ALCOHOL INCREASES MANGANESE ACCUMULATION IN THE BRAIN AND EXACERBATES MANGANESE-INDUCED NEUROTOXICITY

Dissertation Presented by

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To

The Bouve’ Graduate School of Health Sciences
in Partial Fulfillment of the Requirements for the Degree of
Doctor of Philosophy in Pharmaceutical Sciences
with specialization in
Pharmaceutics

NORTHEASTERN UNIVERSITY
BOSTON, MASSACHUSETTS

November 2018
**Alcohol increases manganese accumulation in the brain and exacerbates manganese-induced neurotoxicity**

Presented by: Murui Han, Candidate

Date to be presented: 11/27/2018

Time and location: from 1:30 pm to 3:30 pm at Curry Student Center, Room 346.

Thesis Committee:

Chair: Dr. Jonghan Kim  
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Submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree.
Resources available for this project:

**Laboratory space** (Briefly describe the facilities, their location, and equipment available to you to perform this project):

I have access to laboratories (144, 143, 124 and 241) and the animal facility at 140 The Fenway building. Our lab has apparatus which is in rooms 143 and 116, and software for behavioral experiments. Also, I have the essential materials and equipment to perform the pharmacokinetics experiments, including the isoflurane anesthetic machine, surgical tools and gamma counter. I also have the equipment and chemicals required for conducting the biochemical assays, including western blot apparatus, spectrophotometer and qRT-PCR. Radioisotope is located in X115 and the experiment will be conducted in room 143.

**Animals** (If your project involves live animals, please provide an estimate of the numbers used for each species, and state that you have the proper training or will have completed your training before starting your experiments):

There will be 168 mice (mixed background) needed to complete my proposed research. I have completed the required animal training.

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- Boqiang Yan and Murui Han (2011), “The analysis of IgE and IL-25 in peripheral blood of asthmatic patients”, Chinese Journal of Cellular and Molecular Immunology, 27(6), 691-691.

Scientific abstracts:
- 04/2017: JuOae Chang., Helal Alsulimani, Murui Han, Qi Ye, Fulden Buyukozturk, and Jonghan Kim (2017), “Hands-on Modeling/Simulation Using MATLAB and SimBiology Improves Student Learning in Graduate”, Experimental Biology conference; San Diego, US.
- 11/2016: Murui Han and Jonghan Kim (2016), “Alcohol increases olfactory manganese uptake into the brain and exacerbates neurotoxicity”, Society of Neuroscience conference; San Diego, US.
10/2015: **Murui Han** and Jonghan Kim (2015), “Loss of DMT1 function is associated with elevated brain copper levels and ADHD-like behavior”, Society of Neuroscience conference; Chicago, US.

10/2015: Qi Ye, JuOae Chang, **Murui Han**, Yiting Li, Helal Alsulimani, Archita Venugopal Menon, Richard Deth and Jonghan Kim (2015), “Brain iron loading impairs dopaminergic function and promotes ADHD-like behavior”, Society of Neuroscience conference; Chicago, US.

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<tr>
<td>ALT</td>
<td>Alanine aminotransferase</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood brain barrier</td>
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<tr>
<td>CNS</td>
<td>Central nerve system</td>
</tr>
<tr>
<td>Cu</td>
<td>Copper</td>
</tr>
<tr>
<td>DAT</td>
<td>Dopamine transporter</td>
</tr>
<tr>
<td>D1DR</td>
<td>Dopamine D2 receptor</td>
</tr>
<tr>
<td>D2DR</td>
<td>Dopamine D1 receptor</td>
</tr>
<tr>
<td>DCYTB</td>
<td>Duodenal cytochrome B</td>
</tr>
<tr>
<td>DMT1</td>
<td>Divalent metal transporter 1</td>
</tr>
<tr>
<td>FPN</td>
<td>Ferroportin</td>
</tr>
<tr>
<td>GABA</td>
<td>Gamma-aminobutyric acid</td>
</tr>
<tr>
<td>GABRA1</td>
<td>Gamma-aminobutyric acid receptor subunit alpha-1</td>
</tr>
<tr>
<td>GABRA2</td>
<td>Gamma-aminobutyric acid receptor subunit alpha-2</td>
</tr>
<tr>
<td>GAD</td>
<td>Glutamic acid decarboxylase</td>
</tr>
<tr>
<td>GGC</td>
<td>Gamma-glutamyl cysteine</td>
</tr>
<tr>
<td>GI</td>
<td>Gastrointestinal</td>
</tr>
<tr>
<td>IRE</td>
<td>Iron-responsive elements</td>
</tr>
<tr>
<td>IRPs</td>
<td>Iron regulatory proteins</td>
</tr>
<tr>
<td>MMT</td>
<td>Methylcyclopentadienyl manganese tricarbonyl</td>
</tr>
<tr>
<td>Mn</td>
<td>Manganese</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>Tf</td>
<td>Transferrin</td>
</tr>
<tr>
<td>TfR1</td>
<td>Transferrin receptor 1</td>
</tr>
<tr>
<td>TH</td>
<td>Tyrosine hydroxylase</td>
</tr>
<tr>
<td>V-ATPase</td>
<td>Vacuolar-ATPase</td>
</tr>
<tr>
<td>Zn</td>
<td>Zinc</td>
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ABSTRACT

Environmental and occupational exposure to heavy metals remains one of the major concerns in public health. Manganese (Mn) is an essential element for a number of important functions in the body, such as carbohydrates metabolism, antioxidant function, bone development, wound healing and proper brain functions. However, increasing levels of Mn pollution from mining, welding, and alloy production shows profound neurotoxic effects, which is significantly associated with neurobehavioral deficits and disturbances resembling Parkinson’s disease. Mn absorption is in part mediated by Fe transporters, such as the DMT1, TfR1 and FPN. Interestingly, recent studies have shown that the levels of these Fe transporters are modified by alcohol treatment and that chronic alcohol consumption increases body Fe stores. However, it is largely unexplored whether or not alcohol exposure influences the transport and neurotoxicity of Mn. My investigation has revealed that alcohol consumption in mice increased Mn uptake into the brain after intranasal instillation of Mn in a dose-dependent manner, due to up-regulation of Fe transporters. In addition, the increased Fe transporters was likely due to down-regulation of hepcidin and increased hypoxia response upon alcohol exposure. Moreover, dopamine and GABA levels were decreased in mice after Mn intranasal instillation, and alcohol further reduced levels of dopamine and GABA in the brain of Mn-instilled mice. The expression of dopaminergic and GABAergic proteins was also modified in Mn-instilled mice, which were exacerbated by alcohol exposure. Finally, I used a model of binge drinking to study the effect of acute alcohol exposure on Mn transport. Like sub-chronic alcohol exposure, binge drinking also increased Mn accumulation in the brain. My study suggests that humans who drink alcohol may have a higher risk of Mn neurotoxicity after inhalation of Mn.
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My final thanks go to my family. My parents, Yuegang Han and Xiaoxia Zhang, have provided me unconditional love and warmest support throughout the past years. My husband Qing has always been helpful and extremely supportive. He is not only my family member, but also my best friend. It is their love and accompaniment that help and encourage me to make this achievement. I also would like to thank my baby boy, William, who has always been waiting to give me a hug no matter how late I get home.

It took me six years to get the three letters ‘Ph.D.’ after my name, but I never regret doing it. I enjoyed every moment of it and I will never forget those days at Northeastern University.
CHAPTER 1. INTRODUCTION

1.1. Manganese (Mn)

Manganese (Mn) is a naturally occurring component of the earth’s crust and it is the second most abundant metals on earth. As an essential element that plays important roles in human physiology, it is required for many vital functions, including regulation of blood sugars and cellular energy, bone growth, defense against free radicals, and blood clotting together with vitamin K [1]. Mn also plays an important role in the modulation of the immune system and metabolism of protein, lipid and carbohydrate metabolism [2-5]. In addition, Mn serves as a co-factor for many enzymes such as transferases (e.g. glycosyltransferase and mannosyl-transferase), hydrolases (e.g. tyrosine hydroxylase), integrins, and glutamine synthetase [6]. In particular, Mn superoxide dismutase (Mn-SOD) catalyzes dismutation of superoxide anion to hydrogen peroxide and molecular oxygen [7].

In the brain, Mn is involved in the stellate process production in astrocytes [8], as well as in the metabolism of brain glutamate to glutamine, a step carried out by the astrocyte-specific enzyme: glutamine synthetase [6,9,10]. As a result, a deficiency in Mn decreases the activity of glutamine synthetase and affects glutamate and dopamine and GABA levels [11,12]. Animals and patients with brain Mn deficiency have shown to be more susceptible to epileptic seizures [13,14]. These results indicate that Mn plays an important role in neurobehavioral and neurochemical function [1].

1.1.1. Mn exposure

Although a proper amount of Mn intake is necessary for human health, exposure to high Mn levels is neurotoxic. Mn can be found in steel, dry-cell batteries, glass and fireworks [15]. Several reported cases of Mn toxicity in
humans have involved drinking Mn contaminated water. It should be noted that elevated levels of Mn in drinking water have been reported in a variety of countries worldwide [16]. For example, in 3% of 982 sampled sources, Mn levels were found to exceed the U.S. health reference levels (300 μg/L) [17]. Moreover, the Tar Creek site in northeast Oklahoma has been recognized as a Superfund Site of National Priority [18]. This raises a huge concern for the health of residents of nearby communities.

Although Mn-polluted water causes health problems, only 3-5% of Mn is absorbed by the gut due to extensive hepatic first-pass elimination of orally-ingested Mn [19]. However, high levels of Mn can be achieved via inhalation that directly transport into the brain due to lack of liver metabolism. The inhalation of Mn contaminated air containing Mn particles is the primary source of excessive Mn exposure for the general population in the United States [20]. For those people who are living close to mining activities and industries using Mn, workers who are working in these industries may be especially vulnerable to exposure to Mn nanoparticles [21,22]. Studies show that the concentrations of Mn particles reach 2–22 mg Mn/m$^3$ around the workplace where symptoms of ‘manganism’ are continuously found [20], whereas only 40 ng Mn/m$^3$ of Mn is detected in urban atmosphere [20]. While the workplace is the most common source of excess inhalation of Mn, frequent inhalation of fumes from welding activities can produce a risk of excess Mn exposure leading to neurological disorders [21,22].

1.1.2. Mn neurotoxicity

As a potent neurotoxicant, Mn has been shown to induce permanent neurological disorders such as memory loss and impair emotional behavior and motor function [23], and these symptoms are often preceded by other lesser symptoms, including irritability, aggressiveness, and hallucinations. For example, Mn exposure is significantly associated with neurobehavioral deficits. Mn neurotoxicity resembles Parkinson’s disease that is known as “manganism” with symptoms that include tremors, walking abnormalities, and facial muscle spasms [24]. The neurotoxic effects resulting from Mn exposure occur in people in industrial settings, such as workers employed
in ferroalloy, smelting, mining and welding and agricultural workers exposed to Mn-containing pesticides [25]. The use of methylcyclopentadienyl manganese tricarbonyl (MMT), an octane enhancer in gasoline, continues to be a huge concern about potential neurological damage due to inhalable Mn particles after combustion [26]. For instance, there is definitive evidence from human studies suggesting that exposure to Mn dust in mines and factories results in inhalation of high levels of Mn that can lead to a series of serious and ultimately disabling neurological effects [27]. Mn toxicity is also significant in children living near secondary smelters and in people drinking contaminated water.

It has been known that Mn affects neural functions by modifying the turnover of several neurotransmitters. For instance, investigations suggest that alterations in dopamine signaling may drive the effects associated with Mn neurotoxicity [28,29]. It has been understood for a long time that dopaminergic signaling is important in regulating motor functions [30-32]. Abnormal dopamine function is found in brain disorders with motor function deficits, such as Parkinson’s disease [33]. Moreover, several studies have demonstrated that levels of dopamine, the major neurotransmitter that is severely reduced in the patients with Parkinson’s disease, are reduced by Mn exposure through in vivo [34] and in vitro [35] studies. These lines of evidence suggest that Mn exposure, at least in part, reduces dopamine levels in the brain [36].

In addition, modifications of gamma-aminobutyric acid (GABA) have recently been implicated in Mn exposure [37]. GABA is the most abundant inhibitory neurotransmitter in the adult brain and is found in the medium spiny neurons of the striatum, mediating the dopaminergic activity in this region [37]. The relationship between Mn exposure and GABA level has been recently studied. There are conflicting results about Mn accumulation and a corresponding increase or decrease in regional levels of GABA [11]. For example, Anderson et al. suggested that brain Mn accumulation is associated with decreased GABA content in the striatum, probably due to impaired GABA uptake [38], whereas Lipe et al. found a significant increase in GABA level in the cerebellum of the adult rats exposed to high doses of Mn [39]. These data suggest that GABA levels in the brain could be different under
different conditions of Mn exposure, such as dose and timing, in a tissue-specific manner. Therefore, environmental exposure to Mn raises more public health concerns and represents a health hazard of clinical significance.

1.1.3. Mn transport in the body

Mn homeostasis involves a complex network of proteins that mediate the absorption, distribution and elimination of Mn. Mn is absorbed by ingestion, inhalation and dermal permeation. It is rapidly absorbed in the gastrointestinal (GI) tract and in the lung. Oral exposure is the most common route for Mn absorption. Once Mn is ingested, it enters cells through passive diffusion or active transport. Studies have revealed that Mn is transported into cells via a mechanism similar to that of iron (Fe) as well as several other divalent transition metals which compete for uptake into a number of different cell systems [40]. There are multiple transporters that are involved in active Mn uptake into the cells, such as divalent metal transporter 1 (DMT1), zinc transporters ZIP8 and ZIP14, citrate transporter, choline transporter, dopamine transporter (DAT), transferrin receptor 1 (TfR1) and calcium (Ca) channels [41] (Figure 1-1), among which DMT1 and TfR are the primary ones [42]. The divalent Mn$^{2+}$ and trivalent Mn$^{3+}$ are the two major Mn species in the blood. Mn$^{2+}$ is primarily transported into enterocytes by DMT1 (Figure 1-1), while Mn$^{3+}$, which can compete with ferric ion for Tf binding, is mainly thought to enter a cell through a TfR-mediated mechanism. DMT1 and TfR are found in most cells, including neurons, microglia, astrocytes and the endothelial cells of the blood-brain barrier (BBB) [43,44]. It is believed that DMT1 can act as a symporter that is energized by the proton-motive force generated by the vacuolar-ATPase (V-ATPase) which extrudes protons from the cell. The uptake of protons from the extracellular space provides the energy for the transport of Mn$^{2+}$ into the cell [45]. Therefore, uptake can be enhanced by modulating membrane potential. Mn$^{3+}$ is bound to Tf in the blood and internalized via endocytosis into cells once the complex binds TfR. After endocytosis, acidification by the low pH of endosomes, releases Mn$^{3+}$ from the Tf/TfR complex [46]. Mn$^{3+}$ in the endosome is then reduced to Mn$^{2+}$ by ferrireductase and is transported into the cytosol by DMT1 [47].
The export is another fundamental process essential for regulating the cellular levels of metals such as copper (Cu), zinc (Zn), Fe and Mn. For example, genetic defects in export transporters cause imbalance of metal metabolism and lead to hereditary disorders such as Wilson’s disease (deficient in ATP7A) and Menke’s disease (ATP7B deficiency) in the case of Cu [48]. However, compared to Mn import, less is known about Mn transporters/channel proteins that participate in Mn efflux. SLC30A10 is recently annotated as a Mn exporter expressed mainly in the liver and brain, whose mutation can cause several neurological disorders, like Parkinsonism and dystonia [49,50]. In addition, several other transporters have been identified as Mn exporters, including ATPase13A2 (ATP13A2), ferroportin (FPN) and the secretory pathway Ca\(^{2+}\)-ATPase 1 (SPCA1) (Figure 1-1) [51]. FPN is the only known mammalian Fe exporter and it is present on all physiologically relevant Fe-exporting tissues including placenta, macrophages, hepatocytes, and intestinal duodenum. FPN is ubiquitously expressed in neurons actively maintaining their Fe-homeostasis. It is not clear, however, how FPN drives iron transport, i.e. whether it is a uniporter, an antiporter or a symporter [52].

Figure 1-1. Manganese transport is mediated by several transporters.
1.1.4. Mn transport in the brain

While Mn is primarily taken up by enterocytes via DMT1 before reaching the adjacent tissues and blood, a major portion of absorbed Mn is excreted via the bile juice by the liver. The rest travels in the blood circulation and distributes in different tissues, including the brain (Figure 1-2) [47]. Mn is able to cross the BBB [53], however, the mechanisms of influx and efflux of Mn across the BBB are not clearly understood yet. Studies have suggested that a major route of Mn ion influx across the BBB may be mediated by Tf. Store-operated calcium channels have also been implicated in brain Mn influx [54]. The role of DMT1 has been debated, but several studies of Mn uptake support the hypothesis that DMT1 takes part in Mn transport across the BBB [47]. Based on the existing knowledge on the localization of DMT1, the proposed pathway for Mn uptake into the blood stream and across the BBB is depicted in Figure 1-3 [47]. DMT1 is an importer energized by the proton-motive force generated by the Vacuolar-ATPase (V-ATPase), which extrudes protons from the cell. The uptake of protons from the extracellular space provides the energy for the transport of Mn\(^{2+}\) cations into the cell. The V-ATPase-generated proton gradient is also responsible for the acidification of endocytic vesicles. Upon acidification, Mn\(^{3+}\) ions released by the Tf-TfR system are converted to Mn\(^{2+}\) ions available for transport by DMT1 [47] (Figure 1-3).
Figure 1-2. Manganese uptake via DMT1.

Figure 1-3. Manganese uptake across the blood brain barrier by Tf and DMT1.
1.1.5. Olfactory Mn uptake

Earlier studies have revealed the mechanism of olfactory Mn transport in animal brain, from the nose along olfactory neuronal pathways to the olfactory bulb after inhalation or intranasal instillation exposures [55-58]. Firstly, intranasally-instilled Mn is transported to the primary, secondary and tertiary olfactory neurons, which leads to high levels of Mn levels in the areas such as the olfactory bulb, the olfactory cortex, the hypothalamus, the thalamus, the hippocampus, and the habenular complex [56]. Mn is then shown to migrate to whole regions of the brain. Thus, the olfactory route circumvents the BBB and provides a direct pathway for Mn to be delivered into the brain. Thompson et al. [59], using a model of DMT1-deficient rats, demonstrated that olfactory uptake of Mn requires DMT1. The function of DMT1 in olfactory Mn absorption suggests that the transport and neurotoxicity of the metal can be modified by the factors that influence Fe status, such as ethanol.

