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Research Article

Role of Brain-Derived Neurotrophic Factor on Cognition of Hypothyroid Neonatal Rats

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Abstract: Mechanisms underlying the effects of inadequate thyroid hormone availability to the brain, on cognitive functions are not completely understood. The aim of this study is to assess if dysregulation of Brain derived neurotrophic factor (*BDNF*) through its gene methylation and / or brain oxidative stress state have a role .Fifteen female rats were divided into two groups; control and propylthiouracil hypothyroidism induced group. Offspring of each group were divided into three subgroups for assessment at 3, 7 days and 8 weeks. BDNF protein level, DNA methylation status of *BDNF* gene, malondialdehyde (MDA) and total antioxidant capacity (TAC) were measured in hippocampal brain tissue. Cognitive functions were assessed through Morris Water Maze (MWM) task to the subgroups of age 8 weeks. Results showed that BDNF protein level was significantly reduced in the hippocampi of maternal hypothyroidism offspring at the developmental stage (3 day and 7 day groups) (p value= <0.001in both groups), which was significantly associated with *BDNF* gene methylation state. Hippocampal MDA level was significantly increased in pups from hypothyroidism dams at the developmental stage (3 and 7 day groups) ($p<0.001^{*}$ in both groups). MWM task showed that 8 weeks-old maternal hypothyroidism offspring were significantly impaired in their performance (P<0.001) relative to age-matched controls. We conclude that, long-term memory deficits in hypothyroidism maybe caused by the interplay of the DNA methylation of BDNF gene, the excess oxidative stress and deteriorated antioxidant defense system in the brain hippocampus.
Keywords: Hypothyroidism, Cognitive function, BDNF, Oxidative stress, Gene methylation

INTRODUCTION

Thyroid hormones (THs) act on neuronal cyto-architecture, migration and differentiation of neural cells, synaptogenesis, and myelination [1, 2]. Their receptors are widely distributed in the central nervous system (CNS), therefore factors that interfere with thyroid functions or THs actions may produce deleterious effects on brain development in both humans and rodents [3].

An adequate supply of maternal THs must be sustained during pregnancy, to ensure normal neurological development. It regulates early fetal brain development in human and animal models [4, 5]. A complex process is employed for the delivery of THs to the fetus, requiring expression of brain thyroid hormone receptors, materno-fetal thyroid hormone and iodide transport, a system of endocrine feedback, the hypothalamic–pituitary–thyroid axis, and thyroid hormone metabolism by liver and brain deiodinase enzymes to ensure basal levels are sustained [6].

Maternal thyroxin (T4) insufficiency, even if moderate, has serious adverse consequences on the neurodevelopment of the offspring, including irreversible cognitive deficits [4, 5]. This occurs through molecular and functional alterations in the cerebral cortex, hippocampus and cerebellum of the offspring [7]. This mostly happens due to the delayed neuronal differentiation, and decreased both migration and proliferation [8, 9].

In the rodent brain, the striatum and hippocampus, have the highest expression of thyroid hormones receptors throughout the prenatal and neonatal period [10]. Regions involved in hippocampal formation are the dentate gyrus, CA3 and CA1Via mossy fibers, the cells of dentate gyrus project upon the dendrites of CA3 pyramidal cells. At the same time, these cells contribute a major input system (the Schaffer collaterals) to CA1. Neonatal thyroid hormone deficiency has been reported to interfere with the contact between mossy fibers and dendritic excrescences of CA3 pyramidal cells of the rat hippocampus [11]. It also reduces the density of pyramidal cells in the CA3 region [12] and leads to a

decrease in the total number of pyramidal cells in the CA1 region [13].

The neurotrophins have been shown to modulate many aspects of synaptic transmission and neural plasticity [14]. Long-term potentiation (LTP) in the CA1 region of the hippocampus is greatly reduced in BDNF homozygous and heterozygous mutant mice and can be rescued by exogenous *BDNF* [15, 16].

A gap in knowledge still exists concerning the exact mechanisms underlying the effects of hypothyroidism and *BDNF* on the developing brain. Epigenetic changes in the brain, which essentially include DNA methylation and histone modifications [17], have been associated with a range of neurobiological processes including the CNS development, learning, memory, and neurodegeneration [18]. DNA methylation consists of the transfer of a methyl group to position 5 of the cytosine pyrimidine ring of a cytosine guanine dinucleotide (CpG). When such modification happens in the gene promoter, it ultimately blocks the binding of transcription factors causing gene silencing [19].There are at least four *BDNF* gene promoters in the rat, which are differentially activated in response to various types of signaling events [20]. Limited evidence has demonstrated that DNA methylation and demethylation may play a role in regulating the transcriptional activity of Triiodothyronine (T3)-responsive genes [21].

The antioxidant imbalance has a high potential in the pathological consequences of hypothyroidism. Hypothyroidism induces a dysfunction of the respiratory chain in the mitochondria, accelerating the production of free radicals leading to oxidative stress (OS) and reducing the capacity of antioxidative defense [22].

