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Maternal Subclinical Hypothyroidism and its Association with Developing Rat Brain

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ABSTRACT

The effects of thyroid hormone on brain development and function are largely mediated by binding of T3 to its nuclear receptors (TR) to regulate gene expression. Hypothyroidism during the early period of pregnancy has severe consequences for the developing rat brain. The present study examined effects of maternal hypothyroidism (from subclinical to severe) on gene expression in the developing rat brain. Dose-dependent thyroid hormone insufficiency was induced by delivery of methimazole (MMI) to pregnant rats via drinking water from gestation days 3 (GD 3) until sacrifice of pups at PN20.Maternal blood collected for thyroid hormone analysis. QRT-PCR was used to compare Vimentin, Nestin, GFAP, Mag gene expressions in the brain at postnatal day (PN) 20. Pups experiencing thyroid hormone insufficiency induced by delivery of 0, 50, 75 and 100 ppm MMI to the dam. The number of pups at birth, eye closure opening day, daily body weight in dams and pups are measured. BDNF expression were measured by ELISA in brain extract as a function of MMI exposure. The obtained data support the hypothesis that genes driving important developmental processes in developing rodent CNS are sensitive to perturbations of the thyroid axis, and altered pattern of gene expression in developing rat brain indicate that thyroid disease induce structural and functional abnormalities in the developing central nervous system.

Key words: Methimazole, subclinical hypothyroidism, HPT axis, CNS development, gene expression.

INTRODUCTION

The action of thyroid hormones on brain development and function is gaining renewed interest. It has been known for many years that thyroid hormones are very important in mammalian brain development, maturation, influencing many aspects related to neural cell migration, differentiation, glial cell differentiation and signaling[1]. Maternal T_4 insufficiency has serious adverse consequences for the neurodevelopment of the offspring, including irreversible cognitive deficits[2]. Even modest reductions in thyroid hormones during the critical period of brain development produce morphological alterations, synaptic dysfunction, and behavioral impairments[3]. Early maternal hypothyroxinema alters histogenesis and cytoarchitecture of the developing rat brain[4].

The general picture that emerges is that T4 and T3 may enter the brain through monocarboxylate transporter 8 (MCT8) and MCT 10 (SLC16A2 and SLC16A10 genes)[5], The organic anion transporter polypeptides (OATP, especially OATP1C1 and SLCO1C1 gene), the large neutral amino acid transporters (LAT-1 and LAT-2, products of SLC7A5 and SLC7A8 genes)[6-8], and sodium/taurocholate co-transporting polypeptide (SLC10A1, or NTCPgenes[9]. Thyroid hormone action in CNS development is mediated through nuclear receptors (TRs) and integrin $\alpha_v\beta_3$[10] and activation of nuclear receptors modulates the timing of expression of genes controlling process critical for brain development[11]. T4 is converted to the active hormone, T3, in glial cells, astrocytes, and tanycytes, although the main target cells are neurons and mature oligodendrocytes.

T3, acting through the nuclear receptors, exert transcriptional control over a number of target genes. Although many genes have been reported to be altered by perinatal hypothyroidism[12] the subset of TR-regulated genes that is critical for normal brain development has not been elucidated. In vivo T3 regulates gene expression during development from fetal stages, and in adult animals. A large number of genes are under direct and indirect regulation by thyroid hormone. In neural cells T3 may control around 5% of all expressed genes, and as much as one third of them may be regulated directly at the transcriptional level[1]. The purposes of this study are to identify alteration of many critical genes in developing rat brain. Up to our knowledge, limited data are available on thyroid hormone reduction on Nestin, Vimentin, GFAP, MAG gene transcript alteration in different brain regions. In the present study qRT-PCR was utilized to identify some critical gene transcripts alteration in developing rat brain. Our finding suggest that maternal thyroid hormone insufficiency could affect early CNS development via dysregulation of critical genes.

MATERIAL AND METHODS

All experimental procedures involving animals were performed following the Pasteur Institute of Iran guidelines. Pregnant wistar rats (n=60), weighting 200-260 g, were obtained from Pasteur Institute of Iran on gestation day (GD) 3 and housed individually in standard hanging cages under controlled temperature (24±1 °C). The housing rooms were maintained on a 12:12 light -dark cycle, and animals were permitted free access to standard laboratory chow and tap water. Body weights of dams were monitored from Body weights of dams were monitored from GD0 to GD23 and body weight of pups were determined from PN0

to PN20. Successful delivery day monitored in each treatment group. Beginning on GD3 and continuing until postnatal day (PN) 20,dams were rendered hypothyroid by addition of 0, 50, 75 or 100 ppm of the thyroid hormone synthesis inhibitor MMI (Daroupakhsh, Iran) to the drinking water (n=12/dose group), and a group of rats received 50 ppm MMI in drinking water and 200 µg L-T4 (Daroupkhash, Iran) by gavage (n=12/dose group). Thyroid hormone(TH), l-thyroxine (Daroupkhash, Iran) was dissolved in 0.9% sodium chloride containing 10 mM KOH at a concentration of 200 µg/ml. Drinking MMI solution was replaced daily with fresh MMI solution. MMI not only enters the bloodstream, but also passing through mother's breast milk to the rat pups as well. These dose of MMI could produce a severe state of hypothyroidism (based on serum hormones, body weight gains, and profound developmental delays). In each experiment a pregnant rat was transferred to plastic hanging cages and was permitted free access to food and tap water. All animal treatment began on GD 3 and continuing until weaning of the pups (PN 20). The day of birth was designated PN0. Exposure to MMI terminated when pups were weaned on PN20.

