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Mutation in HFE gene decreases manganese accumulation and oxidative stress in the brain after olfactory manganese exposure

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Abstract

Increased accumulation of manganese (Mn) in the brain is significantly associated with neurobehavioral deficits and impaired brain function. Airborne Mn has a high systemic bioavailability, and can be directly taken up into the brain, making it highly neurotoxic. While Mn transport is in part mediated by several iron transporters, the expression of these transporters is altered by the iron regulatory gene HFE. Mutations in the HFE gene are the major cause of the iron overload disorder hereditary hemochromatosis, one of prevalent genetic diseases in humans. However, whether or not HFE mutation modifies Mn-induced neurotoxicity has not been evaluated. Therefore, our goal was to define the role of HFE mutation in Mn deposition in the brain and resultant neurotoxic effects after olfactory Mn exposure. Mice carrying H67D HFE mutation that is homologous to H63D mutation in humans and their control wild-type mice were intranasally instilled with MnCl₂ with different doses (0, 0.2, 1.0 and 5.0 mg/kg) daily for 3 days. Mn levels in the blood, liver and brain were determined using inductively-coupled plasma mass spectrometry (ICP-MS). H67D mutant mice showed significantly lower Mn levels in blood, liver and most brain regions, especially in the striatum, while mice fed iron overload diet did not. Moreover, mRNA expression of ferroportin, an essential exporter of iron and Mn, was up-regulated in the striatum. In addition, the levels of isoprostane, a marker of lipid peroxidation, were increased in the striatum after Mn exposure in wild-type mice, but unchanged in H67D mice. Together, our results suggest that H67D mutation provides decreased susceptibility to Mn accumulation in the brain and associated neurotoxicity induced by inhaled Mn.

Keywords

ferroportin; hemochromatosis; intranasal instillation; isoprostane; oxidative stress; striatum

INTRODUCTION

Mutations in the HFE (High iron or Fe) gene are the major cause of hereditary hemochromatosis (HH), the most common iron (Fe) overload disorder in the North American Caucasian population^{1,2}. The two most prevalent HFE missense variants are C282Y and H63D. The C282Y mutation is primarily associated with increased iron levels in

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serum and several organs, such as liver and heart³, which predisposes to liver cirrhosis and cardiomyopathy. The H63D mutation, on the other hand, is associated with the development of several neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis³. In particular, the H63D variants are found worldwide in European (30.4%), North American (27.4%), African/Middle Eastern (16.3%) and Asian (2.8%) populations⁴. Notably, Nandar et al. demonstrated that mice carrying H67D knock-in mutation, which is homologous to H63D variant in humans, display not only hepatic iron overload, but also a trend of elevated iron levels in the brain⁵, which could contribute to oxidative brain damage and memory deficits⁶. Recently, we have shown that H67D mutation in mice increases repetitive behavior along with impaired dopamine signaling pathway⁷. These results indicate that increased brain iron stores could promote neurobehavioral deficits. However, the distribution of iron in different brain regions upon HFE mutation has not been explored.

It has been well-recognized that loss of HFE function results in abnormal expression of iron transporters, which consequently promotes systemic iron overload. For example, HFE deficiency show up-regulation of divalent metal transporter 1 (DMT1), a major importer of iron, in the duodenum of both humans and mice^{8, 9}. Also, loss of HFE function increases the expression of ferroportin (FPN) in the liver in humans and mice¹⁰. However, less information is known about iron transporters in the brain upon HFE mutation. Importantly, Nandar et al. showed decreased expression of iron importers, including DMT1 and transferrin, in the whole brain in H67D mutant mice⁵, suggesting that HFE mutation could alter iron transport into the brain. However, it is unclear whether or not the export of iron from the brain is modified in H67D mutation.