Interestingly, there is evidence showing that OS might be increased in conditions where BDNF is decreased [23]. Moreover, some studies have reported that thyroid hormones have a profound effect on antioxidant defenses of rat brain during development [24, 25].

In this study, we hypothesized that experimental prenatal hypothyroidism might lead to dysregulation of BDNF protein of the off spring and thus would be a possible mechanism by which hypothyroidism permanently affects the brain development. Furthermore, such dysregulation of BDNF protein might be caused by changes in the DNA methylation status of *BDNF* gene in the rat hippocampus and /or due to an increase in the OS of brain tissues.

The aim of this work is to assess if maternal thyroid hormone insufficiency affects early CNS development in the offspring via dysregulation of BDNF protein. To assess DNA methylation status of *BDNF*, gene is a possible mechanism for such dysregulation. To assess the brain OS state as another possible mechanism for such dysregulation.

MATERIALS AND METHODS

This study was conducted on 15 female pregnant Spragu Dawley albino rats weighing between 200-250 grams. Ethical committee approval was obtained and animals were treated according to the ethical guidelines of Alexandria University. They were divided into two groups:

- Hypothyroidism induced group; experimentally-induced hypothyroidism pregnant female rats by receiving propylthiouracil (PTU) 4 parts per million (Sigma-Aldrich, St. Louis, MO) dissolved in distilled H2O .PTU treatment was started at the time the dam and stud were first placed together and continued until delivery . There is no specific regimen to induce maternal hypothyroidism. PTU may be started before the occurrence of pregnancy [26], or in already pregnant female rats [27]. While other studies started PTU treatment at the same time of mating [28]. In the present study the latter regimen was employed to avoid the possible effect of hypothyroidism on the female fertility. Hypothyroidism is frequently associated with indirect increases in circulating prolactin that in turn increase the risk of anovulation in women [29]. Furthermore, virgin rats treated with PTU presented irregular cycles, spontaneous pseudopregnancies and altered circulating ovarian hormones after the third estrous cycle that resulted in mammary development similar to that of mid pregnancy. In addition, rats mated 8 days after the start of antithyroid treatment produce smaller litters [30].
- Control group; control pregnant female rats that received distilled water [28]. Hypothyroidism was proved by measuring the thyroid stimulating hormone (TSH), free T3 and freeT4 levels using enzyme-linked immunosorbent Assay (ELISA) before delivery [31]. After giving birth, thirty of the offspring of each group were divided into 3 subgroups (n=10).
- Offspring of control dams group (OCG) were sub-divided into; sacrificed at day 3 (OCG3), sacrificed at day 7 (OCG7) and sacrificed at 8 weeks (OCG8).
- Offspring of hypothyroidism induced dams group (OHG) were sub-divided into; sacrificed at day 3 (OHG3), sacrificed at day 7 (OHG7) and sacrificed at 8 weeks (OHG8).

BDNF protein level was measured at the given time (3, 7 days and 8 weeks) using ELISA technique in the hippocampus [28, 32]. Additionally, DNA methylation status of *BDNF* gene was assessed by methylation specific polymerase chain reaction (MSP-PCR) after bisulfite modification of the extracted DNA [21]. Total antioxidant capacity (TAC) and oxidative damage parameter, Malondialdehyde (MDA) were assayed in

tissue lysate [33, 34]. Furthermore, Morris water maze (MWM) task was only performed to offspring [27] at age of 8 weeks (OCG8 and OHG8). As the rat hippocampal formation that participates in learning and memory, particularly that of a spatial nature, undergoes anatomical and neurophysiological maturation during the first 2 months of life. Rats less than 40 days of age are impaired in spatial navigation tasks [35]. Finally, TSH, free T3and freeT4 levels were estimated in all mentioned groups.

Blood samples were collected after the rats were sacrificed by cervical dislocation. Mixed arteriovenous blood was collected from the neck wound. Blood for serum samples was centrifuged (1500×g, 10 min, 4◦C), and the supernatant was aliquoted and stored at −80◦C.

Morris Water Maze task:

Training procedures

Each rat received four trials per day during three daily acquisition sessions. A trial was started by gently immersing the rat in the pool, facing the wall of the tank. There were four different starting positions (assigned North, West, South and East), each of which was used once in random order in a series of four trials. Starting positions were at the borders between the quadrants, and the escape platform was always in the same quadrant. Each rat was given 90 sec to locate and escape onto the platform. Once on the platform, it was allowed to stay there for 30 sec. If the rat failed to find the platform within 90 sec, it was guided to the platform by the experimenter and was also allowed to stay on the platform for 30 sec. After each trial, the rat was allowed to rest for 10sec in a cage next to the maze, whereupon the next trial was started. After completion of the fourth trial, the rat was gently dried with a towel and returned to its home cage. Between two sessions of individual rats, the water was stirred well to avoid possible olfactory tracks [36].

Probe trial procedure

After the fourth trial of the third daily session, the 'probe trial' was conducted on the fourth day. The platform was removed, and the swimming path of the rat was tracked for 60 sec. In the probe trial, all rats started from the south-west quadrant, i.e. opposite to the quadrant where the escape platform had been previously placed [36].