1.1.6. Mn and Fe relationship

It has been discovered that Mn transport can be mediated by several Fe transporters. The intimate relationship between Fe and Mn in human nutrition has been recognized for many years, but the exact mechanism of interaction between Fe and Mn still remains elusive. Accumulating evidence indicates that the absorption of Mn is altered by body Fe status. For example, Fe-deficient anemia increases intestinal absorption of Mn, whereas Fe overload conditions decreases Mn uptake [60,61]. DMT1 is a major transporter that is responsible for intestinal Fe absorption as well as Mn absorption. Its expression is modified by body Fe status [62]. Studies using exogenous expression of DMT1 have indicated that the transporter mediates the uptake of Mn as well as Fe [63]. Divalent Mn and Fe ions are thought to compete for uptake via DMT1 at the apical surface of duodenal enterocytes. In vitro, over-expression of DMT1 in cultured cells results in increased uptake of both Fe and Mn [64]. In support,
it has been reported in human subjects that elevated levels of Mn levels in the blood are correlated with lower serum ferritin levels [65], lower dietary intake of non-heme Fe [66], and higher levels of circulating soluble TfR [67]. Mena et al. [68] also displayed that men and women with Fe deficiency absorb more Mn (as measured by oral $^{54}$MnCl$_2$ administration) than control subjects. Moreover, Thompson et al. [59] showed that DMT1 is localized to both the microvilli and end feet of sustentacular cells of the olfactory epithelium. In addition, the absorption of intranasally-instilled Mn is enhanced in anemic rats, which is associated with increased DMT1 expression in the olfactory epithelium [59]. Together, these results suggest that the neurotoxicity of Mn can be modified by Fe status due to Fe-responsive regulation of Fe transporters.

1.2. Iron (Fe)

Fe is essential for the development and proper function of the brain, including myelination [69], monoamine metabolism [70] and regulation of nitric oxide synthase [71]. Fe is a critical cofactor for tyrosine hydroxylase (TH) and tryptophan hydroxylase, which are enzymes for dopamine and serotonin synthesis, respectively. Fe also regulates homeostasis of GABA [72]. In addition, a recent investigation has demonstrated that Fe supplementation enhances brain synaptic plasticity by activation of N-methyl-D-aspartate (NMDA) receptor, a receptor associated with memory function [73]. In summation, this suggests that altered Fe status in the brain significantly modulates neurotransmission pathways and neural activities.

1.2.1. Fe imbalance and associated disorders

Fe deficiency leads to abnormal cognitive function and behavioral deficits, especially in the early stage of life. For example, Lozoff et al. have noted that the formerly Fe-deficient children exhibit reduced visual-spatial memory function and delays in cognitive processing even though their anemic status was corrected later by Fe
therapy [74]. Diminished mental and motor development is associated with Fe deficiency in infants [75]. In rats, a period of rapid growth occurs in the first 2–3 weeks of postnatal life [76,77], in which Fe demand is very high. Consequently, Fe transport into the brain at this stage is dramatically increased through the BBB via TfR-mediated uptake [78,79], whereas adult rats display slow rates of Fe uptake into the brain. Within the brain, Fe is particularly concentrated in the basal ganglia, an area highly influenced by dopamine metabolism [80,81]. In addition to its region-specific distribution, there is a prioritization of brain Fe distribution during development [82,83]. For instance, after a short period of feeding a low-Fe diet, Fe stores significantly decrease in the cortex and striatum during the mid-late neonatal periods in rodents (equivalent to human ages 6–12 months), but not in the thalamus, which becomes more sensitive to dietary Fe during post-weaning Fe deficiency [75].

In contrast to Fe deficiency, Fe accumulation has been implicated in elevated oxidative stress and in the development of age-related neurodegenerative diseases [84-87]. Brain Fe levels increase with age [88,89]; this has been shown to occur mainly in brain regions that are affected by disease states, including Alzheimer’s, Parkinson’s, and Huntington’s diseases [89]. Fe overload also disrupts neurotransmitter homeostasis. For example, Fe infusions into the substantia nigra impair monoaminergic systems, especially the dopaminergic pathway, to promote motor function deficits resembling Parkinson’s disease [90-92]. The effects of Fe overload on learning and memory deficits have been noted in animals [93-97]. Likewise, Fe overload appears to alter anxiety-like behavior and mood [98,99]. Anxious responses, determined by the elevated plus maze, are observed in adult rats receiving daily intraperitoneal injections of Fe [99]. Other behavioral impairments have been found in rats fed diet containing 20,000 ppm Fe for 12 weeks [98].

1.2.3. Fe transport

Fe homeostasis, in particular, is maintained by dynamic networks of active and passive Fe transport proteins and their regulators that permit essential use while minimizing toxicity of this redox-active metal [100]. No known
regulatory mechanisms of Fe excretion exist [101], and systemic Fe levels are thus primarily controlled through rigorous regulation of dietary Fe absorption [100,101] (Figure 1-4). Similar to Mn, Fe from the diet is reduced by duodenal cytochrome B (DCYTB) before being taken up by DMT1 at the apical site of the duodenal enterocyte. At the basolateral site of the enterocyte, FPN exports Fe into the blood stream where it is oxidized again and binds to Tf. The Tf bound Fe can be transported to the bone marrow for erythrocytes production or for tissue uptake by TfR1. Among Fe regulating proteins, hepcidin is the critical element that controls Fe homeostasis. Hepcidin is a peptide hormone that is primarily produced by the liver. The synthesis of hepcidin synthesis is associated with Fe status [102]. Studies have shown that increased level of hepcidin reduces Fe absorption by the down-regulation of DMT1 and FPN [103]. Moreover, different physiological and pathological conditions such as hypoxia [104], inflammation [105] and metabolic [106,107] processes can modulate hepcidin levels resulting in alteration of Fe availability and absorption. Taken together, since Mn transport is influenced by Fe status, factors that affect Fe levels may also affect Mn transport, such as ethanol.
1.3. Ethanol and its neurotoxicity

In 2012, 87.6% of individuals who were 18 years of age or older, reported that they had consumed alcohol at some point in their lifetime; 71% reported that they had taken alcohol in the past year; and 56.3% in the past month [108]. Ethanol consumption is considered to cause toxicity, by impairing motor function, loss of memory, and confusion [109-112]. People who drink heavily over a long period of time may also experience alcohol addiction, which is a huge concern for human health [113-115]. Ethanol is a central nervous system (CNS) depressant and shares many of the effects of other CNS depressants, such as sedatives, hypnotics and anesthetic agents [116]. Low blood concentrations of ethanol lead to a feeling of euphoria or disinhibition [117]. Alcohol is known to alter membranes as well as ion channels, enzymes, and receptors. Alcohol also binds directly to the...
receptors for acetylcholine, serotonin, GABA, and the NMDA receptors for glutamate. In particular, dopaminergic neurons are extremely sensitive to alcohol. Several studies have demonstrated a dose-response relationship between alcohol intake and dopamine release in the nucleus accumbens [118]. It is also discovered that alcohol can also affect dopamine receptors, especially dopamine D1 (D1DR) and D2 (D2DR) receptors [119,120]. In addition, GABAergic neurotransmission and GABA receptors have long been implicated in mediating the pharmacological effects of alcohol [121]. Several studies demonstrate that alcohol triggers the inhibitory effect of GABA by interacting with GABA receptors, and thus modifies GABAergic metabolism [117]. Together, alcohol induces neurotoxicity by affecting brain function by interacting with several neurotransmitter systems.

1.3.1. Alcohols affects body Fe status

In human studies, alcoholic patients have been reported to exhibit enhanced intestinal Fe absorption [122]. Studies indicate a significant up-regulation of the duodenal DMT1 and FPN proteins in 129/SvJ mice exposed to 10 or 20% ethanol for 1 week [123]. Similarly, C57BL/6 mice treated with ethanol for 1 week displayed significant up-regulation of both duodenal DMT1 and FPN proteins [123]. Furthermore, recent reports defined a role of alcohol in hepcidin expression and intestinal Fe transport [124]. Alcohol-mediated down-regulation of hepcidin expression in the liver leads to increased expression of DMT1 and FPN in the duodenum, which is abolished by the injection of hepcidin peptide. These findings suggest that alcohol up-regulates Fe transporters, including DMT1, which could consequently increase the uptake of Mn. Body Fe stores can also be altered by other factors, such as gene mutations and different lifestyle. Interestingly, several investigations have demonstrated that the increased Fe levels are observed in alcohol consumption [123]. The underlying molecular mechanism has been identified as follows. Hepcidin maintains body Fe status by binding two major Fe transporters, DMT1 and FPN, and facilitating their intracellular degradation [103,125]. Importantly, alcohol consumption inhibits hepcidin production in the liver [126]. Consequently, DMT1 and FPN in the duodenum are up-regulated, leading to an
increase in Fe absorption [127]. These findings suggest that alcohol could modify Fe transporters. However, whether or not alcohol consumption affects Mn transport and homeostasis is largely unexplored.

1.4. Hypothesis and Specific Aims

Mn is an essential element for a number of important functions in the body, such as carbohydrates metabolism, antioxidant function, bone development, wound healing and proper brain functions. However, excessive Mn accumulation in the brain induces neurotoxicity. For example, industrial and environmental exposure to Mn is closely associated with motor deficits and psychiatric disorders in human studies. Several case studies have also found that increasing levels of manganese pollution from mining, welding, and alloy production could induce Mn-related emotional liability and motor function impairment. Mn absorption is in part mediated by Fe transporters, such as the DMT1, TfR1 and FPN. Interestingly, recent studies have shown that the levels of these Fe transporter are modified by alcohol treatment and that chronic alcohol consumption increases body iron stores. However, it is largely unexplored whether or not alcohol exposure influences the transport and neurotoxicity of Mn. My recent investigation has revealed that ethanol consumption in mice increased Mn uptake into the brain after intranasal instillation of Mn in a dose-dependent manner. Therefore, it is possible that humans who drink alcohol may have a higher risk of Mn neurotoxicity after inhalation. The hypothesis of this proposal is that alcohol increases Mn deposition into the brain and thus exacerbates Mn-induced neurotoxicity. Overall, the focus of this dissertation is to better understand the mechanism by which alcohol consumption increases Mn uptake as a potential risk factor for metal-induced neurotoxicity. I accomplished the objective by addressing the following aims:

**Specific Aim 1:** To determine the effect of alcohol on the Mn transport in mice with sub-chronic alcohol drinking.
Tissues including blood, duodenum, liver and brain were collected from mice that were treated with alcohol for 4 weeks (Chapter 2). Metal levels of Mn, Fe, Cu and Zn were analyzed in the blood, liver and brain. Western blot analysis and real-time qPCR were used to quantify metal transporters including DMT1, ZIP14, TfR1, FPN and SLC30A10 in the duodenum, liver and brain.

**Specific Aim 2:** To characterize the influence of alcohol on Mn transport in mice after intranasally-instilled with Mn.

In order to investigate if alcohol further increases Mn deposition into the brain after Mn inhalation, I characterized Mn transport and associated transporters in mice after intranasal instillation of Mn upon alcohol consumption (Chapter 3). Metal levels of Mn, Fe, Cu and Zn were analyzed in the blood, liver and brain. Western blot analysis and real-time qPCR were used to quantify metal transporters including DMT1, ZIP14, TfR1, FPN and SLC30A10 in the duodenum, liver and brain.

**Specific Aim 3:** To investigate the molecular regulation of metal transport upon alcohol exposure.

Possible molecular regulations (Chapter 4) were evaluated in alcohol drinking mice after Mn intranasal instillation. Hepcidin levels in the duodenum, liver and blood were quantified by real-time qPCR. In addition, hypoxia-related genes were evaluated in the brain. Finally, ALT and GGC were evaluated in the liver to determine if liver dysfunction was induced by alcohol.

**Specific Aim 4:** To determine neurotoxicity in after intranasally-instilled with Mn.

Dopamine and GABA levels were evaluated as neurotoxicity markers using HPLC in Mn-instilled alcohol drinking mice (Chapter 5). Dopaminergic and GABAergic proteins were also analyzed in the brain of these mice by Western blot.

**Specific Aim 5:** To determine Mn transport upon acute alcohol exposure using a binge drinking model.
Mn levels (by ICP-MS) and associated transporters including DMT1, ZIP14, TfR1, FPN and SLC30A10 (by qRT-PCR and Western blot) were characterized in mice after binge drinking (Chapter 6). Possible mechanisms of molecular regulation upon binge drinking were also evaluated, such as hepcidin levels, hypoxia-related genes and liver function markers.
CHAPTER 2. EFFECT OF ALCOHOL EXPOSURE ON THE HOMEOSTASIS OF MANGANESE AND METAL TRANSPORTERS IN MICE

2.1. Introduction

Manganese (Mn) homeostasis involves a complex network of proteins that mediate the transport and metabolism of Mn. Accumulating evidence indicates that the absorption of Mn is altered by body Fe status. For instance, Fe-deficient anemia increases the intestinal absorption of Mn, whereas Fe overload conditions decrease Mn uptake [60,61]. Conversely, high levels of Mn inhibit Fe uptake [128]. These observations have been explained by the fact that Mn and Fe share common transporters, including those responsible for intestinal absorption (e.g. DMT1), tissue uptake (e.g. TfR1), and export (e.g. FPN) of Fe and Mn [63]. In fact, DMT1 and TfR1, are the primary transporters for Mn uptake [42]. Divalent Mn is primarily transported into enterocytes by DMT1, while trivalent Mn is mainly transported by TfR1 [42]. In addition, the expression of Fe transporters is modified by body Fe status [62]. Thus, changes in body Fe status could influence Mn transport. Several studies have supported this idea. For example, individuals with Fe deficiency absorb more Mn than control subjects [68], likely due to up-regulation of Fe transporters upon Fe deficiency. In addition, Thompson et al. [59] demonstrated that Fe deficiency increases the olfactory uptake of Mn after intranasal instillation of Mn, which is correlated with increased DMT1 expression in the olfactory epithelium.

Body Fe storage can also be altered by other factors, such as gene mutations and different lifestyle. Interestingly, several investigations have demonstrated that alcohol consumption results in increased Fe levels [123]. Later, the underlying molecular mechanism has been identified as follows. Hepcidin, a master regulator of Fe transport, is synthesized in the liver and released into the circulation when Fe levels are increased [124]. This 25-amino acid peptide maintains body Fe status by binding two major Fe transporters, FPN and DMT1, and facilitating their
intracellular degradation [103,125]. Importantly, alcohol consumption inhibits hepcidin production in the liver [126]. Consequently, FPN and DMT1 in the duodenum are up-regulated, leading to an increase in intestinal absorption of Fe [127]. These findings suggest that alcohol could modify Mn transport by altering the expression of Fe transporters. However, whether or not alcohol consumption affects Mn transport and homeostasis is largely unexplored. Therefore, in this chapter, I quantified the levels of Mn in the blood, liver and brain of mice after alcohol treatment by drinking water as well as the expression levels of the major Fe transporters, including DMT1, TfR1, ZIP14 and FPN.

2.2. Methods

2.2.1. Animals. Animal protocols were approved by the Division of Laboratory Animal Medicine (DLAM) and the Northeastern University-Institutional Animal Care and Use Committee (NU-IACUC). Animals were maintained on a 12:12 hour light-dark cycle and given water ad libitum provided by DLAM.

2.2.2. Sub-chronic ethanol treatment. To quantify the effect of alcohol on Mn, mice (mixed background of C57BL/6 and 129Sv/J strains; male and female; 8-9 weeks old) were divided into four groups based on the ranked body weight. Mice were treated with water, or ethanol (2.5%, 5% and 10%; v/v) for a total of 4 weeks. The higher dose of ethanol was chosen based on Harrison-Findik et al. [123] who reported that alcohol up-regulates Fe transporters (e.g. DMT1) and total body Fe stores in 129/SvJ mice. I also included lower doses of ethanol to explore the alcohol dose-response relationship of Mn accumulation in the brain. The scheme of sub-chronic ethanol treatment is shown in Figure 2-1.
2.2.3. Time course of metal levels during ethanol exposure. To explore the effect of alcohol on Mn status over time, mice (mixed background of C57BL/6 and 129Sv/J strains; male and female; 8-9 weeks old) were divided into ten groups. Mice were treated with water, or ethanol (10%; v/v) for a total of 0-4 weeks, and euthanized every week starting from 0-week to 4-week during ethanol treatment. The exposure and sampling schemes are shown in Figure 2-2.
2.2.4. Euthanasia and tissue collection. After the treatment, animals were euthanized by an overdose of isoflurane, followed by exsanguination and collection of tissues, including blood, liver, duodenum and brain. The duodenum was scraped to obtain the epithelium fractions. The serum was harvested from the blood. All tissues were flash-frozen in liquid nitrogen and stored at -80°C until analysis.

2.2.5. Western blot analysis. The whole brain, as well as liver and duodenum from mice with alcohol exposure were homogenized on ice in RIPA buffer (100 mM Tris, 0.2% SDS, 2% NP40 and 1.0% sodium deoxycholate, pH 7.5) containing protease inhibitor (Complete Mini, Roche; Nutley, NJ, USA). Samples were centrifuged at 16,000 g for 6 min at 4°C. After determination of protein levels by Bradford assay, samples (40 µg of proteins)
were electrophoresed on a 10% SDS-polyacrylamide gel and transferred to either polyvinylidene difluoride (Millipore, Billerica, MA) or nitrocellulose (GE Healthcare, Piscataway, NJ) membrane. After blocking with 5% non-fat milk, the membrane was incubated in rabbit antibody against FPN (1:200; Novus Biologicals, Littleton, CO), TfR1 (1:1,000; Invitrogen, Waltham, MA), ZIP14 (1:1,000; Abcam, Cambridge, MA) and SLC30A10 (1:200; Santa Cruz Biotech, Dallas, TX). As a loading control, the immunoblot was incubated with mouse anti-actin (1:5,000; MP Biomedicals, Solon, OH). The blots were incubated with donkey anti-rabbit secondary antibody (1:1,000; GE Healthcare), donkey anti-goat antibody (1:1,000; Santa Cruz Biotech) or sheep anti-mouse antibody conjugated with HRP (1:5,000; GE Healthcare), followed by chemiluminescence (ECL West Dura, Thermo Scientific) and scanned using Chemidoc System (ChemiDoc XRS, Bio-Rad, Hercules, CA). Relative intensities of protein bands normalized to actin was determined using Image Lab (Bio-Rad, version 4.1).

2.2.6. Real-time qPCR. RNA was isolated from snap-frozen tissues, such as whole brain, liver and duodenum scrapping using TRI reagent (Sigma-Aldrich, St. Louis, MO, USA) as per the manufacturer’s instructions. RNA (1 µg) was reverse transcribed into cDNA, which was used for real-time polymerase chain reaction assays. The iScript™ reverse transcription supermix and iTaq™ universal SYBR® green supermix were obtained from Bio-Rad (Hercules, CA). Primers were obtained from Eurofins, MWG Operon (Huntsville, AL) and sequences of each primer were shown in Table 2.1. Amplification of the DNA was programmed at 95°C for 2 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min. The expression level of each gene was normalized to that of actin and analyzed by the comparative Ct method (2^ΔΔCt) [129].
### Table 2-1. Primer sequences of metal transporters.