It has been well-demonstrated that altered iron status disrupts manganese (Mn) homeostasis^{11–14}. Mn is an essential metal, serving as a cofactor for a number of metalloenzymes vital to brain function, including superoxide dismutase, glutamine synthetase and arginase^{15, 16}. However, abnormally high Mn levels in the brain can induce oxidative stress and cause neuronal damage^{17, 18}. Airborne Mn exposure has higher risks in Mn-associated neurotoxicity than oral exposure, because the hepatic first-pass elimination through bile secretion provides a protective mechanism against the toxicity of ingested Mn. However, an inhalation route bypasses this homeostatic excretion and thereby increases systemic absorption of Mn¹⁹. This has raised public concerns because high levels of Mn in the brain are associated with various neurological problems, such as memory deficits, impaired motor function and psychotic behaviors that resemble Parkinson-like symptoms²⁰. People in occupational settings of mining, dry battery production and organochemical fungicide use are at higher risk of Mn toxicity^{20–22}.

Mn transport is mediated by multiple mechanisms. To date, several Mn-specific transporters have been identified, including SLC30A10, ATP13A2, and SPCA1²³. In particular, the role of SLC30A10 in Mn transport and associated neurotoxicity has been recently examined. Patients with SLC30A10 mutation display increased Mn levels in the brain²⁴. Conversely, overexpression of SLC30A10 protects primary neuronal cells against Mn-induced neurotoxicity²⁵. Mn is also transported by several iron transporters, including DMT1, transferrin receptor (TfR), zinc-regulated transporter/iron-regulated transporter-like protein

14 (ZIP14) and FPN²³. Therefore, altered expression of iron transporters affects Mn transport and metabolism. For example, Belgrade rats that carry DMT1 mutation exhibit impaired systemic Mn absorption¹¹. When exposed to Mn by intranasal instillation of the metal, Belgrade rats show reduced Mn accumulation in the basal ganglia and hippocampus¹². In addition, iron deficiency up-regulates DMT1 expression in the intestine and olfactory epithelium^{12, 26} and increases blood Mn levels^{13, 14}. Moreover, mice deficient in FPN show impaired intestinal Mn absorption, resulting in lower Mn levels in liver and blood²⁷. These lines of evidence indicate that iron transporters play an important role in Mn homeostasis.

Despite high prevalence of HH and toxicity associated with inhaled Mn, there are only a few studies concerning the transport and toxicity of Mn in the brain in HFE deficiency. Our previous pharmacokinetic study²⁸ showed an increased uptake of Mn into the brain of HFE-knockout mice after intranasal instillation of ⁵⁴MnCl₂. In contrast, the steady-state levels of Mn in blood are lower in both HFE-knockout mice and humans with HFE variants²⁹. These results indicate that loss of HFE function could enhance absorption as well as clearance of Mn. In addition, we found that HFE-knockout mice are resistant to memory deficits and emotional dysfunction induced by chronic intranasal Mn instillation compared with Mn-treated wild-type mice, suggesting that HFE deficiency could modify Mn-associated neurobehavioral problems^{30, 31}. However, it is yet to be determined whether H67D mutation, a prevalent mutation in HH patients, alters Mn deposition in the brain and consequently modifies neurotoxicity after acute olfactory exposure. In this study, we intranasally instilled MnCl₂ to wild-type and H67D mice to characterize the influence of HFE mutation on brain distribution and neurotoxicity of Mn.

MATERIALS AND METHODS

Ethics statement

This study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Northeastern University Animal Care and Use Committee.

Animals

H67D knock-in mice⁵ and their control wild-type mice were kindly provided by Dr. James Connor (Penn State University, PA, USA). H67D mutation (C199G) in mice is homologous to H63D mutation in humans and recapitulates H63D-related hemochromatosis^{3, 5}. All mice used for these studies were on the mixed background of C57BL/6 and 129Sv/J strains⁵. Wild-type and H67D mutant mice (8-week-old; male and female) were fed facility chow (Prolab Isopro RMH 3000, LabDiet; 96 mg manganese and 380 mg iron per kg diet) and given water *ad libitum*. For olfactory exposure to Mn, mice were intranasally-instilled (0.08 mL/kg) with manganese chloride (MnCl₂) dissolved in double distilled water daily for 3 days. Four doses of MnCl₂ were selected for the study: 0 (saline only), 0.2, 1 and 5 mg/kg body weight. For the dietary iron overload mice, weanling wild-type mice (3–4 weeks old) were fed iron overload diet (10,000 mg iron/kg, as carbonyl iron; TD.09077, Harlan Teklad, Madison, WI, USA) or control diet (50 mg iron/kg, TD.07800, Harlan Teklad) for 4