Determination of Plasma TSH, T3, T4 assays by serological assay: Circulating levels of thyroid hormone and TSH in serum are analyzed. Blood was collected directly from the maternal orbital sinus in PN 20. Blood samples were centrifuged at 3000g for 20 min at room temperature to obtain the plasma. Plasma was used to analyze the level of TSH, and T3, fT4 and Total T4. Serum levels of total T3 (tT3) and total T4 (tT4) and free T4 (fT4) were measured by ELISA (Padtangostar, Tehran, Iran) and Serum levels of TSH was measured by rat TSH ELISA kit (LifeSpanBioSciences) according to the manufacture's instruction.

BDNF expression assay: BDNF expression was determined with Rat Brain derived Neurotrophic Factor (BDNF) ELISA kit (Zellbio, Germany) according to the manufacture's instruction. For each treatment group 10 neonates, and in 100 ppm hypothyroid group 6 neonates, were sacrificed for mature BDNF analysis. Brains quickly removed and weighted up and added PBS (pH7.4, 100 mM) and homogenized the sample thoroughly by homogenizer (totally 100 mg tissue/1ml buffer). The PBS contain anti-protease cocktail (Sigma). Then centrifuged (at 6000 RPM) for approximately 10 minutes. After collecting the supernatant, experiment conducted immediately. Aliquots of each sample lysate were analyzed in duplicate for total protein by The Lowry protein assay and sample mature BDNF protein expression was determined in duplicate in each experimental set of plates.

Quantitative Real-Time PCR: The identification of transcripts changed as a function of MMI treatment in each experimental group, as a function of MMI treatment in each experimental group was performed using Reverse-transcriptase chain reaction (RT-PCR). In each treatment group, Six offspring (n=6) were scarified at PN20. Brains were quickly removed and cortex, hippocampus, medulla oblongata and bulbus olfactorius dissected. Total RNA was prepared individually from multiple brain regions using RNXTM-Plus (Cinnagen, Iran) according to the manufacture's instruction. The concentration of RNA was determined by measuring the absorbance at 260 nm (A260) in spectrophotometer (SHIMADZU, UV-1800. Switzerland). The 260/280 ratio was calculated that was greater than 1.6. Then RNA stored at -70°C.

Identification of transcripts were changed as a function of MMI treatment in each experiment group was performed using Reverse transcriptase polymerase chain reaction (RT-PCR). In each treatment group 6 offspring (n=6) were scarified at PN20. Brains were quickly removed and cortex, hippocampus, medulla oblongata and bulbus olfactorius dissected. Total RNA was prepared individually from multiple brain regions using RNXTM-Plus (Cinnagen, Iran). First strand cDNA was synthesized from 5µg total RNA from one animal using first strand cDNA synthesis (Cinnagen, Iran). Expression levels of the following 12 genes in cDNA samples were quantified by Step One Real-Time PCR Systems and normalized using SYBER Green dye. Each sample was run in triplicate for the target and reference genes and the reporter dye signal was normalized using the passive reference dye, ROX, to eliminate volume-handling error. PCR primers were designed in Oligo 7.0 primer analysis software (Molecular Biology Insights) and blasted inhttps://ncbi.nlm.nih.gov. Sequences of the PCR Primers are listed in Table 1.

Table1: Primer sequences and characteristics for quantitative RT- PCR

Gene Symbole	Direction	Sequences of the PCR primers	Function	Gene bank ID
GFAP	Forward	AATTGCTGGAGGGCGAAGAA	Type III intermediate filament (IF) protein	NM_017009.2
	Reverse	CAGGCTGGTTTCTCGGATCT		
Nestin	Forward	GCTCAGATCCTGGAAGGTGG	Type VI intermediate filament (IF) protein	M34384.1
	Reverse	AAAGCCAAGAGAAGCCTGGG		
Vimentin	Forward	TGCGGCTGCGAGAAAAATTG	Type III intermediate filament (IF) protein	NM_031140.1
	Reverse	CGTTCAAGGTCAAGACGTGC		
Mag	Forward	TTGGAGCGGAATCAGGAGAC	Type 1 transmembrane proteinglycoprotein	NM_017190
	Reverse	CAGAGGCAGGGTGGTAAGG		
HPRT	Forward	CCAGCGTCGTGATTAGTG	Housekeeping Gene	S79292.1
	Reverse	CGAGCAAGTCTTTCAGTCC		

RT-PCR data analysis: RT-PCR data analysis was performed using the comparative Ct method ($2^{-\Delta\Delta CT}$). The ΔCt value was calculated as the Ct_{target}- $Ct_{reference}$, using HPRT gene as the reference gene to calculate relative mRNA levels. HPRT was chosen as it is a highly expression constitute gene whose expression does not change as a function of hypothyroidism (Cheng T. et al., 2012). The ΔΔCt value was calculated as the ΔCt test sample-ΔCt calibrator sample, where the mean of the 0 ppm sample ΔCt values was used for the calibrator. Fold changes were calculated as $2^{-\Delta\Delta Ct}$.

