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ORIGINAL PAPER

The effects of prenatal methylmercury exposure on trace element and antioxidant levels in rats following 6-hydroxydopamine-induced neuronal insult

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Abstract Methylmercury (MeHg) is a metal toxin found commonly in the environment. Studies have shown severe neurotoxic effects of MeHg poisoning especially during pregnancy where it crosses the foetoplacental and the blood brain barrier of the foetus leading to neurodevelopmental deficits in the offspring. These deficits may predispose offspring to neurodegenerative diseases later in life. In this study we investigated the effects of prenatal methylmercury exposure (2.5 mg/L in drinking water from GND 1-GND 21) on the trace element status in the brain of adolescent offspring (PND 28). Total antioxidant capacity (TAC) was measured in their blood plasma. In a separate group of animals that was also exposed prenatally to MeHg, 6-hydroydopamine (6-OHDA) was administered at PND 60 as a model of neuronal insult. Trace element and TAC levels were compared before and after 6-OHDA exposure. Prenatal MeHg treatment alone resulted in significantly higher concentrations of zinc, copper, manganese and selenium in the brain of offspring at PND 28 ($p < 0.05$), when compared to controls. In contrast, brain iron levels in MeHg-exposed adolescent offspring were significantly lower than their controls ($p < 0.05$). Following 6-OHDA exposure, the levels of iron, zinc, copper and manganese were increased compared to sham-lesioned offspring ($p < 0.05$). Prenatal MeHg exposure further increased these trace element levels thereby promoting toxicity ($p < 0.05$). Total antioxidant capacity was not significantly different in MeHg and control groups prior to lesion. However, following 6-OHDA administration, MeHg-exposed animals had a significantly lower TAC than that of controls ($p < 0.05$). Brain TAC levels were higher in adult male rats than in female rats during adolescence however male rats that had been exposed to MeHg in utero failed to show this increase at PND 74. Prenatal MeHg exposure results in trace element dyshomeostasis in the brain of offspring and reduces total antioxidant capacity. This may reflect a mechanism by which methylmercury exerts its neurotoxicity and/or predispose offspring to further neurological insults during adulthood.

Keywords Methylmercury · Prenatal · Neurodevelopment · Trace elements · Antioxidants

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Introduction

Excess metals have long been proposed to be neurotoxic and in the young, may induce neurodevelopmental defects (Giménez-Llort et al. 2001; Crichton et al. 2008; Ferraro et al. 2009). This proposal stems from the ability of the brain to concentrate metal ions leading to its abnormal accumulation in various brain regions (Bush 2000). The compartmentalization of ions has been shown to be harmful to the central nervous system when under inefficient homeostatic control (Bush 2000).

Methylmercury (MeHg) is an environmental pollutant which in higher than normal concentrations, is hypothesized to be detrimental to brain structure and function (Ferraro et al. 2009; Franco et al. 2009). For instance, prenatal MeHg intoxication has been associated with neurodevelopmental disorders such as mental retardation, as well as motor and cognitive dysfunction (Giménez-Llort et al. 2001; Daré et al. 2003; Johansson et al. 2007; Ferraro et al. 2009). The mechanism by which MeHg mediates these toxic effects remains unclear. One suggested explanation refers to the ability of MeHg to impair the antioxidant potential of the brain (Nascimento et al. 2008; Franco et al. 2009; Farina et al. 2011a). Franco et al (2009) showed that MeHg reduces the

activity of the glutathione peroxidase enzyme (GPx) in both an in vitro and in vivo model. MeHg binds readily to glutathione due to its affinity for thiol (-SH) groups. MeHg promotes the formation of free radicals such as reactive oxygen species (ROS) which also impair the glutathione antioxidant system and promote cell death by apoptosis (Nascimento et al. 2008; Farina et al. 2011a).

In addition there is some evidence that mercury may disrupt the trace element balance in the brain (Muto et al. 1991; Feng et al. 2004; Zhang et al. 2007). Muto et al (1991) showed an increase in zinc and copper levels in the brain following MeHg treatment. This occurs because MeHg has a stronger binding affinity to sulfhydryl groups than other trace elements thereby displacing them from their active sites leading to their accumulation (Limke et al. 2004; Zhang et al. 2007; Aliaga et al. 2010). Previous studies have shown that developmental exposure to organic mercury compounds such as mercury (II) chloride ($HgCl_2$), results in higher copper and zinc levels as well as reduced iron levels in the brain of rat offspring (Feng et al. 2004; Zhang et al. 2007). These alterations are suggested to promote neurotoxicity and subsequently the development of neurodegenerative diseases.

One of the challenges in our current understanding of metal-induced toxicity is the latency in the manifestation of metal-related diseases. A possible explanation for this delay may be due to what is referred to as silent toxicity – a phenomenon described as "a biochemical or morphological injury which remains clinically unapparent unless unmasked by experimental or natural processes" (Giordano and Costa 2012). Under circumstances of silent toxicity a subsequent neurotoxic injury is therefore required to trigger the onset of disease. Interestingly other trace elements such as selenium have been shown to have a protective effect against MeHg toxicity (Newland et al. 2006; Ralston et al. 2007, 2008).

The aims of the present study were therefore to investigate the effects of prenatal MeHg exposure on the concentrations of a variety of trace elements in the brain, as well as the total antioxidant capacity of the brain in adolescent offspring. We also assessed the impact of prenatal MeHg exposure on the consequences of a subsequent neurotoxin later in life with respect to the levels of these trace elements and the antioxidant status of the brain.