weeks^{32–34}, and intranasally instilled with saline or 1 mg MnCl₂/kg daily for 3 days. We selected a dose of 1 mg/kg because it was the minimum dose required to show the difference in Mn accumulation in the brain between wild-type and H67D mutant mice.

Tissue collection

After Mn treatment, mice were euthanized by isoflurane overdose, followed by exsanguination to harvest blood, liver and brain. The brain samples were microdissected to collect different brain regions, including the olfactory bulb, prefrontal cortex, striatum, hippocampus, cerebellum and cortex. All tissues were flash-frozen in liquid nitrogen and stored at –80°C until analysis.

Metal analysis

Wet digestion method⁷ was used to prepare samples, including blood, liver and microdissected brain tissues (n = 3–5 per group). Briefly, samples were digested in 20% nitric acid⁷ (Trace grade, Fisher Scientific; Pittsburgh, PA, USA) for 1 hour at 125°C in a dry block heater, which resulted in mild boiling. After complete digestion, the samples were diluted 8–10 times with metal-free double-distilled water. Metal concentrations were determined by inductively coupled plasma mass spectrometry (ICP-MS) (Varian 810/820MS, Bruker, Billerica, MA, USA) and calculated as µg/g tissue.

Quantitative RT-PCR analysis

Total RNA in the striatum was extracted using TRIzol reagent (Sigma-Aldrich, St. Louis, MO, USA). Quantitative real-time PCR was performed in Bio-Rad real-time PCR systems (Bio-Rad, Waltham, MA, USA) using the iTaq™ Universal SYBR® Green Supermix (Bio-Rad). Primer sequences were obtained from published studies: DMT1 with iron-responsive element (+IRE)³⁵, DMT1 without iron-responsive element (–IRE)³⁶, ZIP14³⁷, FPN³⁸ and β actin³⁹. Reactions were performed in a 10 µl mixture containing specific primers of each gene, cDNA and iTaq™ Universal SYBR® Green Supermix. Amplification conditions were as follows: 95°C for 2 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. The relative mRNA expression level was calculated by the threshold cycle (CT) value of each PCR product and normalized to that of β actin by using comparative CT method⁴⁰. Results were presented in relative percent to values from saline treated wild-type mice.

Isoprostane analysis

Isoprostane was chosen as a marker of oxidative stress associated with Mn exposure^{41, 42}. Free F2-isoprostanes in the liver and striatum were determined by an 8-isoprostane enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instruction.

Statistical analysis

Values reported were expressed as means ± SEM. Two-way ANOVA was performed using SigmaPlot (version 12.3; Systat Software Inc., San Jose, CA, USA) to determine individual main effects (i.e. Mn exposure effect and H67D effect) and an interaction effect between

HFE mutation and olfactory Mn exposure. Post-hoc multiple comparisons were performed by the Holm-Sidak method. Differences were considered significant at $p < 0.05$.

RESULTS

H67D mutation displays decreased manganese accumulation in blood and liver after intranasal instillation

To investigate the effect of H67D mutation on Mn deposition after olfactory exposure, two-month-old mice were intranasally instilled with a MnCl_2 solution at a dose of 0, 0.2, 1 or 5 mg/kg daily for 3 days. Levels of Mn and Fe in the blood and liver were quantified by ICP-MS analysis (Figure 1 and Supplementary Table 1). There was a dose-dependent increase in Mn concentrations in the blood (Mn effect, $p = 0.028$) and liver ($p = 0.001$) (Figure 1A), with a plateau effect on Mn accumulation at 5 mg/kg dose. Notably, H67D mutant mice showed a significant decrease in Mn concentrations in the blood compared with dose-matched wild-type mice (H67D effect; $p < 0.001$). Mn accumulation in the liver was also reduced in H67D mice compared with wild-type mice (H67D effect; $p < 0.001$). As expected, mice with H67D mutation displayed significantly increased iron accumulation in the blood and liver among all Mn doses tested ($p < 0.001$) (Figure 1B). These results suggest that H67D mutation could decrease tissue distribution of Mn after intranasal instillation.