Statistical analysis: Statistical significance was determined by one-way ANOVA followed by TOKEY's post-hoc test. All numerical data were expressed as mean± standard error of the mean (SEM). Pairwise comparisons were performed by Mann-Whitney U-test between groups (GraphPad PRISM version 7.0 Software, San Diego, CA, USA). If ρ-values were less than 0.05, differences were considered statically significant.

RESULTS

Determination of Plasma TSH, T3, T4 assays by serological assay: As a typical thyroid hormone synthesis antagonist, Methimazole induces hypothyroidism by inhibiting thyroid hormone iodination. MMI significantly in dose-dependent manner reduced T3 and T4, but TSH levels was increased. Thyroid hormones were reduced in a dose- dependent manner in dams in response to 0, 50, 75, 100 ppm, of MMI. fT4 was dose-dependently reduced at 50 ppm, 75 ppm, 100 ppm MMI dose groups on PN20 [F(4,55)=34.18, P<0.0001)](Fig. 1A). tT4 was dose-dependently reduced at 50 ppm, 75 ppm, 100 ppm MMI dose groups on PN20 [F(4, 55)=146.5, P<0.0001)](Fig.1B). T3 was also reduced on PN20 [F(4,55), P<0.0001] (Fig.1C). TSH was dose-dependently increased on PN20 [F(4,55)=1187, P<0.0001](Fig.1D). TSH was dose-dependently increased on PN20[F(4,55)=1187, P<0.0001](Fig.1D).

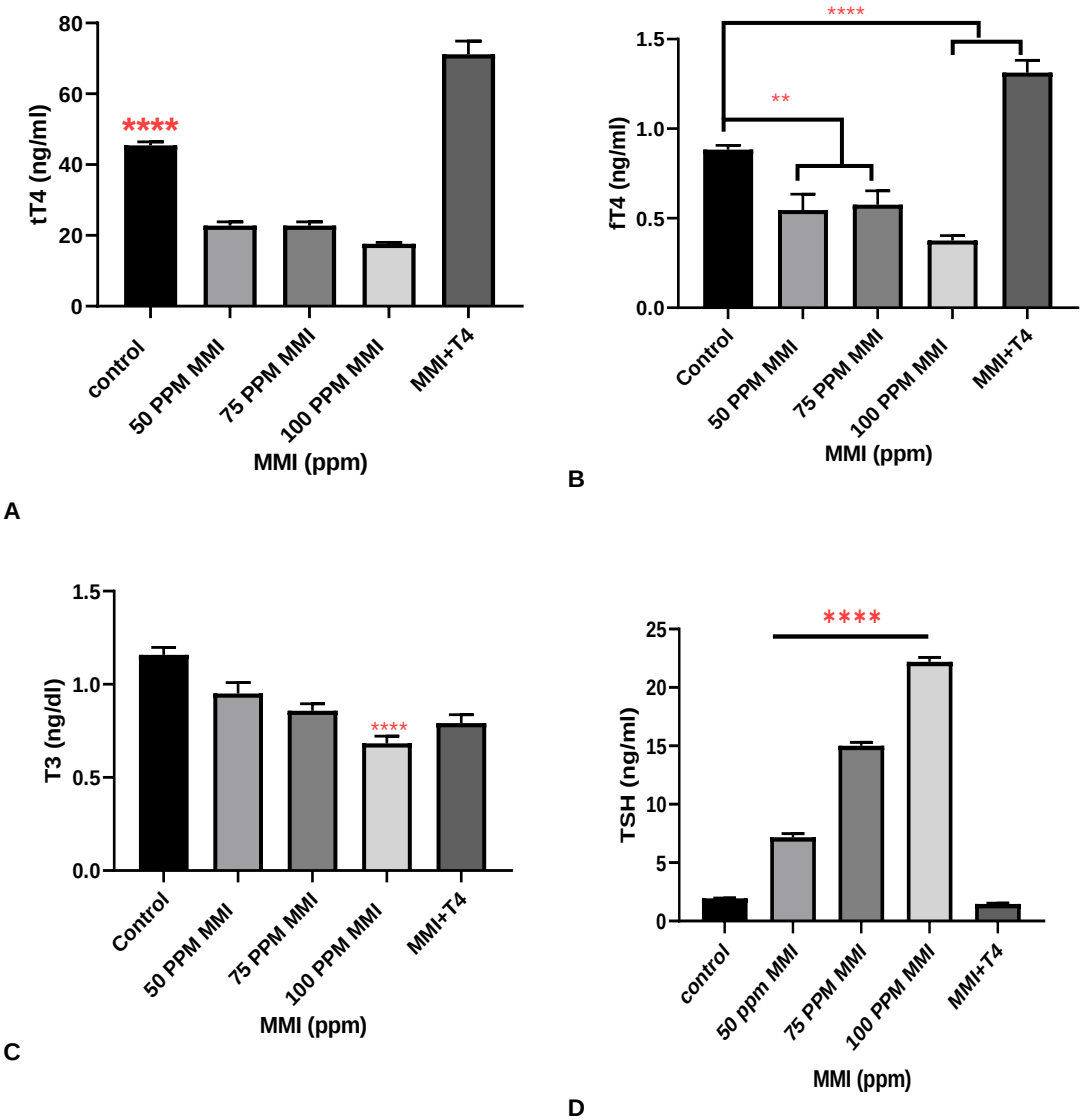


Fig1.Group Mean TSH, T4, and T3 levels in the Wistar rats at PN20. Error bars represent SEM. All data are shown as mean±SEM. *P<0.05, ** P< 0.01,*** P< 0.001, ****P<0.0001

The number of pups at Birth: A significant association exist between the number of pups at birth and maternal thyroid hormone. Significant reduction in the number of pups at birth was observed in hypothyroid rats relative to control [F(4, 35)=785.1 ρvalue<0.0001].