Materials and methods

Animal handling and treatment procedure

Male and female Sprague-Dawley rats were obtained from the Biomedical Resource Centre at the University of KwaZulu-Natal and were housed under a 12 h light/dark cycle (6:00–18:00), with food and water ad libitum. All experiments were conducted with the approval of the University of KwaZulu-Natal Animal Ethics Research Committee (Ethical Clearance number: 090/12/Animal).

Synchronization Female rats were housed in pairs in order to synchronize their oestrus cycles. The rat oestrus cycle is usually between 4 and 5 days long and is divided into 4 phases, namely: - pro-oestrus, oestrus, met-oestrus and di-oestrus (Hubscher et al. 2005; Westwood 2008). During oestrus, the oestrogen concentration is high making it the ideal phase for pregnancy. Vaginal smears were taken daily to check for synchronization of cycles. Briefly, saline (100 μl) was used to flush the vagina of female rats using a micropipette. Vaginal cells were collected, smeared on a glass slide and allowed to air-dry. Once dry, slides were prepared for staining using the Shorr stain method (Shorr 1941; Hartman 1944), fixed and viewed under a light microscope.

Breeding Mating took place during pro-oestrus in anticipation of the oestrus phase. Male and female rats were housed in a 1:1 ratio and allowed to mate overnight. Females were checked for the presence of vaginal plugs the following morning and this was deemed positive for pregnancy and therefore gestational day 1 (GND 1). In the absence of a plug, vaginal smears were performed and sperm-positive smears were regarded as GND 1.

MeHg treatment Pregnant females were divided into two groups: - a control group which received untreated drinking water and an experimental group which was exposed to methylmercury chloride (2.5 mg/L, Sigma, St. Louis MO, U.S.A.) in drinking water from GND 1 to GND 21 after which MeHg-contaminated water was replaced with normal drinking water. MeHg purity was approximately 99.5 % (Sigma Aldrich Certificate of Analysis). Water intake and body weight were measured daily for each animal. A water control bottle was placed in an empty cage to control for water loss by spillage. MeHg exposure amounted to ±0.25 mg/kg/day based on body weight and daily water intake. This dose was chosen as an intermediate to doses in previous publications (Coccini et al. 2000; Gralewicz et al. 2009; Guo et al. 2013) to mimic a low, chronic dose of exposure. MeHg pollution of water resources in South Africa range from below the detection limit, <0.02 ng/L to±2.66 ng/L (Williams et al. 2010, 2011) depending on the site and duration of exposure. Sites closer to anthropogenic sources had higher aqueous MeHg concentration as well as high sediment MeHg levels and high concentrations in fish and other invertebrates (Williams et al. 2010, 2011). These contribute to the elevated levels in the aquatic food chain where recent studies have shown that the MeHg levels in fish are approaching the US EPA guidelines (300 ng/g ww). Brain mercury levels were not measured in this study but are expected to be in the range 2–4 μg/ml based on a study with a similar dose (Ishitobi et al. 2010).

Postnatal handling On postnatal day 21 (PND 21) pups were weaned and placed in a separate cage from the dam. The female offspring were separated into 2 groups: - 1) Offspring which were exposed to MeHg in utero (MeHg, $n=7$) and 2) Offspring which were not exposed to MeHg (Control, $n=7$). Male offspring were allowed to mature until PND 60 when 6-OHDA lesion took place (see below). Female offspring were sacrificed on PND 28 by decapitation. Blood plasma was collected for total antioxidant capacity (TAC) analysis while whole brain tissue was collected for trace element quantification. Whole brain was collected under sterile conditions using plasticware to prevent leaching of metals from dissecting equipment. The tissue was blotted on filter paper, weighed and stored at −20 °C until further analysis.

Behavioural tests Behaviour was assessed to identify motor dysfunction. This occurred both pre-lesion (PND 58) as well as post-lesion (PND 74) for comparative analysis of the neurotoxin effect to be made. The forelimb akinesia (step) test and the limb-use asymmetry (cylinder) test were conducted. Groups were randomly assigned such that the experimenter was blind to the type of treatments.

- The forelimb akinesia (step) test

  This test examines movement initiation (Mabandla and Russell 2010). The animal was held by its torso such that the hindquarters and forelimb not being tested were elevated by the experimenter resulting in the weight of the animal being supported by the forelimb being tested. The animal was then propelled forward on a non-smooth surface and the adjusting step made by the forelimb was measured using a ruler attached adjacently. This was done 3 times per limb and an average was calculated for each limb.
- The limb-use asymmetry (cylinder) test

  This test examines forelimb use during explorative behaviour (Meredith and Kang 2006; Mabandla and Russell 2010). The animal was placed in a plexiglass cylinder (20 cm diameter and 30 cm height) for 5 min and its behaviour was videotaped and subsequently assessed. The animal was tested for wall exploration, contact with the wall as well as landing after wall contact, for both forelimbs (Mabandla and Russell 2010). Animals were assessed for percentage limb-use of the impaired (contralateral) limb by using the following equation:-

$$\%\ limb\ use\ of\ impaired = \left(\frac{impaired + \frac{1}{2} both}{impaired + unimpaired + both} \right) \times 100$$

Where, impaired refers to the limb contralateral to the neurotoxin-injected (lesioned) hemisphere and unimpaired refers to the limb ipsilateral to the lesioned hemisphere. Both, refers to the use of both the impaired and unimpaired limbs during exploratory activity (Tillerson et al. 2001).

