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SEX-DEPENDENT CHANGES IN CEREBELLAR THYROID HORMONE-DEPENDENT GENE EXPRESSION FOLLOWING PERINATAL EXPOSURE TO THIMEROSAL IN RATS

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Mammalian brain development is regulated by the action of thyroid hormone (TH) on target genes. We have previously shown that the perinatal exposure to thimerosal (TM, metabolized to ethylmercury) exerts neurotoxic effects on the developing cerebellum and is associated with a decrease in cerebellar D2 activity, which could result in local brain T3 deficiency. We have also begun to examine TM effect on gene expression. The objective of this study was to expand on our initial observation of altered cerebellar gene expression following perinatal TM exposure and to examine additional genes that include both TH-dependent as well as other genes critical for cerebellar development in male and female neonates exposed perinatally (G10-G15 and P5 to P10) to TM. We report here for the first time that expression of suppressor-of-white-apricot-1 (SWAP-1), a gene negatively regulated by T3, was increased in TM-exposed males (61.1% increase), but not in females; ($p<0.05$). Positively regulated T3-target genes, Purkinje cell protein 2 (Pcp2; $p=0.07$) and Forkhead box protein P4 (FoxP4; $p=0.08$), showed a trend towards decreased expression in TM-exposed males. The expression of deiodinase 2 (DIO2) showed a trend towards an increase in TM-exposed females, while deiodinase 3 (DIO3), transthyretin (TTR), brain derived neurotrophic factor (BDNF) and reelin (RELN) was not significantly altered in either sex. Since regulation of gene splicing is vital to neuronal proliferation and differentiation, altered expression of SWAP-1 may exert wide ranging effects on multiple genes involved in the regulation of cerebellar development. We have previously identified activation of another TH-dependent gene, outer dense fiber of sperm tails 4, in the TM exposed male pups. Together, these results also show sex-dependent differences between the toxic impacts of TM in males and females. Interestingly, the genes that were activated by TM are negatively regulated by TH, supporting our hypothesis of local brain hypothyroidism being induced by TM and suggesting a novel mechanism of action TM in the developing brain.

Key words: *cerebellum, suppressor-of-white-apricot-1 (SWAP-1), thimerosal, thyroid hormone, brain derived neurotrophic factor, reelin*

INTRODUCTION

Thyroid hormone (TH) is critical for brain development; its deficiency during the perinatal period is associated with abnormalities in brain structure and function (1). Many factors, both genetic and environmental may contribute to TH deficiency. Of interest to this study is the contribution of TH-disrupting effect of mercury, and specifically thimerosal (TM - an ethyl mercury-containing preservative included in some vaccines administered to mothers and infants), on TH status. Surprisingly, no data on plasma TH levels following TM exposure have been reported and very few studies have explored the effect of methyl mercury (MetHg) on TH plasma levels. Studies in mice have shown that although the levels of TH in maternal and fetal plasma were not affected by short gestational exposure to MetHg, fetal brain deiodinase type 2 (D2) activity was increased (2). On the other hand, MetHg inhibited D2 activity both in neuroblastoma (3) and rat pituitary tumor cells *in vitro* (4). We have recently reported a decrease in cerebellar D2 activity following the perinatal TM exposure in SHR rats (5). Importantly, a majority of the active TH hormone in the brain is due to the activity of D2, a selenoenzyme that converts the pro-hormone thyroxine (T4) to the active hormone, 3',3,5-triiodothyronine (T3) (6); a relatively small proportion of brain T3 is transported from the plasma. Thus, it is possible that brain TH levels are altered by TM exposure, while plasma levels remain unchanged. Interestingly, T3 produced by D2 in the brain and T3 derived from the plasma are involved in the regulation of distinct gene subpopulations (7). Specifically, a deficiency of D2 results in the up-regulation of genes negatively regulated by TH (7). Thus, a decrease in D2 activity is likely to result in local hypothyroidism within the brain, and contribute to both TM and MetHg neurotoxicity through altered expression of specific subpopulation of TH-dependent genes negatively regulated by T3.