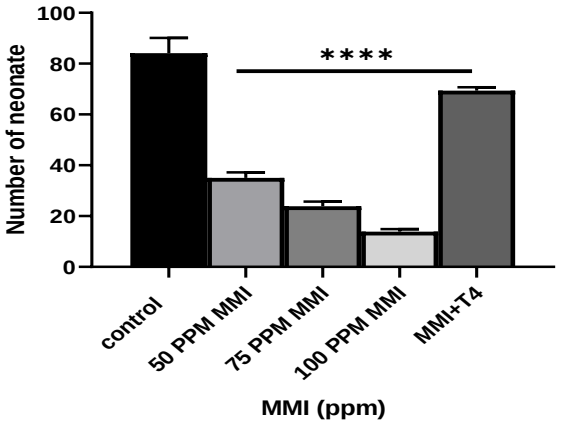


Fig 2. Significant reduction in the number of pups at birth was observed in hypothyroid rats relative to control [F(4, 35)=785.1 ρvalue<0.0001]. ****P<0.0001

Neonatal eye-closure opening day: A delayed prenatal eye closure and postnatal eye opening took place in the treated rats. Our result displayed significantly delay in eye-closure opening day as a function maternal thyroid hormone deficiency relative to control [F(4, 226) Pvalue< 0.0001].

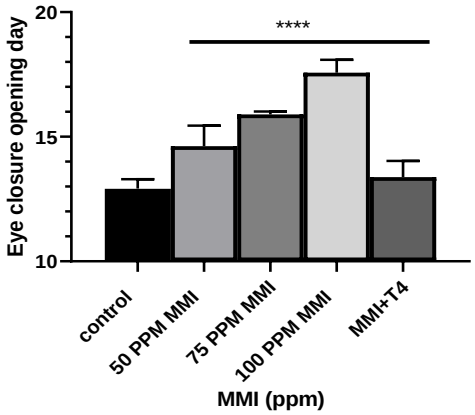


Fig 3. Significant delayed in prenatal eye closure opening took place in the treated rats[F(4, 226) ρvalue< 0.0001].****P<0.0001

Body weight in dams and pups: Significant alternation in dam weights were detected in response to MMI in treated groups relative to control (F=85.891 ρvalue<0.0001). At GD15 significant reduction in body weight were observed in dams In comparison to control group[Dose×Age interaction, F=5.976 ρvalue<0.0001] and extending to the early postnatal period. Offspring maintained on MMI displayed a slower growth rate (F=146.230 ρvalue<0.0001), and significant reductions in body weight were evident by PN3 and became more profound with age [Dose×Age interaction, F=70.072, Pvalue<0.007] and extending to the PN20.

Table2. Mean±SEM body weight of dams. Significant alternation in dam weights were detected in response to MMI in treated groups relative to control (F=85.891ρvalue<0.0001).Significant main effects of Dose and Age in repeated measures ANOVA were further evaluated by TUKEY'spost-hoc test.

	GD0	GD3	GD7	GD10	GD15	GD17	GD19	GD20	GD21	GD22
Control	222.500±10.193	225.500±10.942	233.333±11.523	234.000±25.161	258.833±12.698	272.333±18.097	292.917±23.761	302.167±25.222	311.417±26.071	224.000±14.863
50 PPM MMI	215.250±14.623	218.000±14.844	221.250±15.621	224.083±16.903	233.417±19.523	236.417±19.126	238.000±20.676	243.043±20.676	226.583±11.547	226.583±11.547
75 PPM MMI	197.583±8.543	198.583±8.027	202.667±8.616	210.417±10.415	218.250±10.575	222.417±13.898	222.417±15.096	226.500±17.901	229.167±20.910	235.083±27.830
100 PPM MMI	216.667±9.490	214.833±9.897	219.250±12.447	228.333±17.839	244.417±27.828	247.250±30.427	255.083±37.826	258.583±42.621	262.500±46.802	218.500±7.452
MMI+T4	196.000±10.788	198.000±11.189	202.455±16.305	214.727±18.363	229.818±29.047	240.636±37.524	246.364±46.128	200.727±8.521	200.727±8.521	200.727±8.521

Table3. Mean±SEM body weight of pups. Significant reductions in body weight were seen in pupsin response to 50 ppm, 75 ppm and 100 ppm MMI. Body deficits (mean±SEM) were evident by PN 3 in the offspring that became more apparent with age. Significant main effects of Dose and Age in repeated measures ANOVA were evaluated by TUKEY'spost-hoc test.[Dose×Age interaction, F=70.072, ρvalue<0.007]*P<0.05.