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<th>Gene</th>
<th>Sequence</th>
<th>Reference</th>
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<td>CGTACCGCTGGGACTGA</td>
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<tr>
<td></td>
<td>Reverse</td>
<td>GTCATCTGGACACACCACTGAGTCA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>[130]</td>
</tr>
<tr>
<td>TfR1</td>
<td>Forward</td>
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<tr>
<td></td>
<td>Reverse</td>
<td>AGATACATAGGGCGACAGGAA</td>
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<tr>
<td></td>
<td></td>
<td>[131]</td>
</tr>
<tr>
<td>FPN</td>
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<td></td>
<td>Reverse</td>
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<td></td>
<td></td>
<td>[132]</td>
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<td>SLC30A10</td>
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<td>Reverse</td>
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<tr>
<td></td>
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<td>[133]</td>
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<td>Zip14</td>
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</tbody>
</table>

#### 2.2.7. Metal analysis. Inductively-coupled plasma mass spectrometry (ICP-MS) was used to determine metal levels in blood and tissues, such as liver and brain. Briefly, wet tissues (brain, liver and blood) were weighed and digested in 1 ml of acid solution (20% nitric acid for brain and liver; 10% nitric acid for blood; Trace metal grade; Fisher Scientific) for 1 hour at 125°C in a dry bath. Following the digestion, double-distilled water was added to the samples, to a total volume of 10 mL, followed by filtration through a syringe filter; the amount of essential transition metals (e.g. Fe and Mn) were then quantified using ICP-MS. Metal concentrations in the samples were determined by an external calibration method based on ICP-MS standard solutions (ICP-MS calibration standard 3-A; High Purity Standards, City, State) that range from 0 to 5,000 ng/mL. Data were presented as ppm.

#### 2.2.8. Statistical analysis. In the sub-chronic alcohol drinking experiments, there is one factor (alcohol) and four groups in total. Therefore, comparisons between these groups were performed by one-way ANOVA. In the time course study, statistical analysis was performed for water or alcohol drinking groups at each time point, therefore, Student’s *t*-test was employed. Differences were considered significant at *p* < 0.05. Values were reported as means ± SEM.
2.3. Results

2.3.1. Physiological parameters in mice with alcohol consumption. To explore whether the growth is affected in mice treated with alcohol, body weight and food/water consumption were measured (Figure 2-3). There was no difference in body weight between mice with water drinking and alcohol drinking. In addition, food consumption and water consumption showed were similar in mice exposed to alcohol compared to water drinking controls over four weeks of treatment. Therefore, these results suggest that the growth of mice with alcohol drinking (10%, v/v) for 4 weeks was normal.

![Physiological parameters](image)

**Figure 2-3. Physiological parameters of alcohol drinking mice.**

Mice (mixed background of C57BL/6 and 129Sv/J strains; male and female; 8-9 weeks old) were treated with ethanol (10%; v/v) or water for 4 weeks. Body weight (Figure 2-3A), food intake (Figure 2-3B) and water drinking (Figure 2-3C) were measured weekly and showing as means± SEM. Data were analyzed using Student’s t-test. * p < 0.05.
2.3.2. Systemic metal levels are elevated in mice with alcohol consumption. In alcohol-treated mice, Fe levels in the blood and liver were elevated in a dose-dependent manner (Figure 2-4 and 2-5; ethanol effect, p=0.006 (blood) and p<0.001 (liver)). Similarly, Mn levels in the blood and liver also increased after alcohol exposure (Figure 2-4 and 2-5; ethanol effect, p=0.047 (blood), p=0.031 (liver)). However, copper (Cu) and zinc (Zn) levels did not change upon alcohol exposure in either blood or liver. Taken together, these results suggest that alcohol exposure increases body Mn and Fe stores.

Figure 2-4. Metal levels are elevated in the blood of mice with alcohol consumption.
Mice (mixed background of C57BL/6 and 129Sv/J strains; male and female; 8-9 weeks old) were treated with ethanol 2.5 %, 5% or 10 %; v/v) or water for 4 weeks. After euthanasia, Mn, Fe, Cu and Zn levels in the blood were determined by inductively coupled plasma mass spectrometry (ICP-MS). Data were presented as means ± SEM (n= 7-8 per group) and were analyzed using one-way ANOVA. * p < 0.05.
Figure 2-5. Metal levels are elevated in the liver of mice with alcohol consumption.
Mice (mixed background of C57BL/6 and 129Sv/J strains; male and female; 8-9 weeks old) were treated with ethanol (2.5 %, 5% or 10 %; v/v) or water for 4 weeks. After euthanasia, Mn, Fe, Cu and Zn levels in the liver were determined by inductively coupled plasma mass spectrometry (ICP-MS). Data were presented as means ± SEM (n= 7-8 per group) and were analyzed using one-way ANOVA. * p < 0.05.

2.3.3. Metal levels are elevated in the brain of mice with alcohol consumption. Both Fe and Mn in the brain increased with alcohol in a dose-dependent fashion (Fig 2-6; ethanol effect, p=0.011 (Mn), p=0.041 (Fe)). Again, neither Cu nor Zn levels were altered by alcohol exposure. Our results demonstrate that alcohol exposure modifies not only systemic homeostasis of Mn and Fe, but also the transport of these two metals in the brain.
Figure 2-6. The levels of metals in the brain from mice with alcohol treatment.

Mice (mixed background of C57BL/6 and 129Sv/J strains; male and female; 8-9 weeks old) were treated with ethanol (2.5%, 5% or 10%; v/v) or water for 4 weeks. After euthanasia, Mn, Fe, Cu and Zn levels in the brain were determined by inductively coupled plasma mass spectrometry (ICP-MS). Data were presented as means ± SEM (n = 7-8 per group) and were analyzed using one-way ANOVA. *p < 0.05.

2.3.4. Kinetics of systemic metal levels in mice with alcohol consumption. Next, I explored the effect of the duration of alcohol exposure on levels of Mn and Fe. In the blood, no difference was found in mice with water drinking from 0 to 4 weeks (Figure 2-7). Both Mn (p = 0.035) and Fe (p = 0.004) were increased significantly starting from the third week of alcohol treatment, while Cu and Zn did not change over four weeks of treatment. Similar results were found in the liver (Figure 2-8), where both Mn (p = 0.049) and Fe (p = 0.013) were increased significantly starting from the second week of alcohol treatment with no change of Cu and Zn for the four weeks of treatment. These results indicate that alcohol simultaneously increases Mn and Fe levels in the tissues as early as 2-3 weeks of alcohol treatment.
Figure 2-7. Kinetics of metal levels in the blood mice with alcohol consumption.

Mice (mixed background of C57BL/6 and 129Sv/J strains; male and female; 8-9 weeks old) were treated with ethanol (10%; v/v) or water for 1-4 weeks. After euthanasia, blood was collected for metal analysis by ICP-MS. Data were presented as means ± SEM and were analyzed using one-way ANOVA. * p < 0.05.
Figure 2- 8. Kinetics of metal levels in the liver mice with alcohol consumption.

Mice (mixed background of C57BL/6 and 129Sv/J strains; male and female; 8-9 weeks old) were treated with ethanol (10%; v/v) or water for 1-4 weeks. After euthanasia, liver was collected for metal analysis by ICP-MS. Data were presented as means ± SEM and were analyzed using one-way ANOVA. * p < 0.05.

2.3.5. Kinetics of metal levels in the brain of mice with alcohol consumption. To characterize the time course of brain metal homeostasis during alcohol exposure, I measured the metal levels in the brain of mice that treated with ethanol (10%; v/v) or water for a total of 0 to 4 weeks (Figure 2-9). In the brain, both Mn and Fe levels increased significantly at the fourth week of alcohol treatment, while copper and zinc status did not change over the four weeks of treatment. These results indicated that alcohol up-regulates Mn and Fe levels in the brain at the fourth week of treatment.
Figure 2-9. Kinetics of metal levels in the brain mice with alcohol consumption.

Mice (mixed background of C57BL/6 and 129Sv/J strains; male and female; 8-9 weeks old) were treated with ethanol (10%; v/v) or water for 1-4 weeks. After euthanasia, brain was collected for metal analysis by ICP-MS. Data were presented as means ± SEM and were analyzed using one-way ANOVA. * p < 0.05.

2.3.6. Fe transporters are elevated in the duodenum and liver of mice with alcohol consumption. To evaluate whether increased Fe and Mn levels in the body were due to up-regulation of their transporters in response to alcohol consumption, I determined the protein and/or mRNA expressions of DMT1, Tfr1, ZIP14, FPN and SLC30A10 in the duodenum and liver of mice that were exposed to alcohol (10%, v/v) for four weeks compared to water drinking controls by western blot analysis (Figure 2-10) and real-time qPCR (Figure 2-11). Firstly, the protein levels of Fe importers including Tfr1 (Figure 2-10; 92% increase in the duodenum, p=0.047; 127% increase in the liver, p=0.003) and ZIP14 (Figure 2-10; 73% increase in the duodenum, p=0.046; 55% increase...
in the liver, p=0.049) as well as metal exporter FPN (Figure 2-10; 150% increase in the duodenum, p<0.001; 92% increase in the liver, p=0.027) were up-regulated upon alcohol exposure in the duodenum and liver, respectively. Consistent with protein expressions, mRNA levels of Fe importers DMT1 (Figure 2-11; 23% increase in the duodenum, p=0.033; 17% increase in the liver, p=0.042), TfR1 (Figure 2-11; 30% increase in the duodenum, p=0.024; 88% increase in the liver, p=0.041) and FPN (Figure 2-11; 48% increase in the duodenum, p=0.017; 81% increase in the liver, p=0.030) were also up-regulated in the duodenum and liver, respectively. However, Mn specific exporter, SLC30A10 was not altered in protein or mRNA levels in the duodenum or liver of mice with alcohol drinking. Combined, these data suggest increased Fe and Mn levels in the body of mice with alcohol treatment is possibly due to increased absorption and tissue uptake by up-regulation of Fe transporters.
Figure 2-10. Metal transporters in the duodenum and liver of mice after alcohol consumption.

Mice (mixed background of C57BL/6 and 129Sv/J strains; male and female; 8-9 weeks old) were treated with ethanol (10%; v/v) or water for 4 weeks. After euthanasia, duodenum and liver were collected for protein quantification. TfR1, ZIP14, FPN and SLC30A10 levels in the duodenum (Figure 2-10A) and liver (Figure 2-10B) were quantified by western blot analysis. Data were presented as means ± SEM (n= 4 per group) and were analyzed using Student’s t-test. * p < 0.05.
Figure 2-11. mRNA of metal transporters in the duodenum and liver of mice after alcohol consumption. Mice (mixed background of C57BL/6 and 129Sv/J strains; male and female; 8-9 weeks old) were treated with ethanol (10% v/v) or water for 4 weeks. After euthanasia, duodenum and liver were collected for mRNA quantification. DMT1, Tfr1, FPN and SLC30A10 levels in the duodenum (Figure 2-11A) and liver (Figure 2-11B) were quantified by real-time qPCR. Data were presented as means ± SEM (n= 4 per group) and were analyzed using Student’s t-test. * p < 0.05.

2.3.7. Fe transporters are elevated in the brain of alcohol drinking mice. To further evaluate if Fe and Mn transporters in the brain are modified in the brain after alcohol consumption, protein and/or mRNA expression of
DMT1, TfR1, ZIP14, FPN and SLC30A10 in the whole brain of mice with 4-wk of alcohol (10%; v/v) or water drinking were determined. Firstly, the protein levels of Fe importers including TfR1 (Figure 2-12; 94% increase; p=0.017) and ZIP14 (Figure 2-12; 124% increase; p=0.014) as well as metal exporter FPN (Figure 2-12; 90% increase; p<0.001) were up-regulated upon alcohol exposure. Consistent with protein expressions, mRNA levels of Fe importers, DMT1 (Figure 2-13; 62% increase; p=0.048), TfR1 (Figure 2-13; 53% increase; p=0.044) and FPN (Figure 2-13; 56% increase; p=0.043) were also up-regulated. Although the protein levels of SLC30A10 were not altered in the brain of mice with alcohol drinking, mRNA levels of the exporter were increased upon alcohol exposure (71% increase; p=0.035). Collectively, my results indicate that up-regulation of Fe transporters is a potential mechanism by which increased the amount of Mn is transported into the brain with alcohol consumption.

**Figure 2-12.** Protein expressions of metal transporters in the brain of mice after alcohol consumption.
Mice (mixed background of C57BL/6 and 129Sv/J strains; male and female; 8-9 weeks old) were treated with ethanol (10%; v/v) or water for 4 weeks. After euthanasia, whole brain was collected for protein quantification.
TfR1, ZIP14, FPN and SLC30A10 levels in the brain were quantified by Western blot. Data were presented as means ± SEM (n= 4 per group) and were analyzed using Student’s t-test. * p < 0.05.

Figure 2-13. mRNA expressions of metal transporters in the brain of mice after alcohol consumption.
Mice (mixed background of C57BL/6 and 129Sv/J strains; male and female; 8-9 weeks old) were treated with ethanol (10%; v/v) or water for 4 weeks. After euthanasia, whole brain was collected for mRNA expression analysis. DMT1, TfR1, FPN and SLC30A10 levels in brain were quantified by real-time qPCR. Data were presented as means ± SEM (n= 4 per group) and were analyzed using Student’s t-test. * p < 0.05.

2.4. Discussion

Increasing number of studies show that body Fe stores is increased by alcohol exposure. Patients with alcoholic liver diseases frequently exhibit increased Fe accumulation in the blood and liver. [134-136]. Moreover, Ioannou et al. demonstrated increased body Fe stores was also found in those who even only consumed moderate level of alcohol [137]. These results are consistent with my study that elevated Fe content in the blood and liver of alcohol-
treated mice in a dose-dependent manner. In addition, my results clearly demonstrate that alcohol consumption increased Mn accumulation and Fe stores, but not Cu or Zn, in tissues. I ruled out a possibility that increased Fe and Mn levels in alcohol treated mice are due to increased food/water consumption in alcohol drinking mice compared to alcohol drinking controls. In addition, no change in Cu/Zn levels in the brain and tissues of mice with alcohol drinking indicates that the difference of Fe and Mn was unlikely from different food/water intake. I further investigated Fe and Mn transporters in mice upon alcohol exposure.

To select the best exposure duration of alcohol treatment, I conducted a kinetic study of alcohol treatment in mice over four weeks. Although Mn and Fe started to accumulate in the liver and blood since the second week of alcohol treatment, the metal levels in the brain significantly increased only after four weeks of alcohol treatment, suggesting that Mn and Fe are loaded in the body earlier than brain upon alcohol drinking. Since my purpose is to mainly investigate the metal transport in the brain and metal-induced neurotoxicity, I chose to expose animals with four weeks of alcohol in the later experiments.

I found increased duodenal protein and/or mRNA expression of DMT1, TfR1, ZIP14 and FPN in alcohol-exposed mice, suggesting increased Mn and Fe absorption. Congruently, Harrison-Findik et al. [123] reported a significant up-regulation of duodenal DMT1 and FPN proteins in mice exposed to 10 or 20% ethanol for 1 week. Although we were not be able to detect proteins of DMT1 due to antibody quality issues, the mRNA of DMT1 was up-regulated in mice with alcohol drinking compared to water drinking controls. There are several lines of evidence that mRNA levels are correlated with changes in the expression of its protein [138]. For example, Zoller et al. demonstrated that both mRNA and protein levels of DMT1 are decreased in the duodenum of patients with primary Fe overload hemochromatosis, unchanged in people with secondary Fe overload, and increased in patients with Fe deficiency [138]. Therefore, it is predicted that the protein expression of DMT1 should also be up-regulated in the duodenum of mice with alcohol drinking. Moreover, I demonstrated up-regulation of DMT1, TfR1 and ZIP14 in the liver of alcohol-exposed mice, suggesting increased Mn and Fe uptake into the liver.
Similarly, Kohgo et al. demonstrated that hepatic TfR1 protein is up-regulated in patients with alcoholic liver disease as well as in rat primary hepatocyte culture with alcohol treatment [139]. Mixed results exist on ZIP14 expression in alcohol exposure. While decreased ZIP14 protein along with Zn deficiency is found in mice fed Lieber-DeCarli ethanol liquid diet for up to 8 weeks [140], alcohol consumption is associated with endoplasmic reticulum stress [141], which enhances the expression of ZIP14 [142]. Unchanged Zn levels in the blood and liver of alcohol drinking mice in my study could be due to dynamic regulation of other Zn transporters, including ZIP4, ZIP7, ZIP8, ZIP14, and ZnT7 [143]. It should be noted that elevated Fe and Mn could be due to increased absorption and/or decreased excretion of these metals. Up-regulation of metal importers in the duodenum suggests increased Fe and Mn absorption in my study. Future studies are needed to directly determine the contribution of the absorption and excretion of Mn, Fe, and Zn during alcohol exposure to the steady-state levels of these metals by characterizing the pharmacokinetics of their radioisotopes.

2.5. Conclusions

In this chapter, I investigated the effect of alcohol on Fe and Mn transport in various tissues. My results demonstrate that sub-chronic alcohol consumption increases Mn and Fe levels in the body and brain, possibly due to up-regulated metal transporters. Therefore, individuals who consume more alcohol may have a higher risk of Mn-induced neurotoxicity. To explore whether alcohol further increases Mn accumulation after Mn inhalation, Mn transport in mice exposed to alcohol after Mn intranasal instillation was examined in the following chapter.
CHAPTER 3. THE EFFECT OF ALCOHOL ON OLFACTORY TRANSPORT OF MANGANESE AFTER INTRANASAL INSTILLATION OF THE METAL

3.1. Introduction

Environmental and occupational exposure to heavy metals remains one of the major public health concerns. Although Mn is an essential element for a number of important functions in the body, such as carbohydrates metabolism, antioxidant function, bone development, wound healing and proper brain function, Mn in excess shows profound toxic effects on the brain. It is significantly associated with neurobehavioral deficits and disturbances resembling Parkinson’s disease, as evidenced by deficits in memory and motor function, and is associated with the development of psychiatric disorders [23]. For example, the Tar Creek site in northeast Oklahoma has been recognized as a Superfund Site of National Priority. Over 2900 acres, more than 150 million tons of mining waste known as “chat” containing Mn, lead, and zinc, contaminate air and drinking water, raising a serious concern for the health of residents of nearby communities [144]. Moreover, Mn toxicity is significant in children living near secondary smelters [145], people drinking contaminated water, workers employed in mining and Mn ore processing, and agricultural workers exposed to Mn-containing pesticides [146]. The use of methylcyclopentadienyl Mn tricarbonyl (MMT), an octane enhancer (HiTEC3000, Afton), in gasoline has gained great attention regarding potential neurological damage due to inhalable Mn particles after combustion [147]. Mn intoxication has also been observed in children under long-term parenteral nutrition and in patients with chronic liver diseases [148].

Mn neurotoxicity occurs frequently in people who inhale Mn-contaminated air, such as workers in mining, welding and smelting [25]. It has been demonstrated that inhaled Mn can be directly transported into the brain. Several studies in animals have indicated the mechanism of olfactory Mn transport into the brain from the nose.
along olfactory neuronal pathways to the olfactory bulb after inhalation or intranasal instillation exposures [55-58]. In addition, my study in chapter 2 showed that alcohol consumption increased Mn and Fe level in the body including brain, possibly through up-regulation of Fe transporters. To evaluate whether the levels of Mn further increase after Mn intranasal instillation upon alcohol sub-chronic drinking, I quantified the levels of Mn and its transporter levels in Mn-instilled, alcohol-drinking mice.

3.2. Methods

3.2.1. Animals. Animal protocols were approved by the Division of Laboratory Animal Medicine (DLAM) and the Northeastern University-Institutional Animal Care and Use Committee (NU-IACUC). Animals were maintained on a 12:12 hour light-dark cycle and given water *ad libitum* provided by DLAM.