In the present study we examined this hypothesis, by assessing the effect of TM exposure on both positively and negatively regulated TH-dependent gene expression in the cerebellum. While recently (5), we reported on the effect of TM on two cerebellar genes - the Odf4 gene which was activated in males, and the cold inducible RNA binding protein (Cirbp) gene that was not affected by TM exposure (5) - present report includes data on nine additional genes that include both TH-dependent genes as well as other genes critical for cerebellar development. We report here for the first time up-regulation of a gene negatively regulated by T3

Table 1. Primers for qRT PCR.

Gene name	Gene Bank Accession No.	Primers:
SWAP-1	NM_001034924.1	F: GAGGAGCTCGAAGCTAAGCA R: TGCATCTGGAAGAGGGTTTT
FOXP4	NM_001108788.1	F: TGCCTCCATAGGACCAAGTC R: CTGGTCAGGGTGTTCTAGGC
Pcp2	NM_001107116.1	F: CATGGATGACCAGCGTGTAA R: GGTTGAGGGCTGAGTGTCC
DIO2	NM_031720.3	F: GATGCTCCCAATTCCAGTGT R: TGAACCAAAGTTGACCACCA
DIO3	NM_017210.3	F: CTGTGCTCTGGTTCTGGACA R: CGCAACTCAGACACCTGGTA
BDNF-1	EF125680.1	F: GCGGCAGATAAAAAGACTGC R: GCAGCCTTCCTTCGTGTAAC
RELN	NM_080394.2	F: GCCAACTGGTGGACACTTTT R: AAGGTCACCACAGGAAGTGG
NCAM1	NM_031521.1	F: AGAGCATCGTGAATGCCACT R: CCATCCTTTGTCCAGCTCAT
L1CAM	NM_017345.1	F: ATTTGGCAAGCCAGATTTTG R: CTTCTGGCAAGGCTTTGAAC
TTR	NM_012681.2	F: TCGTCAGTAACCCCCAGAAC R: CCGAGTTGCTAACACGGTTT
CycloA	IQ222826.1	F: AGCACTGGGGAGAAAGGATT R: AGCCACTCAGTCTTGGCAGT

and involved in intron/exon splicing, suppressor-of-white-apricot-1 gene (SWAP-1)(15). Since gene splicing is vital to neuronal proliferation and differentiation, altered expression of this gene may exert wide ranging effects on multiple genes involved in the regulation of cerebellar development. The results also show sex-dependent differences between the toxic impacts of TM in males and females. In addition two other genes showed a trend towards inhibition in TM-exposed males, and one more gene showed a trend towards an increase in TM-exposed female neonates. Interestingly, the genes that were activated by TM are negatively regulated by TH, supporting our hypothesis of local brain hypothyroidism being induced by TM.

MATERIALS AND METHODS

Animals and treatment

Timed-pregnant spontaneously hypertensive rats (SHR) rat dams, purchased from Charles River Breeding Laboratories (Germantown, NY) on gestational day (G) 5 (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) received thimerosal (TM; Sigma-Aldrich, St Louis, MO) 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) received an equal volume of saline solution injections. During the gestational exposure, the average dam's mass was 182 g on G7 and increased to 223 g on G15; during postpartum exposure, dams mass was approximately 200 g. The dose of TM given to dams was an order of magnitude higher than the dose administered to mouse pups (8), primate infants (9), or the dose given in vaccines to human infants (8, 10). The dose of 200 µg/kg was selected to compensate for the placental transfer during pregnancy and the plasma-milk transfer of TM during nursing (5). Furthermore, while the TM content in infant vaccines is ~0.3 µg/dose, the content of TM in older children and adults reaches 25 µg/dose (11). Others, assessing tissue distribution of mercury have used a dose of 320 µg/kg (12), while the lethal dose Met-Hg in rats is 8 mg/kg (13). Most importantly, no overt signs of TM toxicity were observed in dams exposed during pregnancy (G10-G15). The relative gain in maternal body mass did not differ significantly between the TM exposed and control dams, and TM pups were slightly heavier.

