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ORIGINAL PAPER

Maternal Thimerosal Exposure Results in Aberrant Cerebellar Oxidative Stress, Thyroid Hormone Metabolism, and Motor Behavior in Rat Pups; Sex- and Strain-Dependent Effects

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Abstract Methylmercury (Met-Hg) and ethylmercury (Et-Hg) are powerful toxicants with a range of harmful neurological effects in humans and animals. While Met-Hg is a recognized trigger of oxidative stress and an endocrine disruptor impacting neurodevelopment, the developmental neurotoxicity of Et-Hg, a metabolite of thimerosal (TM), has not been explored. We hypothesized that TM exposure during the perinatal period impairs central nervous system development, and specifically the cerebellum, by the mechanism involving oxidative stress. To test this, spontaneously hypertensive rats (SHR) or Sprague–Dawley (SD) rat dams were exposed to TM (200 μg/kg body weight) during pregnancy (G10–G15) and lactation (P5–P10). Male and female neonates were evaluated for auditory and motor function; cerebella were analyzed for oxidative stress and thyroid metabolism. TM exposure resulted in a delayed startle response in SD neonates and decreased motor learning in SHR male (22.6%), in SD male (29.8%), and in SD female (55.0%) neonates. TM exposure also resulted in a significant increase in cerebellar levels of the oxidative stress marker 3-nitrotyrosine in SHR female (35.1%) and SD male (14.0%) neonates. The activity of cerebellar type 2 deiodinase, responsible for local intrabrain conversion of thyroxine to the active hormone, 3′,3,5-triiodothyronine (T3), was significantly decreased in TM-exposed SHR male (60.9%) pups. This coincided with an increased (47.0%) expression of a gene negatively regulated by T3, Odf4 suggesting local intracerebellar T3 deficiency. Our data thus demonstrate a negative neurodevelopmental impact of perinatal TM exposure which appears to be both strain- and sex-dependent.

Keywords Ethylmercury · Rat · Cerebellum · Oxidative stress marker 3-nitrotrosine (3-NT) · Type 2 deiodinase (D2)

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Introduction

Environmental toxicants such as heavy metals [1] including mercury Hg [2, 3] have been identified as factors exerting a range of harmful neurological and cognitive effects in humans and experimental animals, and have been implicated in the etiology of a number of neuropsychiatric disorders. The major environmental organic compounds of mercury include methylmercury (Met-Hg) and ethylmercury (Et-Hg). The main exposure to Met-Hg comes from contaminated fish through bioaccumulation of both organic and inorganic of Hg environmental contamination.

Met-Hg accumulates in both fetal and neonatal brains potentially affecting neurodevelopment [4]. Met-Hg has been shown to cross the placenta [5] and can be transferred from plasma to mothers' milk [6]. It is a known trigger of oxidative stress [7, 8] and both an endocrine [9, 10] and antioxidant defense system [11, 12] disruptor. Gestational exposure to Met-Hg in mice results in increased lipid peroxidation and reduced developmental increase in GSH in the brain [13].

While much is known about the effects of Met-Hg, on the other hand, little is known about the developmental neurotoxicity of Et-Hg, a metabolite of thimerosal (TM), used as a preservative in DPT vaccines in the USA until 1999. TM is still found in flu vaccines administered to pregnant women and infants in the USA and in DPT vaccines in developing countries. While the World Health Organization based its decision on the safety of TM in vaccines on epidemiological studies, it has indicated that the data were derived from well-nourished, full-term infants, and these data cannot necessarily be extrapolated to preterm or malnourished infants [14]. As with other environmental toxins, both health status and genetically determined sensitivity to mercury may be crucial factors in determining the overall outcome of exposure during the developmental period.

The present study was undertaken to address the hypothesis that TM exposure during the perinatal period impairs cerebellar development by the mechanism involving oxidative stress. TM was administered during G10–G15 corresponding to the period of cerebellar Purkinje cell birth and simulating flu (and other TM containing) vaccines given to pregnant women during the beginning of the second trimester of pregnancy [15] and P5–P10 corresponding to the period of granule cells proliferation and a critical period of brain development; TM administration during that time simulates vaccination during the second and the third trimester of pregnancy [15, 16]. To test this hypothesis, we examined the effect of TM on neurodevelopmental milestones, auditory functions, and motor learning. We also examined cerebellar levels of the oxidative stress marker 3-nitrotyrosine (3-NT) and the type 2 deiodinase (D2), a selenoenzyme that converts the prohormone thyroxine (T4) to the active hormone, 3′,3,5-triiodothyronine (T3) and is responsible for most of the T3 supply within the brain [17]. These effects were examined in male and female neonates to test for the sex-dependent nature of these effects and in two strains of rats with different thresholds to oxidative stress, spontaneously hypertensive rats (SHR) and Sprague–Dawley (SD), to test for genetically dependent sensitivity to Hg. We report here that Hg exposure in the form of TM results in a variety of neurodevelopmental deficits, altered cerebellar oxidative stress, and deiodinase activity, which are manifested in a strain- and sex-dependent manner.

Materials and Methods

Animals and Treatment

Timed-pregnant SHR or SD rat dams purchased from Charles River Breeding Laboratories (Germantown, NY, USA) on gestational day (G)7 (G1 defined as the first day after co-housing of males and females on which the female is found to have either a sperm plug or a sperm-positive vaginal smear) were individually housed under standard vivarium conditions (12:12 h light cycle, at 21–24°C). Standard laboratory chow and water were available ad libitum. Following a period of recovery from the stress of shipment, selected SHR dams (n=3) and SD dams (n=6) received TM (Sigma-Aldrich, St. Louis, MO, USA) at a dose of 200 μg/kg body weight (BW) via subcutaneous injections from G10 through G15, and then again from postnatal day (P)5 through P10; control SHR dams (n=3) and SD dams (n=3) received an equal volume of saline solution injections.

