, ethanol causes little oxidative stress in the brain,

although it induces significant oxidative stress in the liver. However, we found that IL-6 in the cerebellum and MCP-1 and IL1β in the cerebral cortex are induced by ethanol exposure, suggesting that proinflammation occurs in the mouse brain. These proinflammatory cytokines may partially contribute to ethanol-induced neurodegeneration. ER stress is a hallmark and an underlying mechanism of many neurodegenerative diseases. We showed that binge ethanol exposure induces ER stress manifested by an increase of ER stress markers, such as GRP78, p-eIF2α, CHOP, ATF6, and XBP1s. Interestingly, the timeline of ethanol-induced ER stress is correlated well with that of ethanol-induced neurodegeneration. Moreover, ethanol activates an ER-resident caspase, caspase-12, which is usually activated by ER stress [129, 143]. Cleaved caspase-12 may activate other caspases including caspase-3, and caspase-9 to induce cell apoptosis [144, 145]. The activation of caspase-12 led us to hypothesize that ER stress may be involved in ethanol-induced neurodegeneration. This hypothesis is supported by our *in vitro* study showing that 4-PBA, an ER stress inhibitor, significantly reduces ethanol-induced ER stress as well as the death of neuronal cells. Together, these data suggest that ER stress at least partially mediates ethanol-induced neurodegeneration.

The effect of ethanol on ER stress in the mature brain is different from that in the developing brain. Our previous study indicated that ethanol rapidly triggers neurodegeneration and ER stress in the developing brain; ethanol-induced ER stress in the neonatal brain is evident after 4-8 hours of exposure and a drastic caspase-3 activation and neurodegeneration are observed after 8 hours of exposure [115]. In the adult brain, however, ethanol-induced neurodegeneration and ER stress are only found after 5 days of exposure to a much lesser extent. These results imply that the adult brain has a more mature

UPR system that can better cope with ER stress.

The underlying mechanisms for ethanol-induced ER stress are unclear. Acetaldehyde, a metabolite of ethanol can form adducts with proteins, nucleic acids, and lipids, which may affect protein folding [146]. Ethanol can also cause dysregulation of Ca^{2+} homeostasis in the ER and consequently results in dysfunction of Ca^{2+}-dependent chaperones or enzyme that leads to ER stress [146]. Additionally, ethanol metabolism generates ROS and thereby disrupts the redox status in the ER that leads to ER stress [146]. Neuroinflammation has also been shown to initiate ER stress in the brain [147-149]. mTOR belongs to the family of serine/threonine kinases. It exists in two complexes, mTORC1 and mTORC2, that have different subunit compositions and undertake distinct cellular tasks [131]. It has been reported that ethanol downregulates mTOR signaling to promote autophagy in the developing brain, and cultured astrocytes and neurons [150, 151]. The current study showed that binge ethanol exposure downregulates mTOR signaling both *in vivo* and *in vitro*. However, we did not observe a significant increase in autophagy in the brain following ethanol exposure (data not shown). Instead, inhibition of mTOR signaling by rapamycin induces ER stress in neuronal cells, whereas inhibition or activation of ER stress can not reverse ethanol-induced inhibition of mTOR signaling. This data suggests that mTOR signaling is upstream of ethanol-induced ER stress and ER stress may act as a hub that converges signals from inflammatory response, oxidative stress, and mTOR signaling.

While the effects of binge ethanol exposure on behavioral deficits in rats were well discussed in previous studies [37, 152, 153], only a few studies focused on binge ethanol exposure-induced behavioral deficits in adult mice. Some studies showed that binge

ethanol exposure can lead to behavioral deficits, such as impaired spatial learning and memory, particularly in adolescent rats [154, 155]. However, we did not observe significant behavioral alterations in our ethanol gavaged mice. There are several possibilities for this finding. First, the extent of neurodegeneration under this ethanol paradigm might be not adequate to elicit behavioral alterations. Second, ethanol-induced changes in behaviors might be recovered because the current behavioral tests were performed 3-5 days after ethanol exposure. Third, depending on the animal model and timing/dosage of ethanol exposure, ethanol can also induce neurogenesis during or after ethanol exposure [111, 156]. Our results showed binge ethanol exposure increased neurogenesis/proliferation in the subgranular zone and subventricular zone after 10 days of ethanol exposure (data not shown), manifested by the increase of doublecortin (DCX) and Ki-67. The new neurons produced by the increase of neurogenesis may compensate for the neuronal deficit elicited by binge ethanol exposure.

