HHS Public Access

Author manuscript

Environ Toxicol. Author manuscript; available in PMC 2023 September 01.

Published in final edited form as:

Environ Toxicol. 2022 September ; 37(9): 2167–2177. doi:10.1002/tox.23583.

Sex-dependent metal accumulation and immunoexpression of Hsp70 and Nrf2 in rats' brain following manganese exposure

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Abstract

Manganese (Mn), although important for multiple cellular processes, has posed environmental health concerns due to its neurotoxic effects. In recent years, there have been extensive studies on the mechanism of Mn-induced neuropathology, as well as the sex-dependent vulnerability to its neurotoxic effects. Nonetheless, cellular mechanisms influenced by sex differences in susceptibility to Mn have yet to be adequately characterized. Since oxidative stress is a key mechanism of Mn neurotoxicity, here, we have probed Hsp70 and Nrf2 proteins to investigate the sex-dependent changes following exposure to Mn. Male and female rats were administered intraperitoneal injections of $MnCl_2$ (10mg/kg and 25mg/kg) 48 hourly for a total of 8 injections (15 days). We evaluated changes in body weight, as well as Mn accumulation, Nrf2 and Hsp70 expression across four brain regions; striatum, cortex, hippocampus and cerebellum in both sexes. Our results showed sex-specific changes in body-weight, specifically in males but not in females. Additionally, we noted sex-dependent accumulation of Mn in the brain, as well as in expression levels of Nrf2 and Hsp70 proteins. These findings revealed sex-dependent susceptibility to Mn-induced neurotoxicity corresponding to differential Mn accumulation, and expression of Hsp70 and Nrf2 across several brain regions.

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Authors' Contribution: Conceptualization – OMI; Animal treatments – OMI, PM, AJA; Mn bioavailability – JB, TS; Western blotting – OMI, PM; Data analysis and interpretation – OMI, JDI, OKI, MA; Manuscript draft – OMI, JDI; Manuscript editing – MA. All authors read and approved the final draft.

Conflict of Interests: The authors declare no conflict of interests.

Keywords

Manganese; brain; oxidative stress; male; female

Introduction

Manganese (Mn) is an essential trace metal for many physiological processes in the human body. It acts as a cofactor for a variety of enzymes, and it mediates/regulates synthesis, bone metabolism, brain function amongst others. However, in excessive quantity, Mn can accumulate in the brain leading to its dysfunction and a parkinsonian-like syndrome, called manganism (Miah et al. 2020). Exposure to Mn mainly occurs through the air (Kornblith et al. 2018), food, and drinking water (Luo et al. 2020). Following exposure, Mn accumulates in different brain regions, mainly the basal ganglia (which includes the striatum), hippocampus, and cerebral cortex (Akingbade et al. 2021; Cheng et al. 2018; Cordova et al. 2012; Lao et al. 2017; Ou et al. 2017). Previous studies found that chronic exposure to Mn led to adverse dose-dependent effects on neurobehavioral function in rats, with overt morphological changes and neurodegeneration in different brain regions including the hippocampus, striatum, cortex, and cerebellum (Akingbade et al. 2021; Caito and Aschner 2015; Ma et al. 2020; Sidoryk-Wegrzynowicz and Aschner 2013; Stanwood et al. 2009).

Oxidative stress plays an important role in Mn-induced toxicity in neurons (Akingbade et al. 2021; Peres et al. 2016) and glia cells (Gorojod et al. 2017; Ke et al. 2019). Oxidative stress and disrupted redox status are key events in Mn neurotoxicity and likely associated with Mn-induced mitochondrial dysfunction (Akingbade et al. 2021; Miah et al. 2020). Heat shock proteins (HSPs) including Hsp70 are molecular chaperones that regulate protein synthesis as well as preserving structural integrity of existing proteins. In pathological conditions, they exhibit several functions, including structural renovation of denatured proteins as well as attenuation of protein aggregation (Powers et al. 2010; Sõti et al. 2005). Oxidative stress caused by metal exposure can further result in protein aggregation. In addition, metals are able to trigger perturbed protein interactions. These protein dysfunction can consequently lead to HSPs upregulation (Avila et al. 2016). Particularly, Mn overexposure has been reported to increase Hsp70 levels in non-nervous as well as brain tissues in experimental models (Cheng et al. 2018; Moyano et al. 2020; Zhu et al. 2016; Zhu et al. 2013). Similarly, Nrf2, a key regulator of the endogenous antioxidant response to oxidative stress can be modulated by metal exposures. Nrf2 triggers the transcription of endogenous antioxidant enzymes such as glutathione, catalase, superoxide dismutase amongst others, through its interaction with the antioxidant responsive element (ARE) (Dong et al. 2017; Jeong et al. 2006; Wang et al. 2019). Several studies have shown that Mn-induced oxidative stress can significantly upregulate the expression of Nrf2 proteins in cells and that Nrf2 pathway modulate Mn-induced neuronal damage (Li et al. 2011a; Li et al. 2011b; Moyano et al. 2020).

Sex-dependent disparities in susceptibility to heavy metals, such as Mn, have received some attention in several studies. A cohort study in Denmark associated Mn-exposed female

children with attention deficit hyperactivity disorder (ADHD) to a greater extent than males (Schullehner et al. 2020). Elsewhere, Broberg et al. (2019) reported sex-dependent sensitivity to Mn exposure on neurobehavioral outcomes in children. Further, Oulhote et al. (2014a) reported that females have significantly higher blood Mn levels than males. Evidence from other studies indicate that these differences might be a result of slight variation in genetics (Broberg et al. 2019) and metabolism across sexes (Bouchard et al. 2018; Dion et al. 2018). However, few reports exist on sex-dependent responses to Mn neurotoxicity. Furthermore, while the role of Nrf2 and Hsp70 in Mn neurotoxicity have been reported, there appears to be lack of attention on the influence of sex on Hsp70 and Nrf2 expression and function in Mn neurotoxicity. Hence, here we evaluated sex-specific changes to brain Hsp70 and Nrf2 expression, as well as variations in Mn accumulation across specific brain regions in response to Mn exposure.