The neonates in TM-exposed and control groups were tested for neurodevelopment milestones and auditory and motor functions between birth and P20 and the cerebellar tissue derived from these animals on P21 was analyzed for oxidative stress by measuring 3-NT levels, D2 activity, and gene expression. Maternal weight was monitored daily from the onset of treatment. Neonates were counted, sexed, and weighed on P1/P2. Neonatal weight was monitored from birth until euthanasia on P21. All procedures were approved by the Institutional Animal Care and Use Committee at Harvard Medical School.

Neurodevelopmental Milestones

Neonatal neurodevelopmental milestones were assessed between birth and P21 (weaning) separately in male and female offspring derived from TM-exposed and control dams. Assessments included testing their righting response (rollover time), auditory (startle) response, and eye opening. Righting response was measured on P3–P5 as the time required for a rat pup to right itself when placed in a supine position. Onset of the startle response—a sign of acquiring hearing ability—was measured on P12–P14 in terms of head movement response to the sound of pen tapped against a glass surface. Eye opening was recorded between P12 and P14.

Motor Functions

Motor functions were measured using a rotarod. One/two males and one/two females per litter were chosen at random for the rotarod training test and these selected pups were followed over the period of 9 days, starting on P12 on a rotarod with an accelerating speed setting through P20 according to the procedure described earlier [18, 19]. Using this paradigm, motor learning was measured by increasing the speed of rotation and evaluating the same neonates over time; the pups tested on a rotarod from P12 through P20 represented “trained pups”. The “trained group” included 7

control SHR pups from three separate litters (3 males, 4 females), 34 control SD pups from three litters (18 males, 16 females), 6 TM-exposed SHR pups from three litters (3 males, 3 females), and 77 TM-exposed SD pups from six litters (41 males, 36 females). Each neonate was subjected to one trial on a rotarod rotating at incremental speeds in the range of 2–20 rpm during 5-min intervals. The length of time the animal remained on the rotarod and the rotational speed were recorded. If all animals were able to remain on the rotarod during a 5-min interval, the speed of rotation was increased. The cerebellar tissue derived from these pups was fixed and not used for subsequent analysis, as the training itself may alter parameters measured in this study such as brain levels of 3-NT.

Remaining pups from each exposure group were kept as rotarod-naïve until P20 and were then tested on a rotarod set at a maximum speed of 20 rev/5 min. The pups tested for the first time represented "untrained pups". The "untrained" group included 6 control SHR pups from three litters (3 males, 3 females), 34 control SD pups from three litters (12 males, 10 females), 6 TM-exposed SHR pups from three litters (3 males, 3 females) and 54 TM-exposed SD pups from six litters (30 males, 24 females).

Cerebellar Tissue

On P21 all pups were euthanized by decapitation. The cerebella, from the pups tested on the rotarod only on P20, including the cerebellum, were rapidly dissected, frozen on dry ice, and stored at −80°C for further analysis.

Analysis of Cerebellar 3-NT Levels

The 3-NT levels were measured in the cerebellar homogenates prepared from frozen tissue according to the procedure previously described [20]. Briefly, the individual cerebella were homogenized in a phosphate buffer containing detergents and protease inhibitors. The supernatants were collected by centrifugation at 16,000×*g* for 30 min at 4°C. 3-NT in the supernatants was measured using aliquots (equivalent to 25 mg tissue) derived from individual male and female cerebellar samples (n=3–6 litters); 3-NT was assayed with a commercially available 3-NT ELISA kit (Percipio Biosciences, Inc., Foster City, CA, USA). The ELISA plates were read at 450 nM. Data on 3-NT levels of individual cerebella were then pooled and the means expressed in picomoles per gram of tissue.

Analysis of Cerebellar D2 Activity

D2 activity was measured in the homogenates derived from individual male and female cerebellar samples by quantifying ^{125}I-release from a ^{125}I labeled T4 tracer (5,700 mCi/mg, Perkin Elmer Life Sciences, Boston, MA, USA) as described previously [21]. For the assays, 50 μg of protein were incubated for 4 h at 37°C with 1 nM T4 and 20 mM DTT. Background levels of deiodination were determined under identical conditions using 100 nM unlabeled T4. Data on D2 levels of individual cerebella were then pooled and expressed as means.

In Vitro Effect of Thimerosal Exposure in Mouse Embryonic Stem Cells

Cultures of Mouse Cortex E14 Neurospheres (StemCell Technologies, Vancouver, BC Canada) were grown in the NeuroCult NSC Proliferation Kit (StemCell Technologies) according to the manufacturer's directions. After two passages, on day 4 in culture, TM was added to a final concentration of 1×10^{-7} M (170 nM); on day 6 in culture, both the control and TM neurospheres were harvested, dissociated using the NeuroCult Chemical Dissociation Kit (StemCell Technologies), and counted.

Analysis of Cerebellar TH-dependent Gene Expression

Cerebellar mRNA was isolated from randomly selected male and female cerebella (n=4) per group using Trizol (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Quantitative real-time PCR was used to measure gene expression levels and was performed as described previously [19]; SuperScript VILO (Invitrogen, Carlsbad CA, USA) was used for cDNA synthesis following the manufacturer's instructions. Sequences of primers used are: rat Cyclophilin A 5′-AGCACTGGGGAGAAAGGATT-3′ and 5′-AGCCACTCAGTCTTGGCAGT-3′; rat Cirpb 5′-TCAGCTTCGACACCAATGAG-3′ and 5′-GTATCCTCGGGACCGGTTAT-3′; rat Odf4 5′-TTTTCCTCACCCTCCTGTTG and 5′-TGCAAGTAGCGTTGATGGAG-3′. PCR gene expression data was analyzed, corrected for cyclophilin expression, and then pooled means were reported.