In summary, binge ethanol exposure induces ER stress and neurodegeneration in adult mice. The induction of ER stress contributes to ethanol-induced neurodegeneration. Mechanistically, ethanol-induced ER stress is regulated at least partially by mTOR signaling. It remains to be revealed whether ER stress indeed contributes to ethanol-induced neurodegeneration *in vivo*. Additionally, the mechanisms for ethanol-induced ER stress have not been completely elucidated. For example, the pathways/intermediates bridging mTOR signaling and ER stress are unknown. Also, does ethanol-induced neuroinflammation contribute to the induction of ER stress or vice versa? A better understanding of the role of ER stress in ethanol-induced neurodegeneration may offer novel approaches in the prevention and treatment of ethanol-associated neurotoxicity.

6.2 MANF, ER Stress, and Ethanol-induced Neurodegeneration

In my second project, using a third-trimester equivalence mouse model and a CNS-specific *Manf* knockout mouse model, we have demonstrated that MANF is neuroprotective against ethanol-induced neurodegeneration through ameliorating ER stress and the neuroprotective role of MANF is likely applied to other ER stress-characterized neurodegenerative diseases. Moreover, the results from a whole transcriptome RNA sequencing on brain samples from *Manf* cKO mice and control mice provide evidence about the involvement of MANF in ER stress regulation and shed light on the potential targets that mediate the ER stress-buffering capacity of MANF in ethanol-induced neurodegeneration.

A third-trimester equivalent mouse model is used in our study to mimic prenatal ethanol exposure that typically occurs in FASDs. Due to a developmental timing shift between humans and rodents, the developmental period of the rodent brain in PD 1-10 is equivalent to the third trimester in humans when brain growth spurt occurs [157]. The original model was established by Olney's group and then became widely used to induce neurodegeneration in infant mice [133]. We modified the dose applied to infant mice from 5 g/kg to 3g/kg. We found this dose resulted in a BEC of 185 ± 10 mg/dl and minimal neuronal apoptosis in control mice. As discussed in **Section 6.1**, we believe the BEC in the modified ethanol exposure paradigm is more relevant to alcoholic drinking during pregnancy and more importantly, the resulting neuronal apoptosis is not saturated so that the exacerbating effects of MANF deficiency on neuronal apoptosis can be detected. Besides, we choose to investigate the role of MANF in ethanol neurotoxicity in the developing brain for the following two reasons. First, the expression level of MANF varies

over time during brain development; it has its highest expression during the developmental stage and declines afterward. We believe that the beneficial effects of MANF on ER stress and ethanol-induced neurodegeneration is more predominant in the developing brain than in the mature brain. Second, as discussed in **Section 6.1**, ER stress is responsive to ethanol exposure in the developing brain to a greater extent than in the mature brain.

We have investigated the effects of MANF on ER stress and neurodegeneration not only in ethanol-induced neurodegeneration but also in TM-induced neurodegeneration. TM is a widely used ER stress inducer by blocking N-linked glycosylation in the ER. Similar effects were present in both pathological conditions showing that MANF deficiency exacerbates ER stress and neurodegeneration, which suggests that the beneficial effects of MANF may be ubiquitous to all ER stress-characterized neurodegenerative diseases. Nevertheless, we did observe some differences regarding the types of cells that were undergoing apoptosis between these two conditions. It appeared that most of the apoptotic cells in TM-induced neurodegeneration are immature neurons as opposed to the apoptotic cells being mature neurons in ethanol-induced neurodegeneration. Since MANF is widely expressed both in neurons and glial cells and the beneficial effects of MANF are likely applied to all brain cells. Hence, we speculate that the treatments (ethanol or TM) determine cell-type-specific toxicity. Although MANF deficiency can exacerbate their harmful effects, it remains aligned with their toxic specificity.

MANF is an ER-resident protein even though secretory MANF has been identified in circulation and interstitial tissue [97]. It is well established that MANF is an ER stress-responsive protein, induced mainly through transcriptional activation by ATF6 [132]. Our results indicate that MANF functions as an ER stress-buffering agent to ameliorate ER

stress and as a result, MANF deficiency causes a deterioration of ER stress. While the exact mechanisms that underlie the buffering effects of MANF on ER stress remain unclear, several mechanisms have been proposed. A study recently reported that the SAP domain of MANF interacts with BiP, antagonizing BiP-mediated nucleotide change, and thereby stabilizes BiP-client complexes to facilitate protein folding [158]. Second, MANF contains a C-XX-C motif and has been reported to be involved in disulfide bond formation, a critical step in protein folding in the ER [94]. Alternatively, one group demonstrated that MANF is a structurally unique redox-sensitive chaperone that can restore protein folding in the ischemic heart [159].