Methods

Animals

Ten-week-old Sprague Dawley rats with average weight of 343±3.77 g for males and 211±3.14 g for females obtained from Charles Rivers Laboratories, USA, were used for the study. All animal experimental protocols were performed in strict accordance to the guidelines of the National Institute of Health for the care and use of laboratory animals and approved by the IACUC at the Albert Einstein College of Medicine (#20171008). Animals were kept under 12-hour light-dark cycle with free access to regular food and water.

Male and female rats (n = 6/sex) were randomly assigned to receive intraperitoneal (i.p.) injections of either saline vehicle (as controls), or 10 mg/kg or 25 mg/kg $MnCl_2$ ($MnCl_2$ was dissolved in saline), every 48 hours for 15 days, for a total of 8 injections. Doses and route of administration are based on our prior report (Morcillo et al. 2021). These doses are relevant to human exposure scenarios as Mn toxicity has been shown in individuals who have ingested water containing high levels of Mn at dose ≥10 mg (Keen et al. 2013). Additionally, this dosing paradigm of Mn injections has been shown to permit accumulation of detectable Mn levels in the brain whilst reducing its effects on other organs (Santos et al. 2012a; Santos et al. 2012b). Furthermore, administration of $MnCl_2$ by i.p. produces steady-state blood concentrations of about 1000 ng Mn/100 ml and is similar to levels obtained via oral gavage and intratracheal routes (Roels et al. 1997). Body weight were monitored twice weekly. At day 16, rats were euthanized via isoflurane inhalation, brains were rapidly excised and the striatum, cortex, hippocampus and cerebellum were isolated for subsequent analysis.

Mn bioavailability by inductively coupled plasma – mass spectrometry

Quantification of Mn concentration in the striatum, cortex, hippocampus and cerebellum was determined via inductively coupled plasma – mass spectrometry (ICP-MS). Brain tissues were processed via a MARS 6 microwave digestion system (CEM GmBH, Germany), using a 15 min ramp to 200 °C and kept for 20 min at 200 °C. Samples were then diluted to final concentrations of 3.25% HNO3 and 1 μg/L Rh with ultrapure H_2O. ICP-MS was performed on the Agilent 8800 ICP-QQQ (Agilent Technologies Deutschland GmbH & Co.

KG, Germany). The following parameters were used for ICP-MS analysis: 1550 W plasma Rf power, Ni-cones, MicroMist nebulizer at 1.08 L Ar/min, and Scott-type spray chamber. The following ratios of mass-to-charge and gas modes were used: (Q1 ➔ Q2): He-mode, Mn (55 ➔ 55) and Rh (103 ➔ 103) (internal standard). Results were validated via the certified reference material ERM-BB 422 (fish muscle). Alongside Mn concentration, we also measured levels of other essential metals including iron (Fe), copper (Cu), and zinc (Zn).

Western blotting

Isolated brain regions were homogenized in cold lysis buffer of 2 ml volume per 500 mg tissue. Lysis buffer contains 970 µl RIPA buffer (Sigma Aldrich, USA; #R0278), supplemented with 10 µl protease inhibitor cocktail (ThermoFischer Scientific, USA; #1861278), 10 µl each of phosphatase inhibitor cocktails 2 (Sigma Aldrich, USA; P5726) and 3 (Sigma Aldrich, USA; #P0044). Homogenized tissues were centrifuged at 10000 rpm for 10 min at 4 °C. The supernatant was collected and protein content was quantified using the Pierce BCA Protein Assay Kit (ThermoFisher Scientific, USA; #23227). Lysates were heated at 100 oC for 5 minutes in 1:1 of Laemmli buffer solution containing 950 µl 2X Laemmli sample buffer (Bio-Rad, USA; #1610737) + 50 µl β-mercaptothanol (Bio-Rad, USA; #1610710). After heating, samples were centrifuged for 1 min, and cooled on ice for 5 min. Then, 10 µg of protein were resolved on 4–20 % sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE, Bio-Rad, USA; #4561096) and transferred to a nitrocellulose membrane. Blocking was performed in Tris buffer saline-Tween-20 (TBS-T) buffer containing 5 % bovine serum albumin (BSA, Sigma Aldrich, USA; #A3059) for 1 h at room temperature. The blots were incubated in primary antibodies diluted in 5% BSA in TBS-T buffer overnight at 4 °C. Primary antibodies used were rabbit anti-Hsp70 (Cell Signaling Technology, USA; #4872), rabbit anti-nrf2 (Cell Signaling Technology, USA; #12721) and mouse anti-β-actin (Sigma Aldrich, USA; A1978). Next, membranes were incubated in appropriate HRP-conjugated secondary antibody, and visualized by chemiluminescent method using SuperSignal™ West Pico Plus Kit (ThermoFischer Scientific, USA; #34579). The densitometric values were quantified using ImageJ software (https://imagej.nih.gov/ij/), and normalized using β-actin as loading control.

Statistics

Data were analyzed on GraphPad Prism 8 software (GraphPad Inc, USA) by two-way analysis of variance (ANOVA) followed by Bonferroni's *post-hoc* tests. Values of $p < 0.05$ were considered statistically significant.

Results

$MnCl_2$ treatment caused a dose-dependent weight loss in male, but not in female rats

Two-way ANOVA revealed significant interaction [$p < 0.001$; $F_{(2,30)} = 16.74$] between sex and treatment on body-weight. Similarly, there was significant sex effect [$p < 0.001$; $F_{(1,30)} = 36.78$], and treatment effect [$p < 0.001$; $F_{(2,30)} = 15.49$]. Further, *post-hoc* analysis revealed significantly increased body weight-only in male rats following 10 mg/kg ($p < 0.05$) and 25 mg/kg ($p < 0.001$) $MnCl_2$ treatment compared with control. In addition, body-weight was

significantly higher ($p < 0.001$) in male than in female rats for control and the 10 mg/kg $MnCl_2$ groups (Fig. 1).