Statistical Analysis

The data presented here is derived from three to six litters per treatment group; both the number of litters and the number of male and female pups per groups is presented in the context of each analysis. When applicable, a two-way ANOVA was run with main effect of treatment and sex, and the interaction between treatment and sex. If a statistically significant interaction was found, test of treatment within each sex was carried out. Because of the small sample size, power was too low for normality test. These comparisons were thus repeated using Wilcoxon rank sum test and the results were nearly identical. All values are reported as a

mean ± the standard error of the mean. For all statistical tests, $p<0.05$ was considered significant.

Results

Effect of TM Exposure During Pregnancy on Gain in Maternal Body Weight

For perinatal exposure, pregnant and lactating dams received 12 subcutaneous injections (Fig. 1) for a cumulative dose of 120 μg/dam. When given to a human infant of an approximate weight of 5 kg as a 0.5 ml vaccine three different times, the cumulative dose is 15 μg. While the dosage of TM per unit of body mass in the present study was about 10 times higher than that used in humans, the lethal dose of Met-Hg at which fetal reabsorption could be detected in rats was 8 mg/kg [22, 23]. There were no overt signs of TM toxicity in SHR dams exposed during pregnancy (G10–G15). The relative gain in maternal body mass did not differ significantly between the TM exposed and control dams of either rat strain (data not shown).

Perinatal TM Exposure and Neonatal Weight Gain

SHR pups were much smaller than SD pups at birth (Table 1), and in general we observed a much greater mortality in TM-exposed SHR neonates (24%) than in TM-exposed SD neonates (4.3%); in SHR neonates attrition occurred during the first 3 days after birth, while in SD pups it was mostly accounted by still births or deaths within first few hours after birth As shown in Table 1, on P2, the mass of TM-exposed SHR neonates was unexplainably significantly increased in both male (16.4% increase) and in female pups (11.9% increase; $p<0.01$). The growth rate of SHR pups was significantly suppressed by TM exposure both in male and female pups and by P20 the weight of TM-exposed neonates was slightly lower in both male and female pups.

In SD neonates, neonatal weight on P2 was not affected by TM exposure in either males or female neonates. Furthermore, the growth rate of TM-exposed SD pups was not affected and there was no difference in weight between the TM exposed and control male or female pups at P21.

TM Exposure Does Not Affect Cerebellar Mass

While SHR pups were much smaller both at birth and on P21 (Table 1), cerebellar mass of SHR neonates at P21 was comparable to SD cerebellar mass. It appeared to be increased in TM-exposed neonates, but the increase was not statistically significant. Cerebellar mass of perinatally TM-exposed SD neonates assessed on P21 was not different (Table 1).

Effect of TM Exposure on Rollover Time

Unexplainably, the rollover time on P4 was shorter in SHR TM-exposed male pups (59%; $p<0.05$); females also showed a tendency to decrease (13%) but this effect was not significant (Table 2). Notably, this also correlates with a higher cerebellar and body mass at birth (Table 1) in TM-exposed SHR pups. The rollover time was not affected by the TM exposure in SD male or female pups.

The Effect of TM Exposure on the Onset of the Auditory Response

The maturation of the auditory functions was tested by startle response (Table 2). In the SHR pups exposed to TM, the startle response measured on P14 showed a small suppression in males 12.2% but not in female pups. However, in the SD strain, the percent of male pups showing a startle response was significantly reduced by 27.8% and in female pups by 19.2%.

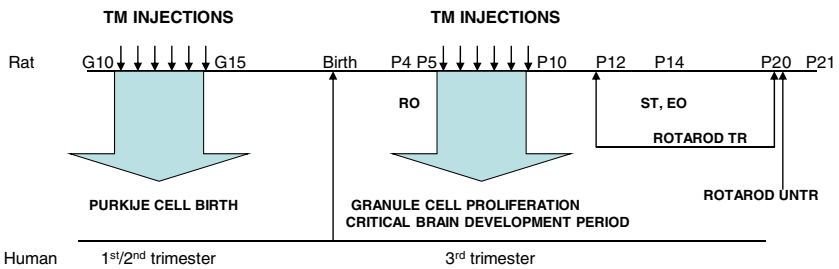

Fig. 1 Schematic representation of thimerosal (TM) exposure and neurobehavioral testing. Timed pregnant spontaneously hypertensive rats (SHR) or Sprague–Dawley (SD) received TM at a dose of 200 μg/kg body weight via subcutaneous injections during G10–G15 and P5–P10; control SHR and SD dams received an equal volume of saline. Rollover time (RO) was measured on P3–P5, startle response (ST), and eye opening were observed on P12–P14. Male and female pups were tested on a rotarod (RT) either on P20 to measure spontaneous motor function in untrained group (ROTAROD UNTR) or between P12 and P20 to assess the learned motor function in trained pups (ROTAROD TR). Animals were euthanized on P21 and cerebella dissected out for biochemical analysis.