On P21 all pups were euthanized by decapitation. The cerebellar tissue derived from these animals was rapidly dissected, frozen on dry ice, and stored at –80°C for further analysis of D2 activity, and gene expression. All procedures were approved by the Institutional Animal Care and Use Committee at Harvard Medical School.

Analysis of cerebellar gene expression

Cerebellar mRNA was isolated using Trizol (InVitrogen, Carlsbad CA) following the manufacturer's instructions. Quantitative real-time PCR (qRT PCR) was used to measure gene expression levels and was performed as described previously (14) with the following modifications: SuperScript VILO (InVitrogen, Carlsbad CA) was used for cDNA synthesis following the manufacturer's instructions. Using qRT PCR we have analyzed the expression of the following TH-dependent genes: suppressor-of-white-apricot-1 (SWAP-1; 15), Purkinje cell protein 2 (Pcp2), type 2 deiodinase 2 (DIO2), type 3 deiodinase 3 (DIO3). Forkhead box protein 4 (FoxP4), brain derived neurotrophic factor 1 (BDNF-1), reelin (RELN), neuronal cell adhesion molecule 1 (NCAM1); L1 cell adhesion molecule (L1CAM) and transthyretin (TTR); cyclophilin A

(CycloA) gene was used as a housekeeper gene for normalization. The primers for each of the genes are presented in *Table 1*.

Statistical analysis

The data presented here is derived from at least three litters per treatment group; the D2 and the PCR analyses were done separately using cerebellar tissue from the male and the female. When applicable, a two-way ANOVA was run to determine the relationship between treatment and sex. If a statistically significant interaction was found, a two-sample t-test was carried out. All values are reported as a MEAN ± the standard error of the mean (S.E.M). For all statistical tests, $p<0.05$ level of confidence was considered significant.

RESULTS

Recently we have reported a decrease in D2 activity in TM-treated SHR male neonates (5) suggesting that decreased local T4 to T3 conversion in the cerebellum of these animals could result in lower T3 content within this tissue. To assess if lower D2 (T3) affects downstream T3-regulated gene expression, we determined the mRNA levels of several genes negatively regulated by T3 that have been previously shown to be up-regulated in D2KO mice (7) or during PTU-induced hypothyroidism (16). We compared their expression in cerebella derived from male and the female neonates exposed to TM; data of this comparison is presented in *Fig. 1*. Expression of the SWAP-1 gene was increased by 61.1% in TM-exposed males, but not in females ($p<0.05$; *Fig. 1A* and *1B*). However, the expression of the DIO2, L1CAM and TTR genes was not significantly altered in TM-exposed males (*Fig. 1A*) or females (*Fig. 1B*).

The effect of TM exposure on TH positively regulated gene expression relevant to cerebellar development is presented in *Fig. 2*. Interestingly, two genes, Pcp2 ($p<0.1$) and FoxP4 ($p<0.1$) show a trend towards inhibition only in male pups exposed to TM while expression in TM-exposed females remained unchanged (*Fig. 2A*). Expression of DIO3, BDNF-1, and reelin was unchanged between control and TM-exposed groups in both males and females (*Fig. 2A*, *2B*).

DISCUSSION

The safety of TM used as a preservative in a number of vaccines administered to both expecting mothers and infants, such as influenza, tetanus, encephalitis, meningococcal, and its contribution to neurodevelopmental disorders remain controversial issues. We have recently reported on the effect of perinatal exposure of TM on CNS development in rats. The decreased motor learning in TM-exposed SHR males coincided with decreased cerebellar D2 enzyme activity and increased expression of the negatively T3-regulated gene Odf4 (5). The decrease in D2 activity is likely to result in a local T3 deficiency, as the majority of brain T3 is derived through a local conversion of T4 by the action of D2 (17). Consequently, in this report we have focused on TM induced changes in cerebellar TH status and its consequences on gene expression.