Recordings

The movements of the rat were recorded by a video camera. The data during the acquisition trials were averaged per rat within each session. Subsequently, the mean time latency to reach the platform and the length of the swimming path to boundary of the platform for each acquisition trial were scored. For the probe trial, the mean time spent in the target quadrant, the mean traveled path and the escape trials were assessed and compared between groups [37].

Tissue samples

Hippocampal tissue samples were stored at -80^{o}C for determination of BDNF protein concentration by ELISA assay and *BDNF* gene methylation state assay by MSP-PCR. OS markers were assessed in tissue lysate using calorimetric method.

BDNF ELISA [28]

Frozen (-80^{o}C) tissue samples were weighed and a 10% homogenate was made with lysis buffer (20.0 mM Tris-HCl, pH 8.0, containing 1.0 mM EDTA, 137.0 mM sodium chloride, 1.0 mM phenyl methyl sulfonyl fluoride, 10.0 µg/µl -aprotonin, 1.0 µg/µl leupeptin, 0.5 Mm sodium ortho-vanadate, 1.0% NP40 and 10% glycerol). The tissue homogenates were centrifuged at 14,000 x g for 30 min at 4^{o}C in a microfuge Eppendorf. Supernatants were stored at -80^{o}C [28]. For normalization of the BDNF results, aliquots of each sample lysate (supernatant) were analyzed in duplicate for total protein by the Lowry's method[38]**.** BDNF levels were determined with ELISA using Rat BDNF-specific polyclonal antibodies according to the manufacturer's instructions (Boster Immunoleader Cat No. EK030. The amount of BDNF protein in each sample was determined in duplicate in each experimental set of plates. The lysate was used for the ELISA assay without prior dilution according to preliminary testing so that sample values fell within the range of the standard curves for total protein and BDNF protein [38, 39].

DNA Methylation Assay (Modified) [21]

Genomic DNA was extracted using a spin column protocol (GeneJET Genomic DNA Purification Kit, (Fermentas. http://www.fermentas.com). DNA samples (1 ng to 2 µg) were modified with sodium bisulfite using the EpiTect Bisulfite Kit (QIAGEN Inc. http://www.qiagen.com). Two primer sets (Bioneer Inc. http:// www.bioneer.com) designed to distinguish between methylated and unmethylated *BDNF* gene sequences were used to carry out two separate PCRs.

Because the structure of *BDNF* gene consists of nine promoters, which were mapping upstream of the nine 5\-exons (eight 5\ non-coding exons, exon I, II, III, IV, V, VI, VII, VIII, and a common exon IX encoding a preproBDNF mRNA), however, exon II mRNA was reported to be the most abundant and can be detected during the developmental stage, thus, only BDNF promoter for exon II was investigated in the present study [21].

Detection of unmethylated *BDNF* DNA was performed using the following primers: forward (5\-GGGTAGTGATTTTGGGGAGGAAGTAT-3\) and reverse (5\-CAACCTCTATACACAACTAAATCCACC-3\). In addition, primer sequences to detect methylated DNA in the *BDNF* promoter for exon II were as follows: forward (5\-GTAGCGATTTTGGGGAGGAAGTAC-

3\') and reverse (5\'-CAACCTCTATACGCGACTAAATCCG-3\') [21].

A total of ≈100 ng of genomic DNA was used in PCR amplification performed in a total volume of 25 µl using the optimized primer volumes. Tubes were transferred to the thermal cycler (Whatman, Biometra, T personal. http://www.biometra.com). PCR was carried out using Muñoz, 2010 protocol [40]. Each sample was assayed in duplicate, one using the methylated primers pair and the other using the unmethylated primers pair. For optimization of PCR, annealing temperature was modified to 55°C. Blank control without DNA was included in each PCR assay as a negative control. Additionally, a positive control for each of methylated and unmethylated *BDNF* gene fragments was used. PCR products were analyzed using 2% agarose gel electrophoresis (Biometra Minicell Power Pack. http://www.biometra.com). PCR products were stained with ethidium bromide and visualized under the UV Transilluminator (Biometra. http://www.biometra.com). GeneRuler100bp DNA ladder served as a reference for DNA fragment size (Fermentas. http://www.fermentas.com).

Markers of oxidative stress

Parameters of OS profile were assayed by colorimetric technique using commercial kits (Biodiagnostic, Egypt) according to the manufacture instructions. The protein content of the supernatants was determined using Lowry's method [38]. TAC (mmol/L) [33] and oxidative damage parameter, MDA (nmol/gm tissue) [34] were assayed in tissue lysate.