Mn quantification in the brain of Mn treated male and female rats

$MnCl_2$ treatment increased Mn levels in the striatum of male and female rats: Two-way ANOVA analysis revealed no significant interaction [$p = 0.5100$; $F_{(2, 30)} = 0.69$] between sex and Mn treatment in the striatum. However, analysis revealed significant sex [$p < 0.01$; $F_{(1, 30)} = 11.30$] and treatment [$p < 0.001$; $F_{(2, 30)} = 73.59$] effect. Bonferroni's *post hoc* test revealed significantly increased Mn levels in the striatum of both male and female rats after exposure to 10 mg/kg (male: $p < 0.001$; female: $p < 0.001$) and 25 mg/kg (male/female: $p < 0.001$) of $MnCl_2$, compared with the respective controls. Similarly, Mn levels in the striatum after 25 mg/kg $MnCl_2$ was significantly higher compared to 10 mg/kg treated rats in both male and female rats. Additionally, at 10 mg/kg, Mn levels in the striatum were significantly higher ($p < 0.05$) in male compared to female rats at an analogous dose. For levels of the other metals measured in the striatum, Fe levels showed no significant changes on interaction nor treatment effects but showed significant sex effect [$p < 0.001$; $F_{(1, 30)} = 18.52$]. Here, Bonferroni's test confirmed significant ($p < 0.05$) Fe increase in males compared to females at 25 mg/kg Mn treatment. No significant effects were seen for Cu levels while Zn showed significant interaction [$p < 0.05$; $F_{(1, 30)} = 0.52$] but not significant sex nor treatment effects (Fig. 2).

$MnCl_2$ treatment increased Mn levels in the cortex of male rats but not in female rats: Two-way ANOVA revealed significant interaction [$p < 0.001$; $F_{(2, 30)} = 36.37$] between sex and treatment on cortical Mn levels. Similarly, there was significant sex effect [$p < 0.001$; $F_{(1, 30)} = 127.56$], and treatment effect [$p < 0.001$; $F_{(2, 30)} = 30.43$]. Multiple comparison with Bonferroni's test showed significantly increased ($p < 0.001$) Mn levels in the cortex of male rats following 10 mg/kg and 25 mg/kg $MnCl_2$ treatment compared with respective controls. No significant differences were observed in female rats. Consequently, $MnCl_2$ treatment at 10 mg/kg and 25 mg/kg caused significantly increased ($p < 0.001$) Mn levels in the cortex of males compared to females. For levels of the other metals measured in the cortex, Fe, Cu, and Zn, no significant interaction, sex nor treatment effect was seen (Fig. 2).

$MnCl_2$ treatment increased Mn levels in the hippocampus of male and female rats: Two-way ANOVA analysis showed no significant interaction [$p = 0.4071$; $F_{(2, 30)} = 0.93$] between sex and treatment on Mn accumulation in the hippocampus. Additionally, there was no significant sex effect [$p = 0.7702$; $F_{(1, 30)} = 0.09$] on hippocampal Mn accumulation, however, a significant treatment effect [$p < 0.001$; $F_{(2, 30)} = 43.79$] was observed. *Post hoc* analysis revealed significantly increased ($p < 0.001$) Mn levels in both male and female rats following 10 mg/kg and 25 mg/kg $MnCl_2$ treatment compared with respective controls. For levels of the other metals measured in the hippocampus, Fe levels showed no significant changes on interaction, nor treatment effects but showed significant sex effect [$p < 0.01$; $F_{(1, 30)} = 11.45$]. Here, Bonferroni's test confirmed significant Fe increase in males compared to females at 10 mg/kg Mn treatment. Cu levels showed significant interaction [$p < 0.01$; $F_{(2, 30)} = 5.58$] and treatment effect [$p < 0.05$; $F_{(2, 30)}$

= 3.59] but no significant sex effects. Bonferroni's test showed significant ($p < 0.05$) Cu increase at 10 mg/kg compared to control and 25 mg/kg treatment in males, and also significant increase ($p < 0.05$) in Cu levels in males compared to females at 10 mg/kg. For Zn levels, no significant interaction nor treatment effect is seen however significant sex effect [$p < 0.001$; $F_{(1, 30)} = 17.25$] is observed. However, further Bonferroni's test did not confirm significant sex effect for any treatment groups (Fig. 2).

$MnCl_2$ treatment increased Mn levels in the cerebellum of male and female rats: Two-way ANOVA analysis showed significant interaction [$p < 0.05$; $F_{(2, 30)} = 3.44$] between sex and Mn treatment in the cerebellum. In addition, ANOVA analysis shows significant sex [$p < 0.001$; $F_{(1, 30)} = 16.04$], and treatment [$p < 0.001$; $F_{(2, 30)} = 82.85$] effect. Multiple comparison using Bonferroni's test revealed significantly increased Mn levels after exposure to 10 mg/kg (male: $p < 0.001$; female: $p < 0.01$) and 25 mg/kg (male/female: $p<0.001$) $MnCl_2$, compared with respective controls. Furthermore, female rats treated with 25 mg/kg $MnCl_2$ showed significantly higher ($p < 0.001$) Mn levels in the cerebellum compared to 10 mg/kg treatment. Additionally, at 10 mg/kg, Mn levels significantly increased ($p < 0.001$) in the cerebellum of male compared to female rats.

For levels of the other metals measured in the cerebellum, Fe levels showed no significant interaction, sex nor treatment effects. Cu levels showed no significant changes on interaction nor treatment effects but showed significant sex effect [$p < 0.01$; $F_{(1, 30)} = 12.00$]. Here, Bonferroni's test confirmed significant ($p < 0.05$) Cu increase in females compared to males at 25 mg/kg Mn treatment. Similarly, Zn showed only significant sex effect [$p < 0.001$; $F_{(1, 30)} = 13.52$]. Bonferroni's test confirmed significant ($p < 0.05$) Cu increase in females compared to males in the control groups (Fig. 2).

Hsp70 expression in the brain of $MnCl_2$ treated male and female rats

Striatal Hsp70 expression was increased in male and female rats after $MnCl_2$ treatment: Two-way ANOVA revealed no significant interaction [$p = 0.8137$; $F_{(2, 30)} = 0.21$] between sex and treatment on striatal Hsp70 expression. In contrast, there was a significant sex [$p < 0.05$; $F_{(1, 30)} = 5.09$], and treatment [$p < 0.001$; $F_{(2, 30)} = 11.13$] effect. *Post hoc* analysis with Bonferroni's test revealed significantly increased Hsp70 expression in males following $MnCl_2$ treatment at 10 mg/kg ($p < 0.05$) and 25 mg/kg ($p < 0.001$) compared to controls. However, in females, Hsp70 expression was significantly increased ($p < 0.05$) only at 25 mg/kg (Fig. 3).