3.2.2. Intranasal Mn instillation. To mimic Mn inhalation in humans, mice (mixed background of C57BL/6 and 129Sv/J strains; male and female; 8-9 weeks old) were intranasally-instilled with MnCl₂ solutions (0.5 or 5 mg/kg as Mn) or saline for three weeks. The dose of Mn (5 mg/kg) and exposure time (3 weeks) were chosen based on my previous study [149] as well as other reports ranging from 0.2 mg/kg to 10 mg/kg [150,151]. The treatment scheme of Mn intranasal instillation is shown in **Figure 3-1**.
3.2.3. Ethanol and Mn treatments. To evaluate the effect of alcohol consumption on olfactory Mn transport, mice (mixed background of C57BL/6 and 129Sv/J strains; male and female; 8-9 weeks old) were treated with water, or ethanol (10%; v/v) for a total of 4 weeks. Since the second week of alcohol treatment, MnCl₂ solutions (5 mg/kg as Mn) or saline were intranasally instilled for 3 weeks. The treatment scheme of Mn intranasally-instilled mice upon ethanol consumption is shown in Figure 3-2.
3.2.4. **Euthanasia and tissue collection.** After the treatment, animals were euthanized by an overdose of isoflurane, followed by exsanguination and collection of tissues, including blood, liver, duodenum and brain. The duodenum was scraped to obtain the epithelium fractions. All tissues were flash-frozen in liquid nitrogen and stored at -80°C until analysis.

3.2.5. **Western blot analysis.** The whole brain, as well as liver and duodenum from mice with alcohol exposure were homogenized on ice in RIPA buffer (100 mM Tris, 0.2% SDS, 2% NP40 and 1.0% sodium deoxycholate, pH 7.5) containing protease inhibitor (Complete Mini, Roche; Nutley, NJ, USA). Samples were centrifuged at 16,000 g for 6 min at 4°C. After determination of protein levels by Bradford assay, samples (40 µg of proteins) were electrophoresed on a 10% SDS-polyacrylamide gel and transferred to either polyvinylidene difluoride (Millipore, Billerica, MA) or nitrocellulose (GE Healthcare, Piscataway, NJ) membrane. After blocking with 5% non-fat milk, the membrane was incubated the membrane was incubated in rabbit antibody against FPN (1:200; Novus Biologicals, Littleton, CO), TfR1 (1:1,000; Invitrogen, Waltham, MA), ZIP14 (1:1,000; Abcam, Cambridge, MA) and SLC30A10 (1:200; Santa Cruz Biotech, Dallas, TX). As a loading control, the immunoblot was incubated with mouse anti-actin (1:5,000; MP Biomedicals, Solon, OH). The blots were incubated with donkey anti-rabbit secondary antibody (1:1,000; GE Healthcare), donkey anti-goat antibody (1:1,000; Santa Cruz Biotech) or sheep anti-mouse antibody conjugated with HRP (1:5,000; GE Healthcare), followed by chemiluminescence (ECL West Dura, Thermo Scientific) and scanned using Chemidoc System (ChemiDoc XRS, Bio-Rad, Hercules, CA). Relative intensities of protein bands normalized to actin was determined using Image Lab (Bio-Rad, version 4.1).

3.2.6. **Real-time qPCR.** RNA was isolated from snap-frozen tissues, such as whole brain, liver and duodenum scrapping using TRI reagent (Sigma-Aldrich, St. Louis, MO, USA) as per the manufacturer’s instructions. RNA (1 µg) was reverse transcribed into cDNA, which was used for real-time polymerase chain reaction assays. The iScript™ reverse transcription supermix and iTaq™ universal SYBR® green supermix were obtained from Bio-
Rad (Hercules, California). Primers were obtained from Eurofins, MWG Operon (Huntsville, AL) and sequences of each primer were shown in table 2.1. Amplification of the DNA was programmed at 95°C for 2 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. The expression level of each gene was normalized to that of actin and analyzed by the comparative Ct method ($2^{-\Delta Ct}$) [129].

Table 3-1. Primer sequences of metal transporters.

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<th>Gene</th>
<th>Sequence</th>
<th>Reference</th>
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<tr>
<td>DMT1</td>
<td>Forward: CGTACCGCCTGGGACTGA</td>
<td>[130]</td>
</tr>
<tr>
<td></td>
<td>Reverse: GTCATCTGGACACCACCTGAGTCA</td>
<td></td>
</tr>
<tr>
<td>TfR1</td>
<td>Forward: TGGGTCTAAGTCTACAGTGGC</td>
<td>[131]</td>
</tr>
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<td></td>
<td>Reverse: AGATACATAGGGCGACAGGAA</td>
<td></td>
</tr>
<tr>
<td>FPN</td>
<td>Forward: TTGCAGGAGTCATTGCTGCTA</td>
<td>[132]</td>
</tr>
<tr>
<td></td>
<td>Reverse: TGGAGTTCTGCAACCACATTGAT</td>
<td></td>
</tr>
<tr>
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<td>[133]</td>
</tr>
<tr>
<td></td>
<td>Reverse: AGCTCTGATGTCTGCTCATCTGTTGA</td>
<td></td>
</tr>
</tbody>
</table>

3.2.7. Metal analysis. Inductively-coupled plasma mass spectrometry (ICP-MS) was used to determine metal levels in blood and tissues such as liver and brain. Briefly, wet tissues (brain, liver and blood) were weighed and digested in 1 ml of acid solution (20% nitric acid for brain and liver; 10% nitric acid for blood; Trace metal grade; Fisher Scientific) for 1 hour at 125°C in a dry bath. Following the digestion, double-distilled water was added to the samples, to a total volume of 10 ml, followed by filtration through a syringe filter; the amount of essential transition metals (e.g. Fe and Mn) were then be quantified using ICP-MS. Metal concentrations in the samples were determined by an external calibration method based on ICP-MS standard solutions (ICP-MS calibration standard 3-A; High Purity Standards) that ranging from 0 to 5,000 ng/ml. Data were presented as ppm.

3.2.8. Statistical analysis. In this study, statistical analysis of Mn instillation study was performed by one-way ANOVA; analysis of Mn instillation with alcohol exposure study was performed by two-way ANOVA, followed...
by the post-hoc method, Holm-Sidak. Differences were considered significant at $p < 0.05$. Statistical analysis values were reported as means ± SEM.

### 3.3. Results

**3.3.1. Mn levels are increased in the brain of mice intranasally-instilled with Mn.** To confirm that Mn can be taken up from the olfactory epithelium into the brain, the metal levels in the brain of mice with Mn intranasal instillation were examined (Figure 3-3). Mn levels in the brain was increased by 25% in mice instilled with 0.5 mg/kg of MnCl$_2$, and by 108% in mice instilled with 5 mg/kg of MnCl$_2$, compared with saline-instilled control mice ($p<0.001$). Fe levels in the brain did not alter in mice intranasally-instilled with 0.5 mg/kg or 5 mg/kg of Mn solution. My results indicate that Mn can be taken up directly into the brain by Mn intranasal instillation.

![Metal levels in the brain](image)

**Figure 3-3. The levels of Mn and Fe in the brain of Mn intranasally instilled mice.**

Mice (mixed background of C57BL/6 and 129Sv/J strains; male and female; 8-9 weeks old) were intranasally instilled with MnCl$_2$ solution (0.5 mg/kg or 5 mg/kg) or saline daily for 3 weeks. After euthanasia, Mn and Fe
levels in the brain were determined by inductively coupled plasma mass spectrometry (ICP-MS). Data were presented as means ± SEM (n= 7-8 per group) and were analyzed using one-way ANOVA. * p < 0.05.

3.3.2. Alcohol consumption increases Mn levels in the brain of mice after intranasal instillation of Mn. To evaluate whether alcohol exposure affects olfactory transport of Mn into the brain, mice that were exposed to ethanol by drinking water (10%) were intranasally instilled with Mn, and metal levels were quantified in different tissues. In the blood (Figure 3-4) and liver (Figure 3-5), although ethanol drinking increased Fe and Mn status, Mn instillation did not modify metal levels compared to the saline-instilled control group. These results indicate that intranasal administration of Mn does not change systemic Mn status. In contrast, intranasal Mn instillation increased Mn levels in the brain by 150%, compared to the saline-instilled group (Figure 3-6; p=0.047), which were further increased by 53% upon alcohol exposure (p=0.021). However, Fe levels in the brain increased upon alcohol exposure, but not by Mn intranasal instillation (ethanol effect, p<0.001). Cu and Zn levels were not altered by either Mn instillation or alcohol treatment.
Figure 3-4. Metal levels are elevated in the blood of mice with alcohol consumption.

Mice (mixed background of C57BL/6 and 129Sv/J strains; male and female; 8-9 weeks old) were treated with water, or ethanol (10%; v/v) for a total of 4 weeks. Since the second week of alcohol treatment, MnCl₂ solutions (5 mg/kg as Mn) or saline were intranasally instilled for 3 weeks. After euthanasia, Mn, Fe, Cu and Zn levels in the blood were determined by inductively coupled plasma mass spectrometry (ICP-MS). Data were presented as means ± SEM (n=7-8 per group) and were analyzed using two-way ANOVA. * p < 0.05.

Figure 3-5. Metal levels are elevated in the liver of mice with alcohol consumption.

Mice (mixed background of C57BL/6 and 129Sv/J strains; male and female; 8-9 weeks old) were treated with water, or ethanol (10%; v/v) for a total of 4 weeks. Since the second week of alcohol treatment, MnCl₂ solutions (5 mg/kg as Mn) or saline were intranasally instilled for 3 weeks. After euthanasia, Mn, Fe, Cu and Zn levels in the liver were determined by inductively coupled plasma mass spectrometry (ICP-MS). Data were presented as means ± SEM (n=7-8 per group) and were analyzed using two-way ANOVA. * p < 0.05.
Figure 3-6. Elevated metal levels in the brain of alcohol drinking mice after Mn intranasal instillation.

Mice (mixed background of C57BL/6 and 129Sv/J strains; male and female; 8-9 weeks old) were treated with water, or ethanol (10%; v/v) for a total of 4 weeks. Since the second week of alcohol treatment, MnCl₂ solutions (5 mg/kg as Mn) or saline were intranasally instilled for 3 weeks. After euthanasia, Mn, Fe, Cu and Zn levels in the brain were determined by inductively coupled plasma mass spectrometry (ICP-MS). Data were presented as means ± SEM (n= 7-8 per group) and were analyzed using two-way ANOVA. * p < 0.05.

3.3.3. Alcohol drinking up-regulates protein expression of metal transporters in mice. To examine if increased Mn and Fe concentrations in alcohol-exposed mice are associated with increased expression of metal transporters, I characterized protein levels of metal transporters by western blot analysis (Figure 3-7 to Figure 3-9). In the duodenum (Figure 3-7) and liver (Figure 3-8), alcohol exposure increased expression of TfR1, ZIP14 and FPN compared to the water-drinking control group. However, Mn instillation did not alter expression of these transporters in the duodenum or liver. Similarly, TfR1, ZIP14 and FPN were up-regulated in the brain of alcohol-exposed mice compared to the water control group (Figure 3-9; p=0.008 (TfR1), p=0.028 (ZIP14), p=0.005
(FPN)), while Mn instillation did not alter the expression of these transporters. In addition, the expression of an Mn-exporter SLC30A10 was not altered by alcohol exposure or Mn instillation in the duodenum, liver or brain. These results suggest that ethanol consumption up-regulates the activity of Fe/Mn transporters and thereby increases Mn and Fe levels in blood, liver and brain.

**Figure 3-7. Metal transporters in the duodenum of alcohol drinking mice with Mn intranasal instillation.**

Mice (8-9 weeks old) were given facility water or ethanol-containing water (10%; v/v) for a total of 4 weeks. Since the second week of alcohol treatment, MnCl₂ solutions (5 mg/kg) or saline were intranasally instilled for three weeks prior to euthanasia, followed by tissue collection. The protein levels of Fe and Mn transporters including transferrin receptor 1 (TfR1), SLC39A14 (Zip 14), ferroportin (FPN) and SLC30A10 in the duodenum were evaluated by Western blot analysis. The expression level of each protein was normalized to that of actin.
Data were analyzed using two-way ANOVA and shown as ratios to water drinking mice with saline instillation (mean ± SEM; n=4/group).

Figure 3-8. Metal transporters in the liver of alcohol drinking mice after Mn intranasal instillation.

Mice (8-9 weeks old) were given facility water or ethanol-containing water (10%; v/v) for a total of 4 weeks. Since the second week of alcohol treatment, MnCl$_2$ solutions (5 mg/kg) or saline were intranasally instilled for three weeks prior to euthanasia, followed by tissue collection. The protein levels of Fe and Mn transporters including transferrin receptor 1 (TfR1), SLC39A14 (Zip 14), ferroportin (FPN) and SLC30A10 in the liver were evaluated by Western blot analysis. The expression level of each protein was normalized to that of actin. Data were analyzed using two-way ANOVA and shown as ratios to water drinking mice with saline instillation (mean ± SEM; n=4/group).
3.3.4. Alcohol drinking up-regulates mRNA expression of metal transporters in mice. To examine if mRNA levels of metal transporters are correlate with protein up-regulation upon alcohol exposure with Mn intranasal instillation, I characterized mRNA expressions of metal transporters by real-time qPCR (Figure 3-10 to Figure

**Figure 3-9. Metal transporters in the brain of alcohol drinking mice after Mn intranasal instillation.**

Mice (8-9 weeks old) were given facility water or ethanol-containing water (10%; v/v) for a total of 4 weeks. Since the second week of alcohol treatment, MnCl$_2$ solutions (5 mg/kg) or saline were intranasally instilled for three weeks prior to euthanasia, followed by tissue collection. The protein levels of Fe and Mn transporters including transferrin receptor 1 (TfR1), SLC39A14 (Zip 14), ferroportin (FPN) and SLC30A10 in the brain were evaluated by Western blot analysis. The expression level of each protein was normalized to that of actin. Data were analyzed using two-way ANOVA and shown as ratios to water drinking mice with saline instillation (mean ± SEM; n=4/group).
In the duodenum (Figure 3-10) and liver (Figure 3-11), alcohol exposure increased mRNA levels of DMT1, TfR1 and FPN compared to the water-drinking control group. However, Mn instillation did not alter expression of these transporters in the duodenum or liver. Similarly, mRNA levels of DMT1, TfR1 and FPN were up-regulated in the brain of alcohol-exposed mice compared to the water control group (Figure 3-12, p=0.048 (DMT1), p=0.021 (TfR1), p=0.032 (FPN)), while Mn instillation did not alter the expression of these transporters. In addition, consistent with protein levels, the expression of an Mn-exporter SLC30A10 was not altered by alcohol exposure or Mn instillation in the duodenum or liver. Interestingly, mRNA of SLC30A10 was increased upon alcohol exposure and Mn intranasal instillation respectively, and further increased in the brain of Mn-instilled alcohol drinking mice. Taken together, these results suggest that in agreement with protein expression, ethanol consumption up-regulates the mRNA of Fe/Mn transporters.

![mRNA levels of metal transporters in the duodenum](image)

Figure 3-10. Duodenal mRNA of metal transporters of Mn instilled alcohol drinking mice.
Mice (8-9 weeks old) were given facility water or ethanol-containing water (10%; v/v) for a total of 4 weeks. Since the second week of alcohol treatment, MnCl₂ solutions (5 mg/kg) or saline were intranasally instilled for three weeks prior to euthanasia, followed by tissue collection. The mRNA levels of Fe and Mn transporters including divalent metal transporter 1 (DMT1), transferrin receptor 1 (TfR1), ferroportin (FPN) and SLC30A10 in the duodenum were evaluated by real-time qPCR analysis. The mRNA level of each protein was normalized to that of actin. Data were analyzed using two-way ANOVA and shown as ratios to water drinking mice with saline instillation (mean ± SEM; n=4/group).

**Figure 3-11. **Hepatic mRNA of metal transporters of Mn-instilled alcohol drinking mice.

Mice (8-9 weeks old) were given facility water or ethanol-containing water (10%; v/v) for a total of 4 weeks. Since the second week of alcohol treatment, MnCl₂ solutions (5 mg/kg) or saline were intranasally instilled for three weeks prior to euthanasia, followed by tissue collection. The mRNA levels of Fe and Mn transporters
including divalent metal transporter 1 (DMT1), transferrin receptor 1 (TfR1), ferroportin (FPN) and SLC30A10 in the liver were evaluated by real-time qPCR analysis. The mRNA level of each protein was normalized to that of actin. Data were analyzed using two-way ANOVA and shown as ratios to water drinking mice with saline instillation (mean ± SEM; n=4/group).

**Figure 3- 12. Brain mRNA of metal transporters of Mn-instilled alcohol drinking mice.**

Mice (8-9 weeks old) were given facility water or ethanol-containing water (10%; v/v) for a total of 4 weeks. Since the second week of alcohol treatment, MnCl₂ solutions (5 mg/kg) or saline were intranasally instilled for three weeks prior to euthanasia, followed by tissue collection. The mRNA levels of Fe and Mn transporters including divalent metal transporter 1 (DMT1), transferrin receptor 1 (TfR1), ferroportin (FPN) and SLC30A10 in the brain were evaluated by real-time qPCR analysis. The mRNA level of each protein was normalized to that
of actin. Data were analyzed using two-way ANOVA and shown as ratios to water drinking mice with saline instillation (mean ± SEM; n=4/group).

3.4. Discussion

Increased Mn accumulation in the brain is significantly associated with neurological disorders and impaired brain function [23,152]. In Chapter 2, I demonstrated that alcohol increases Mn and Fe levels in the body and in the brain by up-regulation of Fe transporters. In the present chapter, I showed that after Mn instillation, alcohol further promoted Mn deposition in the brain. To investigate whether increased Mn accumulation in the brain was due to altered Fe and Mn transporters in Mn-instilled alcohol drinking mice, the expression of metal importers (including TfR1, DMT1 and ZIP14) and exporters (FPN and SLC30A10) was evaluated.

While both alcohol and Mn are neurotoxic, little is known about the interaction effect of alcohol and Mn. A number of studies have revealed that Mn can be directly transported into the brain after intranasal instillation [57,151]. For example, several animal studies showing that olfactory Mn transport can be directly transported into the brain from the nose along olfactory neuronal pathways to the olfactory bulb after inhalation or intranasal instillation exposures [55-58]. In agreement with their studies, I confirmed that Mn can be taken up by intranasal instillation into the brain by showing a dose-dependent Mn accumulation in the brain after intranasal instillation of Mn. In addition, the olfactory transport of Mn did not affect Fe levels in the brain in my study, which is in good agreement with Molina et al. who demonstrated no significant changes of non-heme Fe levels in the liver or brain in mice with Mn exposure by drinking water from gestation to postnatal day 24 [153]. In support, Alsulimani et al. showed that in mice with Mn drinking water, Mn levels significantly increased with no change in Fe levels in the liver after five weeks of treatment [154]. In contrast, Rossander-Hulten et al. indicated a direct competitive inhibition of Mn on Fe absorption from the gut lumen using radioisotopes [128], and Huang et al. [155] reported a significant decrease in liver Fe levels in rats after 30 days of Mn exposure by intraperitoneal injection of 6.0 mg
Mn/kg. These mixed results suggest that different doses and/or routes of Mn exposure could affect Fe status differently.