Our RNA sequencing data further confirmed the involvement of MANF in ER stress regulation. Apart from the ER stress markers examined by immunoblots, it has identified a number of targets that are ER-resident protein responsible for the folding and trafficking of newly synthesized protein. These targets carry on a series of functions ranging from protein chaperoning to protein disulfide isomerization. It should be noted that these proteins could be direct targets of MANF or indirect targets that are indicative of the deterioration of ER stress. Of note, most of the upregulated transcripts are ER-resident chaperones or protein disulfides (PDIs) that can improve an adaptation to ER stress, whereas most of the downregulated transcripts (UBE2J2 and UBXN6) are ubiquitin-conjugating enzymes that are involved in proteasomal degradation [160, 161]. We speculate that MANF deficiency may disrupt the ubiquitination system and consequently causes ER stress manifested by an abrupt increase of ER stress-related transcripts. Further experiments are needed to identify their physical interaction and functional connection with MANF.

In summary, we have provided evidence, with loss-of-function of MANF, that MANF is neuroprotective against ER stress-characterized neurodegenerative disease through ameliorating ER stress. Nevertheless, direct evidence about the effects of MANF on ethanol- or TM- induced ER stress and neurodegeneration from the gain-of-function of MANF is needed to draw a clear-cut conclusion. Besides, although MANF is an ER-resident protein, it can also secrete out of cells in stressful conditions [162]. Therefore, future studies are needed to examine the potential distinct function of intracellular MANF and extracellular MANF. Lastly, MANF is expressed in both neurons and glial cells. Our preliminary data indeed reflects the effects of MANF ablation in the entire CNS, including neurons and glial cells. It would be interesting and rewarding to delineate the contribution of cell type (neuron or astrocyte) derived MANF on ethanol-induced neurodegeneration. A better understanding of the role of MANF in ethanol-induced neurodegeneration may reveal MANF as a critical player in ER stress regulation, thereby laying a foundation for targeting MANF as a therapeutic approach in the treatment of FASDs or AUD.

6.3 MANF and Neurogenesis

In my third project, we have demonstrated that MANF deficiency promotes neurogenesis in the SVZ and SGZ of the mouse brain by immunostaining of DCX and Ki-67 as well as BrdU incorporation. Besides, we have shown that two crucial transcriptional factors (NeuroD1 and Sox2) that are tightly linked with NSC multipotent and proliferative capacities are increased in the brain of *Manf* cKO mice. Mechanistically, MANF was found to inhibit cell cycle progression, which might be one of the mechanisms that underlie the increase of neurogenesis in *Manf* cKO mice.

Neurogenesis was first reported to exist in the dentate gyrus of adult mouse brain

decades ago [105]. It is now widely accepted that adult neurogenesis occurs at least in two regions, i.e., subgranular zone and subventricular zone in humans and rodents [104, 106, 107]. MANF was first identified in an astrocyte cell line in search of astrocyte-derived neurotrophic factor [91]. Subsequent studies found MANF is neuroprotective, especially to dopaminergic neurons *in vivo* and *in vitro* [91, 96]. It has also been shown that MANF is involved in neurite outgrowth and neuronal migration [95]. Mechanistically, MANF promotes cell survival by interacting with proapoptotic proteins such as Bax and Bak to prevent their proapoptotic properties or by promoting mTOR signaling to increase the transcription of prosurvival genes [93, 94, 163]. As a neurotrophic factor, we reckoned that MANF might participate in neurogenesis in the developing and mature mouse brain. As opposed to our anticipation that MANF promotes neurogenesis, we observed an increase of neurogenesis both in the SGZ and SVZ of *Manf* cKO mice. We think it may be because MANF inhibits cell proliferative activity even though it promotes cell survival. Indeed, numerous studies in cancer biology have reported that the gain-of-function of MANF inhibits cell proliferation and reduces aggressive tumor properties [100, 164]. In light of these reports and our data, we have examined a number of markers that are associated with cell cycle progression. It turned out that MANF deficiency upregulates mitotic markers (p-HH3 and cyclin A2) and meantime downregulates cell cycle inhibitors (p15 and p27). Besides, we found the transcriptional factor, i.e., Sox2, is also upregulated in the brain of *Manf* cKO mice. Sox2 has been reported to promote neurogenesis by repressing pathways that promote cell quiescence such as p53 pathway and cell-cycle inhibitor p21 [165]. Collectively, all pieces of information, when assembled, seem to indicate that MANF deficiency increases neurogenesis by promoting NSC proliferation.