Statistical analysis [41, 42]

Data were fed to the computer and analyzed using IBM *SPSS software package version 20.0.* Qualitative data were described using number and percent. Quantitative data were described using mean and standard deviation. Comparison between Unmethylated and Methylated genes categorical variables was tested using Fisher's Exact test. The distributions of quantitative variables were tested for normality. Comparison between two independent population were done using independent t-test comparison between different periods using ANOVA with repeated measures and Post Hoc test was assessed using Bonferroni adjusted. Correlations between two quantitative variables were assessed using Pearson coefficient. Significance test results were quoted as two-tailed probabilities. Significance of the obtained results was judged at the 5% level.

RESULTS

MWM task results

The OCG8 and OHG8 were examined on cognitive abilities known to depend on the hippocampal function using the MWM task. The repeated measures ANOVA was conducted to compare between the 3 days of training in both groups, while the student t-test was performed to analyze the difference between both groups during the test phase on the 4th day.

The 2 experimental groups showed a general decrease in the latencies to escape on the hidden platform (escape latency) over the course of the 3 days training (control: $F=51.632$, $p<0.001$, hypothyroid: $F=68.979$, $p<0.001$) (Fig. 1A). Similarly, the swimming distance to reach the platform was significantly decreased in both OCG8 and OHG8 (where $F=35.334$, $F=119.225$; respectively at $p<0.001$) (Fig. 1A, B).

However, the OHG8 were significantly impaired in their performance ($p<0.001$) as regard the escape and distance latencies relative to age-matched controls (OCG8). Indicating that while these animals were able to learn the task, they did not perform as good as controls. The net decreased escape latency in the acquisition phase (subtraction of the escape latency in 3rd day from that in the 1st day) of OHG8 (28.03±1.51s) was lower than OCG8 (41.47±3.66s).

In the probe trial test done on the 4th day, OHG8 traveled in the quadrant where the hidden platform was previously placed, significantly less time and less distance than OCG8 ($p<0.001$ for both groups) (Figure 1C,D). In a similar way, during the 60-s swim of the probe test, OHG8 tried significantly more time to escape from the swimming pool compared with their corresponding control offspring OCG8($p<0.001$) (Fig. 1 E).

These probe trial results confirmed further the poor performance of maternal hypothyroidism offspring during the training period. This may be probably due to deficits in the spatial learning and subsequently memory deficit to locate the place where the hidden platform was present.