6-Hydroxydopamine (6-OHDA) lesion On post-natal day 60 (PND 60), the neurotoxin 6-OHDA was injected unilaterally into the medial forebrain bundle as a model of neuronal insult (Deumens et al. 2002; Blandini et al. 2008). Male offspring (Control and MeHg groups, $n=7$) were first anaesthetized with sodium pentobarbital (50 mg/kg i.p., Sigma, St. Louis MO, U.S.A.). After, the rat was placed in the stereotaxic frame (David Kopf Instruments, Tujunga CA, U.S.A.). The skull was exposed by making a midline incision with a scalpel. A small burr hole was drilled at the following coordinates: 4.7 mm lateral to midline and 1.6 mm caudal to bregma (Mabandla and Russell 2010). At these coordinates, a Hamilton needle was slowly inserted into the brain tissue 8.4 mm below the skull, to inject a fresh solution of 6-OHDA (5 μg/4 μl dissolved in 0.2 % ascorbic acid; Sigma, St. Louis MO, U.S.A.). The 6-OHDA solution was injected into the right medial forebrain bundle at a rate of 0.5 ml/min. The needle was kept in its position for a further 3 min after 6-OHDA infusion and thereafter the needle was gradually removed. Sham-lesioned animals were injected with saline instead of 6-OHDA. The hole was covered with sterilized oxidized cellulose and the wound sutured thereafter. During recovery, the animals were warmed using heating pads to prevent hypothermia. They were returned to their home cages after full recovery from the surgical procedure (±2 h post-lesion).

Tissue collection On postnatal day 75 (PND 75), animals were decapitated and blood plasma and whole brain were collected (as described for PND 28) for measurement of total antioxidant capacity (TAC) and trace elements respectively.

Biochemical analysis

Trace element analysis

Trace element levels were measured by Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) using a method adapted from Levy et al. 2001. Briefly, whole brain tissue (1 g) was homogenized in 2 N hydrochloric acid (HCl) (7 ml) using a Misonix Sonicator XL2000-010 (Newtown CT, USA) until a smooth homogenate was obtained. Samples were then treated with 70 % perchloric acid (1 ml) and incubated at 50 °C for 24–36 h in a water bath. Following incubation, samples were centrifuged at 3500 rpm for 1 h and thereafter filter-syringed through a 0.45 μm pore size filter. Samples and standards were then analysed on the Perkin Elmer Optima 5300 DV Optimal Emission Spectrometer (Waltham MA, USA).

Total antioxidant capacity (TAC)

The TAC is a measure of the collective capacity of biomolecules from a sample to exert antioxidant activity. Whole blood was centrifuged at 3500 rpm for 10 min using a Hermle Labortechnik GmbH centrifuge (Wehingen, Germany). Plasma was collected and analysed for TAC using the OxiSelect™ Total Antioxidant Capacity (TAC) Assay kit (Cell Biolabs Inc., San Diego CA, USA) according to the manufacturer's instructions.

Statistical analysis

All data was analysed using the software programme GraphPad Prism (Version 5) and was tested for normality (Kolmogorov-Smirnov test for normality). For non-parametric data, the Kruskal Wallis test was used for comparison of more than 2 groups. The Wilcoxin matched paired test and the Mann-Whitney U test were used for comparison between 2 individual groups. For parametric data, the One-way ANOVA was performed with Tukey's Multiple Comparison test. Results were considered significant when a p-value <0.05 was obtained.

Results

Water intake during pregnancy

Pregnant rats exposed to MeHg (2.5 mg/L) showed no significant difference in the daily water intake compared to that of the controls (control 29.43±2.9 ml/day vs. MeHg 29.81± 2.26 ml/day). Average water loss by spillage amounted to 4.8 ml/day and this was corrected for in the result.

Brain weight of juvenile offspring

Offspring of MeHg-treated rats had a significantly greater brain mass when compared to non-exposed pups (Fig. 1; $p<0.05$) at PND 28.

Behavioural analysis

a) Step test

There was both a 6-OHDA and MeHg effect on the step length following lesion. Prenatal exposure to MeHg per se did not affect step length (Fig. 2: control vs. MeHg pre-lesion), while 6-OHDA injection resulted in a significant increase in step length (Fig. 2: * control pre-lesion vs. control post-lesion, ** MeHg pre-lesion vs. MeHg post-lesion; $p<0.0005$). This effect of 6-OHDA was exacerbated in animals pre-exposed to MeHg (Fig. 2: $^{\#}$ control post-lesion vs. MeHg post-lesion; $p<0.05$).

b) Cylinder test

Offspring exposed to MeHg showed decreased locomotor activity compared to control offspring in both the impaired (Fig. 3, * control pre-lesion vs. MeHg pre-lesion; $p<0.05$) and unimpaired limbs (Data not shown). 6-OHDA treatment had an effect in decreasing the percentage limb-use in control (Fig. 3, ^{a} control pre- vs. post-lesion; $p<0.05$) and MeHg offspring (Fig. 3, ^{b} MeHg pre- vs. post-lesion; $p<0.05$). We also observed a significantly lower percentage limb use in the MeHg offspring post-lesion compared to that of controls (Fig. 3, ** control post-lesion vs. MeHg post-lesion $p<0.05$).

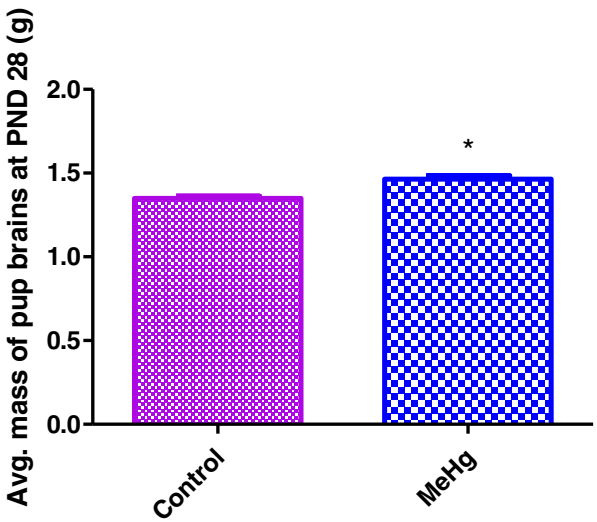

Fig. 1 Graph comparing average brain mass (g) of rats exposed to MeHg or untreated drinking water at PND 28 ($n=7$ per group). * $p<0.05$; significantly different from control (Mann-Whitney U test)