Table 1 Effect of perinatal TM exposure on neonatal growth and cerebellar weight

Strain	Sex	Treatment group (*n*)	Neonatal mass on P2 (g)	Neonatal mass on P21 (g)	Relative growth (P21–P2)/P2 (%)	Cerebellar mass on P21 (mg)
SHR	Male	C (3)	5.35±0.05	27.60±1.20	4.16±0.27	161.13±21.38
		TM (3)	6.23±0.19 **	26.70±1.30	3.28±0.14*	173.64±3.39
	Female	C (3)	5.30±0.10	27.00±0.70	4.10±0.23	153.75±9.75
		TM (3)	5.93±0.2**	26.60±2.4	3.48±0.19*	173.06±1.47
SD	Male	C (3)	10.07±0.38	49.83±1.18	3.96±0.0.08	171.11±6.57
		TM (7)	10.04±0.24	51.73±1.87	4.15±0.15	173.46±2.96
	Female	C (3)	9.57±0.26	48.37±0.67	4.06±0.15	165.83±10.25
		TM (7)	9.46±0.28	48.50±1.92	4.22±0.08	161.67±3.53

The neonatal mass on P2 was increased in TM-exposed SHR males and females, but the growth rate was actually decreased. Neonatal mass was not affected by TM exposure in SD neonates. Cerebellar mass was not significantly affected by TM exposure in either strain. In the analysis of neonatal and P21 mass and the cerebellar weight, litter was used as a unit of observations; the numbers presented here are averages across litters

C controls, *TM* thimerosal-exposed, *l* number of litters, *n* number of neonates

*$p<0.05$; **$p<0.01$

TM Exposure Does Not Affect Eye Opening

Eye opening in TM-exposed SHR males showed a tendency to be delayed, while no effect was observed in TM-exposed SHR females. No effect of TM exposure on eye opening was observed in the SD strain (Table 2).

TM Exposure Impairs Motor Learning

Effect of TM Exposure on Spontaneous Motor Function on P20

To assess the spontaneous motor behavior, a subset of rotarod "naïve" rats was tested on P20 on a rotarod set to a maximum setting of 20 rpm/5 min; this subset is referred to as "untrained pups" (Fig. 2). TM exposure did not affect falling latency in untrained SHR male (Fig. 2a) or female pups (Fig. 2b). Similarly, perinatal TM exposure did not affect falling latency in untrained SD male (Fig. 2c) or SD female pups (Fig. 2d).

Effect of TM Exposure on Motor Learning

To assess the effect of TM on motor learning, selected male and female neonates from each litter were tested on a rotarod daily commencing on P12 and continuing until P20; this subset is referred to as "trained pups" (Fig. 2).

Overall, trained SHR pups had poorer performance on a rotarod, and their falling latency was shorter than that observed in SD pups (Fig. 2). TM-exposed SHR male pups

Table 2 Effect of perinatal TM exposure on neurodevelopmental milestones

Strain	Sex	Treatment group (*n*)	Rollover time (s)	Startle response (% responders)	Eye opening (% of pups)
SHR	Male	C (3)	14.2±7.0	73.3±6.7	100.0±0
		TM (3)	6.4±1.5 *	64.3±18.0	83.3±16.7
	Female	C (3)	14.6±2.9	87.5±12.5	100.0±0
		TM (*n*=3)	12.7±2.9	91.7±8.3	100.0±0
	Male	C (*n*=3)	7.1±2.8	74.1±25.9	66.7±33.3
SD		TM (7)	6.9±2.5	53.5±7.8 *	78.2±10.6
	Female	C (3)	11.4±2.0	90.5±9.5	85.7±14.3
		TM (7)	13.5±2.2	73.1±9.1 *	83.9±8.1

The rollover time on P4 was shorter in SHR TM exposed male, but not female neonates; it was not affected in SD neonates. TM exposure did not affect the number of SHR neonates of either sex responding to auditory stimulus; but it reduced the number of responding SD males and females. Eye opening was delayed in SHR rats by ~3 days. On P17, TM exposed SHR male, but not female neonates, showed a tendency towards delayed eye opening on. No effect of TM exposure on eye opening was observed in SD pups on P14. In the analysis of rollover, startle response and eye opening, litter was used as a unit of observations; the numbers presented here are averages across litters