Experiment					
	PN0	PN3	PN7	PN10	PN20
Control (n=88)	9.9943± 0.05330	10.9886±0.10660	12.8068±0.98094	18.7614±2.0850	40.8409±6.35879
50 ppm MMI (n=37)	9.9459±0.22924	10.9189±0.34387	12.5946±0.86472	13.7432±1.26722	31.9189±10.03709
75 ppm MMI (n-28)	9.6964±0.34263	10.6071±0.61399	11.5357±0.79266*	12.3750±1.11076*	21.1071±5.13044*
100 ppm MMI (n=14)	9.5000±0.27735	9.8929±0.48748*	10.3571±0.71867*	11.5357±1.59885*	19.8571±3.48308*
MMI + L-T4 (n=64)	10.0154±0.12403	11.0000±0.0000	13.1719±0.88290	17.3906±3.35470	27.6562±3.24267*

BDNF expression assay: Significant differences in BDNF expression were detected in developing rat brain as the main effect of MMI dose. One-way ANOVA statistical analysis for each group revealed Control levels of BDNF expression were highest in developing rat brain at PN20 and much lower in group that exposed to 100 PPM MMI [F(3, 32)=3.144 , ρ_{value}=0.039].

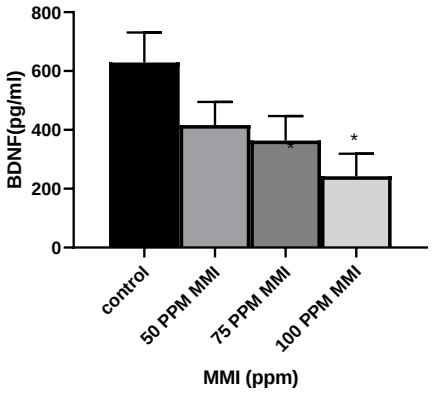


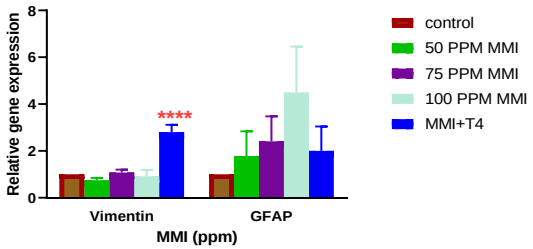
Fig 4.Effect of MMI on mature BDNF levels in the Wistar rat's pups at PN20. As shown in this diagram BDNF (pg / ml) in the total brain of the test groups relative to the control were decreased and in the groups that exposed to 100ppm MMI its decreasing was significant.($F(3,32)=3.144$ $\rho_{value}=0.0386$ n=36) .* p<0.05

Quantitative Real-time PCR assay: Although relative gene expression of Myelin –associated glycoprotein mRNA were increased as a function of exposure to MMI, Step-down ANOVA analysis for each group revealed no significant difference in Myelin-associated glycoprotein mRNA were detected in developing rat brain as a main effect of dose at PN20 [$F(4, 95)=2.130$, $\rho_{value}=0.083$].

Relative gene expression of Nestin mRNA were increased as a function of exposure to MMI, Tukey HSD ANOVA statistical analysis for each group revealed significant difference as a main effect of 100 PPM MMI relative to control [$F(4,95)= 3.446$, $\rho_{value} =0.011$] but no significant difference in relative gene expression of Nestin mRNA between 50 PPM MMI and 75 PPM MMI groups relative to control were detected (Respectively $\rho_{value}=1$ and 0.204). Step-down ANOVA analysis revealed a significant difference as a function of MMI between those group exposed to 100 PPM MMI and 50 PPM MMI ($P_{value}= 0.038$).

Relative gene expression of vimentin mRNA were significant [$F(4,95)= 18.422$, $\rho_{value} \leq 0.000$], Following Tukey HSD ANOVA statistical analysis for each group revealed no significant difference as a main effect of MMI at 50 MMI, 75 MMI and 100 MMI dose group relative to control (Respectively $\rho_{value}=0.898$, 0.999 and 0.998).

Relative gene expression of GFAP mRNA were increased as a function of MMI. Following Tukey HSD ANOVA statistical analysis showed no significant difference in GFAP mRNA in developing rat brain as a main effect of dose at PN20 [$F(4, 95)= 1.184$, $\rho_{value}= 0.323$).



A

B

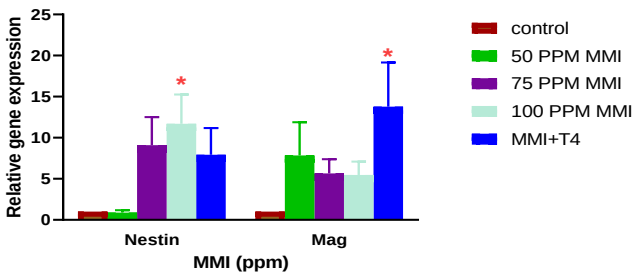


Fig 5. Maternal thyroid status and gene expression in developing rat brain. Relative gene expression values are normalized to the 0 ppm value . Values are expressed as mean ± SEM.*P<0.05, ** P< 0.002,*** P< 0.001, ****P<0.0001

Glial fibrillary acidic protein (GFAP) is the classical marker used to identify differentiated astrocytes and Astrocyte maturation is followed by a switch between vimentin and glial fibrillary acidic protein (GFAP) expression. Nestin is a cytoskeletal intermediate filament initially characterized in neural stem cells[13][12][11] and is implicated in the radial growth of the axon.