$MnCl_2$ treatment increased cortical Hsp70 expression in female rats but not males: Two-way ANOVA revealed significant interaction [$p < 0.01$; $F_{(2, 30)} = 5.95$] between sex and treatment on cortical Hsp70 expression. Similarly, there was significant sex effect [$p < 0.01$; $F_{(1, 30)} = 12.95$], and treatment effect [$p<0.001$; $F_{(2, 30)} = 13.56$]. Bonferroni's *post hoc* analysis showed significantly increased Hsp70 expression only in cortex of female rats following 10 mg/kg ($p < 0.05$) and 25 mg/kg ($p < 0.001$) $MnCl_2$ treatment compared to respective control. In addition, at 25 mg/kg $MnCl_2$ treatment, cortical Hsp70 expression was significantly higher ($p < 0.001$) in females compared to males (Fig. 3).

$MnCl_2$ treatment increased hippocampal Hsp70 expression in male and female rats: Two-way ANOVA showed no significant interaction [$p = 0.1763$; $F_{(2, 30)} = 1.84$] between sex and treatment. While there was significant treatment effect [$p < 0.001$; $F_{(2, 30)} = 15.82$], there was no significant sex effect [$p = 0.0971$; $F_{(1, 30)} = 2.93$]. *Post hoc* analysis showed significantly increased ($p < 0.01$) hippocampal Hsp70 expression in both males and females following 25 mg/kg $MnCl_2$ treatment. Additionally, in female rats only, 25 mg/kg $MnCl_2$ treatment showed significantly higher ($p < 0.001$) hippocampal Hsp70 expression compared to 10 mg/kg treatment (Fig. 3).

$MnCl_2$ treatment did not alter cerebellar Hsp70 expression in male and female rats: Two-way ANOVA of cerebellar Hsp70 expression showed no significant interaction [$p = 0.87$; $F_{(2, 29)} = 0.14$] between sex and treatment. Similarly, there was no significant sex effect [$p = 0.8889$; $F_{(1, 29)} = 0.02$] and treatment effect [$p = 0.3238$; $F_{(2, 29)} = 1.17$]. Bonferroni's *post hoc* analysis revealed no significant differences in Hsp70 expression across sex and treatments groups (Fig. 3).

Nrf2 expression in the brain of $MnCl_2$ treated male and female rats

$MnCl_2$ treatment increased striatal Nrf2 expression in male and female rats: Two-way ANOVA of striatal Nrf2 expression showed no significant interaction [$p = 0.8285$; $F_{(2, 27)} = 0.19$] between sex and treatment. In addition, no significant sex effect [$p = 0.0798$; $F_{(1, 27)} = 3.31$] was observed, however, there was a significant treatment effect ($p < 0.001$; $F_{(2, 27)} = 13.05$). Bonferroni's *post hoc* analysis in males, revealed significantly increased Nrf2 expression following 10 mg/kg ($p < 0.05$) and 25 mg/kg $MnCl_2$ ($p < 0.001$) treatment compared to control. On the other hand, in female rats, only at 25 mg/kg $MnCl_2$ treatment a significant increase ($p < 0.05$) was ascertained in Nrf2 expression compared to controls (Fig. 4).

$MnCl_2$ treatment increased cortical Nrf2 expression in female rats only: Two-way ANOVA of cortical Nrf2 expression revealed significant interaction [$p < 0.05$; $F_{(2, 30)} = 3.78$] between sex and treatment. In contrast, there was no significant sex [$p = 0.8395$; $F_{(1, 30)} = 0.04$], and treatment effect [$p = 0.0520$; $F_{(2, 30)} = 3.27$]. Bonferroni's *post hoc* analysis revealed significantly higher ($p < 0.01$) Nrf2 expression in females after 25 mg/kg of $MnCl_2$ compared to respective control.

Hippocampal Nrf2 expression is not significantly changed after $MnCl_2$ treatment in male and female rats: Two-way ANOVA of hippocampal Nrf2 expression showed no significant interaction [$p = 0.5960$; $F_{(2, 29)} = 0.53$] between sex and treatment. Similarly, no significant sex [$p = 0.3553$; $F_{1, 29)} = 0.88$] or treatment [$p = 0.0720$; $F_{(2, 29)} = 2.88$] effects were noted. Bonferroni's *post hoc* analysis revealed no significant difference in Hsp70 expressions across sex and treatment groups (Fig. 4).

$MnCl_2$ treatment increased cerebellar Nrf2 expression in female rats: Two-way ANOVA of cerebellar Nrf2 expression revealed no significant interaction [$p = 0.1875$; $F_{(2, 30)} = 1.77$] between sex and treatment. Additionally, no significant sex effect [$p = 0.7165$; $F_{(1, 30)} = 0.13$], however, a significant treatment effect [$p < 0.01$; $F_{(2, 30)} = 6.61$]

is observed. Bonferroni's *post hoc* analysis revealed no significant difference between the $MnCl_2$ treated groups compared to respective control. However, $MnCl_2$ treatment at 25 mg/kg in female rats led to significantly higher ($p < 0.01$) Nrf2 expression compared to the group treated with 10mg/kg $MnCl_2$.

Discussion

There is evidence that sex can influence susceptibility to Mn-induced neurotoxicity (Bailey et al. 2019; Dorman et al. 2004; Llop et al. 2013; Vorhees et al. 2014; Zheng et al. 2000). In this study, we demonstrate sex- and structure-specific changes in brain Nrf2 and Hsp70 protein expression levels following Mn exposure.