With respect to metal transporters, my results demonstrated that there were no significant changes in either protein or mRNA levels of metal importers (e.g. TfR1 or ZIP14) or exporters (e.g. FPN) upon Mn intranasal instillation. These findings are in agreement with Ye et al. [156] who demonstrated no significant changes in striatal mRNA levels of Mn importers (ZIP14) and exporter (FPN) after intranasal instillation of MnCl$_2$ up to 5 mg/kg for 3 days. However, Ye et al. [156] did not find changes in the mRNA levels of DMT1+IRE or DMT1-IRE, the two isoforms of DMT1 with or without the Fe-responsive element. In contrast, the mRNA levels of DMT1 in the whole brain were increased in my study after Mn-intranasal instillation for 3 weeks. The difference between the two studies could be due to a difference in the duration of Mn exposure: acute exposure of 3-day Mn instillation into the brain may not be enough to regulate the mRNA levels of DMT1. Alternatively, brain region-specific DMT1 regulation could be another possibility. In support, Siddappa et al. demonstrated that the expression of hippocampal DMT1 is up-regulated in rats with Fe deficiency, whereas the level of striatal DMT1 upon Fe deficiency was not altered [157]. As an Mn exporter, SLC30A10 mediates Mn efflux in the brain [50]. Although I was not be able to detect proteins of DMT1 due to the absence of validated antibodies, it has been shown that mRNA levels are parallel by comparable changes in the expression of its protein [138]. Therefore, increased Mn levels by intranasal instillation of Mn could be possibly due to up-regulation of DMT1 in the brain. Since DMT1 is expressed in the olfactory epithelium and could play a critical role for nasal transport of metals [59,158,159], a future study is necessary to determine whether alcohol modifies the levels of these Mn importers in the olfactory epithelium as well as other brain regions. Notably, the mRNA level of SLC30A10 in the brain after Mn intranasal instillation was increased in response to high Mn levels in the brain. Since studies reported that high level of Mn leads to up-regulation of SLC30A10 in both mRNA and protein levels [160], I speculate that exposure schedule of three weeks of Mn instillation could be sufficient to up-regulate gene expression of SLC30A10, but not its protein levels. Therefore,
it may suggest that alterations in gene expression of SLC30A10 occurs earlier than changes in protein levels in response to Mn exposure.

I also tested the effect of alcohol exposure on Mn uptake into the brain in mice after Mn-instillation, which represents inhalation of the metal in humans. Interestingly, I observed that after Mn instillation, alcohol promoted Mn deposition into the brain. In line with increased Mn levels, DMT1 was further increased in the brain of Mn-instilled alcohol-drinking mice from saline-instilled alcohol-drinking group as assessed by qRT-PCR, suggesting that brain Mn uptake could be further increased after Mn instillation and alcohol drinking. Although protein levels of metal importers (TfR1 and ZIP14) did not further up-regulate from those in mice with alcohol drinking, the mRNA level of DMT1 was further increased in the brain of Mn-instilled alcohol-drinking mice from saline-instilled alcohol-drinking group. With respect to metal exporters, both FPN and SLC30A10 were further increased in Mn-instilled alcohol drinking mice from saline-instilled alcohol drinking mice. The up-regulation of Mn exporters could be the consequence in response to high Mn levels in the brain. In support, Yin et al. [161] demonstrated that in an inducible human embryonic kidney (HEK293T) cells, FPN levels were significantly increased after 6 h of Mn exposure. Furthermore, they also found the up-regulation of FPN in the cerebellum and cortex of mice after Mn exposure [161]. Combined, these results indicate that the up-regulated metal importers are possibly responsible for increased Mn deposition in the brain of Mn-instilled alcohol-drinking mice compared with Mn-instilled water-drinking mice.

3.5. Conclusions

In the present chapter, I demonstrated that alcohol promotes the olfactory Mn uptake into the brain, likely through up-regulation of Fe transporters. My study identified that alcohol, an important lifestyle factor, can significantly modify the transport and neurotoxicity of inhaled Mn, which has a direct impact on occupational health and safety. For example, welders who are routinely exposed to high levels of Mn, despite protective equipment, may be at a
higher risk of Mn neurotoxicity with alcohol consumption. To my knowledge, this is the first report to determine the impact of alcohol exposure on olfactory transport of Mn. The potential mechanisms by which alcohol increases Fe transporters were explored in Chapter 4.
CHAPTER 4. THE MOLECULAR REGULATION OF METAL TRANSPORT UPON ALCOHOL EXPOSURE

4.1. Introduction

Hepcidin is a master regulator that controls whole-body Fe homeostasis. It regulates systemic Fe metabolism by binding to FPN and DMT1 to induce the degradation of these transporters [162]. The synthesis of hepcidin is affected by Fe status [102]. For example, in patients with anemia disorder, the low Fe levels in the body inhibit hepcidin expression in the liver, leading to up-regulation of Fe transporter levels and ultimately increased Fe levels [162]. On the other hand, in individuals with Fe overload, hepcidin synthesis is up-regulated in response to high Fe levels in the body, resulting in down-regulation of Fe transporters and thus decreased Fe levels [162]. In addition to Fe status, different physiological and pathological conditions, such as hypoxia [104], inflammation [105], liver damage [163,164] and metabolic processes [106,107], can modulate the expression of hepcidin, which results in the alteration of Fe absorption and transport.

Emerging evidence has suggested that hypoxia alters hepcidin status by binding HIF to the hepcidin promoter, and thus inhibit hepcidin expression [165]. It is well understood that alcohol intake stimulate oxygen consumption and consequently cause hypoxia in tissues [166], including brain [167], which leads to up-regulation of HO-1 and activation of hypoxia response by up-regulation of HIF [168-171]. In addition, Fe transporters (e.g. DMT1, TfR1 and FPN) contain hypoxia-response elements (HREs) [172] to which hypoxia-inducible factors (HIFs) can bind, thereby regulating the gene expression of Fe transporters [173]. Therefore, under hypoxia condition, major Fe transporters, including TfR1, DMT1 and FPN, are activated by HIFs in order to increase Fe uptake for erythropoiesis and delivery of oxygen to hypoxic cells [165,174]. It is known that hypoxia influences Fe status, however, whether or not Mn transport is modified by hypoxia induced by alcohol exposure is largely unexplored.
In this chapter, I elucidated the molecular regulations of Mn transport upon alcohol exposure and Mn instillation, by evaluating hepcidin, hypoxia response and potential liver damage in Mn intranasally-instilled alcohol drinking mice.

4.2. Methods

4.2.1. Animals. Animal protocols were approved by the Division of Laboratory Animal Medicine (DLAM) and the Northeastern University-Institutional Animal Care and Use Committee (NU-IACUC). Animals were maintained on a 12:12 hour light-dark cycle and given water *ad libitum* provided by DLAM.

4.2.2. Cell culture. All cells were cultured at 37°C under a 5% CO₂ atmosphere in 75-cm² tissue culture flasks (Corning, NY) and passed when confluent. The J774 macrophage cell line was kindly provided by Dr. Mansoor Amiji (Department of Pharmaceutical Sciences, Northeastern University) and maintained in DMEM medium containing 10% FBS with 10,000 I.U/mL penicillin, 10,000 μg/mL streptomycin and 25 μg/mL amphotericin.

4.2.3. Ethanol and Mn treatments. Animal treatments were the same as Chapter 3. For cell culture, the J774 cells were seeded and cultured into 6-well plates with 10⁵ cells/well. Cell culture medium (2-3 mL/well) was changed every two days. On day 5, three wells of cells were treated with low ethanol containing medium (0.5% alcohol) and three wells were treated with high ethanol containing medium (2.5% alcohol), while the other three wells were treated with normal medium. Twenty-four hours after the treatment, cells were washed once with cold PBS (1 mL/well) and scraped for collection. Low-dose alcohol (0.5%) was chosen because it is a pharmacologically relevant concentration with binge drinking *in vivo*, whereas 2.5% alcohol was chosen to examine the dose-response of alcohol treatment.
4.2.4. **Euthanasia and tissue collection.** After the treatment, animals were euthanized by an overdose of isoflurane, followed by exsanguination and collection of duodenum, liver and brain. The tissues were flash-frozen in liquid nitrogen and stored at -80°C until analysis.

4.2.5. **Real-time qPCR.** RNA was isolated from snap-frozen tissues, such as whole brain, liver and duodenum scrapping, as well as J774 cells using TRI reagent (Sigma-Aldrich, St. Louis, MO, USA) as per the manufacturer’s instructions. RNA (1 µg) was reverse transcribed into cDNA, which was used for real-time polymerase chain reaction assays. The iScript™ reverse transcription supermix and iTaq™ universal SYBR® green supermix were obtained from Bio-Rad (Hercules, California). Primers were obtained from Eurofins, MWG Operon (Huntsville, AL) and sequences of each primer were shown in Table 2.1. Amplification of the DNA was programmed at 95°C for 2 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. The expression level of each gene was normalized to that of actin and analyzed by the comparative Ct method (2^-ΔCt) [129].

### Table 4-1. Primer sequences of metal transporters and hypoxia-related genes.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLC30A10</td>
<td>Forward: TGTCTCCTGCTTCTCCTCCTTG</td>
<td>[133]</td>
</tr>
<tr>
<td></td>
<td>Reverse: AGCTCTGTAGTCTGTCTCATCTCTGTTGA</td>
<td></td>
</tr>
<tr>
<td>DMT1</td>
<td>Forward: CGTACCCGCTGGGACTGA</td>
<td>[130]</td>
</tr>
<tr>
<td></td>
<td>Reverse: GTCATCTGGACACACCACTGAGTCA</td>
<td></td>
</tr>
<tr>
<td>TfR1</td>
<td>Forward: TGGGTCTAAGTCTACAGTGGC</td>
<td>[131]</td>
</tr>
<tr>
<td></td>
<td>Reverse: AGATACATAGGGCGACAGGAA</td>
<td></td>
</tr>
<tr>
<td>FPN</td>
<td>Forward: TTGCAGGAGTCATTGCTGCTA</td>
<td>[132]</td>
</tr>
<tr>
<td></td>
<td>Reverse: TGGAGTTCTGCACACCACATTGAT</td>
<td></td>
</tr>
<tr>
<td>Hepcidin</td>
<td>Forward: TGTCTCCTGCTCCTCCTCCTTGG</td>
<td>[175]</td>
</tr>
<tr>
<td></td>
<td>Reverse: AGCTCTGTAGTCTGTCTCATCTGTTGA</td>
<td></td>
</tr>
<tr>
<td>Heme oxygenase 1 (HO-1)</td>
<td>Forward: GGTGATGGCCTTCCTGTGACC</td>
<td>[176]</td>
</tr>
<tr>
<td></td>
<td>Reverse: AGTGAGGCCCCATACCAGAAG</td>
<td></td>
</tr>
<tr>
<td>Hypoxia-inducible factor 1-alpha (HIF-1α)</td>
<td>Forward: TGATGTGGGTTGCTGTTGTC</td>
<td>[177]</td>
</tr>
<tr>
<td></td>
<td>Reverse: TTGTGTTGGGGCAGTACTG</td>
<td></td>
</tr>
<tr>
<td>Hypoxia-inducible factor 2-alpha (HIF-2α)</td>
<td>Forward: CAGGCAGTATGCTGGCTAATTCCAGTT</td>
<td>[177]</td>
</tr>
<tr>
<td></td>
<td>Reverse: CTCTCCTCATTCTGGGATCTGGGACT</td>
<td></td>
</tr>
</tbody>
</table>
4.2.6. **Liver function analysis.** Alanine aminotransferase (ALT) activity in the serum and liver homogenates were measured using a colorimetric assay (Abcam, Boston, MA) according to the instruction provided. Gamma-glutamyl transferase (GGT) activity in the liver was measured using Diagnostic kit (Sigma Chemical Company, St. Louis, MO) according to the instruction provided.

4.2.7. **Histopathological examination.** Liver tissues were fixed with 10% neutral formalin and embedded in paraffin. Tissue sections of 5 μm were cut and stained with hematoxylin and eosin.

4.2.8. **Statistical analysis.** In the experiments of sub-chronic alcohol drinking with Mn intranasal-instillation, comparisons among four groups with two factors (alcohol and Mn) were performed by two-way ANOVA. With respect to binge drinking in which two groups were tested, two-sample t-test was employed. Differences were considered significant at \( p < 0.05 \). Statistical analysis values were reported as means ± SEM.

4.3. **Results**

4.3.1. **Hepcidin levels are decreased in the duodenum from alcohol-drinking mice.** Since hepcidin plays an important role in iron absorption by regulating the expression of iron transporters, I tested whether alcohol modifies hepcidin with or without intranasal Mn instillation in the duodenum (**Fig 4-1**). In the duodenum, there was no significant difference in hepcidin mRNA expression between Mn-instilled and saline-instilled control groups, whereas alcohol exposure significantly down-regulated hepcidin levels (\( p=0.017 \)). This result suggests that alcohol increases iron absorption possibly through up-regulation of Fe transporters and down-regulation of hepcidin in the duodenum.
Figure 4-1. Duodenal hepcidin expression of alcohol drinking mice after Mn intranasal instillation. Mice (mixed background of C57BL/6 and 129Sv/J strains; male and female; 8-9 weeks old) were treated with water, or ethanol (10%; v/v) for a total of 4 weeks. Since the second week of alcohol treatment, MnCl₂ solutions (5 mg/kg as Mn) or saline were intranasally instilled for 3 weeks. After euthanasia, the mRNA levels of hepcidin in the duodenum were analyzed by qRT-PCR. The expression level of each gene was normalized to that of actin according to the comparative Ct method (2⁻ΔCt) and analyzed using two-way ANOVA. Data were shown as ratios to water drinking mice with saline instillation (mean ± SEM; n=4/group).

4.3.2. Hepcidin levels are decreased in the liver from alcohol-drinking mice. To investigate whether the up-regulated Fe transporters in the liver are correlated with the expression of hepcidin production, I tested hepcidin levels in the liver of alcohol drinking mice with or without intranasal Mn instillation (Fig 4-2). In the liver, there was no significant difference in hepcidin mRNA expression between Mn-instilled and saline-instilled control groups, whereas alcohol exposure significantly down-regulated hepcidin levels (p=0.039). This result suggests
that alcohol increases iron tissue uptake possibly through up-regulation of Fe transporters by down-regulation of hepcidin in the liver.

**Figure 4-2. Hepcidin in the liver of mice with alcohol consumption after Mn intranasal instillation.**
Mice (mixed background of C57BL/6 and 129Sv/J strains; male and female; 8-9 weeks old) were treated with water, or ethanol (10%; v/v) for a total of 4 weeks. Since the second week of alcohol treatment, MnCl₂ solutions (5 mg/kg as Mn) or saline were intranasally instilled for 3 weeks. After euthanasia, the mRNA levels of hepcidin in the liver were analyzed by qRT-PCR. The expression level of each gene was normalized to that of actin according to the comparative Ct method ($2^{-\Delta Ct}$) and analyzed using two-way ANOVA. Data were shown as ratios to water drinking mice with saline instillation (mean ± SEM; n=4/group).

**4.3.3. Hepcidin levels are decreased in the liver from alcohol-drinking mice.** In the brain, however, hepcidin expression decreased by 25% after Mn intranasal instillation compared with saline-instilled control mice (Figure 4-3). Hepcidin expression was also down-regulated by 56% after alcohol consumption compared to the water-
drinking control group. Notably, the extent of decreased hepcidin expression due to ethanol exposure was even greater in Mn-instilled mice (95% decrease) than in saline-treated mice.

**Figure 4-3. Hepcidin in the brain of mice with alcohol consumption after Mn intranasal instillation.**

Mice (mixed background of C57BL/6 and 129Sv/J strains; male and female; 8-9 weeks old) were treated with water, or ethanol (10%; v/v) for a total of 4 weeks. Since the second week of alcohol treatment, MnCl$_2$ solutions (5 mg /kg as Mn) or saline were intranasally instilled for 3 weeks. After euthanasia, the mRNA levels of hepcidin in the brain were analyzed by qRT-PCR. The expression level of each gene was normalized to that of actin according to the comparative Ct method ($2^{-\Delta\text{Ct}}$) and analyzed using two-way ANOVA. Data were shown as ratios to water drinking mice with saline instillation (mean ± SEM; n=4/group).

4.3.4. Alcohol exposure up-regulates hypoxia-related genes in the brain. Accumulating evidence suggest that hypoxia affects Fe status through the transcriptional [172] and post-transcriptional [178] regulation of metal transporters. To evaluate if alcohol induced hypoxia by alcohol exposure, the levels of hypoxia-related gene expression by qRT-PCR in the brain of mice exposed to alcohol and Mn were examined. HIF-1α mRNA levels
were elevated either by Mn instillation (35% increase) or by alcohol exposure (31% increase) (Fig 4-4). Alcohol further increased HIF-1α by 48% after Mn intranasal instillation compared with saline-instilled alcohol drinking mice. Neither HIF-2α nor HIF-1β was altered by alcohol exposure or by Mn instillation. Similar to HIF-1α, HO-1 levels were also up-regulated by Mn instillation (60% increase) and alcohol consumption (93% increase), and further increased by 56% in Mn-instilled alcohol drinking mice. These data suggest that alcohol-induced hypoxia up-regulates metal transporters and thereby promotes Mn deposition in the brain.

**Figure 4- 4. Hypoxia-related mRNAs in mice with alcohol consumption after Mn intranasal instillation.**

Mice (mixed background of C57BL/6 and 129Sv/J strains; male and female; 8-9 weeks old) were treated with water, or ethanol (10%; v/v) for a total of 4 weeks. Since the second week of alcohol treatment, MnCl2 solutions (5 mg /kg as Mn) or saline were intranasally instilled for 3 weeks. After euthanasia, the levels of mRNA expression including hypoxia-inducible factor 1-alpha (HIF-1α), hypoxia-inducible factor 2-alpha (HIF-2α),
hypoxia-inducible factor 1-beta (HIF-2β) and heme oxygenase 1 (HO-1) in the brain were determined by qRT-PCR. The expression level of each gene was normalized to that of actin according to the comparative Ct method ($2^{-\Delta\text{Ct}}$) and analyzed using two-way ANOVA. Data were shown as ratios to water drinking mice with saline instillation (mean ± SEM; n= 4/group).

4.3.5. Alcohol exposure up-regulates hypoxia-related genes in J774 cells. To evaluate whether alcohol can modify metal transporters without hepcidin, we characterized the levels of metal transporters after alcohol treatment by RT-qPCR in J774 macrophages (Fig 4-5). As expected, the level of hepcidin mRNA was not detected in J774 cells. However, metal transporters, including DMT1 (ethanol effect, p=0.033), FPN (ethanol effect, p=0.003) and TfR1 (ethanol effect, p=0.045) were still increased upon alcohol treatment in a dose-dependent manner. Consistent with SLC30A10 in vivo, the Mn-specific exporter did not alter upon alcohol exposure in J774 macrophages. Interestingly, alcohol exposure up-regulated hypoxia related genes, including HIF-1α (ethanol effect, p<0.001), HIF-2α (ethanol effect, p=0.008) and HO-1 (ethanol effect, p<0.001), with no change in HIF-1β (Fig 4-6). These data suggest a hepcidin-independent pathway in which up-regulation in Fe transporters by alcohol exposure is possibly caused by alcohol-induced hypoxia.
Figure 4-5. Metal transporter mRNAs in J774 cells with alcohol.