DISCUSSION

The physiological and developmental effects of thyroid hormones are mainly due to the control of gene expression after interaction of T3 with the nuclear receptors. To understand the role of thyroid hormones on CNS development, knowledge of the genes regulated by T3 during specific stages of development is required.In this study we identified genes regulated by T3 in developing rat brain. By comparing these data with control, the cellular targets of T3 can be identified. This study analyses the specific roles of T3-regulated genes in the different stages of CNS development within the physiological frame of the developmental changes of thyroid hormones in the developing rat brain. Alterations of thyroid gland function during development are known to produce extensive damage to the central nervous system including severe mental retardation.T3 regulates transcriptionally genes encoding extracellular matrix proteins, involved in cell migration and the control of diverse signaling pathways.

Intermediate filament (IF) proteins constitute an extremely large multigene family of developmentally and tissue-regulated cytoskeleton proteins abundant in most vertebrate cell types. IF network can be regulated by phosphorylation, mediated by the RhoA signaling pathway leading to reorganization of IF filamentous structure and alterations of the microfilament organization may have important implications in neural cell functions, the effects of TH on glial cell cytoskeleton could be implicated in essential neural events such as brain development[14, 15].

Astrocytes make up 20 to 50% of the volume of most brain areas and correspond to a heterogeneous class of cells that have many different roles. Glial fibrillary acidic protein (GFAP) is the classical marker used to identify differentiated astrocytes. The level of GFAP expression, however, can vary greatly from one cell to another, between different species, as well as under different conditions. Astrocytes express thyroid hormone receptor

alpha and beta subtypes suggesting that astrocytes are direct target cells of thyroid hormones. Glial fibrillary acidic protein (GFAP) accumulates in astrocytes during development. We have characterized the increase in GFAP mRNA during development of the rat brain by using quantity real time PCR and vimentin and GFAP gene expression in different brain of developing rat brain assessed relative to control. We report that a caudal to rostral gradient of expression, consistent with overall brain maturation and then showed that the level of GFAP transcript in under examination groups relative to control had 100% increased. The level of Vimentin gene transcription compared to control relatively increased.

Astrocyte precursors of the CNS usually express vimentin as the major IF. Astrocyte maturation is followed by a switch between vimentin and glial fibrillary acidic protein (GFAP) expression, with the latter being recognized as an astrocyte maturation marker. Levels of GFAP are regulated under developmental and pathological conditions. Modulators of GFAP expression include several hormones such as thyroid hormone, glucocorticoids and several growth factors such as FGF, CNTF and TGF beta, among others. In the developing rat brain GFAP mRNA was first observed at embryonic day 16 (E16) in the glial limitans of the ventral hindbrain. During brain development message levels increased rostrally and by postnatal day 5 (P5) the entire glial limitans showed a positive signal which persisted into adulthood. GFAP mRNA was also found to accumulate in a caudal to rostral direction within the Purkinje cell layer of cerebellum beginning shortly after birth. By P5 the entire layer was positive and signal in this region could be localized to Bergmann glia by P15. A transient elevation in GFAP mRNA was apparent during the second postnatal week in cerebellum and cerebrum. Using in situ hybridization, a peak in message levels was observed at P15 and could be localized primarily to the deep white matter of cerebellum, to the corpus callosum, and to certain hippocampal fiber tracts. The pattern of GFAP expression in these regions is consistent with the differentiation of interfascicular glia and the appearance of type-2 astrocytes during the initial events of myelination. GFAP mRNA levels in white matter were greatly reduced in the adult. The pronounced regional differences in GFAP mRNA expression during development may reflect the differentiation of subpopulations of astrocytes[16]. Studies of the GFAP gene have already identified several putative growth factor binding domains in its promoter region[17]. Differences between normal and hypothyroid rats were observed starting from postnatal day 4, the vimentin-glial fibrillary acidic protein transition was delayed and most differentiated astrocytes remained in the white matter[18]. Differentiated astrocytes appeared in the superior medullary vellum by postnatal day 2 and reached the white mater and internal granular layer by postnatal day 4. Intermediate filament marker expression was transiently lost from postnatal days 6 to 8 in anterior lobes, without an increased apoptosis. Vimentin expression was replaced by glial fibrillary acidic protein between postnatal days 10 and 32. The differentiated astrocytes were evenly distributed throughout the cerebellar slices, including the internal granular layer[18]. Furthermore, The development of glia in the hippocampal formation of normal and hypothyroid rats was studied using immunocytochemical staining for either glial fibrillary acidic protein (GFAP) or vimentin by Rami A.et al.,(1988). Their results indicated that in the radial glial processes of young hypothyroid rats compared to normal animals GFAP immunoreactivity was lowered. Vimentin immunoreactivity showed that the glial processes were present and therefore immature at least with respect to their cytoskeletal composition. They propose that this early defect in the maturation of the radial glial fibers accounts for the final deficit in the granule cells of the dentate gyrus. Later in development, thyroid deficiency also reduced the density and number of GFAP-labeled astrocytes and the growth of their processes. This observation is in complete disagreement with the glial hypertrophy induced by thyroid deficiency in the cerebellum[19]. These radial glial processes followed the known path of neuroblast migration toward the proliferative zone of the dentate gyrus until the end of the 1st postnatal week. Moreover, The concentrations of glial fibrillary acidic protein (GFAP) and its encoding mRNA in the cerebellum and hippocampal formation were assayed during the development of normal and hypothyroid rats by Faivre-Sarrailh C. etal.,(1991). They showed that in neonatal hypothyroidism induced a significant reduction in the GFAP concentration in both regions from day 14. The reduction was especially marked on day 35 in the cerebellum (-43%) and the hippocampal formation (-55%). The immunocytochemical study of vimentin showed that the developmental disappearance of this protein from the Bergmann and internal astrocytes is greatly delayed in the cerebellum of the hypothyroid rats. The reduction in GFAP concentration together with the delayed vimentin-GFAP transition could explain how astrocyte morphogenesis is impaired by neonatal thyroid deficiency. The GFAP-mRNA concentration in the hippocampal formation was reduced throughout the development of thyroid-deficient rats, while the GFAP-mRNA concentration in the cerebellum first increased between birth and day 14 to reach a peak well above the normal value (+78%) and decreased thereafter to reach 53% of the normal value by day 35. This transient increase in the cerebellar GFAP-mRNA concentration may be related to the astroglial hyperplasia that occurs in these animals[20]. The difference between the developmental profile of GFAP and its encoding mRNA, especially under pathological conditions, indicates that two distinct mechanisms control the synthesis or stability of the protein and its messenger RNA[21]. Whereas, Trentin et al., (1998) have shown that T3 induces cerebellar astrocyte proliferation. This effect is accompanied by alteration in glial fibrillary acidic protein (GFAP) and fibronectin organization. In that study, they report that the C6 glioma cell line, which expresses GFAP and is classified as an undifferentiated astrocytic cell type, is a target for T3 action. The C6 monolayers were treated with 50 nM T3 for 3 days, after which the cells were maintained for 2 days without medium changes. In C6 cells, T3 induced the expression of proteins of 107, 73 and 62 kDa. The hormone also up-regulated protein bands of 100 (+50%), 37 (+50%) and 25.5 kDa (+50%) and down-regulated proteins of 94 (-100%), 86.5 (-100%), 68 (-100%), 60 (-100%), 54 (-33%), 51 (-33%) and 43.5 kDa (-33%). We suggest, on the basis of molecular mass, that the 54-, 51-