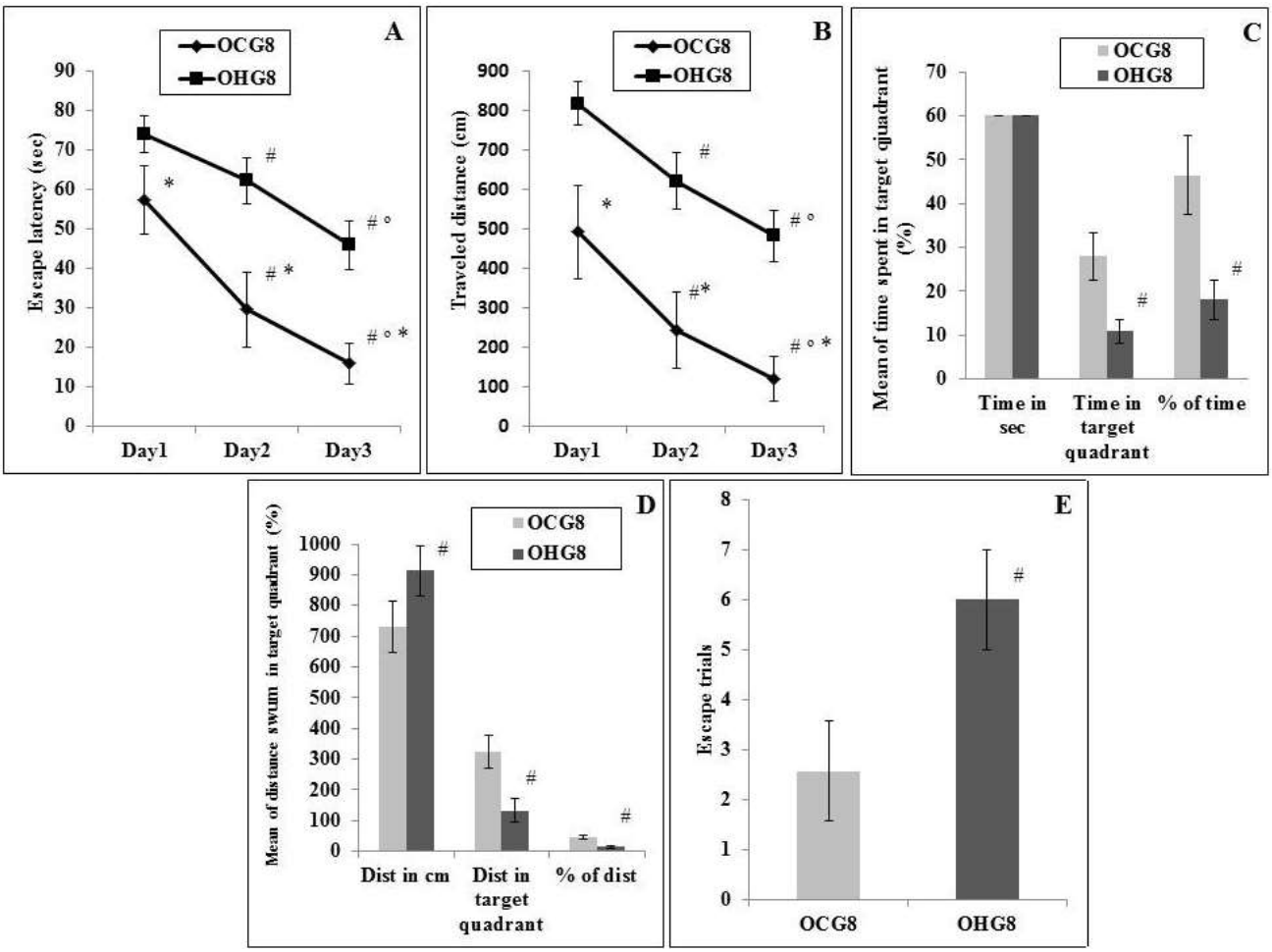

Fig. 1: Spatial learning in the Morris Water Maze test

(A): Escape latency (sec) and (B): distance traveled (cm); to reach the platform during 3 days of training for 8 weeks-aged offspring of control (OCG8) and hypothyroid group (OHG8) using repeated measures ANOVA. # $p \leq 0.05$ vs day 1, ° $p \leq 0.05$ vs day 2; in the same group, * $p \leq 0.05$ vs controls for each corresponding day. (C): Percent of time spent, (D): Percent of distance traveled; in target quadrant, and (E): Number of trials to escape from the circular pool (Student t-test). # $p \leq 0.05$ vs control group. All data are expressed as mean ±SD values for 10 offspring per group.

Thyroid hormones

Manipulation of thyroid status in dams produced the expected effects on circulating levels of TH. Maternal hypothyroidism was proved by measuring the TSH, freeT3 and T4 level by ELISA technique before delivery (Table 1).

Propyl thiouracil (PTU) induces hypothyroidism by inhibiting thyroid iodination and has been used as a typical thyroid hormone synthesis inhibitor [43]. Exposure to 4 parts per million PTU from the time the dam and stud were first placed together until delivery significantly reduced circulating free T3 and free T4 levels in the offspring at the developmental stage (OHG3 and OHG7 day groups, $p < 0.001$ in both groups for the two parameters). In accordance to that, circulating TSH levels were significantly increased in the offspring at the developmental stage (OHG3 and OHG7 groups, $p < 0.001$ in both groups) (Table 2). However, there is no significant difference in the levels of T3, T4and TSH levels between OCG8 and OHG8.

Table (1): Comparison between Thyroid hormones levels (TSH, free T3, free T4) in control & hypothyroid dams)

	Control dams	Hypothyroid dams	p
TSH (mIU/ml)	0.77 ± 0.15	1.63* ± 0.21	<0.001*
Free T3 (pg/ml)	1.95 ± 0.35	0.90* ± 0.14	<0.001*
Free T4 (pg/ml)	14.12 ± 1.92	1.79* ± 0.72	<0.001*

p: p value for p: p value for Student t-test for comparing between control and hypothyroid induced dams regarding the thyroid hormones levels (TSH, free T3, free T4)

*: Statistically significant at $p \leq 0.05$

Table 2: Comparison between the all studied groups according TSH, T3and T4 levels

	OHG3	OCG3	OHG7	OCG7	OHG8	OCG8
TSH(uIU/ml)	1.33 ± 0.09	0.71$^{\#}$ ± 0.10	1.31 ± 0.07	0.73$^{\#}$ ± 0.07	0.26 ± 0.11	0.23 ± 0.09
^{t}p	<0.001*		<0.001*		0.517	
T3(pg/ml)	0.09 ± 0.01	0.26$^{\#}$ ± 0.07	0.11 ± 0.02	0.24$^{\#}$ ± 0.06	1.41 ± 0.14	1.40 ± 0.15
^{t}p	<0.001*		<0.001*		0.878	
T4(pg/ml)	0.81 ± 0.08	1.18$^{\#}$ ± 0.05	0.81 ± 0.06	1.19$^{\#}$ ± 0.05	13.80 ± 2.53	14.40 ± 2.37

Data are expressed using mean ± SD (# Significant difference between OHG3 and OCG3,OHG7 and OCG7,OHG8 and OCG8, ^{t}p: p value for Student t-test

BDNF protein level and gene methylation

Regarding BDNF protein, maternal hypothyroidism significantly reduced BDNF protein level in the offspring' hippocampi at the developmental stage, OHG3 and OHG7 groups, as compared to their controls p ≤0.001 On the other hand, there was no significant difference in the BDNF protein level between OHG8 and their corresponding controls OCG8 (p=0.088) (Fig. 2A).

The results showed a significantly reduced BDNF protein level with *BDNF* gene methylation state. Such effect was demonstrated in OHG3_where BDNF protein showed a mean value of 48.21±15.34 pg/mg protein in the unmethylated gene state and a mean of 30.84±6.08 pg/mg protein in meythylated gene state (p = 0.034*) (Fig. 2B) MSP analysis for *BDNF* gene promoter methylation is shown in Fig. 2C.