J774 cells were treated with water, or ethanol (0.5% or 2.5%) for 24 hours. The levels of mRNA expression including hepcidin, DMT1, FPN, Tfr1 and SLC30A10 were determined by qRT-PCR. The expression level of each gene was normalized to that of actin according to the comparative Ct method (2^(-ΔCt)) and analyzed using one-way ANOVA. Data were shown as ratios to non-alcohol treated control cells (mean ± SEM; n=3/group).
Figure 4- 6. Hypoxia-related mRNAs in J774 cells with alcohol.
J774 cells were treated with water, or ethanol (0.5% or 5%) for 24 hours. The levels of mRNA expression including hypoxia-inducible factor 1-alpha (HIF-1α), hypoxia-inducible factor 2-alpha (HIF-2α), hypoxia-inducible factor 1-beta (HIF-2β) and heme oxygenase 1 (HO-1) were determined by qRT-PCR. The expression level of each gene was normalized to that of actin according to the comparative Ct method (2^-ΔCt) and analyzed using one-way ANOVA. Data were shown as ratios to non-alcohol treated control cells (mean ± SEM; n=3/group).

4.3.6. Increased Mn status after alcohol exposure is not due to liver dysfunction. While alcohol is an established inducer of liver injury, impaired liver function could decrease hepcidin production as well as Mn excretion [124,163], both of which would increase Mn retention in the body and brain. Thus, I evaluated the potential impact of liver damage on elevated Mn levels during alcohol consumption (Figure 4-7 and Figure 4-8). Neither alanine aminotransferase (ALT) nor hepatic gamma-glutamyl transferase (GGT) in the liver was altered after alcohol exposure or Mn instillation (Figure 4-7). Hematoxylin and eosin (H&E) staining (Figure 4-8) analysis demonstrated normal histological appearance in mice treated with alcohol and/or Mn instillation. These results indicate that there is no significant liver injury due to alcohol or Mn exposure, excluding a possibility that Mn accumulation results from alcohol-induced liver damage.
Figure 4-7. Liver function tests in mice with alcohol consumption after Mn intranasal instillation.

Mice (mixed background of C57BL/6 and 129Sv/J strains; male and female; 8-9 weeks old) were treated with water, or ethanol (10%; v/v) for a total of 4 weeks. Since the second week of alcohol treatment, MnCl₂ solutions (5 mg/kg as Mn) or saline were intranasally instilled for 3 weeks. After euthanasia, liver tissues were collected from alcohol-exposed mice with or without intranasal Mn instillation. The concentrations of alanine aminotransferase (ALT) in the serum (Figure 4-7A) and liver (Figure 4-7B) and gamma-glutamyl transpeptidase (GGT) in the liver (Figure 4-7C) were analyzed by colorimetric assays. Data were analyzed using two-way ANOVA and shown as IU/L in the serum and IU/g tissue in the liver (mean ± SEM; n=4/group).
Figure 4- 8. Liver pathology evaluation in mice with alcohol consumption after Mn intranasal instillation.
Mice (mixed background of C57BL/6 and 129Sv/J strains; male and female; 8-9 weeks old) were treated with water, or ethanol (10%; v/v) for a total of 4 weeks. Since the second week of alcohol treatment, MnCl$_2$ solutions (5 mg/kg as Mn) or saline were intranasally instilled for 3 weeks. After euthanasia, H&E staining in the liver with alcohol consumption after Mn intranasal instillation were shown.

4.4. Discussion

A few studies have demonstrated the effects of Mn and alcohol exposure on hepcidin regulation. A case study by Fan et al. suggests that hepcidin is inversely related to blood Mn levels in smelters who are occupationally exposed to Mn [179], which is in agreement with my results of decreased hepcidin in mice after intranasal instillation of Mn. However, the mechanism of how Mn affects hepcidin remains elusive. With respect to the role of alcohol in hepcidin, I have shown that hepcidin is down-regulated in the duodenum, liver and brain of sub-chronic alcohol
drinking mice. Several lines of evidence indicate a direct inhibitory effect of alcohol on hepcidin expression [124,163,180]. For example, alcohol can suppress a DNA-binding activity of the transcription factor CCAAT/enhancer binding protein (C/EBP) alpha in hepatocytes, thereby inactivating hepcidin [180]. Additional evidence suggests that alcohol fails to suppress hepcidin expression in mice with defective toll-like receptor 4 (TLR4), a transmembrane protein involved in innate immune response [181], indicating a vital role of TLR4 in hepcidin homeostasis. While exact molecular mechanisms of the alcohol-hepcidin relationship require further investigation, my study provides another possibility that alcohol-induced hypoxia, evidenced by up-regulation of HIF-1α and HO-1, could serve as a repressor on hepcidin. In support, Peyssonnaux et al. showed that Fe-deficiency induced HIF-1α up-regulation decreases hepatic hepcidin expression in mice by binding to and negatively transactivate the hepcidin promoter, and the down-regulation of hepcidin is partially blunted in hepatocyte-specific HIF-1α–knockout mice [165]. Other groups reported that hypoxia could induce hepcidin suppression through the HIF pathway, but requires erythropoietin induction, which is associated with increased erythropoietic activity [182,183]. Future study is needed to measure the erythropoietin levels and erythropoietic activity in the brain of mice after alcohol exposure. Regarding the liver function, both histochemical and functional data rule out the possibility of decreased hepcidin production due to alcohol-induced liver damage. Collectively, the hepcidin down-regulation by alcohol exposure in my study is likely through the direct effect of alcohol or indirectly effect of alcohol-induced hypoxia.

Since biliary secretion is the main pathway for Mn excretion [184,185], unchanged liver function in mice after sub-chronic alcohol also excludes the possibility of decreased metal secretion by liver damage. I also demonstrated unchanged levels of Cu in the blood, liver and brain of mice after sub-chronic alcohol drinking. Since biliary excretion is the major route for Cu excretion [186], no change in Cu levels after alcohol drinking in my study suggests normal function of the liver for biliary excretion of metals. Although these findings favor a possibility that increased absorption of Fe and Mn could promote elevated levels of these metals, there could be other regulations to modify Mn excretion. Therefore, future studies are needed to directly determine the
contribution of the excretion of Mn and Fe during alcohol exposure to steady-state levels of metals by characterizing the pharmacokinetics of the metals using their radioisotopes.

Both Mn in excess and alcohol intake stimulate oxygen consumption and consequently cause hypoxia in tissues [166], including brain [167], which leads to up-regulation of HO-1 and activation of hypoxia response [168-171]. Transcriptional responses to hypoxia are primarily mediated by HIF. In particular, HIF-1α contributes to an early tissue response to hypoxia [187-189]. Although studies have indicated HIF-2α is involved in changes in duodenal Fe transporters as well as Fe absorption in mice with conditional knockout of either HIF-1α or HIF-2α [190,191], elevated hepatic HIF-1α is observed in chronic alcohol exposure [192,193]. In particular, up-regulation of HIF-1α is widely associated with hypoxia under brain injury, brain tumors and neurodegenerative diseases [194-196], whereas HIF-2α is more found to be altered in the liver [197,198]. Therefore, it is possible that elevated HIF-1α in the brain of Mn-instilled alcohol-exposed mice is likely due to the alcohol dependent, tissue-specific distribution of hypoxia-inducible factors. Increased expression of Fe transporters in tissues after alcohol exposure in my study could be due to direct consequence of hypoxia since these transporters, including DMT1, TfR1 and FPN, contain hypoxia-response elements (HREs) [172]. Consistently, I found that mRNA levels of DMT1, TfR1 and FPN were elevated upon alcohol exposure. In support, an in vitro study using J774 macrophages was performed to examine the hepcidin-independent regulation of Mn transport by alcohol exposure. J774 macrophages was chosen because hepcidin is primarily produced by the liver, and the endogenous hepcidin expression in peritoneal macrophages is very limited. Additionally, metal transporters, such as FPN, are reported to be highly expressed in macrophages [199], where hepcidin-independent regulation of Mn transport upon alcohol exposure can be investigated. Increased mRNA levels of DMT1, TfR1 and FPN with no detection of hepcidin in J774 cells upon alcohol exposure excludes a possibility that hepcidin is required for alcohol-induced up-regulation of metal transporters. Rather, increased levels of metal transporters were associated with HIF-1α, HIF-2α and HO-1 up-regulation in these alcohol-treated cells, suggesting a direct influence of alcohol-induced
hypoxia on the expression of metal transporters. Together, alcohol-induced hypoxia results in up-regulation of Fe transporters and thereby promotes Mn accumulation in the brain, increasing the risk of Mn-mediated neurotoxicity.

4.5. Conclusions

In this chapter, I characterized the potential molecular regulation of Mn and Fe transport upon alcohol. Firstly, my study demonstrated that up-regulation of Fe transporters in the duodenum, liver and brain are likely results from down-regulation of hepcidin levels in those tissues, suggesting a direct inhibitory effect of alcohol on hepcidin. I also showed that alcohol-induced hypoxia likely increases the expression of Fe transporters through up-regulation of hypoxia-inducible factors. Finally, I ruled out a possibility that decreased hepcidin production or decreased excretion is caused by alcohol-induced liver damage. Collectively, it is clear that Fe transporters are up-regulated during alcohol consumption, resulting from combined effects mediated by down-regulation of hepcidin expression and increased hypoxic response.
5.1. Introduction

Mn, an essential trace element, is needed for many vital functions, including metabolic processes and anti-oxidant capacities. In particular, Mn superoxide dismutase (Mn-SOD) catalyzes dismutation of superoxide anion to hydrogen peroxide and molecular oxygen [7]. Although a proper amount of Mn intake is necessary for human health, exposure to high Mn levels is neurotoxic. Mounting evidence suggests that Mn can be directly transported into the brain via inhalation while bypassing liver metabolism. High level of Mn in humans has been widely associated with neurological disorders, such as memory loss, impair emotional behavior and motor function [23]. For example, there is definitive evidence from human studies suggesting that exposure to Mn dust in mines and factories results in inhalation of high levels of Mn that can lead to a series of serious disabling neurological effects [27]. It has been known that Mn affects neural functions by modifying the turnover of several neurotransmitters. For instance, investigations suggest that alterations in dopaminergic and GABAergic signaling may drive the effects associated with Mn neurotoxicity [28,29,37].

Notably, Ellingsen et al. observed the interaction between Mn exposure and alcohol on neurobehavioral outcomes in welders [200]. This study demonstrated that welders with high blood and urine Mn levels per creatinine in the blood and urine had poor performance of several motor function tests, including Grooved Pegboard, Finger Tapping, Simple Reaction Time compared to the referents. Interestingly, welders with alcohol consumption had poorer performances on these motor function tests, indicating an interaction between alcohol and Mn exposure. This study prompted me to investigate the mechanism of Mn transport upon ethanol drinking, together with the possible molecular mechanism underlying motor function deficits upon Mn and ethanol interaction.
It has long been known that dopaminergic signaling is important for regulating motor functions [30-32]. Abnormal dopamine function is found in brain disorders with motor function deficits, such as Parkinson’s disease [33]. Moreover, several studies have demonstrated that levels of dopamine, the major neurotransmitter that is severely reduced in the patients with Parkinson’s disease, are reduced by Mn exposure through in vivo [34] and in vitro [35] studies. These lines of evidence suggest that Mn exposure, at least in part, reduces dopamine levels in the brain [36]. Therefore, I examined dopaminergic signaling in Mn-instilled alcohol drinking mice.

Advances in molecular physiology and toxicology have revealed that abnormal GABAergic function is also associated with Mn neurotoxicity [37]. GABA is the most abundant inhibitory neurotransmitter in the adult brain and is found in the medium spiny neurons of the striatum, mediating the dopaminergic activity in this region [37]. The relationship between Mn exposure and GABA level has been recently studied. There are conflicting results about Mn accumulation and a corresponding increase or decrease in regional levels of GABA [11]. For example, Anderson et al. suggested that brain Mn accumulation is associated with decreased GABA content in the striatum, probably due to impaired GABA uptake [38], whereas Lipe et al. found a significant increase in GABA level in the cerebellum of the adult rats exposed to high doses of Mn [39]. These data suggest that GABA levels in the brain could be different under different conditions of Mn exposure, such as dose and timing, in a tissue-specific manner.

Alcohol is known to alter membranes as well as ion channels, enzymes, and receptors. Alcohol also binds directly to the receptors for acetylcholine, serotonin, GABA, and the NMDA receptors for glutamate. GABAergic neurotransmission and GABA_A receptors in particular have long been implicated in mediating the pharmacological effects of alcohol [121]. It has been well documented that GABA has an inhibitory effect on neuronal activity as it promotes entries of chloride ion in the post-synaptic neuron. Since chloride ions have a negative electrical charge, they make the neuron less excitable. This hypo-excitation effect of chloride ions is amplified when alcohol binds to the GABA_A receptor. There exists a possibility that ethanol may enable the ion
channel to stay open and thus let more chloride ions enter the cell. Together, alcohol triggers the inhibitory effect of GABA and thus modifies GABAergic metabolism, which could potentially predispose to Mn neurotoxicity.

The focus of the present chapter was to examine dopaminergic and GABAergic signaling and to better understand the mechanism by which alcohol consumption increases Mn uptake and thereby represents as a potential risk factor for metal-induced neurotoxicity.

5.2. Methods

5.2.1. Animals. Animal protocols were approved by the Division of Laboratory Animal Medicine (DLAM) and the Northeastern University-Institutional Animal Care and Use Committee (NU-IACUC). Animals were maintained on a 12:12 hour light-dark cycle and given water ad libitum provided by DLAM.

5.2.2. Ethanol and Mn treatments. Same as Chapter 3.

5.2.3. Euthanasia and tissue collection. After the treatment, animals were euthanized by an overdose of isoflurane, followed by exsanguination and collection of brain. The brains were flash-frozen in liquid nitrogen and stored at -80°C until analysis.

5.2.4. Dopamine analysis. The whole brain tissue homogenates were prepared in ice cold 0.2 M perchloric acid (1:10 w/v) containing ascorbic acid (0.2 µM) and EDTA (0.2 µM) followed by centrifuging for 6 min at 15,000×g. An aliquot of the supernatant was further diluted 1:2 in perchloric acid solution and centrifuged for an additional 2 min at 15,000×g. The final supernatant (10 µL) was analyzed using an HPLC system consisting of an ESA 542 autosampler (ESA), an ESA 580 dual-piston pump, an ESA MD-150 column (C18, 150×3.2 mm, 3 µm particles,
120 Å pores), an ESA Coulochem II detector (Model 5200), with a 5020-guard cell and a 5011 coulometric analytical cell. The potentials of the electrochemical cells were EGuardCell +350 mV, E1 -150 mV and E2 +300 mV, all against a palladium reference electrode. Data were acquired from E2 and analyzed by PC/Chrom+ software (H & A Scientific, Greenville, NC). The mobile phase consisted of 90 mM sodium phosphate monobasic monohydrate; 50 mM citric acid monohydrate; 1.7 mM 1-octanesulfonic acid sodium salt hydrate, HPLC grade; 50 µM disodium ethylenediamine tetraacetate dihydrate; and 10% acetonitrile (v/v) in water pre-filtered as described above. The flow rate was 0.4 mL/min. Calibration was by external standards prepared in solutions of the above preservative.

5.2.5. GABA analysis. The brain samples from alcohol exposed mice with Mn intranasal-instillation were homogenized on ice in 10 volumes (w/v) of 0.4 M perchloric acid containing 50 µM EDTA. L-norvaline was added as an internal standard. After neutralization by sodium borate buffer (10 mM, 10- volume), the homogenates were centrifuged at 15,000 g for 15 min at 4°C and the supernatant was derivatized with o-phthalaldehyde (16.4 mM, 20:1, v/v) (Rowley et al. 1995). Derivatization will be conducted 10 minutes before injecting the samples. Mobile phase consisted of aqueous phase (0.1 M monosodium phosphate, 0.5 mM EDTA, pH 4.5) and methanol at a 3:1 ratio (v/v). The flow rate was set to 0.8 ml/min. Sampled were injected (100 µL) into an HPLC system (Shimadzu, Kyoto, Japan) with an ESA HR-80 RP C18 column (80 3 4.6 mm, 3-mm particles, 120 A pores) for GABA analysis. The GABA peak was detected at 344 nm by an UV detector (Shimazu), which was normalized to norvaline.

5.2.6. Western blot analysis. The whole brain from mice with alcohol exposure were homogenized on ice in RIPA buffer (100 mM Tris, 0.2% SDS, 2% NP40 and 1.0% sodium deoxycholate, pH 7.5) containing protease inhibitor (Complete Mini, Roche; Nutley, NJ, USA). Samples were centrifuged at 16,000 g for 6 minutes at 4°C. After determination of protein levels, samples (40 µg of proteins) were electrophoresed on a 10% SDS-polyacrylamide gel and transferred to either polyvinylidene difluoride (Millipore, Billerica, MA) or nitrocellulose
(GE Healthcare, Piscataway, NJ) membrane. After blocking with 5% non-fat milk, the membrane was incubated the membrane was incubated in goat antibody against GABA_A receptor α1 (GABRA1, 1:500, Novus Biologicals), GABA_A receptor α2 (GABRA2, 1:500, Novus Biologicals), glutamate decarboxylase 65 (GAD65, 1:100, Santa Cruz), glutamate decarboxylase 67 (GAD67, 1:100, Santa Cruz Biotech), GABA transporter (GAT, 1:500, Novus Biologicals), dopamine transporter (DAT; 1:100; Santa Cruz Biotech), and mouse antibody against tyrosine hydroxylase (TH; 1:500; Santa Cruz Biotech) and dopamine D2 receptor (D2DR; 1:500; Santa Cruz Biotech). As a loading control, the immunoblot was incubated with mouse anti-actin (1:5,000; MP Biomedicals, Solon, OH). The blots were incubated with donkey anti-goat antibody (1:1,000; Santa Cruz Biotech) or sheep anti-mouse antibody conjugated with HRP (1:5,000; GE Healthcare), followed by chemiluminescence (ECL West Dura, Thermo Scientific) and scanned using Chemidoc System (ChemiDoc XRS, Bio-Rad, Hercules, CA). Relative intensities of protein bands normalized to actin was determined using Image Lab (Bio-Rad, version 4.1).

5.2.7. Statistical analysis. In the experiments of sub-chronic alcohol drinking with Mn intranasal-instillation, comparisons among four groups with two factors (alcohol and Mn) were performed by two-way ANOVA followed by the post-hoc method, Holm-Sidak. Differences were considered significant at p < 0.05. Values were reported as means ± SEM.

5.3. Results

5.3.1. Dopamine levels are decreased in mice upon alcohol exposure with Mn instillation. Dopamine concentrations in the brain of mice with Mn intranasal instillation were significantly decreased compared to saline-instilled control mice (27% decrease, p=0.002). Although alcohol exposure by drinking water did not change dopamine levels in the brain of saline-instilled mice, it synergistically down-regulated 50% of dopamine in the brain of mice with Mn intranasal instillation, compared with Mn-instilled, water drinking mice (Figure 5-1).
**Figure 5-1. Dopamine levels are decreased in the brain of Mn-instilled alcohol drinking mice.**

Mice (mixed background of C57BL/6 and 129Sv/J strains; male and female; 8-9 weeks old) were treated with 10% (v/v) alcohol by drinking water for a total of 4 weeks. Since the second week of alcohol treatment, MnCl₂ (5 mg/kg) or saline was intranasally instilled daily for 3 weeks. After euthanasia, the levels of dopamine in the brain of alcohol-exposed mice with or without intranasal Mn instillation were determined using HPLC. Data were analyzed using two-way ANOVA and shown as ratios to water drinking mice with saline instillation (mean ± SEM; n=4-6/group).