and 43.5-kDa proteins could be the cytoskeletal proteins vimentin, GFAP and actin, respectively. The down-regulation of these proteins may be involved in the effects of thyroid hormone on C6 differentiation[22]. T3 also has a morphological effect on cultured cortical astrocytes with rearrangement of GFAP filaments, and induces proliferation in the cultured cerebellar astrocytes of newborn rats and thyroid hormone prepares the astrocytes to interact with neurons[23]. T3 may induce astrocyte secretion of factor(s) that promotes morphological differentiation in cerebral hemisphere astroglial cultures and stimulates the proliferation of cerebellar astrocytes. Astrocytes obtained from hypothyroid animals were more sensitive to secreted factors than normal cells. These results emphasize the heterogeneity and the importance of glial cells to normal brain development and open new questions about thyroid hormone therapy in hypothyroidism[24]. Rodrigo Martinez et al.,(2005) have reported that cerebellar astrocytes treated with thyroid hormone secrete epidermal growth factor (EGF), which directly induces neuronal proliferation and, indirectly, by increasing synthesis of extracellular matrix proteins, induces neurite outgrowth in vitro. Here, by using a neuron-astrocyte coculture model, they investigated the involvement of cell contact on neuronal proliferation. Culturing of cerebellar neurons on T3-treated astrocyte carpets or conditioned medium derived from them (T3CM) yielded similar results, revealed by a 60% increase in cell population Their data reveal an important role of astrocytes as mediators of T3-induced cerebellar development and partially elucidate the role of cell contact and soluble factors on this process. Also, Gomes et al., (1999) report that thyroid hormone induces astrocytes to secrete growth factors that can interfere with neuronal proliferation via a paracrine pathway[25]