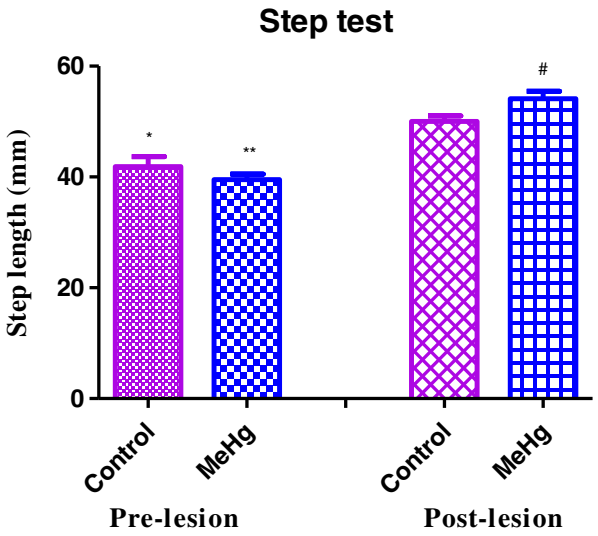

Fig. 2 Graph showing step length of impaired limb of either MeHg-exposed rats or control rats that were exposed to untreated drinking water, before (pre-lesion) and after receiving a unilateral 6-OHDA injection (post-lesion) into their medial forebrain bundle ($n=7$). *$p<0.05$; significantly different from control post-lesion group (Kruskal-Wallis followed by Wilcoxin paired test) ** $p<0.05$; significantly different from MeHg post-lesion group (Kruskal-Wallis followed by Wilcoxin paired test) $^{\#}$ $p<0.05$; significantly different from control post-lesion group (Kruskal-Wallis followed by Mann Whitney U test)

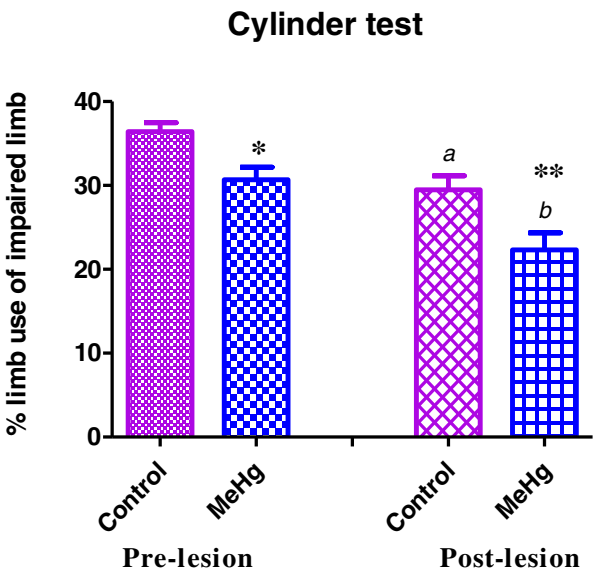

Fig. 3 Graph showing percentage limb-use of impaired limb of either MeHg-exposed rats or control rats that were exposed to untreated drinking water, before (pre-lesion) and after receiving a unilateral 6-OHDA injection (post-lesion) into their medial forebrain bundle ($n=7$). * $p<0.05$; significantly different from control pre-lesion group (Kruskal-Wallis followed by Mann Whitney U test) ** $p<0.05$; significantly different from control post-lesion group (Kruskal-Wallis followed by Mann Whitney U test) ^{a} $p<0.05$; significantly different from control pre-lesion group (Kruskal-Wallis followed by Wilcoxin paired test) ^{b} $p<0.05$; significantly different from MeHg pre-lesion group (Kruskal-Wallis followed by Wilcoxin paired test)

Trace element analysis

Concentrations of zinc, copper, manganese, selenium and iron were quantified in brain tissue at PND 28 as well as at PND 75. Prenatal MeHg treatment resulted in decreased iron levels in the brain of offspring at PND 28 (Fig. 4a; *$p<0.05$). This was accompanied by an increase in zinc, copper, manganese and selenium concentrations with MeHg exposure (Fig. 4b, c, d and e respectively; *$p<0.05$).

Following 6-OHDA neurotoxicity, iron, zinc, copper and manganese concentrations were elevated compared to sham-lesioned animals (Fig. 5a, b, c and d respectively; $^{\#}$ saline vs. control, $p<0.05$). Exposure to MeHg showed a similar increase in all trace element levels (Fig. 5a, b, c and d; ** saline vs. MeHg, $p<0.05$). Iron, zinc, copper and manganese concentrations were further increased when exposed to prenatal MeHg exposure and lesioned with 6-OHDA (Fig. 5a, b, c and d respectively; * control vs. MeHg, $p<0.05$). Selenium levels were very low and close to the detection limit post-lesion.