C controls, *TM* thimerosal-exposed, *n* number of litters

*$p<0.05$

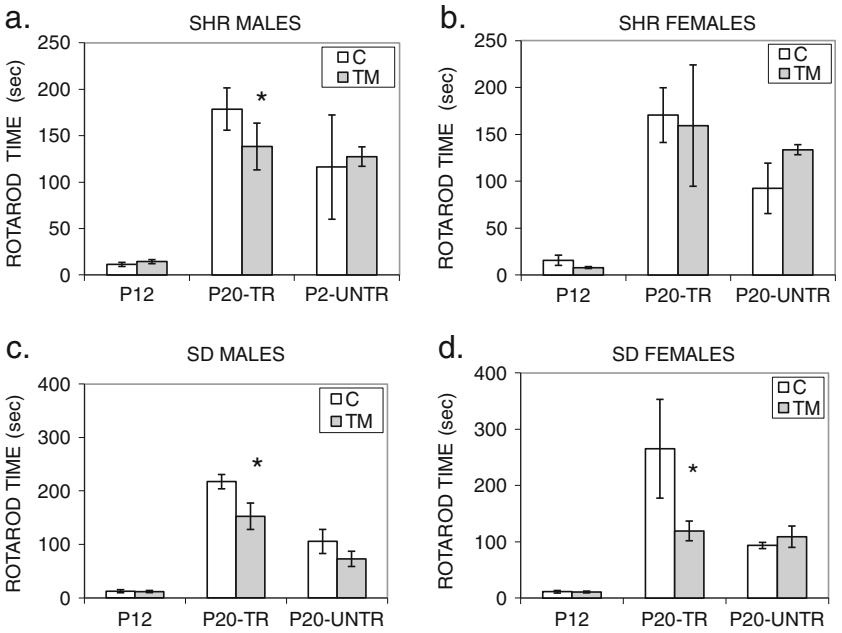

Fig. 2 TM exposure impairs motor learning. Motor performance is presented as falling latency on the rotarod apparatus (seconds). To assess the spontaneous motor behavior a rotarod naïve "untrained pups" (P20-UNTR) were tested on P20 on a rotarod set to a maximum setting of 20 rpm/5 min. For this analysis, litter was used as a unit of observation; data is presented as means±SEM. The untrained pups are represented by: **a** *C* n=3, *TM* n=3; **b** *C* n=3, *TM* n=3; **c** *C* n=3, *TM* n=6; and **d** *C* n=3, *TM* n=6. Perinatal TM exposure did not affect falling latency in SHR males (Fig. 1a) or female pups (Fig. 1b). Similarly, perinatal TM exposure did not affect falling latency in SD male (Fig. 1c) or SD female pups (Fig. 1d). To assess the effect of TM on motor learning, selected male and female neonates from each litter were tested on a rotarod daily commencing on P12 neonates and continued until P20 (P20-TR). The trained pups are represented by: **a** *C* three litters, three males; *TM* three litters, three males; **b** *C* three litters, four females; *TM* three litters, three females; **c** *C* three litters, 18 males; *TM* six litters, 41 males; **d** *C* three litters, 16 females; *TM* six litters, 36 females. The mean falling latency in SHR TM-exposed male pups decreased by 22.6% (Fig. 1a; *p<0.05), and in SHR TM-exposed female pups it decreased by 6.6% (Fig. 1b). The mean falling latency in TM-exposed SD male pups was decreased by 29.8% (Fig. 1c; *p<0.05), and in TM-exposed SD female pups it was decreased by 55% (Fig. 1d; *p<0.05)

falling latency was 77.4% of that observed in control male pups (p<0.05; Fig. 2a), while in TM-exposed female pups there was no difference from the control group (Fig. 2b).

Perinatal TM exposure induced motor impairment in SD pups of both sexes. The mean falling latency in TM-exposed male pups was 70.2% of that observed in control male pups (p<0.05; Fig. 2c), and in TM-exposed female pups it was 45.0% of that observed in control female pups (p<0.05; Fig. 2d).

TM Exposure Results in Increased Cerebellar Levels of Oxidative Stress Marker, 3-NT

The levels of the oxidative stress marker 3-NT were quantified in cerebellar tissue obtained from TM-exposed male and female SHR and SD rat pups on P21 (Fig. 3). The levels of 3NT were increased in both male (6.2%; Fig. 3a) and in female SHR neonates (35.1%; p<0.05; Fig. 3b) exposed to TM. Exposure to TM in SD rats resulted in 14.0% increase in 3-NT in male neonates (p<0.05; Fig. 3c) but there was no significant change in the female neonates (Fig. 3d).

TM Exposure Results in Decreased Cerebellar D2 Activity

The levels of D2 activity were quantified in cerebellar tissue from control and TM-exposed P21 male and female SHR and SD rat neonates (Fig. 4). Perinatal exposure to TM resulted in a very significant 60.9% decrease in cerebellar D2 activity in male (p<0.01; Fig. 4a), but not in female SHR neonates (Fig. 4b). TM exposure also tended to decrease D2 activity in male and female SD neonates (Fig. 4c, d).

TM Exposure Disrupts TH-dependent Gene Expression

The decrease in D2 activity observed in SHR male neonates suggests that there may be less local T4 to T3 conversion in the cerebellum of these animals resulting in a decreased T3 content within this tissue (Fig. 4a). To assess if this affects downstream T3-regulated gene expression, we determined the mRNA levels of two genes negatively regulated by T3 that have been previously shown to be upregulated in D2KO mice [24]. Expression of Cirbp remained unchanged in TM-exposed SHR males and females (Fig. 5a, b). Notably, Odf4

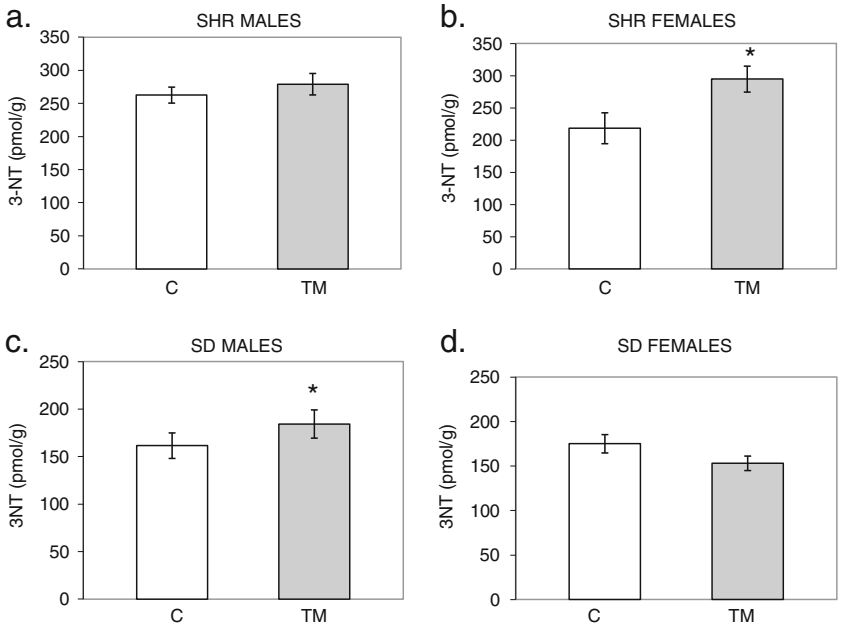

Fig. 3 TM exposure results in increased cerebellar 3-NT levels. Cerebellar 3-NT levels were presented as picomoles per gram tissue. **a** *C* Three litters, six males; *TM* three litters, 14 males. **b** *C* Three litters, seven females; *TM* three litters, seven females. **c** *C* three litters, seven males; *TM* six litters, 16 males. **d** *C* Three litters, six females; *TM* three litters, 15 females. Data are presented as mean ±SEM. The levels of 3NT were increased in both SHR male (**a**, 6.2%) and SHR female neonates (**b**, *$p<0.05$) exposed to TM. Exposure to TM in SD rats resulted in an increase in 3-NT in male neonates (**c**, *$p<0.05$), but there was no significant change in the female neonates (Fig. 2d)

expression was increased by 47.7 % in TM-exposed males ($p<0.05$; Fig. 5a) consistent with the decreased D2 activity found in this group that likely resulted in lower T3 levels within the cerebellum of these animals.