Despite the well recognized role of TH in brain development (1), and its undisputed role in gene regulation, the exact gene targets of TH action remain to be identified. Genome-wide analysis and comparison between euthyroid and hypothyroid mice has identified 204 significantly altered genes including Synaptogamin 12 (Syt12), RE1-silencing transcription factor co-repressor (Rcor), Bcl-associated athanogene 3 (Bag3), cyclinD, Bcl2-associated X protein (Bax), and Purkinje cell protein 2

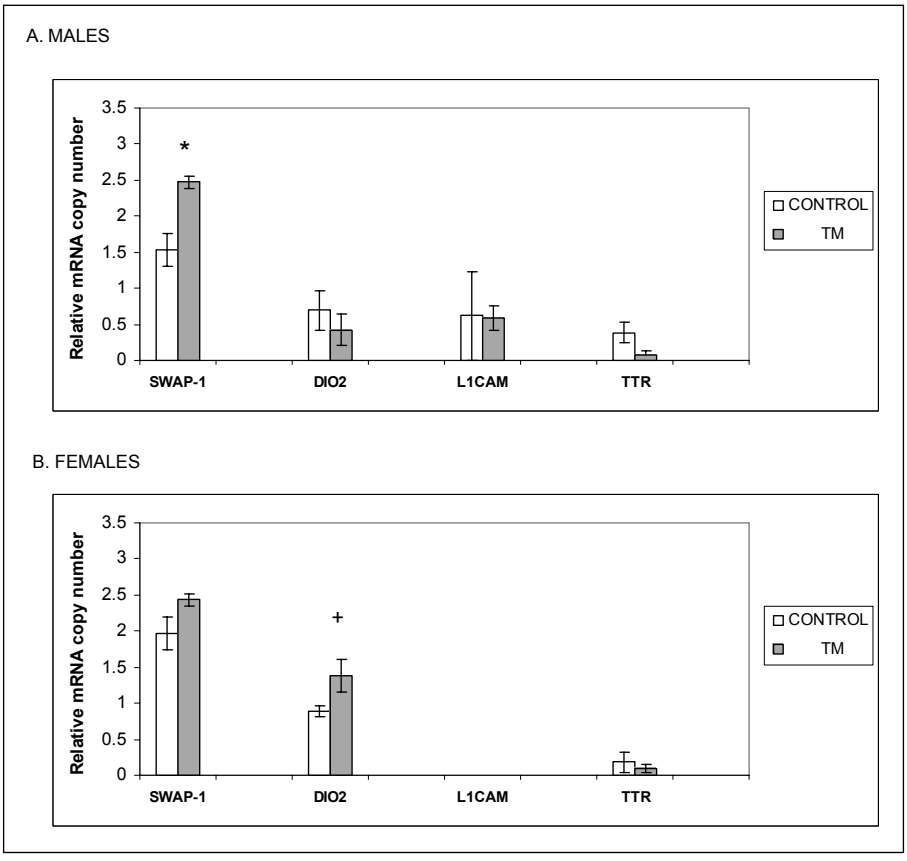

Fig. 1. Effect of thimerosal (TM) on the expression of genes negatively regulated by TH. Gene expression was measured by quantitative real time PCR of RNA using cerebellar tissue derived from male (A) and female (B) P21 SHR rat pups. The results are expressed as relative mRNA copy number. Column value represent mean and standard error bar. The * indicates statistical significance ($p<0.05$) and + indicates a trend ($p<0.01$). Specifically, for males: SWAP, $p=0.005$; DIO2, $p=0.33$; L1CAM, $p=0.9$; TTR, $p=0.24$; for females: SWAP, $p=0.11$; DIO2, $p=0.08$; TTR, $p=0.55$.