H67D mutation decreases manganese deposition in the brain after intranasal instillation

We further measured Mn levels in several brain regions from the mice that received intranasal instillation of MnCl_2 (Figure 2 and Supplementary Table 2). The steady-state concentrations of Mn were increased in a dose-dependent manner in the olfactory bulb (Mn effect; $p = 0.001$), prefrontal cortex ($p < 0.001$), hippocampus ($p < 0.001$), cerebellum ($p = 0.013$) and cortex ($p < 0.001$), but not in the striatum ($p = 0.062$). Similar to blood and liver, the anterior brain regions (olfactory bulb and prefrontal cortex) showed plateau effects at 5 mg/kg. Of note, H67D mice showed significantly reduced Mn accumulation in the olfactory bulb (H67D effect; $p = 0.018$), prefrontal cortex ($p = 0.025$), striatum ($p < 0.001$), hippocampus ($p < 0.001$) and cortex ($p < 0.001$), compared with wild-type mice. Interestingly, the reduction in Mn levels in H67D mutation was the greatest (i.e. 24–45% decreases across four doses) in the striatum among brain regions. In contrast, the hippocampus showed a dose-dependent increase in Mn concentrations in wild-type mice, but in H67D mice (Mn $\times$ H67D interaction effect; $p = 0.013$). Combined, these results indicate that H67D mutation mitigates Mn deposition in the brain after a short-term olfactory exposure.

H67D mutation and Mn instillation alter iron levels in brain regions

We also quantified iron levels in different brain regions to examine if Mn instillation alters brain iron status (Figure 3 and Supplementary Table 2). Iron levels were not altered in the olfactory bulb, prefrontal cortex, striatum, hippocampus and cerebellum upon H67D mutation. However, H67D mutant mice displayed elevated iron levels in the cortex (15–48%, $p < 0.001$), which could contribute to increased iron levels found in the whole brain of H67D mice⁵. In the striatum, Mn instillation slightly decreased iron concentrations in wild-mice in a dose-dependent manner, but not in H67D mice (Mn $\times$ H67D interaction effect; $p =$

0.012). We observed a similar finding in the cerebellum ($\text{Mn} \times \text{H67D}$ interaction effect; $p = 0.044$), but not in other brain regions. These results indicate that Mn exposure alters iron levels in the brain in a region-specific manner, which is nullified by H67D mutation.

Ferroportin is up-regulated in the striatum of H67D mutant mice

To explore if decreased Mn in the brain upon H67D mutation was due to changes in the expression of metal transporters that mediate Mn transport, we quantified mRNA levels of several major metal transporters in the striatum (Figure 4). The striatum was chosen for several reasons. Firstly, Mn levels were consistently lower in H67D mice among all Mn doses, suggesting a pronounced effect of H67D on Mn transport (Figure 2); secondly, the striatum is a brain region sensitive to Mn neurotoxicity⁴³; thirdly, the striatum showed significant neurochemical changes in response to both HFE deficiency and intranasal Mn exposure³⁰. Neither Mn instillation nor H67D mutation affected the expression of DMT regardless of the existence of IRE. In addition, ZIP14 expression trended lower in H67D mice ($p = 0.064$), implying a decreased Mn uptake into the striatum after olfactory Mn exposure. Importantly, H67D mutant mice showed significantly increased expression of FPN in the striatum compared with wild-type mice (H67D effect; $p = 0.002$). The effect of H67D on FPN expression was more pronounced in no Mn (0 mg/kg; $p = 0.001$) or low Mn (0.2 mg/kg; $p = 0.050$) dose groups than higher doses. Also, there was a trend of increased FPN expression upon Mn exposure in wild-type mice, but not in H67D mice ($\text{Mn} \times \text{H67D}$ effect; $p = 0.148$). Together, our data indicate that H67D mutation could enhance FPN-mediated export of Mn in the striatum.