While MANF ablation overall promotes neurogenesis in the mouse brain, it fails to reactivate neurogenesis in aged mice. It has been reported that aging negatively regulates neurogenesis manifested by a sharp and continuous decrease in cell population in both SGZ and SVZ of the aged mouse brain [166, 167]. With aging, NSCs lose their proliferative capability and become more quiescent even though some reports indicate that neurogenesis can be reactivated to a certain extent in aged mice upon stimulation such as exercise or calorie restriction [168, 169]. In humans, using postmortem and fresh tissues, scientists have demonstrated that there is no evidence of neurogenesis in humans after adolescence or there is a sharp decrease of neurogenesis with aging [170]. In summary, recent findings point to the conclusion that neurogenesis is decreased with aging both in humans and rodents. Our data is consistent with these reports showing that MANF ablation cannot rejuvenate the quiescent NSCs that might or might not exist in aged mice. We speculate that MANF may affect the process of neurogenesis, e.g., promote or inhibit neurogenesis, but could not alter the intrinsic programs that underlie or determine NSC fate.

In summary, we have provided evidence showing that MANF affects neurogenesis-that is, MANF deficiency promotes neurogenesis in the mouse brain. With DCX staining in the SGZ and Ki-67 in the SVZ as well as BrdU incorporation, we demonstrate that neurogenesis is indeed increased in the brain of *Manf* cKO mice. Neurogenesis is a complex process involving multiple developmental steps or stages characterized by cell differentiation and lineage specification before they are integrated into neural circuitry. For example, in the hippocampal dentate gyrus, mature granule neurons undergo at least three developmental stages before they are functionally integrated into the hippocampal circuitry. Type1 radial glia-like cells (RGLs) are thought to represent the NSCs population

that possess self-renewal and multipotent capabilities. Intermediate progenitor cells (IPCs, or type 2 cells) are believed to stem from RGL differentiation and possess transient amplifying activities. IPC can differentiate into neuroblasts (type 3) that give rise to mature granule dentate granule neurons [103]. It has been reported that DCX is expressed in neuroblasts but not in RGL or IPC cells [102]. Therefore, it is unclear in which stage or step MANF boosts neurogenesis in the hippocampal dentate gyrus. Likely, Ki-67 is a general cell proliferation maker that can label any cells that are undergoing cell division regardless of the specific stage of lineage differentiation. To decipher the regulatory role of MANF in neurogenesis, the first and foremost important thing is to identify the particular differential stages at which MANF regulates neurogenesis. Markers that are expressed at specific stages of hippocampal neurogenesis and SVZ might be able to undertake this task.

In addition, MANF may affect neurogenesis through other signaling pathways such as the metabolic pathway. Metabolic status has been suggested to be associated with stem cell behavior or activity [103]. Inhibition of fatty acid synthesis through genetic or pharmacological approaches can result in a decrease in stem cell proliferation [103]. A recent study demonstrated that MANF is involved in energy metabolism by inducing insulin resistance in the hypothalamus [99], which could directly or indirectly affect the process of neurogenesis. Besides, although sharing no homology with another neurotrophic factor, i.e., BDNF, MANF may also activate Trk receptor to stimulate stem cell growth or maturation. A better understanding of the role of MANF in adult neurogenesis can enrich our knowledge about neurogenesis, its regulation, and relevance to some neurological and psychiatric diseases that are related to dysregulation of neurogenesis.

6.4 Perspectives and Conclusions

It has been well established that ER stress is functionally linked with brain damage [76]. Depending on diseases and the stimulant, the UPR triggered by ER stress may activate different signaling components and consequently generate distinct outcomes. Initiated as a protective mechanism, UPR induces the upregulation of a bunch of pro-survival factors to maintain protein homeostasis. However, sustained activation of UPR may cause a shift toward promoting cell damage during the symptomatic stage [76]. It still remains unclear what factors, pathways or events mediate this functional shift of UPR from the constructive role to the destructive role.