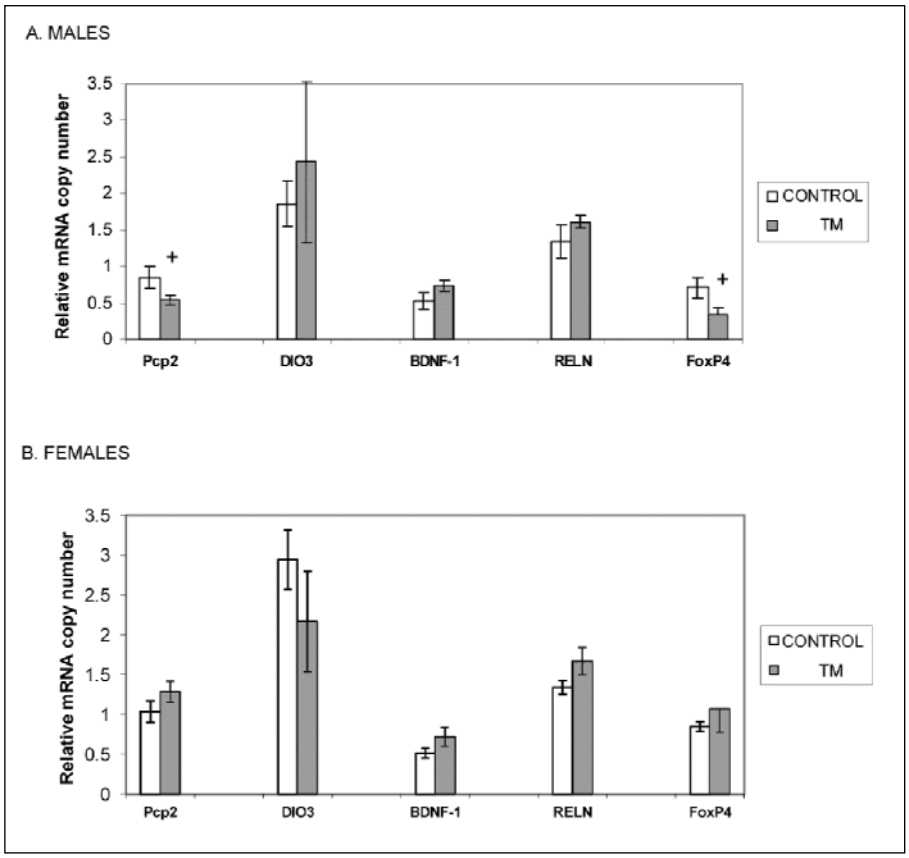

Fig. 2. Effect of thimerosal (TM) on the expression of genes positively regulated by TH. Gene expression was measured by quantitative real time PCR of RNA using cerebellar tissue derived from male (A) and female (B) P21 SHR rat pups. The results are expressed as relative mRNA copy number. Column value represent mean and standard error bar. The + indicates a trend ($p<0.1$). Specifically, for males: Pcp2, $p=0.07$; DIO3, $p=0.64$; BDNF, $p=0.16$; RELN, $p=0.31$; FoxP4, $p=0.08$; for females: Pcp2, $p=0.22$; DIO3, $p=0.32$; BDNF, $p=0.17$; RELN, $p=0.14$; FoxP4, $p=0.40$.

(Pcp2), (18). Subsequent analysis of the effect of hypothyroidism in rat using affymetrix rat neurobiology array RNU34 and a complete array rat 230A, identified 25 differentially expressed genes (19). Screening of TH-responsive gene in the developing mouse cerebellum has confirmed six genes to be positively regulated by the hormone (20).

Except for a few studies, there is little known regarding the impact of mercury compounds on gene expression. Genes that were affected by MetHg during rat embryonic development include heat shock protein 70 mRNA, fibronectin and p16 mRNA (21), while myelin basic protein (Mbp) gene expression was altered by perinatal MetHg exposure (16). Perinatal MetHg exposure in mice resulted in altered expression of 50 genes that included zinc/metal binding protein, transcription regulation, cell division and methylation (22). Studies on the effect of TM on gene expression reported altered expression of metallothionein (MT1) mRNA *in vitro* (23) and *in vivo* (24).

We have recently examined the effect of perinatal TM exposure on two cerebellar TH-dependent genes and reported that while Odf4 gene was upregulated selectively in TM-exposed males, the expression of Cirbp gene was not affected by TM exposure (5).