Total antioxidant capacity (TAC)

Blood plasma was analysed for Total Antioxidant Capacity (TAC). Comparison between MeHg-exposed animals and controls showed no significant differences at PND 28 however, there was a tendency for the MeHg group to have a lower antioxidant capacity (Fig. 6a). At PND 75, TAC was significantly lower in MeHg exposed animals compared to controls (Fig. 6b, $p<0.05$). In control animals TAC was higher in older rats (Fig. 6a and b); $^{\#}$ control PND 28 vs. control PND 75, $p<0.05$) but there was no age-related change in total antioxidant capacity in animals that were exposed to MeHg. Although offspring were not gender-matched at PND 28 compared to PND 75, the female rats at PND 28 were pre-pubescent and therefore there were no hormonal influences on the results. Studies have shown that the onset of puberty occurs at approximately 30 days of age and later before which there is no activity in the reproductive tract (Goldman et al. 2007; Westwood 2008). Gender-specific differences in brain development has been suggested to occur due to the effect of these hormones which exhibit during the peri-adolescent period (PND 28–42) (Spear 2000; Neufang et al. 2009). Thus the gender difference would not have an impact on our results.

Discussion

It has been established that prenatal methylmercury (MeHg) exposure is neurotoxic to the developing foetus (Newland et al. 2006; Carratu et al. 2008; Ferraro et al. 2009; Gralewicz et al. 2009). Previous studies have shown that offspring which have been exposed to methylmercury in utero have neurological deficits such as motor and cognitive

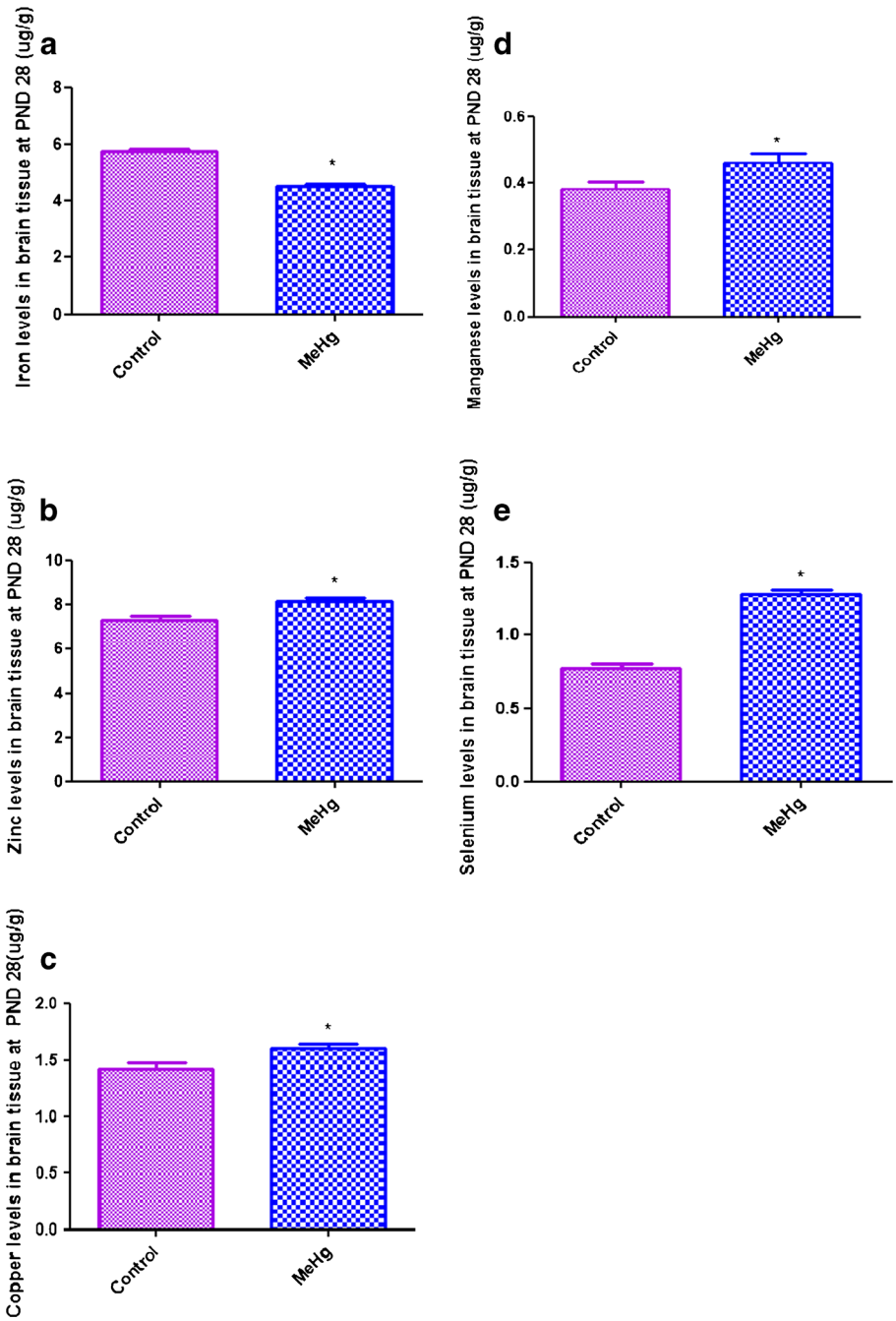

Fig. 4 Graph showing trace element levels in either MeHg-exposed rats or control rats that were exposed to untreated drinking water at PND 28 ($n=7$ per group). Trace elements measured were **a** iron **b** zinc **c** copper **d** manganese **e** selenium * $p<0.05$; significantly different from control (Mann-Whitney U test)

dysfunction (Carratu et al. 2008; Gralewicz et al. 2009). Mechanisms of methylmercury toxicity vary from the generation of reactive oxygen species to the impairment of the glutathione antioxidant system to glutamate dyshomeostasis (Aschner et al. 2007; Nascimento et al. 2008; Farina et al. 2011a). Many studies have emphasized the importance of trace element homeostasis in maintaining brain function (Bush 2000; Feng et al. 2004; Levenson 2005; Valko et al. 2005). The perinatal phase is especially vulnerable to dyshomeostasis and may result in neurological dysfunction (Lozoff et al. 2006; Cordova et al. 2013). In this study we investigated the effect of prenatal MeHg exposure on the trace element status of offspring during adolescence (PND 28). We also examined the Total Antioxidant Capacity (TAC) at this critical period.