Ethanol exposure can induce ER stress both in the developing mouse brain and in the adult mouse brain [115, 171]. It appears that ER stress contributes to ethanol-induced neuronal death demonstrated by the employment of pharmacological approaches to manipulate ER stress in cell culture. This finding is supported by the recent report showing that ER stress also underlies ethanol-induced neuronal cell death in the developing brain [116]. Ethanol exposure can downregulate mTOR signaling, which was previously suggested to contribute to the induction of autophagy [150, 151]. While absent in autophagy induction in our binge drinking animals, the downregulated mTOR signaling seems to mediate the induction of ER stress (Figure 6). Therefore, depending on the ethanol exposure paradigm and disease stages, mTOR signaling plays a critical role either in promoting cell survival through the induction of autophagy or in triggering cell death through the induction of ER stress. The underlying mechanisms behind this functional shift of mTOR in the context of ethanol-induced neuronal death from preventing cell death to favoring cell death remain to be determined.

MANF has pleiotropic effects by participating in a multitude of biological processes [95, 98, 99]. Located in the downstream of UPR signaling, MANF appears to confer neuroprotective effects by preventing the overactivation of ER stress (Figure 6). The exact mechanisms underlying this neuroprotective role remain unknown although multiple mechanisms have been proposed. In our studies, MANF also exerts inhibitory effects on neurogenesis, which is likely though retarding cell cycle progression (Figure 6). This inhibitory effect of MANF on neurogenesis is intuitively contradictory to its neuroprotective role. However, it is possible that the MANF-mediated cell cycle arrest, in concert with its role in ER stress adaptation, renders beneficial effects on neuronal plasticity. The temporary cell cycle arrest by MANF could be programmed intrinsically for the cells to have enough time to fix ER stress and restore protein homeostasis (Figure 6). Hence, MANF might have a dual role in neuroprotection and both are in a constructive manner. In addition, given that MANF is induced by ethanol exposure [115, 116], the inhibitory effects of MANF on neurogenesis might be also associated with the fact that ethanol-induced intoxication can cause a decrease in neurogenesis in the vast majority of cases. Future efforts are needed to systematically delineate the role of MANF-mediated cell cycle alteration in neuronal plasticity modulation and its implication in ethanol-mediated neurogenesis regulation.

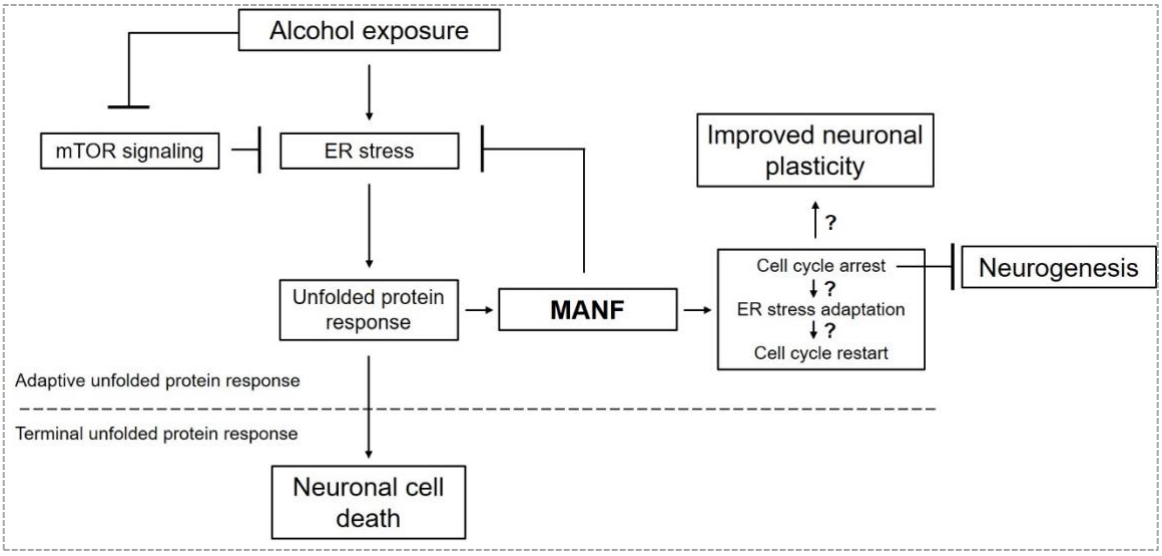


Figure 6. A Working Model for the Regulatory Network Involving mTOR, ER Stress and MANF in Ethanol-induced Neurodegeneration. Ethanol exposure can cause a sustained activation of ER stress that consequently results in neuronal cell death through uncontrolled unfolded protein response. The downregulation of mTOR signaling by ethanol mediates the induction of ER stress. MANF is neuroprotective against ethanol-induced neuronal death through ameliorating the overactivation of ER stress. MANF can also retard cell cycle progression that consequently reduces neurogenesis.

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PUBLICATIONS

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