The present study was designed to further examine the hypothesis that a TM-induced decrease in cerebellar D2 activity could affect TH-dependent gene expression by including nine additional genes. In this regard, mice with a global targeted disruption of the DIO2 gene (D2KO mice) have ~50% less T3 content in their cerebral cortex, cerebellum, and hypothalamus (25). Thus a decrease in D2 activity within the cerebella of TM-exposed male neonates could potentially result in local cerebellar "hypothyroidism", altered TH-dependent gene expression, and abnormal cerebellar development. Previously it has been shown that the expression of genes negatively regulated by T3 was preferentially altered in D2KO mice with some of them being upregulated (7). Thus, to test our hypothesis we selected a few genes previously observed to be up-regulated in D2KO mice and under hypothyroidism (7), as well as positively regulated genes with a specific relevance to cerebellar development, and examined their expression using the quantitative real-time PCR.

Furthermore, since the effect of TM on D2 was sex specific, being more pronounced in SHR males, we compared gene expression in male and in female neonates. Our data indicates that TM exposure associated with up-regulation of some of the TH-dependent genes normally suppressed by TH, is sex-dependent, with males being more affected. Interestingly, hypothyroidism had a greater effect on gene expression in male than in female pups (18).

We report here for the first time that the expression of splicing regulator SWAP gene, negatively regulated by TH, was increased in TM-exposed males (61.1% increase), but not in females ($p<0.05$). The expression of TH-dependent SWAP gene has been previously observed in rat brain during the critical period of development (15). Increased expression of the SWAP-1 gene, negatively regulated by T3, following TM exposure may affect the developmental stage/cell-specific splicing pattern; distinctive splicing patterns have been observed during development of rat forebrain and cerebellum (26). We have previously reported (5) that the expression of the one of the marker genes negatively regulated by T3 found to be altered in D2KO mice, Odf4 (7), is also increased in TM-treated male neonates. The Odf4 gene belongs to a cancer-testis gene family specifically expressed in normal testis, fetal ovary, and different types of cancer (27), as well as in rodent cortex (7). However, the significance of an increased Odf4 expression during cerebellar development in TM-exposed pups is unclear at this point.

Not all of the negatively TH-regulated genes are affected by TM exposure. The DIO2 gene expression is negatively regulated

by T3 (28). However, DIO2 gene expression was not altered by TM exposure in male pups, while D2 enzyme activity was decreased in TM exposed male pups. It is possible that D2 enzyme activity has been inactivated directly by ubiquitination (17). Interestingly, cerebellar DIO2 gene showed a trend towards increased expression in female pups.

Transthyretin (TTR), another gene negatively regulated by TH, was unaffected by TM exposure; interestingly it was not affected by MetHg exposure in mice (16), but was up-regulated by PTU in the male mouse cortex (16). TTR protein is involved in the transport of thyroxine, and also has neuroprotective properties (29). The absence of the effect on TTR expression suggests that the transport of T3 from plasma is not affected by TM exposure, however we cannot rule out the effects of changes in other thyroid hormone transporters (30). Similarly, the expression of L1CAM, shown to be up-regulated under hypothyroid conditions (31) was not affected by TM exposure. We have previously reported (5) that Cirbp gene expression up-regulated in D2KO a mouse (7) was not affected by TM exposure.

We have also examined several genes positively regulated by TH and of special relevance to cerebellar development. Pcp2 is involved in neuronal differentiation (32); a decrease in Pcp2 protein may contribute to the abnormalities in Purkinje cells. Pcp2, showed a trend towards inhibition ($p<0.1$) in male pups. It has been previously reported that Pcp2 was not affected by MetHg exposure in mice, but inhibited in both male and in female PTU-treated mice cortex (16). T3 up-regulates the expression of Pcp2 gene during the first 2 weeks of rat neonatal life (33); a T3 deficiency during that time may contribute to Purkinje cell abnormalities.

Expression of the TH inactivating enzyme DIO3 is positively regulated by T3 (34). The lack of the effect on D3 gene expression is consistent with unaltered D3 enzyme activity in TM exposed rat cerebellum (data not shown). The expression of BDNF-1, another positively TH-regulated gene critical for cerebellar development, was not altered by TM exposure. BDNF expression is decreased in PTU treated mice cerebellum during postnatal development (35), and increased by TH in several regions of the developing brain (36). Similarly, RELN expression was not affected by TM exposure, although it is down-regulated by methimazole-induced hypothyroidism (37).