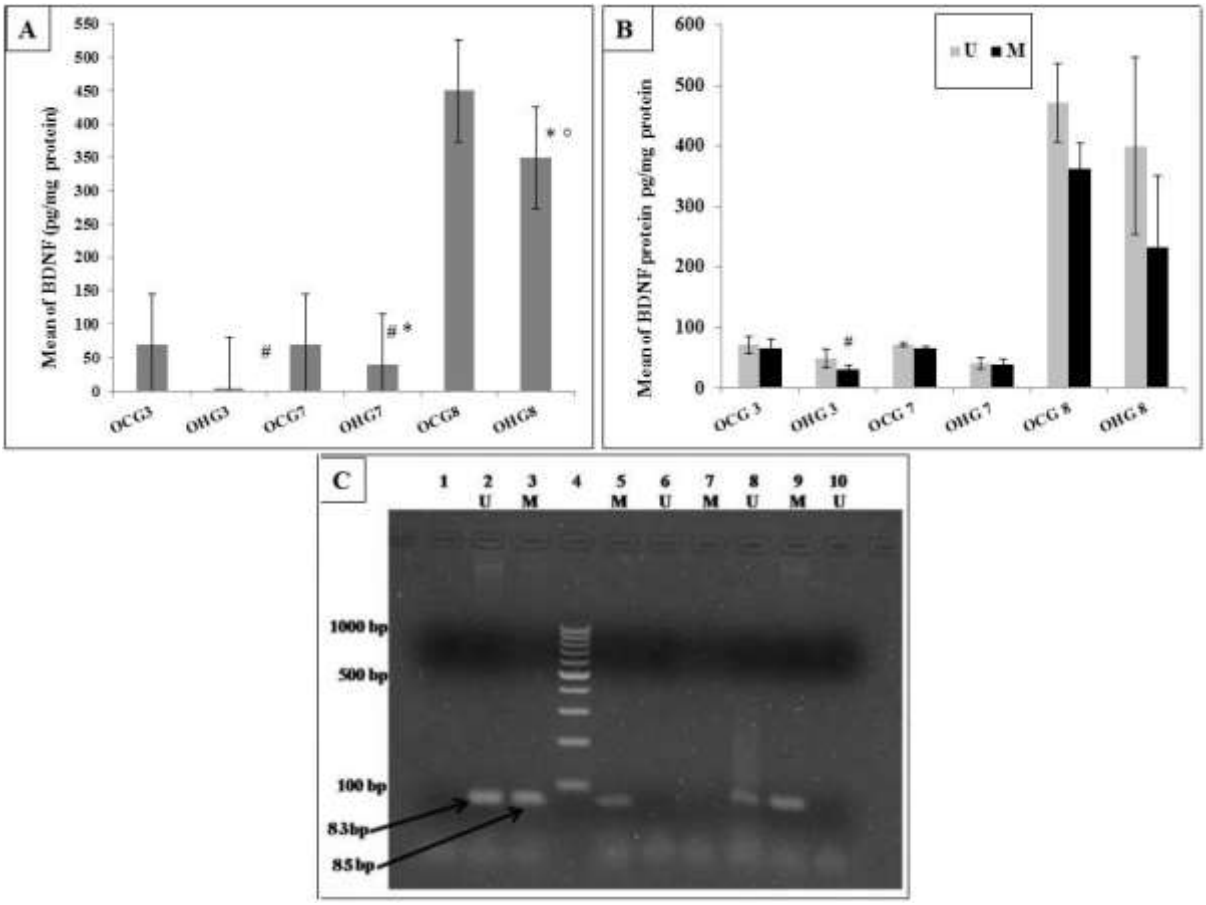

Fig. 2: Effect of PTU-induced neonatal hypothyroidism on the levels of BDNF protein, and BDNF gene methylation, (A): hippocampal BDNF protein levels (pg/mg protein) in offspring of euthyroid (OCG) and hypothyroid groups (OHG) at the age of 3 days, 7 days, and 8 weeks respectively. (Student t-test, n=10 per group). # p≤ 0.05 vs age-matched controls, * p≤ 0.05 vs offspring of hypothyroid group at 3 days, ° p≤ 0.05 vs offspring of hypothyroid group at 7 days. Data are shown as mean ±SD values, OCG3=69.36±13.54, OHG3=3.79±13.40, OCG7=68.30±5.13, OHG7=39.51±8.34, OCG8=449.54 ± 75.16 and OHG8=349.40±154.50. **(B):** Mean values of BDNF protein in relation to BDNF gene methylation state in the hippocampus of offspring of control and hypothyroid groups at the different ages of the study (n=10 per group at each age) # p≤ 0.05 vs unmethylated BDNF gene in 3 days-aged off springs of hypothyroid group. Fisher Exact test was used to analyze BDNF gene methylation. **(C):** MSP analysis of BDNF gene. Lane 1 shows

the negative control for PCR. Lanes 2 and 3 show the positive controls for unmethylated and methylated BDNF gene, respectively. Lane 4 shows the 100bp DNA ladder. Each case is represented with two lanes, one for the methylated BDNF gene amplified fragment (85bp) and the other for the unmethylated BDNF gene amplified fragment (83bp). Two cases with methylated BDNF gene are shown in lanes [5, 6] and [9, 10]. A case with unmethylated BDNF gene is shown in lanes [7, 8].