We observed sex-specific changes in body weight gain following Mn exposure. Mn significantly decreased body weight gain in male rats with increased dosing, but this effect was absent in females. In our results, we see that though males gained weight, Mn treatment significantly reduced the weight gain compared to male control. However, compared to females, it is apparent that since the males already higher body weight (at the same age), hence the weight gain is higher than that of the females. Consistent with our results Kim et al. (2012) previously reported no significant change in body-weight following Mn exposure in female rats, while Schmitz et al. (2019) reported significant decrease weight gain in male rats following Mn exposure, with no significant effect in female rats. In contrast, Zhang et al. (2003) reported significant decrease and increase in the weight of male and female rats, respectively, following Mn exposure. Loss of body-weight has been previously reported in several neuropathologies, both in experimental animal models (Apland et al. 2017; Shih et al. 2019; Wang et al. 2020) and humans (Bachmann and Trenkwalder 2006; Barrett-Connor et al. 1998; Buchman et al. 2005; Djousse et al. 2002). Furthermore, it has been shown that reduced body-weight alters the responsiveness and status of the redox system (García-Sánchez et al. 2020; Milagro et al. 2006). Increased oxidative stress and inflammatory responses, established consequences of Mn neurotoxicity (Miah et al. 2020), have been linked to weight loss by inhibition of appetite and induction of muscle loss. It is thus possible, that these changes could contribute to the weight-loss in males as corroborated in an earlier study (Schmitz et al. 2019). Furthermore, Schmitz et al. (2019), as previously noted that differences between male and female body weights following Mn exposure may be due to association between induction of inflammatory responses and accumulation and distribution of visceral fat in males.

We also examined potential sex-influenced variations in Mn deposition across different brain regions - striatum, cortex, hippocampus, and cerebellum, following Mn exposure. Mn exposure has previously associated with pathologic changes in the several brain regions; the striatum is particularly susceptible to the deleterious effect of Mn (Zhao et al. 2019). Similarly, Mn accumulates in the hippocampus, cerebellum (Hernández et al. 2020; Nkpaa et al. 2022), and cortex (Guilarte 2010; Guilarte et al. 2008). Although it well established that Mn deposition in the brain is region-specific owing to differential expression of important Mn transporters – DMT-1, transferrin, across specific brain regions (Nyarko-Danquah et al. 2020), sex-specific distribution and metabolism of Mn have also been recorded both in humans (Bauer et al. 2017; Wahlberg et al. 2018; Zhou et al. 2020)

and experimental models (Madison et al. 2011; Vorhees et al. 2014). Similarly, we observed differences in Mn levels in male *vs*. female cortex, with elevated Mn levels in males and absent of significant change in females. While brain Mn levels increased in both sexes, we failed to note sex-dependent effects in striatal, hippocampal or cerebellar Mn levels. Our finding on striatal Mn accumulation show the lack-of sex-dependent Mn accumulation corroborates an earlier report by Madison et al. (2011), though their study employed a mouse model. Guilarte et al. (2006) demonstrated increased Mn accumulation in the frontal cortex of male *Cynomolgus macaques*. Finally, Mn levels increased in the hippocampus and cerebellum of exposed male and female rats after Mn exposure (Yamagata et al. 2017). Additionally, we measured levels of other essential metals included Fe, Cu, and Zn, after Mn exposure. We observed no overt and consistent influence on Mn administration on these metals. However, our results do show that Mn triggers sex-dependent response in levels of Fe in the striatum and hippocampus, with Mn administration triggering Fe increase in males compared to females. Our results have not fully supported Anderson and colleagues who have previously shown that Mn supplementation resulted in lowered Fe levels in brains of both male and female rats (Anderson et al. 2007). It is worth noting that a recent report found no significant changes to Fe, Cu and Zn levels in striatum and cortex after treatment with Mn, albeit in mice (Foster et al. 2017).

Since increased Mn level is associated with oxidative stress (Akingbade et al. 2021; Alarifi et al. 2017; Huang et al. 2021). We further explored sex-dependent oxidative response following Mn accumulation via Hsp70 and Nrf2 modulation. Oxidative stress is a key event in many brain pathologies (Erukainure et al. 2019; Ijomone et al. 2018). Reactive oxygen species (ROS)-mediated oxidative stress following Mn exposure is known to activate different cellular signalling pathways involving the activation of the stress-responsive Hsp70 (Cheng et al. 2018; Liu et al. 2021) and antioxidant modulator, Nrf2 (Bahar et al. 2017; Costa-Silva et al. 2018).

The brain is the main target of Mn exposure, with excess Mn accumulating predominantly in the basal ganglia, particularly in the striatum (caudate nucleus, putamen and nucleus accumbens) (Peres et al. 2016). Hence, the striatum is most prone to Mn-induced neurostructural and neurochemical perturbations. This is supported by our present data, showing consistently dose-dependent Mn accumulation in the striatum in both male and female animals. In addition, we show upregulation of striatal Hsp70 and Nrf2 for both doses used in males, but only in higher dose in females. Since we detected no difference in striatal Mn accumulation between sexes, the inability of Mn to elicit cellular stress via Hsp70 and Nrf2 expression at a lower dose in the female brain suggests that Mn may interact with sex-specific hormones (Oulhote et al. 2014b), and that a higher Mn dose may be required to push striatal cells to an oxidative state and altered redox status in females.

In the cortex, upregulated Hsp70 and Nrf2 were observed in females only. Surprisingly, the increase in cortical Nrf2 and Hsp70 in the female cortex were not accompanied by elevated Mn levels. Conversely, males failed to exhibit cortical Hsp70 and Nrf2 changes despite significant Mn accumulation. The sex discrepancy observed in this study could be linked to the presence or absence of the hormones, such as estrogen and β-estradiol. Thakur and Sharma (2007) reported a higher level of ERα (estrogen receptor alpha) mRNA

in the cortex of adult females than male mice. These ERα are activated by β-estradiol, which protected SHSY5Y neuroblastoma cells from cobalt and mercury-induced oxidative stress by increasing the GSH production (Olivieri et al. 2002). Similarly, β-estradiol afford neuroprotection to Mn-induced toxicity in rat cortical primary neuronal and astroglial cultures via the attenuation of oxidative stress and activation of the MAPK/ERK and PI3K/Akt signaling pathway (Lee et al. 2009). Interestingly, administration of β-estradiol in male mice offer neuroprotection in experimental model of PD (Ramirez et al. 2003). The neuroprotective effect of β-estradiol is largely attributed to its interaction with nuclear estrogen receptors. Notably, β-estradiol can modulate gene expression via the regulation of signaling pathways, such as the ERK (Jover-Mengual et al. 2007) and PI3K/Akt (Dominguez et al. 2007). Through the aforementioned mechanism, β-estradiol can protect against Aβ-induced neurotoxicity by increasing the levels of Hsp70 protein (Zhang et al. 2004). Hence, the upregulated Hsp70 and Nrf2 may be a protective feedback response to oxidative stress in the female cortex. A plausible explanation for the lack of changes in Hsp70 and Nrf2 levels in male cortex despite significant Mn accumulation could be that estrogen, through the regulation of antioxidant genes, contributes to important antioxidant defense with lower oxidative damage and higher antioxidant activation in females compared to the male counterpart as shown by Razmara et al. (2007). However, this fails to explain the insignificant change in cortical Mn levels in females and further study is needed to elucidate this observation. Nonetheless, the lack of cellular response in male cortex could suggest an ability to withstand greater levels of Mn deposit compared to female rats.