**5.3.2. Mn-induced dopaminergic dysfunction is exacerbated by alcohol drinking.** In addition to dopamine concentrations, I further examined dopamine related-proteins in the brain of Mn-instilled alcohol-drinking mice. (**Fig 5-2**). The expression of tyrosine hydroxylase (TH), a critical enzyme responsible for dopamine production, was significantly decreased by intranasal instillation of Mn (43% reduction), but not by alcohol consumption. Notably, TH was synergistically decreased by alcohol and Mn in the brain (42% more decrease compared with Mn-instilled, water-drinking mice). These results suggest that Mn instillation decreases dopamine production, which is exacerbated by alcohol exposure. Levels of DAT decreased by Mn instillation (p<0.001) and ethanol
exposure (p=0.038). These results suggest that Mn in excess and ethanol exposure could decrease total dopamine levels in the brain. To maintain extracellular dopamine level, DAT is down-regulated by Mn and ethanol to decrease dopamine reuptake as a compensatory mechanism. While dopamine D1 receptor (D1DR) expression was not altered either by Mn or by ethanol, dopamine D2 receptor (D2DR) was up-regulated by intranasal instillation of Mn (34% increase), which was synergistically increased upon alcohol drinking (24% increase; Mn instillation vs. alcohol/Mn). Taken together, these results demonstrate that dopaminergic neurochemical system is affected by Mn intranasal instillation and worsened by ethanol exposure.

**Figure 5-2. Dopaminergic proteins are altered in the brain of Mn-instilled alcohol drinking mice.**
Mice (mixed background of C57BL/6 and 129Sv/J strains; male and female; 8-9 weeks old) were treated with 10% (v/v) alcohol by drinking water for a total of 4 weeks. Since the second week of alcohol treatment, MnCl₂ (5 mg/kg) or saline was intranasally instilled daily for 3 weeks. After euthanasia, the protein levels of tyrosine hydroxylase (TH), dopamine transporter (DAT), dopamine D1 receptor (D1DR) and dopamine D2 receptor
(D2DR) were evaluated in the brain by western blot analysis. Data were analyzed using two-way ANOVA and shown as ratios to water drinking mice with saline instillation (mean ± SEM; n=4-6/group).

5.3.3. GABA levels are decreased in mice upon alcohol exposure with Mn instillation. Since both Mn exposure and alcohol consumption influence GABAergic neurotransmission, we examined GABA levels in the brain of Mn exposed mice with alcohol treatment (Figure 5-3). GABA concentrations were significantly decreased by intranasal instillation of Mn (37% reduction) but not by alcohol consumption. Notably, GABA levels were synergistically decreased in the brain of alcohol-exposed mice that were instilled with Mn (30% more decrease compared with Mn-instilled, water-drinking mice).

![GABA levels in the brain](image)

**Figure 5-3.** GABA levels are decreased in the brain of Mn-instilled alcohol drinking mice.

Mice (mixed background of C57BL/6 and 129Sv/J strains; male and female; 8-9 weeks old) were treated with 10% (v/v) alcohol by drinking water for a total of 4 weeks. Since the second week of alcohol treatment, MnCl₂ (5 mg/kg) or saline was intranasally instilled daily for 3 weeks. After euthanasia, the levels of GABA in the brain of alcohol-exposed mice with or without intranasal Mn instillation were determined using HPLC. Data were
analyzed using two-way ANOVA and shown as ratios to water drinking mice with saline instillation (mean ± SEM; n=4-6/group).

5.3.4. GABA and GABAergic proteins are altered in mice upon alcohol exposure with Mn instillation. In addition to GABA levels, the proteins related to GABA homeostasis were evaluated in Mn-instilled mice with alcohol exposure (Figure 5-4). Similar to the changes in GABA concentrations, GABRA2 expression were significantly decreased by intranasal instillation of Mn (24% reduction) but not by alcohol consumption. Notably, GABRA2 expressions were synergistically decreased in the brain of alcohol-exposed mice that were instilled with Mn (25% more decrease compared with Mn-instilled, water-drinking mice). Unlike GABRA2, GABRA1 did not change either by alcohol consumption or by Mn instillation. There was no difference in GAT expression among groups. In addition, protein expression of GAD65 and GAD67, the enzymes that catalyze the production of GABA from glutamate, was differentially modified by alcohol and Mn exposures; both alcohol and Mn instillation independently decreased GAD65 levels in the brain, whereas GAD67 levels were significantly down-regulated only when both alcohol and Mn instillation were given.
**Figure 5-4. GABAergic proteins are altered in the brain of Mn-instilled alcohol drinking mice.**

Mice (mixed background of C57BL/6 and 129Sv/J strains; male and female; 8-9 weeks old) were treated with 10% (v/v) alcohol by drinking water for a total of 4 weeks. Since the second week of alcohol treatment, MnCl$_2$ (5 mg/kg) or saline was intranasally instilled daily for 3 weeks. After euthanasia, the protein levels of GABA receptor 1 (GABRA1), GABA receptor 2 (GABRA2), GABA transporter (GAT), glutamic acid decarboxylase 65 (GAD65) and glutamic acid decarboxylase 67 (GAD67) were evaluated in the brain by Western blot. Data were analyzed using two-way ANOVA and shown as ratios to water drinking mice with saline instillation (mean ± SEM; n=4-6/group).
5.4. Discussion

Chronic Mn exposure is associated with impaired dopaminergic function [201,202]. Several lines of evidence have suggested that exposure to Mn induces depletion of dopamine [203-205]. Moreover, I previously reported that intranasal instillation of Mn causes down-regulation of TH in the striatum along with impaired motor function in rats [206]. Although motor coordination is mediated by several neurotransmission pathways, the dopaminergic pathway has been shown to play an important role [207]. Mixed results exist about the impact of ethanol on TH levels. For example, Tran et al. indicated an increase in TH protein and dopamine synthesis in the brain of acute alcohol-exposed zebrafish [208]. Whereas Zhang et al. [209] showed no change in TH expression by immunostaining along with no change in dopamine levels in the ventral tegmental area of rats after 30 days of alcohol exposure, which is in agreement with my findings that no change of TH and dopamine levels in alcohol drinking mice. Therefore, it is possible that my observation of further diminished TH levels in Mn instilled, alcohol drinking mice are due to elevated Mn accumulation in the brain by alcohol exposure. This result suggests a decrease of dopamine synthesis in the brain upon Mn intranasal instillation, and this effect is augmented by ethanol drinking. Consequently, decreased DAT density after intranasal instillation of Mn could result from a compensatory mechanism in response to decreased dopamine levels [210]. Interestingly, I found alcohol exposure decreased DAT expression in the brain, suggesting increased extracellular dopamine levels. Congruently, several studies have demonstrated elevation of extracellular dopamine levels in the brain regions after alcohol exposure [211,212].

With respect to dopamine receptors, Nam et al. earlier showed that motor coordination was significantly decreased in mice along with increased mRNA and protein levels of D2DR in the striatum after 5-day of Mn intraperitoneal injections [213], suggesting that Mn-induced motor deficits could be modulated in part by the expression of D2DR. In line with this finding, experimental evidence suggests that dopamine depletion in the stratum of patients with
Parkinson’s disease is associated with increased D2DR expression in the striatum, possibly due to compensatory mechanisms [214-216], while inconsistent results were reported on D1DR expression from studies of Parkinson’s disease [217-219]. Since D2DR is a primary therapeutic target for the treatment of bradykinesia associated with Parkinson’s disease [220], up-regulation of D2DR expression could be a risk factor for motor deficits. In the present study, while Mn instillation did not modify D1DR, there was an elevation of D2DR after olfactory Mn exposure, and this effect was enhanced by alcohol drinking. Hence, it is plausible that the poorer performance of motor function evaluated by Grooved Pegboard and Finger Tapping by Ellingsen et al. [200] in welders and exacerbated motor impairment in alcohol-drinking welders could be attributed to decreased dopamine synthesis and the subsequent increased D2DR levels. Further studies on the characterization of synaptic neurotransmitters would help to illustrate the effect of Mn instillation and alcohol exposure on monoaminergic signaling pathways.

Recent investigations also have suggested that Mn exposure can induce GABAergic dysregulation, but results of GABA concentrations in the brain upon Mn exposure are inconsistent [221-223]. I observed a decrease in GABA concentrations in the whole brain hemisphere of mice treated with intranasal instillation of Mn. The difference in GABA concentration changes by Mn exposure may result from different doses and duration of exposure and/or routes of exposure to Mn. In addition, the effect of ethanol on GABAergic system has been investigated for years. It is well-recognized that the pharmacological effects of alcohol significantly affect GABAergic neurotransmission [121], however, conflicting results have been reported regarding the effects of ethanol on GABA levels in the brain [224,225]. Moreover, Ledig et al. studied the effect of pre- and postnatal alcohol consumption on GABA levels of various brain regions in the rat offspring and found that GABA levels in the brain respond to alcohol consumption in a region-specific fashion [225]. For example, GABA levels were decreased in the thalamus, pons, cerebellum and hippocampus, unchanged in the posterior colliculus, occipital cortex, temporal cortex, hypothalamus, septum or striatum and increased in the frontal cortex, olfactory bulbs, anterior colliculus and amygdala in rats with pre- and postnatal alcohol exposure [225]. In my study, sub-chronic alcohol drinking did not alter GABA levels in the intact brain hemisphere. However, I did not examine
extracellular levels of GABA, which would reflect the event at the synaptic level and can be associated with neuronal functions more relevantly than tissue levels. Therefore, future studies are needed to directly determine extracellular GABA concentrations using microdialysis. In addition, I observed a further decrease in brain GABA in Mn intranasally-instilled mice after alcohol exposure. Since alcohol did not affect brain GABA levels in my study, I speculate that the more decreased GABA levels in alcohol-exposed mice with Mn instillation, when compared with Mn instillation without alcohol exposure, are the consequence of exacerbated Mn-induced neurotoxicity, possibly due to increased Mn accumulation in the brain by alcohol.

In addition to brain GABA levels, I also examined GABA metabolism in my study. I observed a reduction in GAD, the enzyme that is responsible for GABA synthesis after Mn intranasal instillation, but not in alcohol-exposed mice. However, in alcohol-exposed mice with Mn instillation, both isoforms of GAD (GAD 65 and GAD67) were decreased compared to water-drinking, Mn-instilled mice. The pattern of decreased GAD is consistent with GABA concentration after Mn exposure and alcohol treatment. Therefore, it is reasonable to conclude that decreased GABA levels in the brain of Mn-instilled mice after alcohol drinking is due to decreased GAD without affecting GAT levels. In support of this idea, Tomas-Camardiel et al. showed that Mn treatment led to a decrease in GAD in the globus pallidus of rats exposed to Mn via intraperitoneal injections for 30 days [226]. In addition, a decrease in GABRA2 expression was observed after intranasal instillation of Mn, and further diminished by alcohol exposure without changes in GABRA1. These results suggest that GABAergic signaling was decreased by intranasal Mn instillation, which is further down-regulated by alcohol exposure. While some studies reported that ethanol exposure alters the expression of GABA receptors, for example, GABA receptor binding sites were augmented in patients with alcoholic brain [227], I did not observe the changes of GABRA1 and GABRA2 in protein levels caused by sub-chronic alcohol drinking. These suggest that alcohol treatment alone in my study might not be enough to induce GABAergic system changes. Taken together, these results suggest that Mn-induced GABAergic disruption is exacerbated by alcohol exposure.
5.5. Conclusions

In this chapter, I demonstrated that alcohol exacerbated Mn-induced neurochemical toxicity by demonstrating that dopaminergic and GABAergic signaling were disrupted in Mn-exposed mice by intranasal instillation, and these changes were further exacerbated by alcohol consumption. This study provides potential molecular mechanisms underlying alcohol and Mn interaction by which humans who drink alcohol may have a higher risk of Mn neurotoxicity after inhalation.
CHAPTER 6. MANGANESE TRANSPORT IN A MOUSE MODEL OF BINGE DRINKING

6.1. Introduction

Accumulating studies suggest that binge drinking in humans is prevalent and has serious physiological and psychological consequences [228]. While effects of chronic or sub-chronic alcohol consumption on metal transport and neurotoxicity have been studied by many investigators [229,230], little is known about the effects of acute ethanol consumption on metal transport. Acute consumption of alcohol or binge drinking is more common and in humans than chronic alcoholism [231]. A recent survey from National Epidemiologic Survey on Alcohol and Related Conditions nationwide indicated that 8.5% of more than 13,000 adults consumed to exceed 5/4 binge drinking guidelines of the National Institute on Alcohol Abuse and Alcoholism 1–2 times per week [232]. Binge drinking is a severe risk factor to cause injury, driving accidents and death due to overdose [228]. There are also longer-term risks from repeated episodes of binge drinking that induces neurotoxicity, as well as severe adverse consequences to heart, liver, immune system, bone health and other organ system[228]. To better mimic alcohol drinking style in humans, I employed binge drinking model using mice. Since administration of alcohol by gavage provides a reasonable representation of binge drinking in humans [228], in this chapter I explored the effect of binge drinking on Mn transport in the tissue and brain in mice, as well as the potential molecular mechanisms.

6.2. Methods
6.2.1. **Animals.** Animal protocols were approved by the Division of Laboratory Animal Medicine (DLAM) and the Northeastern University-Institutional Animal Care and Use Committee (NU-IACUC). Animals were maintained on a 12:12 hour light-dark cycle and given water *ad libitum* provided by DLAM.

6.2.2. **Binge drinking.** Mice (mixed background of C57BL/6 and 129Sv/J strains; male and female; 8-9 weeks old) were administered with water or alcohol (5 g/kg, 25% v/v) by oral gavage daily for 7 days.

6.2.3. **Euthanasia and tissue collection.** After the treatment, animals were euthanized by an overdose of isoflurane, followed by exsanguination and collection of brain. The brains were flash-frozen in liquid nitrogen and stored at -80°C until analysis.

6.2.4. **Western blot analysis.** The whole brain, as well as liver and duodenum from mice with binge drinking were homogenized on ice in RIPA buffer (100 mM Tris, 0.2% SDS, 2% NP40 and 1.0% sodium deoxycholate, pH 7.5) containing protease inhibitor (Complete Mini, Roche; Nutley, NJ, USA). Samples were centrifuged at 16,000 g for 6 min at 4°C. After determination of protein levels, samples (40 µg of proteins) were electrophoresed on a 10% SDS-polyacrylamide gel and transferred to either polyvinylidene difluoride (Millipore, Billerica, MA) or nitrocellulose (GE Healthcare, Piscataway, NJ) membrane. After blocking with 5% non-fat milk, the membrane was incubated the membrane was incubated in rabbit antibody against FPN (1:200; Novus Biologicals, Littleton, CO), TfR1 (1:1,000; Invitrogen, Waltham, MA), ZIP14 (1:1,000; Abcam, Cambridge, MA) and SLC30A10 (1:200; Santa Cruz Biotech, Dallas, TX). As a loading control, the immunoblot was incubated with mouse anti-actin (1:5,000; MP Biomedicals, Solon, OH). The blots were incubated with donkey anti-rabbit secondary antibody (1:1,000; GE Healthcare), donkey anti-goat antibody (1:1,000; Santa Cruz Biotech) or sheep anti-mouse antibody conjugated with HRP (1:5,000; GE Healthcare), followed by chemiluminescence (ECL West Dura, Thermo Scientific) and scanned using Chemidoc System (ChemiDoc XRS, Bio-Rad, Hercules, CA). Relative intensities of protein bands normalized to actin was determined using Image Lab (Bio-Rad, version 4.1).
6.2.5. **Real-time qPCR.** RNA was isolated from snap-frozen tissues, such as whole brain, liver and duodenum scrapping using TRI reagent (Sigma-Aldrich, St. Louis, MO, USA) as per the manufacturer’s instructions. RNA (1 µg) was reverse transcribed into cDNA, which was used for real-time polymerase chain reaction assays. The iScript™ reverse transcription supermix and iTaq™ universal SYBR® green supermix were obtained from Bio-Rad (Hercules, California). Primers were obtained from Eurofins, MWG Operon (Huntsville, AL) and sequences of each primer were shown in table 2.1. Amplification of the DNA was programmed at 95°C for 2 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min. The expression level of each gene was normalized to that of cyclophilin and analyzed by the comparative Ct method ($2^{-\Delta\Delta Ct}$) [129].

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMT1</td>
<td>Forward: CGTACCGCCTGGGACTGA</td>
<td>[130]</td>
</tr>
<tr>
<td></td>
<td>Reverse: GTCATCTGGACACCACTGAGTCA</td>
<td></td>
</tr>
<tr>
<td>TfR1</td>
<td>Forward: TGGGTCTAAGTCTACAGTGCC</td>
<td>[131]</td>
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<tr>
<td></td>
<td>Reverse: AGATACATAGGGCGACAGGAA</td>
<td></td>
</tr>
<tr>
<td>FPN</td>
<td>Forward: TTGCAGGAGTCATTGCTGCTA</td>
<td>[132]</td>
</tr>
<tr>
<td></td>
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<td></td>
</tr>
<tr>
<td>SLC30A10</td>
<td>Forward: TGTCTCCTGCTTCTCCTCTTG</td>
<td>[133]</td>
</tr>
<tr>
<td></td>
<td>Reverse: AGCTCTGTAGTCTGTCTGTTGA</td>
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<tr>
<td>hepcidin</td>
<td>Forward: TGTCTCCTGCTTCTCCTCCTTG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reverse: AGCTCTGTAGTCTGTCTGTTGA</td>
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</tr>
<tr>
<td>Heme</td>
<td>Forward: GGTGATGGCTTCTTGTTACC</td>
<td>[176]</td>
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<tr>
<td>oxygenase</td>
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<td>1 (HO-1)</td>
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<td>[177]</td>
</tr>
<tr>
<td></td>
<td>Reverse: TTGTGTGGGGCGCAGTACTG</td>
<td></td>
</tr>
<tr>
<td>Hypoxia-inducible factor 2-alpha (HIF-2α)</td>
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<td>[177]</td>
</tr>
<tr>
<td></td>
<td>Reverse: CTTCTCCATCATCTGGGATCTGGGACT</td>
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</tbody>
</table>

6.2.6. **Metal analysis.** Inductively-coupled plasma mass spectrometry (ICP-MS) was used to determine metal levels in blood and tissues such as liver and brain. Briefly, wet tissues (brain, liver and blood) were weighed and
digested in 1 mL of acid solution (20% nitric acid for brain and liver; 10% nitric acid for blood; Trace metal grade; Fisher Scientific) for 1 h at 125°C in a dry bath. Following the digestion, double-distilled water was added to the samples, to a total volume of 10 mL, followed by filtration through a syringe filter; the amount of essential transition metals (e.g. iron and manganese) were then be quantified using ICP-MS. Metal concentrations in the samples were determined by an external calibration method based on ICP-MS standard solutions (ICP-MS calibration standard 3-A; High Purity Standards) that ranging from 0 to 5,000 ng/mL. Data were presented as ppm.

6.2.7. Liver function analysis. Alanine aminotransferase (ALT) activity in the serum and liver homogenates were measured using a colorimetric assay (Abcam, Boston, MA) according to the instruction provided. Gamma-glutamyl transferase (GGT) activity in the liver was measured using Diagnostic kit (Sigma Chemical Company, St. Louis, MO) according to the instruction provided.