MeHg treatment did not alter water intake during pregnancy however, offspring of dams exposed to MeHg had a greater brain mass than that of control offspring. This is in contrast to previous studies which showed no difference in offspring brain mass following prenatal MeHg exposure (Newland and Reile 1999). This may be explained by contrasting doses of MeHg as well as different dosing regimens, i.e. in our study

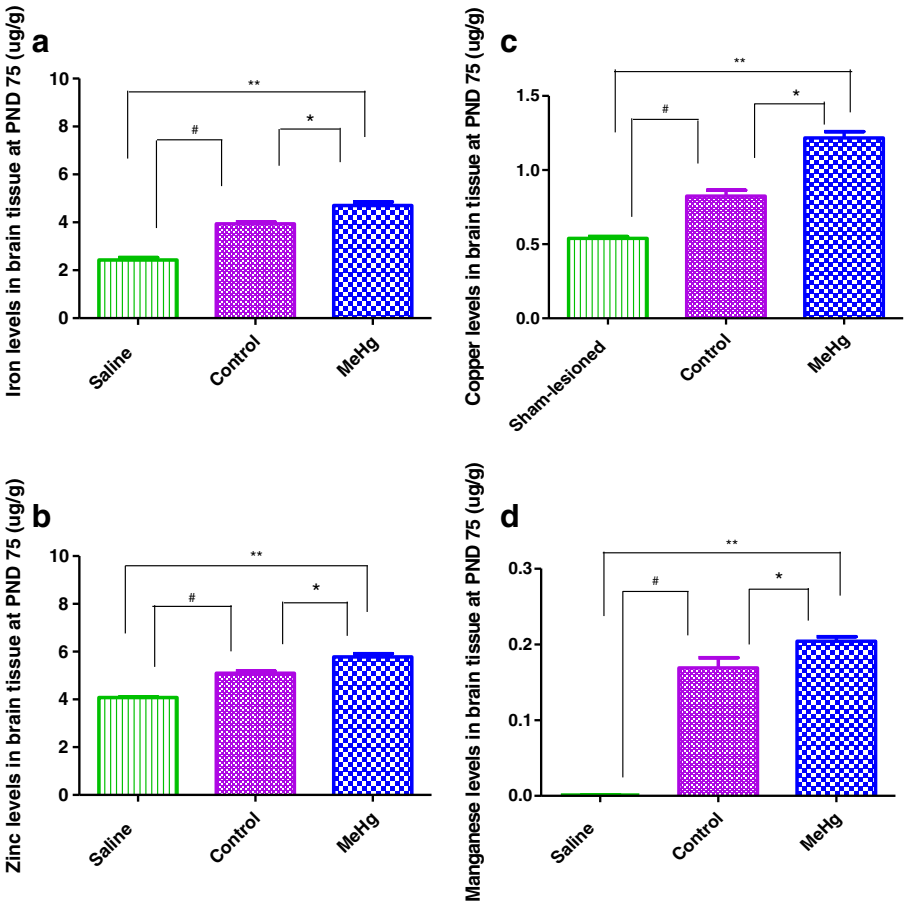

Fig. 5 Graph showing trace element levels in either MeHg-exposed rats ($n=8$) or control rats that were exposed to untreated drinking water ($n=8$) after receiving a unilateral 6-OHDA injection or sham-lesion ($n=5$). Trace elements measured were **a** iron **b** zinc **c** copper **d** manganese $^{\#}p<0.05$; significantly different from saline (Mann-Whitney U test) $^{*}p<0.05$; significantly different from control (Mann-Whitney U test) $^{**}p<0.05$; MeHg significantly different from control (Mann-Whitney U test)

MeHg exposure occurred for the duration of the pregnancy only (GND 1–21) in contrast to exposure from pre-breeding to PND 16 in the above-mentioned study. Furthermore, the brain of offspring was collected at PND 28 in our study differing from Newland and Reile (1999) and Feng et al (2004) where offspring brain were collected at PND 21 and PND 20 respectively. Another study showed that mercury treatment (as $HgCl_2$ exposed from GND 0 to PND 20) in adult males resulted in increased cerebral brain mass similar to our results (Feng et al. 2004). We hypothesize that the observed increase in brain mass of MeHg-treated offspring may be due to cerebral oedema. Yamamoto et al (2012) showed that MeHg treatment increased expression of aquaporin 4 in marmoset model of MeHg toxicity. Aquaporin 4 is the main aquaporin in the mammalian brain and is found in the end-feet of astrocytes making up the blood brain barrier (Pasantes-Morales and Cruz-Rangel 2010; Yamamoto et al. 2012). It is responsible for regulating water balance in the brain and increased expression may result in cerebral oedema (Pasantes-Morales and Cruz-Rangel 2010; Yamamoto et al. 2012). Aquaporin 4 is up-regulated in astrocytes and this may impair astrocyte function and contribute to MeHg toxicity (Yamamoto et al. 2012). Astrocytes are responsible for the support and nutrition of neurons (Sidoryk-Wegrzynowicz et al. 2011). They are responsible for the production and release of critical growth factors, can act as free radical scavengers and are involved in the modulation of glutathione levels (Sidoryk-Wegrzynowicz et al. 2011). MeHg binds readily to astrocytic glutamate transporters preventing glutamate uptake and thereby promoting glutamate excitotoxicity (Nascimento et al. 2008; Farina et al. 2011a). MeHg can also act within the astrocytes by inhibiting glutathione synthesis as well as binding directly to glutathione impairing antioxidant defences (Nascimento et al. 2008). Therefore MeHg readily affects astrocyte function by impairing neuronal support and promoting oxidative stress which can lead to neuronal dysfunction.