Dietary iron overload does not alter manganese accumulation in the brain after intranasal Mn instillation

To examine if reduced Mn levels in the brain of H67D mice result from HFE mutation or iron overload effect, weanling wild-type mice were fed iron overload or control diet for 4 weeks and received intranasal instillation of a MnCl_2 solution (1 mg/kg) daily for 3 days. We chose MnCl_2 1 mg/kg because this dose showed significant changes in Mn levels in the brain, whereas 5 mg/kg dose displayed a saturation effect on Mn accumulation (Figure 2). As expected, the steady-state concentrations of Mn were increased in the liver ($p = 0.029$) and striatum ($p = 0.039$) after intranasal Mn instillation in both control and iron overload mice (Figure 5A). However, mice fed iron overload diet did not decrease Mn accumulation in either liver ($p = 0.902$) or striatum ($p = 0.267$) compared with control mice (Figure 5A). Hepatic iron level in iron overload mice ($925.5 \pm 124.1 \mu\text{g/g}$ liver) was 14 times higher than that in control mice ($61.8 \pm 3.9 \mu\text{g/g}$ liver), verifying that our diet regimen indeed created iron loading condition (Figure 5B). Similar to the case of H67D mutation (Figure 3), iron levels in the striatum were unaltered by iron overload diet (Figure 5B). These results suggest that iron overload alone does not directly alter Mn accumulation after olfactory exposure.

H67D mutation reduces oxidative stress induced by manganese in the striatum

To evaluate if decreased brain Mn due to HFE mutation could ameliorate Mn-induced oxidative stress, we quantified the levels of isoprostanes, a marker of lipid peroxidation (Figure 6). In the liver, Mn instillation increased isoprostane levels, which were not affected by HFE mutation. However, there was an interaction effect between Mn exposure and H67D

mutation ($p = 0.005$) in the striatum; olfactory Mn exposure (5 mg/kg) elevated isoprostane levels in wild-type mice ($p = 0.013$), but not in H67D mice, indicating that H67D could attenuate Mn-associated neurotoxicity. Together, these results demonstrate that H67D mutation is resistant against oxidative stress induced by olfactory Mn exposure.

DISCUSSION

Increased accumulation of Mn in the brain is significantly associated with neurobehavioral deficits and impaired brain function^{44–47}. Airborne Mn is highly neurotoxic due to the ability of direct uptake into the brain, as well as high systemic bioavailability of Mn. Although Mn transport is in part mediated by several iron transporters, whose expression is modified by HFE deficiency, whether or not HFE gene mutation, one of prevalent polymorphisms in humans, modifies Mn-induced neurotoxicity is poorly understood. Therefore, our goal was to define the role of HFE mutation in the distribution of Mn in the brain and resultant neurotoxic effects after olfactory exposure. Using the H67D HFE-mutant mice that recapitulate human H63D mutation, we demonstrated that Mn accumulation was decreased in most brain regions upon HFE mutation, especially in the striatum, which was associated with up-regulation of the metal exporter FPN. We further found that HFE mutation is resistant to Mn-associated oxidative stress in the brain. Together, our results indicate that H67D mutation could enhance Mn efflux from the brain and reduce the susceptibility to Mn-associated neurotoxicity.