6.2.8. Statistical analysis. In the experiments of binge drinking in mice, comparisons among two groups, Student’s t-test was used for the statistical analysis. Differences were considered significant at p < 0.05. Values were reported as means ± SEM.

6.3. Results

6.3.1. Systemic metal levels are elevated in the mice with binge drinking. In mice with binge drinking, Fe levels in the blood and liver were elevated in a dose-dependent manner (Figure 6-1, A and B; p=0.003 (blood), p=0.015 (liver)). Similarly, Mn levels in the blood and liver also increased after alcohol exposure (Figure 6-2, A and B; p=0.017 (blood), p=0.033 (liver)). However, copper (Cu) and zinc (Zn) levels did not change upon binge drinking exposure in either blood or liver. Taken together, these results suggest that exposure of binge drinking increases body Mn and Fe stores.
Figure 6-1. Metal levels are elevated in the blood of mice with binge drinking.
Mice (mixed background of C57BL/6 and 129Sv/J strains strain; male and female; 8-9 weeks old) were treated with binge drinking or water for 7 days. After euthanasia, Mn (Figure 6-1A), Fe (Figure 6-1B), Cu (Figure 6-1C) and Zn (Figure 6-1D) levels in the blood were determined by inductively coupled plasma mass spectrometry (ICP-MS). Data were presented as means ± SEM (n= 7-8 per group) and were analyzed using one-way ANOVA. * p < 0.05.
Figure 6.2. Metal levels are elevated in the liver of mice with binge drinking.
Mice (mixed background of C57BL/6 and 129Sv/J strains strain; male and female; 8-9 weeks old) were treated with binge drinking or water for 7 days. After euthanasia, Mn (Figure 6-2A), Fe (Figure 6-2B), Cu (Figure 6-2C) and Zn (Figure 6-2D) levels in the liver were determined by inductively coupled plasma mass spectrometry (ICP-MS). Data were presented as means ± SEM (n= 7-8 per group) and were analyzed using one-way ANOVA. * p < 0.05.

6.3.2. Metal levels are elevated in the brain of mice with binge drinking. Both Fe and Mn in the brain were increased in mice with binge drinking (Fig 6-3; 14% increase in Mn, p=0.010; 13% increase in Fe, p=0.008). Again, neither Cu nor Zn levels were altered by alcohol exposure. Our results demonstrate that alcohol exposure modifies not only systemic homeostasis of Mn and Fe, but also the transport of these two metals in the brain.
Figure 6-3. Metal levels are elevated in the brain of mice with binge drinking.
Mice (mixed background of C57BL/6 and 129Sv/J strains strain; male and female; 8-9 weeks old) were treated with binge drinking or water for 7 days. After euthanasia, Mn (Figure 6-3A), Fe (Figure 6-3B), Cu (Figure 6-3C) and Zn (Figure 6-3D) levels in the brain were determined by inductively coupled plasma mass spectrometry (ICP-MS). Data were presented as means ± SEM (n= 7-8 per group) and were analyzed using one-way ANOVA.  
*p < 0.05.

6.3.3. Fe transporters are elevated in the duodenum of mice with binge drinking. To evaluate whether metal transporters were up-regulated in response to binge drinking, I determined the protein and/or mRNA expression of DMT1, TfR1, ZIP14, FPN and SLC30A10 in the duodenum and liver of mice with binge drinking by Western blotting (Figure 6-4) and real-time qPCR (Figure 6-5). Similar to sub-chronic alcohol drinking, binge drinking increased the protein levels of Fe importers including TfR1 (Figure 6-4; 593% increase, p=0.014), ZIP14 (Figure 6-4; 109% increase, p=0.021) and metal exporter FPN (Figure 6-4; 86% increase, p=0.048) were up-regulated upon alcohol exposure in the duodenum compared to water-administered control. Consistent with protein expressions, mRNA levels of Fe importers DMT1 (Figure 6-5; 23% p=0.031), TfR1 (Figure 6-5; 30%, p=0.027)
and FPN (Figure 6-5; 48% increase, p=0.006) were also up-regulated in the duodenum of mice with binge drinking compared to water-administered mice. However, Mn specific exporter, SLC30A10 was not altered in protein or mRNA levels in the duodenum of mice with binge drinking. Combined, these data suggest increased Fe and Mn levels in the body of mice with binge drinking, possibly due to increased absorption and tissue uptake by up-regulation of Fe transporters.

**Figure 6-4. Expression of metal transporters in the duodenum of mice after alcohol consumption.**

Mice (mixed background of C57BL/6 and 129Sv/J strains; male and female; 8-9 weeks old) were treated with binge drinking or water for 7 days. After euthanasia, duodenum was collected for protein quantification. TfR1, ZIP14, FPN and SLC30A10 levels in the duodenum (Figure 6-4) were quantified by Western blot. Data were presented as means ± SEM (n= 4 per group) and were analyzed using Student’s t-test. * p < 0.05.
Figure 6-5. mRNA levels of metal transporters in the duodenum of mice after alcohol consumption.

Mice (mixed background of C57BL/6 and 129Sv/J strains strain; male and female; 8-9 weeks old) were treated with binge drinking or water for 7 days. After euthanasia, duodenum was collected for mRNA quantification. DMT1, TfR1, FPN and SLC30A10 levels in the duodenum (Figure 6-5) were quantified by qRT-PCR. Data were presented as means ± SEM (n= 4 per group) and were analyzed using Student t-test. * p < 0.05.

6.3.4. Fe transporters are elevated in the liver of mice with binge drinking. To evaluate whether increased Mn and Fe levels in the liver of mice with binge drinking are due to increased Fe transporters, I determined the protein and/or mRNA expression of DMT1, TfR1, ZIP14, FPN and SLC30A10 in the liver of mice with binge drinking by western blot analysis (Figure 6-6) and real-time qPCR (Figure 6-7). Similar to sub-chronic alcohol drinking, binge drinking increased the protein levels of Fe importers including TfR1 (Figure 6-6; 522% increase, p<0.001), ZIP14 (Figure 6-6; 124%, p=0.049) and metal exporter FPN (Figure 6-6; 94% increase, p=0.036) were up-regulated upon alcohol exposure in the liver compared to water-administered control. Consistent with protein
expressions, mRNA levels of Fe importers DMT1 (Figure 6-7; 17% p=0.042), TfR1 (Figure 6-7; 88%, p=0.039) and FPN (Figure 6-7; 81% increase, p=0.037) were also up-regulated in the liver of mice with binge drinking compared to water-administered mice. However, Mn specific exporter, SLC30A10 was not altered in protein or mRNA levels in the liver of mice with binge drinking. Collectively, these data suggest increased of Fe and Mn levels in the liver of mice with binge drinking, are due to increased tissue uptake by up-regulation of Fe transporters.

![Protein levels of metal transporters in the liver](image)

Figure 6-6. mRNA levels of metal transporters in the liver of mice after alcohol consumption. Mice (mixed background of C57BL/6 and 129Sv/J strains strain; male and female; 8-9 weeks old) were treated with binge drinking or water for 7 days. After euthanasia, liver was collected for protein quantification. TfR1, ZIP14, FPN and SLC30A10 levels in the duodenum (Figure 6-6) were quantified by western blot analysis. Data were presented as means ± SEM (n= 4 per group) and were analyzed using Student’s t-test. * p < 0.05.
Figure 6-7. mRNA levels of metal transporters in the liver of mice after alcohol consumption.

Mice (mixed background of C57BL/6 and 129Sv/J strains strain; male and female; 8-9 weeks old) were treated with binge drinking or water for 7 days. After euthanasia, liver was collected for mRNA quantification. DMT1, TfR1, FPN and SLC30A10 levels in the duodenum (Figure 6-7) were quantified by qRT-PCR. Data were presented as means ± SEM (n= 4 per group) and were analyzed using Student’s t-test. * p < 0.05.

6.3.5. Fe transporters are elevated in the brain of mice with binge drinking. To further evaluate if Fe and Mn transporters in the brain were also up-regulated in the brain after acute alcohol exposure, protein and/or mRNA expressions of DMT1, TfR1, ZIP14, FPN and SLC30A10 in the whole brain of mice with binge drinking or water administration were determined. Firstly, the protein levels of Fe importers including TfR1 (Figure 6-8; 129% increase; p=0.005) and ZIP14 (Figure 6-8; 124% increase; p=0.014) as well as metal exporter FPN (Figure 6-8; 141% increase, p=0.019) were up-regulated upon alcohol exposure. Consistent with protein expressions, mRNA
levels of Fe importers, DMT1 (Figure 6-9; 62% increase; p=0.048), (Figure 6-9; 53% increase; p=0.044) and FPN (Figure 6-9; 56% increase; p=0.043) were also up-regulated. Although the protein levels of SLC30A10 was not altered in the brain of mice with alcohol drinking, mRNA levels of the exporter were increased upon alcohol exposure (71% increase; p=0.035). Collectively, my result indicated that similar to sub-chronic alcohol drinking, binge drinking also increases Mn and Fe absorption and tissue uptake by up-regulation of Fe transporters.

**Figure 6-8. Expression of metal transporters in the brain of mice after alcohol consumption.**
Mice (mixed background of C57BL/6 and 129Sv/J strains; male and female; 8-9 weeks old) were treated with binge drinking or water for 7 days. After euthanasia, brain was collected for protein quantification. TfR1, ZIP14, FPN and SLC30A10 levels in the duodenum (Figure 6-8) were quantified by western blot. Data were presented as means ± SEM (n= 4 per group) and were analyzed using Student’s t-test. * p < 0.05.
**Figure 6-9. mRNA levels of metal transporters in the brain of mice after alcohol consumption.**

Mice (mixed background of C57BL/6 and 129Sv/J strains strain; male and female; 8-9 weeks old) were treated with binge drinking or water for 7 days. After euthanasia, brain was collected for mRNA quantification. DMT1, TfR1, FPN and SLC30A10 levels in the duodenum (Figure 6-7) were quantified by qRT-PCR. Data were presented as means ± SEM (n= 4 per group) and were analyzed using Student’s t-test. * p < 0.05.

6.3.6. Hepcidin levels are decreased in the mice with binge drinking. Since Fe transporters were up-regulated upon acute alcohol exposure, I tested hepcidin levels in the duodenum, liver and brain in mice with binge drinking (Figure 6-10). Consistent with sub-chronic alcohol drinking, hepcidin was decreased in the duodenum (32%, p=0.025), liver (49%, p=0.008) and brain (26%, p=0.041) in mice upon binge drinking. This result suggests that acute alcohol exposure by binge drinking increases iron absorption possibly through up-regulation of Fe transporters and down-regulation of hepcidin.
Figure 6- 10. Hepcidin expression in the duodenum, liver and brain of mice with binge drinking.

Mice (mixed background of C57BL/6 and 129Sv/J strains strain; male and female; 8-9 weeks old) were treated with binge drinking or water for 7 days. After euthanasia, the mRNA levels of hepcidin in the duodenum, liver and brain were analyzed by qRT-PCR. The expression level of each gene was normalized to that of actin according to the comparative Ct method (2-ΔCt) and analyzed using Student’s t-test. Data were shown as ratios to mice with water administration (mean ± SEM; n=4/group).

6.3.7. Binge drinking up-regulates hypoxia-related genes in the brain. To evaluate if acute alcohol exposure induces hypoxia in the brain, the levels of hypoxia-related gene expression by qRT-PCR in the brain of mice with binge drinking were examined (Figure 6-11). HIF-1α mRNA levels increased by 72% (p=0.044) in the brain of mice with binge drinking compared to mice with water administration. Similarly, HO-1 levels were also up-regulated by binge drinking (67% increase, p<0.001) in the brain of alcohol-binged mice compared water treated
group. However, neither HIF-2α nor HIF-1β was altered by binge drinking. These data suggest that acute alcohol exposure induces hypoxia in the brain, and that possibly contribute to the up-regulation of metal transporters and thereby promotes Mn deposition in the brain.

**Figure 6-11. Hypoxia-related mRNAs in the brain of mice with binge drinking.**

Mice (mixed background of C57BL/6 and 129Sv/J strains; male and female; 8-9 weeks old) were treated with binge drinking or water for 7 days. After euthanasia, the levels of mRNA expression including hypoxia-inducible factor 1-alpha (HIF-1α), hypoxia-inducible factor 2-alpha (HIF-2α), hypoxia-inducible factor 1-beta (HIF-2β) and heme oxygenase 1 (HO-1) in the brain were determined by qRT-PCR. The expression level of each gene was normalized to that of actin according to the comparative Ct method ($2^{-\Delta\text{Ct}}$) and analyzed using Student’s t-test. Data were shown as ratios to water administered mice (mean ± SEM; n= 4/group).
6.3.8. Liver dysfunction may also contribute to increased Mn status after binge drinking. We evaluated the potential impact of liver damage on elevated Mn levels after acute alcohol exposure by binge drinking (Figure 6-12). Although alanine aminotransferase (ALT) did not change in the serum or liver of mice upon binge drinking, hepatic gamma-glutamyl transferase (GGT) was increased (47% increase, p=0.031). Therefore, acute alcohol exposure is more likely to induce liver damage than sub-chronic alcohol drinking, which may partially contribute to increased Mn status after binge drinking.

Figure 6-12. Liver function tests in mice with binge drinking.

Mice (mixed background of C57BL/6 and 129Sv/J strains strain; male and female; 8-9 weeks old) were treated with binge drinking or water for 7 days. After euthanasia, liver tissues were collected from mice with binge drinking. The concentrations of alanine aminotransferase (ALT) in the serum (Figure 6-12A) and liver (Figure 6-12B) and gamma-glutamyl transpeptidase (GGT) in the liver (Figure 6-12C) were analyzed by colorimetric
assays. Data were analyzed using Student’s t-test and shown as IU/L in the serum and IU/g tissue in the liver (mean ± SEM; n=4/group).

6.4. Discussion

Previous chapters were focused on the mechanism of Mn transport upon sub-chronic alcohol drinking. However, sub-chronic alcohol exposure by drinking water does not exactly mimic alcohol consumption in humans. Therefore, in this chapter, I attempted to extend the study of the alcohol-Mn relationship to more realistic exposure using an binge drinking model in mice to mimic alcohol exposure conditions in humans: heavy, episodic, voluntary oral consumption of alcoholic beverages.

To better simulate real-life conditions of alcohol drinking in humans, I employed an binge drinking model in mice. There are several animal models currently in use to mimic the human drinking spectrum and severity of liver diseases, including chronic intragastric ethanol administration [233], chronic-plus-binge ethanol administration by National Institute on Alcohol Abuse and Alcoholism (NIAAA) [234]. Chronic intragastric ethanol administration by French et al. causes severe steatosis with inflammation and fibrosis; and the NIAAA model induces a neutrophil-mediated liver injury [234]. Since the purpose of my study was to understand how human drinking patterns affects Mn transport, I chose a relatively mild 7-d intragastric alcohol administration to minimize liver injury. In agreement with long-term alcohol exposure by drinking water, binge drinking decreased hepcidin levels in the duodenum, liver and brain, and increased the expression of Fe transporters (TfR1, ZIP14 and FPN). However, although ALT did not change upon binge drinking in either liver or serum, levels of GGT in the liver were elevated. Of note, my results also revealed the up-regulation of hypoxia-associated genes in binge drinking mice, which would contribute to elevated metal transporters in tissues.
While I demonstrated that there are many common features in Mn transport and Fe homeostasis between sub-chronic alcohol drinking and 7-d binge drinking models, it is worthwhile to discuss a few differential effects of these two methods of alcohol exposure on metal homeostasis. First, levels of Mn and Fe were increased more in sub-chronic alcohol drinking than binge drinking. For example, in mice with sub-chronic alcohol treatment, there was a 20% increase in brain Mn and a 40% in brain Fe, while binge drinking only increased 14% of Mn and 13% of Fe in the brain. Although there is no available study of acute ethanol exposure on metal transport, there are several studies showing differences in acute and chronic ethanol consumption on tissue and brain toxicities [235,236]. Previously, I have demonstrated that hepcidin production was inhibited in mice as early as one week of alcohol treatment compared to water drinking group, and the difference was larger after four-week alcohol treatment than 7-d binge drinking. This could be due to more pronounced up-regulation of Fe and Mn transporters in mice that were exposed to alcohol for 4 weeks. In support, the alcohol kinetics study in Chapter 2 demonstrated that four-week of alcohol treatment elevated Mn and Fe more in the blood, liver and brain compared to one-week of alcohol treatment. Collectively, my study demonstrated Mn and Fe levels were elevated more significantly by sub-chronic alcohol drinking than acute binge drinking, possibly due to different levels of hepcidin and metal transporters in response to acute and sub-chronic alcohol treatment.

Second, gene expression levels in the brain of HIF-1α and HO-1 were up-regulated by both sub-chronic alcohol drinking and binge drinking, suggesting hypoxic conditions. Our result is in agreement with several studies that reported that HIF-1α is up-regulated in both acute binge and chronic alcohol drinking in mice [170,192,237]. However, mixed results were reported about HO-1 levels in response to acute and chronic alcohol exposure [238,239]. It should be noted that HO-1 and HO-2 levels are regulated differently in the duodenum, liver and brain upon acute and chronic alcohol exposure [238], suggesting that chronic and acute alcohol exposure may modify HO via different mechanisms, and the regulation of HO occurs in a tissue-specific manner. Future studies are necessary to evaluate the HIF family and HO in the duodenum and liver, in addition to brain.
Third, I showed an increase of GGT in the liver upon binge drinking, while unchanged GGT was found in the liver of sub-chronic drinking mice. The result suggests that modest liver damage caused by binge drinking, but not sub-chronic alcohol drinking. Several studies found that long-term of alcohol drinking has little effect on liver damage [240], while acute gavage of a single dose or multiple doses of ethanol induces hepatic steatosis [241-244]. It is possible that after sub-chronic alcohol consumption, the mice may have developed tolerance to alcohol. Taken together, my investigation utilizing an binge drinking model demonstrates that humans who consume alcohol acutely may have an increased risk of Mn neurotoxicity when exposed to Mn in our environment as well as in occupational settings.

6.5. Conclusions

Mn levels in various tissues were increased upon acute alcohol exposure (binge drinking) in mice, likely due to up-regulation of Fe transporters. The changes in Fe transporters are associated with decreased production of hepcidin and hypoxia-induced elevation in HIF expression. A mild liver injury could in part contribute to increased Mn retention in the body and brain.
OVERALL CONCLUSIONS

Figure 7-1. Model of Mn-induced neurotoxicity under the influence of alcohol consumption.

Overall, the focus of my study was to investigate the mechanism by which alcohol consumption increases Mn uptake, which represents a potential risk factor for metal-induced neurotoxicity. In addition to sub-chronic alcohol drinking, binge drinking model was also used to study the effect of acute alcohol exposure on Mn transport. My investigation has revealed that ethanol consumption in mice increased Mn uptake into the brain after intranasal
instillation of Mn in a dose-dependent manner, due to up-regulation of Fe transporters that mediate the uptake of Mn as well as Fe. My study further demonstrated that the increased Fe transporters could occur due to down-regulation of hepcidin and increased hypoxia response upon alcohol exposure. Moreover, dopaminergic and GABAergic signaling was disrupted in Mn-instilled mice, which was exacerbated by alcohol exposure. Therefore, my model suggests that humans who drink alcohol may have a higher risk of Mn neurotoxicity after inhalation of Mn.
REFERENCES