TM Induces Cell Apoptosis in Embryonic Stem Cells In Vitro

The effect of in vitro TM exposure on mouse embryonic stem cells is shown in Fig. 6. Addition of TM to a final concentration of 1.7×10^{-7} M, similar to one used in primary rat cerebellar [25] and human blood mononuclear cells in cultures [26], resulted in a 65.5 % reduction ($p<0.05$; Fig. 6) on a number of viable cells within 48 h. The number of viable cells in TM cultures fell below cell number prior to TM addition, indicating the observed reduction in cell number was due to decreased survival of neuronal cells.

Limitations of the Study

The experimental design of the present study incorporates attempts to model both the perinatal Et-Hg/TM exposure

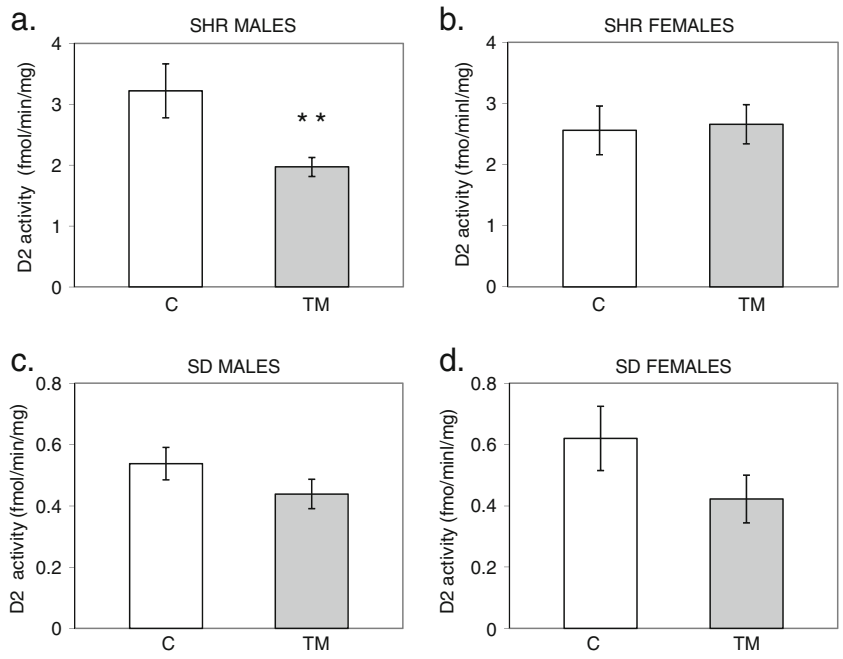

Fig. 4 TM exposure results in decreased cerebellar D2 activity. Cerebellar D2 activity is presented in femtomole per minute per milligram of tissue. **a** *C* three litters, five males; *TM* three litters, 14 males. **b** *C* Three litters, eight females; *TM* three litters, seven females. **c** *C* three litters, four males; *TM* six litters, eight males. **d** *C* Three litters, four females; *TM* six litters, five females. Data are expressed as mean±SEM. Perinatal exposure to TM resulted in decreased cerebellar levels of D2 in SHR male (Fig. 3a; **$p<0.01$) but not in SHR female neonates (Fig. **3**b). On the other hand, D2 was decreased both in TM-exposed SD males (Fig. 3c) and SD female neonates (Fig. 3d)

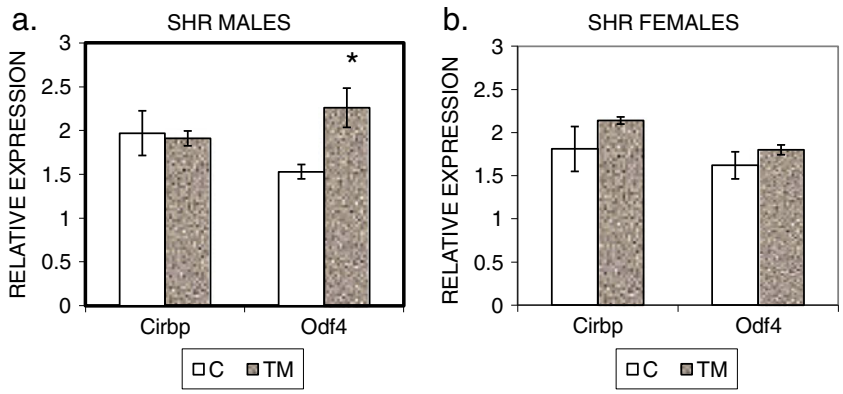

Fig. 5 TM exposure disrupts TH-dependent gene expression. Cerebellar gene expression was measured by quantitative RT-PCR and was normalized to cyclophilin A expression. **a** *C* Cirbp, three litters, four males; *C Odf4*, three litters, four males; *TM Cirbp* three litters four males; *TM Odf4* three litters, four males. **b** *C Cirbp* three litters, four females; *C Odf4* three litters, four females; *TM Cirbp* three litters four females; *TM Odf4* three litters, four females. Data are presented as a relative gene expression (mean±SEM). An increase in Cirbp expression was observed in TM-exposed SHR females (**b**) and an increase in Odf4 expression was observed in TM-exposed SHR male cerebella (**a**, $*p<0.05$)

and exposure to TM-containing vaccines administered during pregnancy on the developing brain. Somehow unorthodox schedule of repeated TM injections attempts to model a scenario of maternal exposure to flu and several other TM-containing vaccines such as hepatitis B, pneumococcal, meningococcal, and rabies vaccines recommended to high-risk mothers. While the present paradigm may exaggerate human exposure, it forms basis for future in-depth studies.