We first investigated whether attenuated Mn accumulation in the striatum was due to altered expression of Mn importers in H67D mutant brain. DMT1 and ZIP14 play an important role in Mn uptake^{12, 48–50}. While mRNA expression of DMT1 in the duodenum and liver is altered in response to body iron status^{51, 52}, Nandar et al. showed a 40% decrease, although insignificant, in the expression of DMT1 in the whole brain of H67D mice⁵. However, our results showed that there were no significant changes in basal expression of DMT1 in the striatum with H67D mutation. Brain region-specific DMT1 regulation could be one possibility. For example, Siddappa et al. demonstrated that the expression of DMT1 is up-regulated in the hippocampus of iron-deficient rats, but remained unchanged in the striatum⁵³. Alternatively, the difference could result from iron-responsive post-transcriptional and/or post-translational regulation of DMT1 protein expression^{54, 55}. Of note, there was a trend of decreased striatal ZIP14 expression, which could have partially contributed to reduced Mn levels in HFE mutant mice. It has been reported that ZIP14 is abundantly expressed in the liver⁵⁶, but also found in some extent in the brain⁵⁷, consistent with our results. Since DMT1 and ZIP14 are expressed in the olfactory epithelium and could play a critical role for nasal transport of metals^{12, 58, 59}, a future study is necessary to determine whether HFE mutation modifies the levels of these Mn importers on the olfactory epithelium as well as other brain regions.

An increasing body of evidence has indicated that FPN mediates Mn efflux in several tissues, including the brain and liver^{27, 60, 61}. Importantly, we found a significant increase in FPN expression in the striatum of H67D mice, which could increase Mn export and contribute to decreased Mn levels in the brain. Our results are consistent with previous findings that HFE deficiency increases the expression levels of FPN mRNA in the liver¹⁰.

Increased mRNA levels of FPN are associated with elevated expression levels of FPN protein⁶². It should be noted that FPN protein levels are also regulated post-translationally by hepcidin⁶³, a small peptide mainly produced by the liver and released into the blood. Hepcidin binds to FPN and induces intracellular degradation of FPN⁶⁴. Hepcidin also regulates DMT1 expression via a degradative pathway⁵⁵. Interestingly, hepcidin protein is widely found in the brain with mRNA levels undetected in rats, leading to a hypothesis that circulating hepcidin could enter the brain⁶⁵. Similarly, we observed that hepcidin mRNA levels in the striatum were below the detection limit regardless of genotype or Mn instillation (data now shown). Alternatively, systemic hepcidin can bind and degrade FPN at the blood-brain barrier (BBB)⁶⁵. Since serum hepcidin levels are decreased in HFE deficiency in both humans and mice^{66, 67}, it is possible that levels of hepcidin protein in the brain as well as BBB are decreased in H67D mice. As a consequence, FPN protein could be up-regulated in the brain of H67D mice due to decreased hepcidin availability in addition to increased FPN mRNA. A future study is necessary to test this possibility by measuring protein levels of FPN and DMT1 as well as hepcidin in the striatum and other brain regions of H67D mice.

Although the affinity of FPN to Mn is lower by three orders of magnitude than that to iron⁶⁸, FPN plays an important role in Mn homeostasis. For example, FPN deficiency impairs Mn transport and metabolism both *in vitro* and *in vivo*^{27, 60, 69}. Moreover, overexpression of FPN decreases Mn accumulation and cytotoxicity in human embryonic kidney cells⁶¹. Our results indicate that H67D mutation has a greater influence on the export of Mn than on the import of the metal in the striatum. We note that up-regulation of striatal FPN in H67D is greater upon no or low dose of Mn instillation compared with higher Mn doses, whereas H67D's effect on decreased Mn levels in the stratum persists among all Mn doses. This suggests a potential transport mechanism independent of FPN. Mn-specific transporters, such as SLC30A10 and ATP13A2^{24, 25, 70} could serve this role, but whether HFE mutation influences the expression of these Mn transporters is unknown. Further studies on the expression of these metal exporters are warranted to provide insights into Mn efflux systems in the brain in the presence of HFE mutation as well as altered iron metabolism. In addition, pharmacokinetic studies after intrastriatal injection will directly confirm the influence of HFE mutation on Mn export from the brain.