Hsp70-mediated oxidative response was accompanied by Mn accumulation in the hippocampus of in males and female rats. This observation is consistent with the findings in (Cheng et al. 2018; Moyano et al. 2020). Conversely, Nrf2 levels remained unchanged, suggesting selective activation of the Hsp70 system to combat neuronal oxidative injury in the hippocampus. Further, the similar oxidative responses in both sexes suggest that the neurotoxic impact of Mn in the hippocampus is non-sex-specific. In the cerebellum, male and female rats did not show significant alterations to Hsp70-mediated oxidative response. However, a significant increase in the activities of Nrf2 was observed in female rats, only after exposure to a higher concentration of Mn. Thus, the sex-specific difference in the cerebellum was observed only in the Nrf2 system. As mentioned above, several sex-specific hormones can play significant roles in these observed variations during oxidative response.

In conclusion, our findings support sex-dependent vulnerability to Mn exposure. We quantified Nrf2 and Hsp70 proteins on the antioxidant system in rats, showing sex-dependent susceptibility to Mn-induced neurotoxicity, particularly in the cortex and cerebellum. Nonetheless, it is still essential to identify the regulators that mediates these differential responses, to better target potential therapeutic modalities.

Funding Information:

OMI was supported by the International Brain Research Organization (IBRO)-International Society for Neurochemistry (ISN) Research Fellowship 2017. MA was supported by National Institute of Health (NIH) R01 ES10563, R01 ES07331 and R01 ES020852. JB and TS were supported by the DFG Research Unit TraceAge (FOR 2558).

Data Availability Statement:

The data that supports the findings of this study are available from the corresponding author, upon appropriate request.

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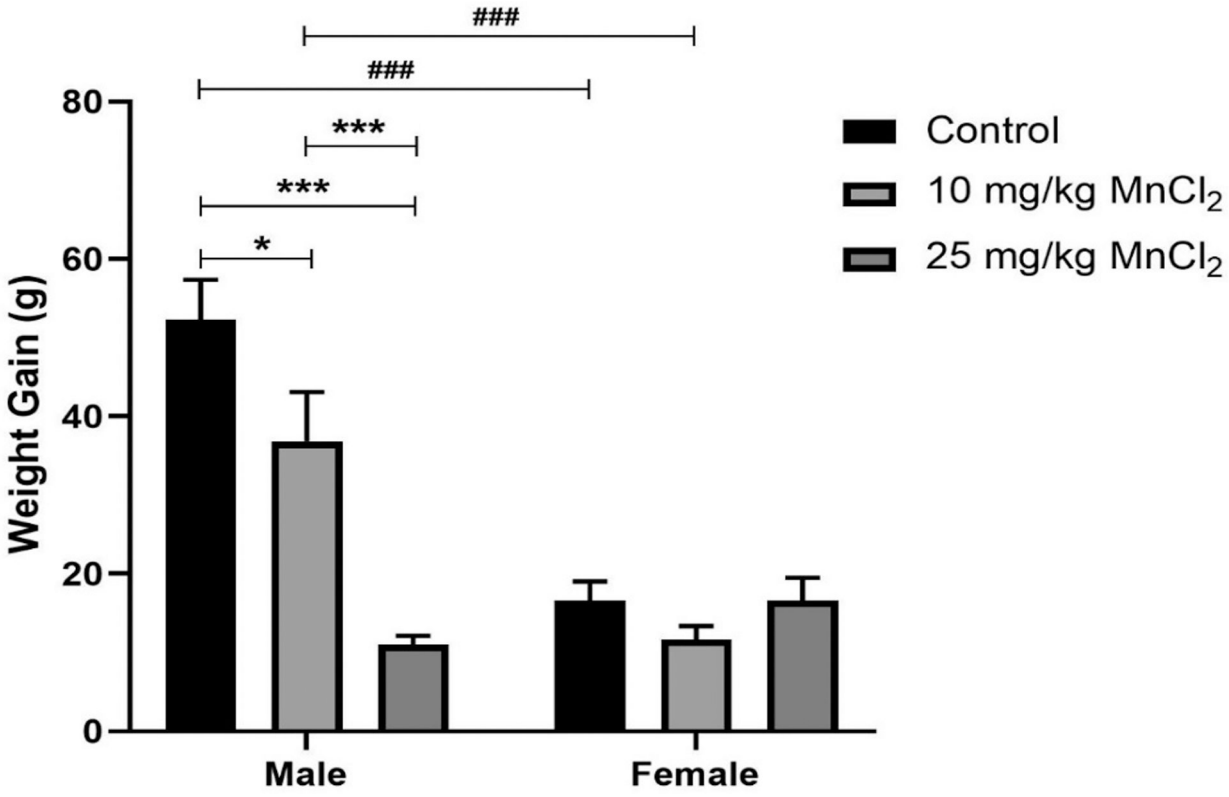

Fig. 1.

Effect of $MnCl_2$ treatment on the body weight gain of male and female rats. There was a dose-dependent decrease in the weight gain of male rats but not in female rats. Data were analyzed by two-way ANOVA followed by Bonferroni's post-test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. $^{###}p < 0.001$ between male and female of same treatment.