Oxidative stress state

Concerning the OS state, hypothyroidism state was accompanied by significantly increased MDA levels in the offspring hippocampi. This difference was demonstrated only in the early life stage where hypothyroidism was shown through thyroid hormones and TSH levels (3 and 7 day groups) ($p<0.001^{*}$ in both groups) (Fig. 3A).

Student T test was conducted to explore the possible effect of *BDNF* gene methylation on BDNF protein, OS markers and THs.

The correlation study revealed a significant negative correlation between BDNF protein level and MDA level in OHG8 ($r = -0.559$, $0.665^{;}$ $p = 0.093$, 0.036 respectively) (Fig. 3C).

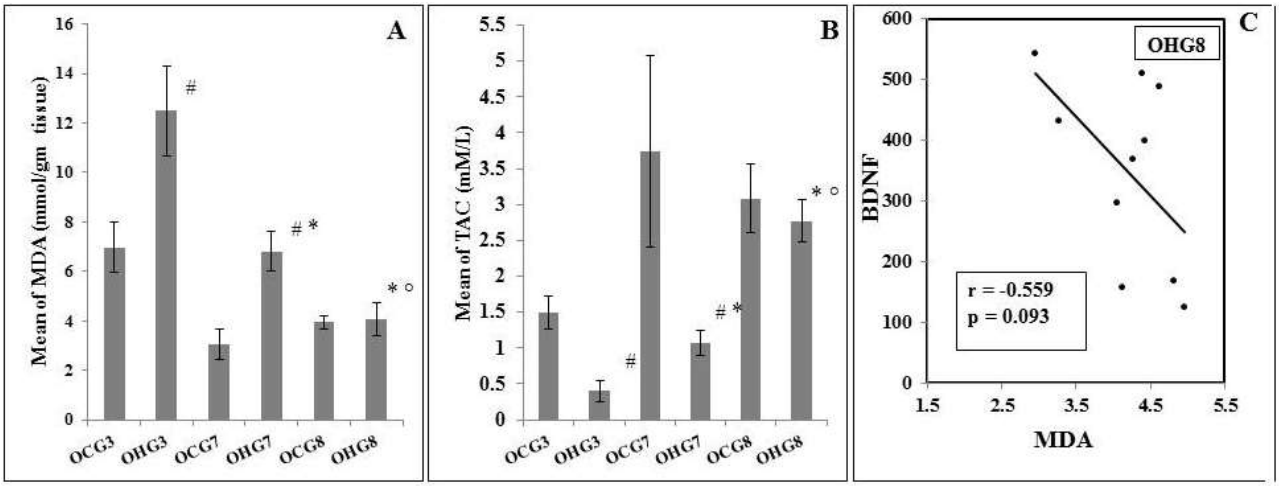

Fig. 3: Effect of PTU-induced neonatal hypothyroidism on hippocampal

(A) Malonaldehyde (MDA) content (mmol/gm tissue), of 3days, 7days and 8weeks-old offspring of control and hypothyroid dams using student t-test. (n= 10 rats per group). Data are presented as mean ±SD ,OCG3=6.96 ± 1.01 , OHG3=12.49 ± 1.83, OCG7= 3.06 ± 0.62, OHG7= 6.81 ± 0.81, OCG8=3.94 ± 0.27, OHG8=4.08 ± 0.67 (B) Total antioxidant capacity (TAC) (mM/L) of 3days, 7days and 8weeks-old offspring of control and hypothyroid dams using student t-test. (n= 10 rats per group). Data are presented as mean ±SD, OCG3=1.49± 0.23, OHG3=0.40 ± 0.15, OCG7=3.74 ± 1.33,OGH7=1.07 ± 0.17, OCG8=3.08 ± 0.48, OHG8=2.77 ± 0.29. # $p<0.05$ vs age-matched controls, * $p< 0.05$ vs 3days offspring of hypothyroid group, and ° $p< 0.05$ vs 7days offspring of hypothyroid group. Data are presented as mean ±SD **(C):** The correlation between hippocampal BDNF protein and MDA content in 8weeks-aged offspring of hypothyroid group was a negative correlation where r (Pearson correlation coefficient) = -0.559 and p=0.093.

DISCUSSION

Thyroid hormones are essential for brain maturation and function throughout life [44]. Previous studies showed that perinatal deficiency of THs or impairment of its signaling severely affect brain development [45 ,46]. But the exact mechanism(s) by which these hormones impair brain development are only partly uncovered.