By including two strains of rats, SD and SHR, with differential sensitivity to environmental triggers of oxidative stress [33–35], we have addressed the individual genetic susceptibility to environmental toxicants, which may ultimately determine the consequences of the exposure to TM. The limited number of SHR dams (due to the financial constrains) as compared to SD dams, could potentially influence the reported outcomes and limit the interpretation of the study.

Another factor that could influence the outcomes of our study is the decision not to cull litters. Litter culling is a controversial practice which offers both advantages and disadvantages. Culling of the rodent litters has been recommended by some [66] to reduce variability in the growth and development of pups. On the other hand, others [67] are highly critical of culling claiming lack of evidence for clear advantage of the process that eliminates 30–45% of pups and risks missing the effects of neurotoxicity, such as pups mortality and malformations. Indeed, in the case of SHR pups the attrition could be observed during the first 3 days.

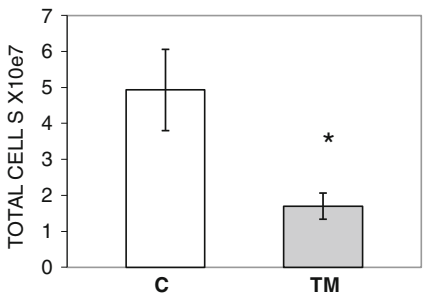

Fig. 6 TM induces cell apoptosis in embryonic stem cells in vitro. Mouse embryonic stem cells are presented as total number of cells per well; data are expressed as mean±SEM. Exposure to TM (1.7×10^{-7} M) resulted in a significant reduction ($*p<0.05$) in the number of viable cells within 48 h

Discussion

Epidemiological surveys have shown that the industrial release of mercury into Minamata Bay in Japan and consumption of Met-Hg contaminated fish resulted in a distinct pathology recognized as Minamata disease affecting neonates and children with the brain being a primary target organ [27]. Met-Hg exposure in expecting mothers due to fish consumption is associated with increased mercury accumulation in the infant brains accompanied by behavioral abnormalities, which include deficits in motor, attention, and verbal performance that are more pronounced in males [28], while the postnatal Met-Hg exposure in humans appears to have no recognizable effects [29].

Hg is transferred through the placenta, with the concentration in fetal blood cells being 30% higher than in maternal blood cells [30]. Studies in mice have shown that gestational exposure (G8–G18) through a diet contaminated with Met-Hg (100 μg/kg) resulted in a deficit in motor behavior and coordination [31]. Hg also enters the milk [32], is taken up by the suckling pups [6], and accumulates in their brains [4, 33]. In rats, postnatal

exposure (P1–P30) resulted in impairments in motor coordination and learning [34]. It is important to point out that our exposure paradigm overlaps with the critical period of cerebellar development in rats (P5–P10; Fig. 1).

While many toxicological studies have documented the developmental neurotoxicity of Met-Hg, few studies have explored the impact of Et-Hg/TM on the developing central nervous system (CNS). The immediate objective of the present study was to assess the neurodevelopmental consequences of perinatal Et-Hg/TM exposure, while the ultimate goal has been to gain insight into the potential impact of TM-containing vaccines administered during pregnancy on the developing human brain. Thus, the experimental design of the present study incorporates attempts to model several aspects relevant to both the short and the long term objectives. The paradigm of repeated TM injections attempts to model administration of flu and other thimerosal-containing vaccines such as hepatitis B, pneumococcal, meningococcal, and rabies vaccines recommended to the high-risk mothers. Thus, while the multiple TM administrations may exaggerate human exposure, they form the basis for future more in-depth studies relevant to a single flu vaccine during pregnancy. This study also addresses the issue of the individual genetic susceptibility to environmental toxicants, by including two strains of rats, SD and SHR, with differential sensitivity to environmental triggers of oxidative stress [35–37]. Furthermore, the study addresses sex-dependent nature of environmental impacts on the developing CNS by including a separate analysis of TM effects in male and in female neonates. This study also attempts to link the environmental exposure to TM during G10–G15 and P5–P10 not only to specific periods of human pregnancy, i.e., the beginning of the second trimester and the third trimester, respectively, but also to critical developmental events, i.e., Purkinje cell birth and granule cell migration.

Our results indicate that perinatal TM exposure delays auditory maturation [38] and impairs motor learning [34], consistent with previous results observed in rodents exposed to Met-Hg. Our data show that the percentage of both male and female SD neonates displaying a startle response at 14 days with TM exposure was significantly decreased, indicating a deficit in auditory development. We further found that spontaneous motor coordination in neonates of either sex or strain measured on P20 was not affected by perinatal TM exposure. However, notably, TM exposure does appear to affect learned motor function. In the SHR pups, which appear to be less coordinated over all, learned motor function was impaired in male pups. On the other hand, the learned motor coordination was impaired in SD rats of both sexes, with a larger effect in female neonates. While comparative studies on the effects of TM are lacking, our results are in agreement with the previously published studies of the Met-Hg effect on learning [22, 31, 34].

Impaired cerebellar development due to Hg exposure has been suggested by several studies. Combined gestational and neonatal Met-Hg exposure in rats results in increased height of Purkinje cells [39], while postnatal exposure is associated with neuronal degeneration and astrocytosis in the cortex, striatum, and the cerebellum [34]. In neonatal cerebellar neurons in culture, both Met-Hg and TM increase intracellular calcium concentration and exert a cytotoxic effect on granule cells [40]. Our in vitro results involving embryonic neuronal stem cells indicate that the immature neurons are very sensitive to TM, with exposure resulting in decreased cell proliferation/survival (Fig. 6).