We have also examined the expression of FoxP4, member of FoxP gene family of transcription regulators involved in brain development (38), and implicated in Hashimoto's induced hypothyroidism (39); members of Fox gene family have been implicated in TH-induced differentiation (40). FoxP4 showed a trend towards inhibition in TM exposed male pups ($p<0.1$). Since FoxP4 is expressed in migrating and mature Purkinje cells and appears to be essential for maintenance of Purkinje cell dendritic arborization (41), its down-regulation may impact cerebellar development. Another member of this gene family, FoxP2, has been linked to regulation of language and speech in humans (38).

Overall, perinatal TM exposure affects the negatively regulated TH-dependent genes to a greater extent than the positively regulated genes, which is consistent with a deficiency of D2-derived T3. Furthermore, our data indicate that the effect of TM exposure on cerebellar gene expression is more pronounced in males than in females. Our behavioral and biochemical data (5) and present gene expression data also 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 (42) and in animals (43) showing that the developing males appear to be more sensitive to Hg exposure. This is consistent with earlier observation of higher sensitivity to TM in male *vs*. female mice (44) and a selective renal retention of inorganic HG in males (45).

While present report focuses on TM-induced changes in TH-dependent gene expression, other mechanisms of TM neurotoxicity, both at the transcriptional and posttranscriptional levels, should not be dismissed. Recently (46) described TM-induced reduction of GABA- and NMDA-evoked currents in hippocampal cultured neurons. Although, TM appears to interact directly with GABA and NMDA receptor complexes it is possible that TM may also alter NMDA-receptor gene expression as has been shown for MetHg (47). Another possible mechanism involved in TM neurotoxicity may be related to TM-induced oxidative stress. We and others have shown that TM induces oxidative stress both *in vivo* (5) and *in vitro* (48). Oxidative stress, affects vascular endothelial cells (49) and several downstream signaling pathways involved in modulation of neurogenesis (50). Specifically, chronic sublethal hypoxia in mice, resulting in increased oxidative stress, leads to inhibition of genes involved in synaptic and glial maturation and neurotransmission (Madri 2009) and results in developmentally inappropriate synaptogenic pattern that may contribute to motor and cognitive abnormalities. It is possible that these, as well as other, mechanisms converge in altering gene expression linked to TM-related neurotoxicity.

The effect of TM exposure on cerebellar gene expression presented here may be more pronounced than in other brain regions, as the cerebellum has been shown to be the most sensitive region to mercury toxicity (51). It is also possible that the timing of TM exposure, may contribute to the magnitude of the observed effects. Nevertheless, data presented here indicates that gene expression is altered in TM-exposed rat neonates, and further supports the concept of developmental neurotoxicity of TM. Furthermore, the genes that were activated by TM all are negatively regulated by TH, supporting our hypothesis of local brain hypothyroidism being induced by TM. Future studies are needed to assess the safety of TM as additive to vaccines given to pregnant women and infants.

In conclusion, our data indicate that perinatal TM exposure results in altered TH-dependent gene expression. Especially noteworthy is the up-regulation of SWAP-1 gene negatively regulated by T3 and involved in intron/exon splicing. Since regulation of gene splicing is vital to neuronal proliferation and differentiation, altered expression of SWAP-1 and perhaps other splicing regulator genes could have broad effects on many genes involved in the regulation of cerebellar development.

In light of our recent report of decreased D2 activity in TM exposed rat pups, presented here data support the hypothesis that TM may alter gene expression by lowering the local intra-cerebellar TH level. Consistent with a decreased D2 activity and local brain hypothyroidism is the up-regulation of genes negatively regulated by TH in the TM exposed rat neonates. The results also show sex-dependent differences in gene expression, with male being more sensitive to the effects of TM exposure. The differential regulation of TH-dependent gene expression, consistent with brain TH deficiency, defines a novel mechanism of toxicity of mercury compounds, and specifically TM in the developing brain, and warrants further studies on safety of TM in human vaccines during critical developmental periods.

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