Nestin is a protein that in humans is encoded by the NES gene. Nestin (acronym for neuroectodermal stem cell marker) is a type VI intermediate filament (IF) protein. Nestin is a cytoskeletal intermediate filament initially characterized in neural stem cells[13] and is implicated in the radial growth of the axon. The putative stem cell/quiescent neural progenitor (QNP), also referred to as the Type 1 cell, is a radial glial-like cell that expresses glial fibrillary acidic protein (GFAP), the intermediate filament protein nestin, as well as the transcription factor and stem cell marker Sox2[26]. QNPs are slowly dividing cells that undergo asymmetric divisions to generate the transiently amplifying neural progenitors/Type 2a cells that are immunopositive for nestin, but no longer express GFAP or Sox2. Amplifying neural progenitors divide relatively rapidly to generate the Type 2b cells, which are neuroblasts expressing Nestin as well as the microtubule-associated protein doublecortin (DCX). Type 2b cells have been reported to retain the ability to undergo limited cell division[26]. At this stage neuroblasts also express the pro-neurogenic basic helix-loop-helix transcription factor NeuroD that is involved in neuronal cell fate acquisition. The Type 2b cells migrate into the GCL, and form DCX-positive Type 3 cells that lose their immunopositivity for nestin.Type 2b cells have been reported to retain the ability to undergo limited cell divisions[27]. In this study we report that hypothyroid rat groups showed an increased in number of nestin process in different brain of hypothyroid developing rat brains at PN20 with respect to control animals. No significant differences in Nestin mRNA were detect in groups which exposed to 50 PPM MMIwith respect to control animals. Using immunohistochemistry to identify the intermediate filament nestin, Juan Ramon Martinez-Galan et al.,(2004) have studied the possible influence of fetal and neonatal hypothyroidism on neocortical neuronal migration by arresting the normal development of the radial glial scaffold.By embryonic day 21 (E21), hypothyroid animals had a significant decrease in the number of nestin immunoreactive processes in the presumptive visual cortex. By postnatal day 5 (P5), hypothyroid animals showed a significant increase in the number of glial processes in relation with controls, although only in the upper layers of the visual cortex. Moreover, by P10, there was a marked increase in the number of radial glial processes in hypothyroid rats in superficial and deep zones of the visual cortex with respect to control animals. Their data indicate an important delay in the formation of the radial glial scaffold during the embryonic stage in hypothyroid animals that was interestingly accompanied by the later presence of abundant nestin immunoreactive fibers at P10. This impairment in the evolution of radial glia during development might be affecting the normal neuronal migratory pattern in the neocortex of hypothyroid rats[28].The latter finding in the subclinical model is consistent with our findings of effect of MMI at any dose level tested in preweanling rat (PN20); unfortunately, animals in the current study were not assessed prior to PN20.

It is well-established that thyroid hormone is required for the terminal differentiation of oligodendrocyte precursor cells (OPCs) into myelinating oligodendrocytes by inducing rapid cell-cycle arrest and constant transcription of pro-differentiation genes[29]. During development, myelinating oligodendrocytes only appear after the formation of neural circuits, indicating that the timing of oligodendrocyte differentiation is important. Since fetal and post-natal serum thyroid hormone levels peak at the stage of active myelination, it is suspected that the timing of oligodendrocyte development is finely controlled by thyroid hormone. Lack of thyroid hormone does not change the expression of the oligodendrocyte progenitor-specific gene, the platelet derived growth factor receptor α[30]. To assess the role of thyroid hormone on myelin gene expression, we have studied the effect of hypothyroidism on the steady state levels of myelin-associated glycoprotein (MAG) mRNA in developing rat brain during PN20. As studied by qRT PCR, Hypothyroid rats showed a delay of several days in the onset of mRNA expression, increasing thereafter at the same rate as in normal animals, and eventually reaching similar values. When individual brain regions were analyzed, we found strong regional differences in the effect of hypothyroidism. The Hippocampus and cerebral cortex were most affected, with messenger levels more accumulate than other regional brain. In caudal regions differences between control and hypothyroid rats were not evident at PN20. As showed previously, MAG mRNA increased from days 10-15 onwards, reaching constant levels by days 20-25[31]. The effect of hypothyroidism on the accumulation of the protein correlated with mRNA

levels. By run on analysis, they found no differences in transcriptional activities of the MAG gene in normal, hypothyroid, or T4-treated rats. Therefore, the effects of hypothyroidism on MAG mRNA and protein levels were most likely caused by decreased mRNA stability. We propose that thyroid hormone contributes to enhanced myelin gene expression by affecting the stability of newly transcribed mRNA in the early phases of myelination[32]. To assess the role of thyroid hormone on myelin gene expression, they have studied the effect of hypothyroidism on the mRNA steady state levels for the major myelin protein genes: myelin basic protein (MBP), proteolipid protein (PLP), myelin-associated glycoprotein (MAG) and 2′:3′-cyclic nucleotide 3′-phosphodiesterase (CNP) in different rat brain regions, during the first postnatal month. Ibarrola et al.,(1997) found that hypothyroidism reduces the levels of every myelin protein transcript, with striking differences between the different brain regions. Thus, in the more caudal regions, the effect of hypothyroidism was extremely modest, being only evident at the earlier stages of myelination. In contrast, in the striatum and the cerebral cortex the important decrease in the myelin protein transcripts is maintained beyond the first postnatal month. Therefore, thyroid hormone modulates in a synchronous fashion the expression of the myelin genes and the length of its effect depends on the brain region. On the other hand, hyperthyroidism leads to an increase of the major myelin protein transcripts above control values[30]. This is well supported by the hypomyelinating phenotypes exhibited by patients with congenital hypothyroidism, cretinism. During development, myelinating oligodendrocytes only appear after the formation of neural circuits, indicating that the timing of oligodendrocyte differentiation is important. Since fetal and post-natal serum thyroid hormone levels peak at the stage of active myelination, it is suspected that the timing of oligodendrocyte development is finely controlled by thyroid hormone. The essential machinery for thyroid hormone signaling such as deiodinase activity (utilized by cells to auto-regulate the level of thyroid hormone), and nuclear thyroid hormone receptors (for gene transcription) are expressed on oligodendrocytes

CONCLUSION

As demonstrated by animal studies, the lack of maternal TH leads to irreversible deficits in brain cytoarchitecture and development. Furthermore, data from prospective study revealed an increased risk of seizure disorders, autism spectrum disorder, attention deficit hyperactivity disorder, and other psychiatric conditions among patients born to mothers with thyroid dysfunction

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