We note that decreased Mn concentrations in H67D mutation were not correlated with increased iron levels across brain regions. For example, the cortex showed significant higher levels of iron at basal state (MnCl₂ 0 mg/kg) in H67D mice (Figure 3), but Mn levels were not different between the two genotypes (Figure 2). Conversely, the striatum showed similar basal levels of iron between wild-type and H67D mice, whereas Mn concentrations were significantly lower upon H67D mutation. In parallel, both H67D mutant and dietary iron loaded mice exhibited increased iron levels in the liver, but only H67D mutant mice showed reduced Mn concentrations (Figures 1 and 5). Therefore, attenuated Mn accumulation in the brain and liver of H67D mice is independent of iron loading. Our results are in agreement with a previous finding that iron-treated Wistar rats do not display a change in either iron or Mn levels in the brain⁷¹. Thus, our results suggest that H67D mutation affects Mn transport, in part, via up-regulation of FPN. In addition, Chua et al. studied iron-loaded rats during lactation and found increased⁵⁴ Mn uptake and higher steady-state concentrations of Mn in

the brain⁷². Since H67D mice display genetic iron overload, it is also possible that iron loading during development could have altered protein expression necessary for Mn transport in the brain. Further investigation using gestational/lactational iron loading models will address this question.

Isoprostanes have been used as a lipid peroxidation marker for Mn-induced oxidative stress^{41, 42}. In the striatum, decreased isoprostane levels in H67D mutation were associated with reduced Mn concentrations after Mn exposure, suggesting that HFE mutation could provide a resistant mechanism to oxidative stress in the brain induced by inhaled Mn. While the nuclear factor E2-related factor 2 (Nrf2) triggers the expression of antioxidant enzymes⁷³, previous studies demonstrated that Mn treatment increases Nrf2 levels in the brain via adaptive response to metal-induced oxidative stress⁷⁴. In addition, Nandar et al. showed that H67D mutant mice also increase Nrf2 expression in the brain⁵. Therefore, it is anticipated that Mn-exposed H67D mice would have higher Nrf2 expression compared with Mn-exposed wild-type mice and potentially up-regulate antioxidant enzymes in the striatum, which accounts for decreased isoprostane in H67D mutation. Future studies will focus on the investigation of molecular mechanisms and consequences of oxidative stress, including protein carbonylation, hemeoxygenase-1 and alanine aminotransferase, as well as antioxidant enzyme activities upon HFE mutation. In addition, other types of chemicals that increase oxidative stress (e.g. diquat) and heavy metals can be treated in H67D mice; these experiments will differentiate if reduced isoprostane levels in Mn-exposed H67D mice as compared with Mn-exposed wild-type mice were due to enhanced antioxidant reserves or decreased Mn accumulation in the brain upon H67D mutation. The latter can further be confirmed by the use of FPN inhibitors (e.g. intrastriatal injection of synthetic hepcidin⁷⁵).

We also note that the levels of isoprostane after Mn instillation were not significantly different in the liver of H67D mice compared with wild-type mice. While H67D mutation exhibits iron overload in both liver and brain, iron stores in the liver are by far greater than those in the brain^{5, 7, 76}. Since iron overload can promote the production of reactive oxygen species, increased hepatic iron levels in H67D mice could potentiate Mn-induced oxidative stress, but brain iron stores are insufficient to do so. We previously reported that HFE deficiency attenuates neurobehavioral and neurochemical dysfunction induced by intranasal Mn instillation^{30, 31}. In the current study we demonstrated that HFE mutation decreases brain Mn levels, increases FPN expression and down-regulates Mn-induced oxidative stress. These findings provide the potential mechanism by which HFE mutation modulates Mn-associated neurotoxicity and contribute to a better understanding of molecular basis for iron-manganese interactions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

DMT1	divalent metal transporter 1
FPN	ferroportin
HFE	high iron or Fe
HH	hereditary hemochromatosis
IRE	iron-responsive element
Mn	manganese
ZIP14	zinc-regulated transporter (ZRT)/iron-regulated transporter (IRT)-like protein 14

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SIGNIFICANCE TO METALLOMICS STATEMENT

Manganese (Mn) and iron share several metal transporters for cellular uptake and tissue distribution, and the expression of metal transporters is modified by altered iron status. Here we report that mice with genetic iron loading demonstrate increased expression of a metal exporter ferroportin and decreased Mn accumulation across most brain regions after intranasal instillation of Mn, thereby attenuating Mn-induced oxidative stress in the brain. Our investigation provides improved understanding of genetic influence on the transport and neurotoxicity of airborne manganese.