The importance of trace elements in brain development is well established (Shanker 2008; Dauncey 2009). Our data showed major differences in levels of iron, copper, zinc, manganese and selenium in brains of MeHg-exposed offspring compared to controls. Results showed that iron levels were significantly decreased at PND 28 with prenatal MeHg exposure. Iron is critical during prenatal and early postnatal period due to the massive uptake needed for formation of neural circuits (Lozoff et al. 2006). Thus iron deficiency can lead to abnormal brain development. Iron deficiency has been strongly associated with cognitive and motor deficits due to its primary effect on the hippocampus and striatum (Lozoff et al.

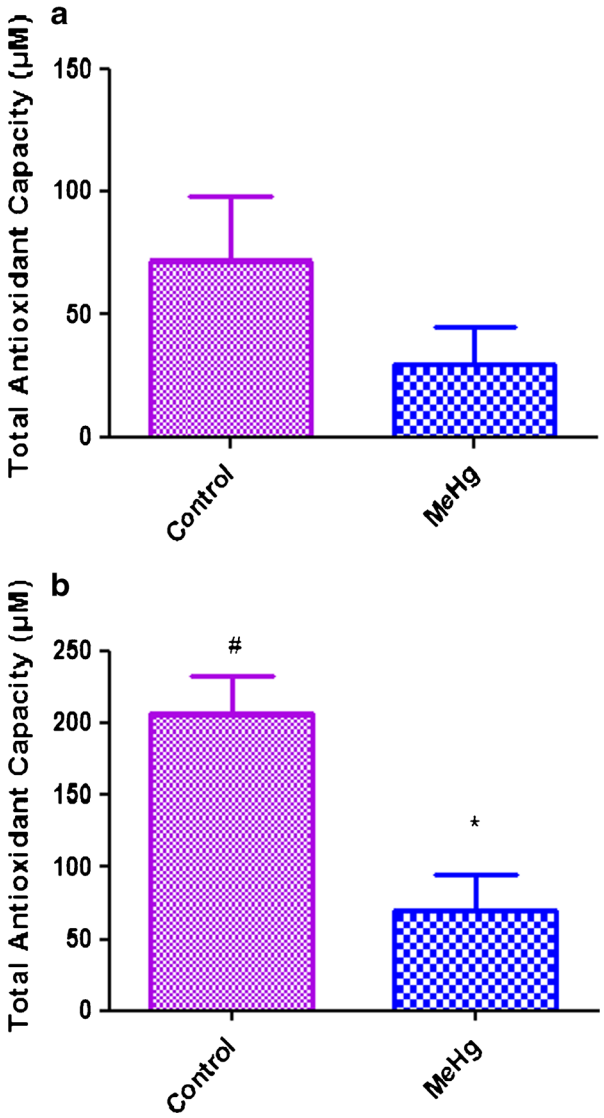

Fig. 6 Graph showing total antioxidant capacity (µM) in MeHg-exposed rats or control rats that were exposed to untreated drinking water ($n=7$ per group) and **b** after 6-OHDA lesion at PND 75 ($n=7$ per group). * $p<0.05$; significantly different from control PND 75 (Mann-Whitney U test) $^{\#}$ $p<0.05$; significantly different from control PND 28 (Mann-Whitney U test)

2006). Iron deficiency can also result in alterations of other essential elements (Oladiji 2003). Oladiji (2003) showed increased concentrations of copper and zinc with iron deficiency. This result may explain the increased levels of both copper and zinc in our study both before and after 6-OHDA-induced lesion. Elevated zinc, copper and iron levels have been strongly associated with neurodegenerative diseases (Ide-Ektessabi and Rabionet 2005; Kozlowski et al. 2009). Our study showed significantly higher manganese levels with MeHg treatment. Manganese toxicity has also been associated with iron deficiency (Cordova et al. 2013). Thus the observed increases in copper, zinc and manganese levels may be mediated in part by the decrease in iron levels. Extremely high manganese levels leads to the neurodegenerative disorder manganism and some studies suggest that this may predispose to the development of other neurodegenerative diseases (Milatovic et al. 2009; Weiss 2011). Copper, zinc and manganese are essential elements in normal brain development (Feng et al. 2004; Dauncey 2009; Milatovic et al. 2009). However, alterations in their homeostasis can lead to excessive accumulation which may be toxic, leading to abnormal neuronal functioning (Feng et al. 2004; Cordova et al. 2013). Copper is a co-factor for many enzymes and is therefore essential for proper enzymatic function (Gaetke and Chow 2003). Excess copper can be toxic by promoting the formation of free radicals thereby promoting oxidative stress (Valko et al. 2005). Copper binds readily to glutathione and forms a Cu(I)–$[GSH]_2$ complex which may react with oxygen molecules to promote superoxide radical formation (Aliaga et al. 2010). Aliaga et al (2010) showed that mercury ions can exacerbate this process leading to further oxidative damage. Zinc has been shown to be both neurotoxic and neuroprotective in the brain (Valko et al. 2005). Zinc deficiency and excess zinc readily induces apoptosis however evidence also exists that zinc has antioxidant potential (Chen and Liao 2003). This emphasizes the importance of zinc homeostasis for proper neuronal function. Manganism occurs with excess manganese levels, leading to rapid accumulation especially in the basal ganglia (Cordova et al. 2013). Manganism is characterized by motor deficits with symptoms similar to that of Parkinson's disease. Therefore alterations in the homeostasis of these elements may contribute to neurotoxicity in offspring of MeHg-exposed animals. Franco et al (2009) showed that treatment with MeHg results in the inhibition of selenoproteins such as glutathione peroxidase (GPx) in a mouse model of neurotoxicity. Inhibition of Gpx prevents its antioxidant function promoting oxidative damage. It has been suggested that exposure to MeHg results in sequestration of selenium thereby causing selenium deficiency (Ralston et al. 2007). Supplementation of selenium in the diet has been shown to combat this selenium deficiency. Studies have shown that selenium is protective against MeHg toxicity (Ralston et al. 2007; Meinerz et al. 2011). When compared to control offspring, our data showed significantly higher selenium levels in MeHg-exposed animals. This is in contrast to previous studies where selenium levels in offspring were relatively unaffected by gestational mercury exposure (Feng et al. 2004; Newland et al. 2006). This difference may be explained by the different mechanisms of MeHg toxicity. We propose that MeHg impairs the neurocircuitry during development affecting the proper mechanisms for selenium homeostasis leading to excess selenium levels from dietary sources.