Like many other environmental toxicants, Hg accumulated in the developing brain following either in vivo Met-Hg exposure [41] or in vitro exposure to Met-Hg or Et-Hg [41–43], induces oxidative stress that leads to a cascade of other changes including decreased neurogenesis, increased neuronal apoptosis and impaired synaptic plasticity in the neonatal brain. Further, gestational exposure to Met-Hg in mice results in increased lipid peroxidation via interference brain GSH levels [13], while gestational exposure (G12–G14) in rats to Met-Hg (5 mg/kg) induces oxidative stress and reduces the antioxidant enzyme superoxide dismutase in the hippocampus [44]. However, a direct quantification of oxidative stress is impractical because free radicals including reactive oxygen species are short-lived. An alternative approach is to monitor stable end-products of oxidative stress damage. 3-NT is a well-accepted marker of oxidative stress found in over 50 different pathologies [45] including Alzheimer's and Parkinson's diseases [46, 47] and autism [20]. Results of our present study indicate that perinatal TM exposure also increases cerebellar oxidative stress, as assessed by 3-NT measurement. The effect is more pronounced in SHR pups, with the increase observed in both male and female neonates, but significant only in female pups. In SD neonates, increased 3-NT levels were only observed in males.

We also found that TM exposure significantly decreased D2 activity in the cerebellar tissue of SHR male neonates, and also showed a tendency to decreased D2 activity in male and female SD neonates. The D2 enzyme catalyzes the activation of the pro-hormone T4 to the biologically active T3, and plays a key role in the local control of thyroid hormone levels within a tissue [48]. Further, it has been estimated that greater than 80% of the T3 found within the brain comes from local T4 to T3 conversion by D2 [17], and mice with a global targeted disruption of the *Dio2* gene [D2KO mice] have ~50% less T3 content in their cerebral cortex, cerebellum, and hypothalamus [49]. Thus, a decrease in D2 activity within the cerebella of TM-exposed SHR male neonates potentially could result in local cerebellar "hypothyroidism". Such local decrease in

TH adds yet another possible mechanism of action to the mercury compounds already qualified as endocrine disruptors [2, 3, 9, 10].

Furthermore, D2KO mice have been found to have significantly increased expression of numerous genes that are negatively regulated by T3 [24]. Consistent with this, we find that expression of the one of the T3-responsive marker genes found to be altered in D2KO mice, Odf4, is also increased in TM-treated SHR male neonates, representing the first evidence of altered TH-dependent gene expression following TM exposure. This suggests that Et-Hg may affect local brain T3 levels that are critical for normal neurodevelopmental processes, in turn impacting cerebellar development and impairing cerebellum-dependent auditory and motor functions. Indeed, our data on auditory and motor functions support the hypothesis of T3 deficiency in TM-exposed rat neonates. Interestingly, impairment of auditory and motor functions is also observed in propylthiouracil-induced hypothyroid conditions in rats [50], and D2KO mice have mildly reduced motor function and learning deficits in some, but not all, tests [49]. Auditory impairment can be observed in D2 knockout mice, however this is due to defects in cochlear development [51].

While the mechanism by which D2 activity is decreased in TM-treated neonates is unclear, Met-Hg also interacts with selenium [52] and can inhibit function of selenoproteins such as the deiodinases [53]. We have also shown that TM exposure increases levels of oxidative stress, which has been found previously to decrease expression of the *Dio2* gene [54]. Lastly, exposure of neuronal cells to Met-Hg [55] or neuroblastoma cells to TM [56] results in a depletion of GSH which is both an antioxidant and a cofactor of deiodinases [57–59], thus cerebellar D2 activity might be impaired due to a lack of reducing co-factor. Additionally, T3 regulates GSH levels in the developing brain and treatment of astrocyte cultures with T3 results in increased GSH levels and improved antioxidative defense, suggesting that TH plays a positive role in maintaining GSH homeostasis and protecting the brain from oxidative stress [60]. Thus, is it is also possible that a decrease in D2 activity could further amplify the effects of oxidative stress.

Our behavioral and biochemical data indicate that the effects of perinatal TM exposures are sex-dependent. These findings are at least in part in agreement with earlier observations both in humans [28] and in animals [61] showing that the developing males appear to be more sensitive to Hg exposure. The present study indicates that learned motor coordination is impaired more significantly both in SD and SHR males, but is unaffected in SHR females. Our results also indicate that perinatal TM exposure induces an increase in cerebellar oxidative stress in SD males but not females; however in SHR rats, the increase is greater in females. Perinatal exposure to TM resulted in decreased cerebellar D2 activity in male, but not in female SHR neonates, and this decrease was correlated with a disruption of T3-dependent gene expression in SHR males.

In addition to the sex-dependent effect of TM exposure on the developing CNS, the effect is also distinct in different rat strains. Other studies reported strain differences in Hg effect on sensitivity to pain [62] and renal HG toxicity [63]. This difference may be attributed to genetically dependent susceptibility to environmental toxicants including Hg, although the mechanisms involved are poorly understood [64]. Our results show higher levels of the brain oxidative stress marker 3-NT in SHR than in SD rats, which is consistent with previous reports [35–37], that may be due, at least in part, to a different degree of activation of inflammatory processes between the two strains [65].

Conclusions

Our data indicate that maternal TM exposure results in a delayed auditory maturation and impaired motor learning in rat pups. Factors that may contribute to these abnormalities include increased cerebellar oxidative stress and decreased D2 activity resulting local intracerebellar T3 deficiency and altered TH-dependent gene expression. Indeed, provided here is the first evidence of altered TH-dependent gene expression following TM exposure. Our data thus demonstrate a negative neurodevelopmental impact of perinatal TM exposure, which appears to be both strain- and sex-dependent. Although, additional studies are needed, data derived from TM exposure in rats may provide clues relevant to understanding neurodevelopmental consequences of TM exposure in humans.

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