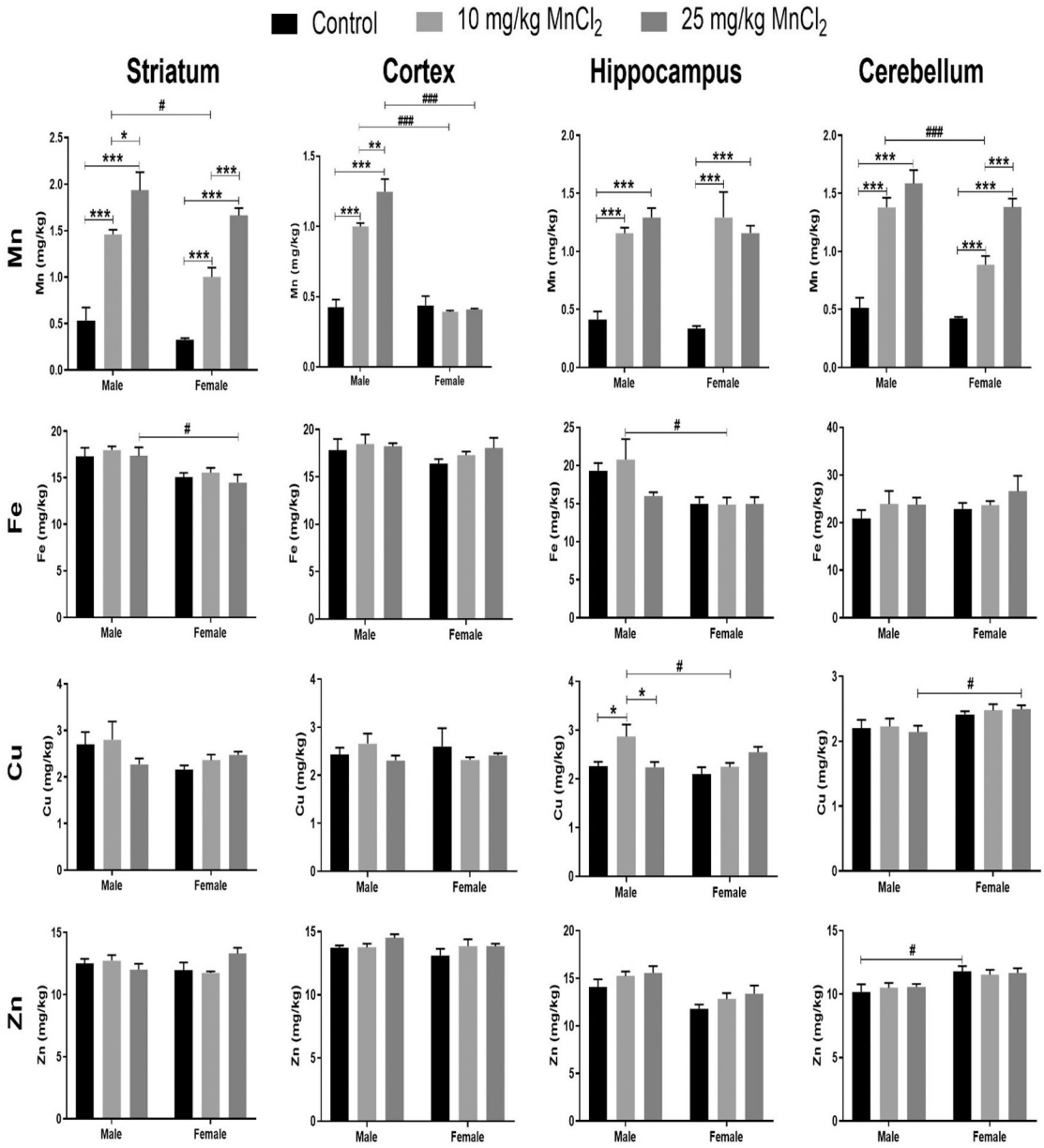

Fig. 2.

Effect of $MnCl_2$ treatment on levels of Mn and other metals in the striatum, cortex, hippocampus and cerebellum of male and female rats. Data were analyzed by two-way ANOVA followed by Bonferroni's post-test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ between male and female of same treatment.