There were also imbalances in trace element concentrations after animals were subjected to a subsequent neurotoxic insult. 6-Hydroxydopamine treatment led to increases in iron, zinc, copper and manganese concentrations compared to sham-lesioned animals. Gestational exposure to MeHg resulted in

higher brain iron levels than controls after 6-OHDA-induced neurotoxicity. Iron toxicity has been linked to neurodegenerative diseases such as Parkinson's disease (He et al. 1996; Graham et al. 2000). Post-mortem analysis of Parkinson's disease patients showed elevated iron levels in brain tissue (He et al. 1996; Graham et al. 2000). Iron promotes the formation of reactive oxygen species (ROS) via the Fenton reaction. Excessive accumulation of ROS may lead to oxidative stress and neuronal cell death. Manganese levels were extremely low in sham-lesioned animals and close to the detection limit. Unpublished data from our lab showed a progressive decline in manganese levels with age as measured on postnatal day 28, postnatal day 60 and postnatal day 75. This reflects the decrease in manganese requirement in adulthood as compared to the developmental stage. Copper, zinc and manganese levels were also significantly higher following 6-OHDA exposure with the MeHg group having higher levels than control animals. Copper and zinc are redox metals and therefore imbalances in redox cycling promote the generation of ROS and oxidative stress which leads to apoptosis (Barnham and Bush 2008; Crichton et al. 2008). Both copper and zinc have been associated with Alzheimer's disease while manganese toxicity results in manganism (Crichton et al. 2008; Kozlowski et al. 2009; Milatovic et al. 2009). Selenium levels were not detectable following 6-OHDA neurotoxicity in both control and MeHg groups. This may be due to 6-OHDA-induced oxidative stress which up-regulates selenium-dependent enzymes such as glutathione peroxidase thereby resulting in selenium deficiency (Schweizer et al. 2004). Thus tissue homeostasis of trace elements are essential for proper brain functioning. Alterations in their levels may contribute to MeHg neurotoxicity which could have further implications should the offspring be exposed to a subsequent neurotoxic insult.

One of the major mechanisms of MeHg toxicity is the disruption of antioxidant defences (Nascimento et al. 2008; Farina et al. 2011b). MeHg has been shown to impair the glutathione antioxidant system by binding readily to glutathione leading to glutathione depletion (Farina et al. 2011b; Kaur et al. 2011). MeHg also disrupts the antioxidant enzymes glutathione peroxidase (Franco et al. 2009; Farina et al. 2011a). In our study we examined the effect of developmental MeHg toxicity on the total antioxidant capacity of offspring. Results showed that MeHg exposed offspring had a tendency to have a lower antioxidant capacity than that of control animals however these results were not statistically significant. Since the Total Antioxidant Capacity (TAC) kit is not a direct measurement of antioxidant levels, damage to the antioxidant enzymes might not have been detected with this method at PND 28. Alternatively the absence of change in the TAC could be due to compensatory mechanisms since MeHg was given prenatally and as MeHg is metabolized and cleared from the body, the antioxidant levels of the offspring may have normalized by PND 28. Our results also showed that TAC levels were higher in adult control animals compared to control animals at PND 28 but there was no difference in animals that were exposed to MeHg at the different ages. Sullivan and Newton (1988) showed that serum antioxidant levels were higher in adult rats than neonates. Our data similarly showed higher plasma TAC levels in adult offspring compared to adolescents. Animals exposed to MeHg did not show any difference in plasma TAC levels. This suggests that MeHg interferes with the development of the antioxidant system rendering the offspring more susceptible to future insult. This hypothesis is supported by the 6-OHDA result which showed that following exposure to 6-OHDA, animals that were exposed to MeHg prenatally had a significantly lower antioxidant capacity than that of controls. MeHg toxicity has been strongly associated with the development of oxidative stress and studies have shown decreased glutathione concentrations (Kaur et al. 2006; Ni et al. 2010). This may account for the reduction in total antioxidant capacity. Although prenatal exposure to MeHg did not show a significant change in the total antioxidant capacity at PND 28, when exposed to a subsequent insult via 6-OHDA, TAC was severely reduced.

In conclusion, our data showed that developmental MeHg toxicity can disrupt the homeostasis of essential trace elements leading to deficiency or excessive accumulation. This promotes toxicity leading to neuronal dysfunction. When exposed to a subsequent neuronal insult, MeHg toxicity was exacerbated and TAC was reduced. This may have implications for the offspring in adulthood by increasing their susceptibility to neurotoxic insults. Thus our study provides a correlation for prenatal MeHg in promoting foetal basis of adult diseases by trace element dyshomeostasis and impaired antioxidant capacity.

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Conflict of interest The authors declare that there are no conflicts of interest.

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