Figure 1. Manganese and iron levels in blood and liver

Blood and liver samples were collected from mice intranasally instilled with MnCl₂ (0, 0.2, 1.0 and 5.0 mg/kg) daily for 3 days. Manganese (**A**) and level (**B**) concentrations in the blood and liver were quantified by inductively coupled plasma mass spectrometry (ICP-MS). Empty and closed circles represent wild-type and H67D mutant mice, respectively. Data were presented as mean $\pm$ SEM ($n = 4-5$ per group), and significant differences were analyzed using two-way ANOVA. * $p < 0.05$, Mn-instilled wild-type vs. Mn-instilled H67D mice.

Figure 2. Regional distribution of manganese in the brain

Different brain regions were collected from mice intranasally instilled with MnCl₂ (0, 0.2, 1.0 and 5.0 mg/kg) for 3 days. Manganese concentrations in the olfactory bulb, prefrontal cortex, striatum, hippocampus, cerebellum and cortex were quantified by ICP-MS. Empty and closed circles represent wild-type and H67D mutant mice, respectively. Data were presented as mean ± SEM (n = 3–5 per group), and significant differences were analyzed using two-way ANOVA. * p < 0.05, Mn-instilled wild-type vs. Mn-instilled H67D mice.

Figure 3. Regional distribution of iron in the brain

Different brain regions were collected from mice intranasally instilled with MnCl₂ (0, 0.2, 1.0 and 5.0 mg/kg) for 3 days. Iron concentrations in the olfactory bulb, prefrontal cortex, striatum, hippocampus, cerebellum and cortex were quantified by ICP-MS. Empty and closed circles represent wild-type and H67D mutant mice, respectively. Data were presented as mean $\pm$ SEM (n = 3–5 per group), and significant differences were analyzed using two-way ANOVA. * $p < 0.05$, Mn-instilled wild-type vs. Mn-instilled H67D mice.

Figure 4. Expression levels of metal transporters in the striatum

The striatum was collected from mice instilled with MnCl₂. The mRNA levels of metal transporters were quantified by real-time PCR analysis. Empty and closed bars represent wild-type and H67D mutant mice, respectively. Data were presented as mean $\pm$ SEM (n = 4 per group), and significant differences were analyzed using two-way ANOVA. * p < 0.05, Mn-instilled wild-type vs. Mn-instilled H67D mice.

Figure 5. Manganese levels in the liver and striatum in dietary iron overload mice

Weanling mice were fed control or iron overload diet for 4 weeks, followed by intranasal instillation of MnCl_2 (0 and 1 mg/kg) daily for 3 days. Manganese (A) and iron (B) concentrations in the liver and striatum were quantified by ICP-MS. Empty and hatched bars represent wild-type and dietary iron overload mice, respectively. Data were presented as mean $\pm$ SEM ($n = 4-5$ per group), and significant differences were analyzed using two-way ANOVA. * $p < 0.05$, Mn-instilled wild-type vs. Mn-instilled iron-loaded mice.

Isoprostanes

Figure 6. Isoprostane levels in the liver and striatum

Liver and striatum were homogenized to determine free F₂-isoprostane levels. Empty and closed bars represent wild-type and H67D mutant mice, respectively. Data were presented as mean $\pm$ SEM ($n = 4$ per group), and significant differences were analyzed using two-way ANOVA. * $p < 0.05$, Mn-instilled wild-type vs. Mn-instilled H67D mice.