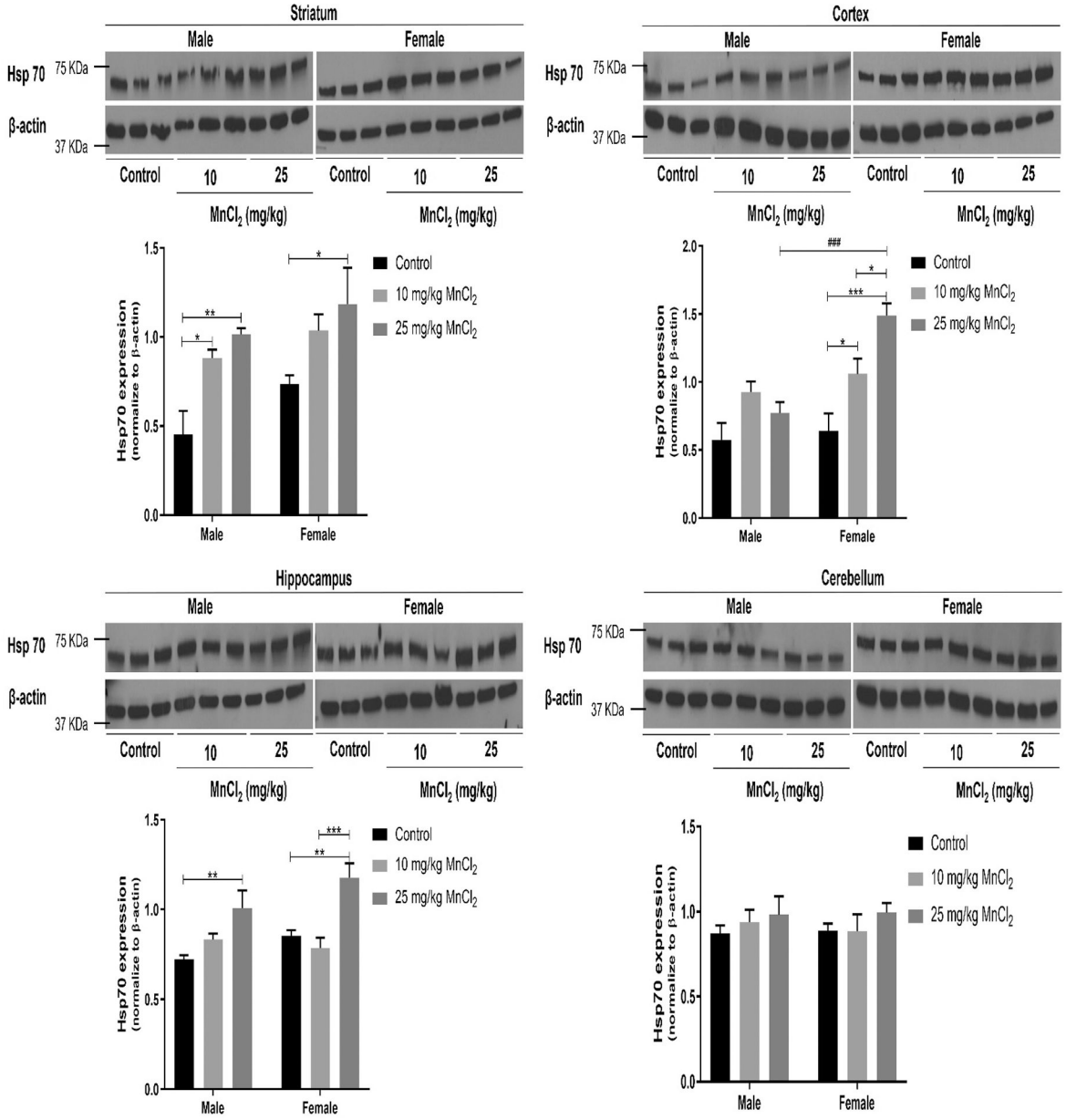

Figure 3:

Effect of $MnCl_2$ treatment on Hsp70 expression in the striatum, cortex, hippocampus and cerebellum of male and female rats. Data were analyzed by two-way ANOVA followed by Bonferroni's post-test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. $^{###}p < 0.001$ between male and female of same treatment.

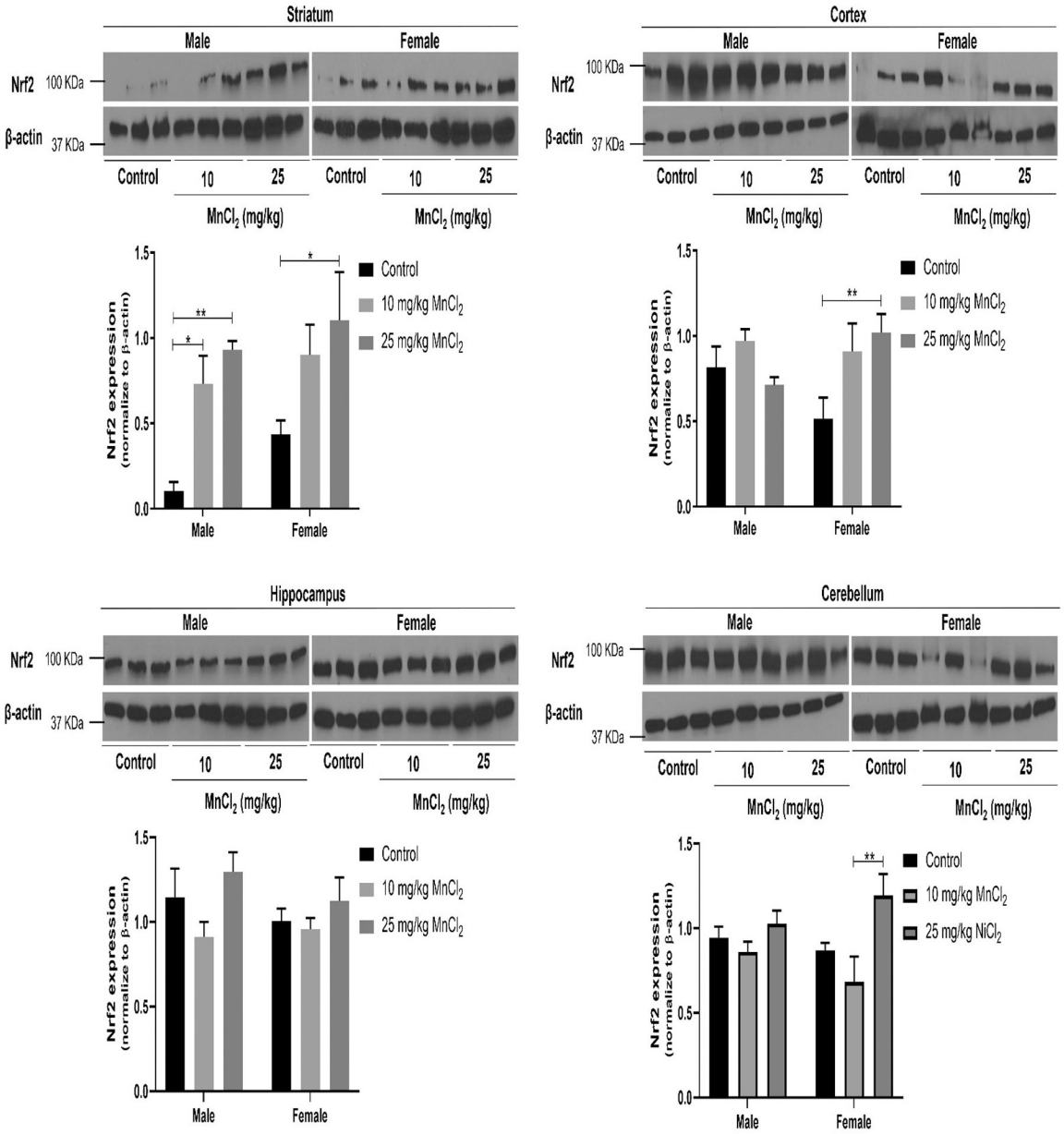

Figure 4:

Effect of $MnCl_2$ treatment on Nrf2 expression in the striatum, cortex, hippocampus and cerebellum of male and female rats. Data were analyzed by two-way ANOVA followed by Bonferroni's post-test. *$p < 0.05$, **$p < 0.01$.