In the present study, we demonstrated the possibility of BDNF protein dysregulation through its gene methylation and / or the brain OS state to have a role in the mechanism(s) of the cognitive function impairment in hypothyroidism.

To impair thyroid function in a manner comparable with earlier studies [47], we administered PTU to dams. PTU has been shown to induce functional brain deficits [48, 49], even at doses that produce only a relatively mild, transient hypothyroidism in the mothers [48, 50]. In the current work, PTU showed effects on the offspring in the early developmental stages (OHG3 and OHG7 groups). On the other hand, the offspring returned to euthyroid state by the age of 8 weeks (Table 2).

The PTU effect may have been more pronounced in the offspring because the rat thyroid gland does not start to develop until shortly before birth, so the fetus is critically dependent on maternal T4 [51, 52].

The hippocampus is the part of the brain that is related to cognitive ability, including learning and memory ability [53]. The CA1 area in the hippocampus

is important for spatial learning. The memories are coded and incorporated, and then stored plastically by synapse [54]. The hippocampus is dependent on adequate supplies of THs during development and adulthood. In the present study, we focused on the developing rat hippocampus which was confirmed to be one of the highly sensitive regions to THs status [55]. Chakraborty *et al*. [28] reported that PTU treatments altered neurotrophin levels in the early postnatal brain in a region-specific manner: hippocampal neurotrophin levels were reduced, whereas cerebellum and brain stem neurotrophin levels remained unchanged.

In accordance to this, our results demonstrated that maternal hypothyroidism significantly reduced BDNF protein level in the hypothyroid offspring hippocampi at the developmental stage (OHG3 and OHG 7). Alteration in BDNF levels in early life contributes to the adverse neurodevelopmental effects that occur after prenatal hypothyroidism as cognitive and behavioral alterations in the rats offspring were shown when tested through the MWM task at the age of 8 weeks. In this task, the time to reach the hidden platform in offspring born to normal & hypothyroid mothers became shorter as the number of training trials increased. However, the offspring of the hypothyroid dams group had longer escape latency in the acquisition phase and shorter duration in target quadrant in the probe trial phase than that of age-matched control ones. This impairment in learning capacity was associated with thyroid dysfunction and a reduced level of BDNF at the early developmental stage of 3 and 7 days, although euthyroid state and normal BDNF protein levels were reached by the age of 8 weeks.

These findings suggest that THs deficiencies during the gestational and early postnatal period result in severe neurological deficits that last even if these hormonal and protein levels returned back to normal levels later in life.

In agreement with our data, Zhang *et al*. [56] reported similar cognitive deficits in offspring born to mothers received low and excessive iodine during gestation, where the rat offspring undergone MWM task on postnatal day40-44. Also, Lui *et al*. [54] and Wang *et al*. [57] reported that maternal subclinical hypothyroidism decreased BDNF expression in rat pup hippocampi and impaired spatial learning; offspring required more time during the MWM task to find the hidden platform, compared with offspring from normal control mothers.

In our study, the cognitive neurobehavioral dysfunction of the offspring was further confirmed by the longer distance swum by the offspring of the hypothyroid group to reach the hidden platform during the training period when compared to normal control rats. In the test phase, the distance swum in the quadrant where the platform was present was less in the offspring of the hypothyroid dams group than in the control group.

The total long distance swum by the rats during either the acquisition or the test phase of MWM task revealed that there was no locomotor impairment. Consistent with this finding, Ge J F *et al*. [58] demonstrated that subclinical hypothyroidism did not affect the motor functions in rats, inspite of the impaired learning and memory ability in the MWM task.

Consistent with the study by Opazo *et al*. [59], our data showed that the latencies of all groups had decreased with the increasing of training trials. These data suggest that progeny of maternal hypothyroidism offspring need more training for a prolonged period to establish or reinforce neuronal connections required for spatial learning.

Transient PTU-dependent reduction in hippocampal BDNF in early postnatal life of rats which was followed by a period of relatively normal BDNF levels were in accordance to Lasley et al, who found no detectable effect of PTU on hippocampal BDNF, 14 d after birth [47]. MacLusky *et al*. [60] and Matthews *et al*. [61], added that hormonal effects during early development often remain latent until later in life, when they may reemerge as changes in hormone sensitivity.

In a trial to reveal the possible factors affecting the BDNF protein levels after maternal hypothyroidism, *BDNF* gene promoter methylation and OS states were explored.

Regarding *BDNF* gene promoter methylation, the present study showed that this state was accompanied by a significantly lower BDNF protein level in 3 day hypothyroid offspring (Fig. 2B). DNA methylation and histone acetylation are major epigenetic modifications that play critical roles in gene expression reprogramming during development and differentiation. DNA methylation is an essential mechanism for the normal development of different organisms including mammals and plants, and has been implicated in the silencing of gene expression [62]. Sui *et al*. [21] and Xiaohui *et al*. [63], reported that epigenetic modification of *BDNF* gene might be a mechanism for the devastative effects of perinatal hypothyroidism on the CNS. They added that some thyroid hormone-responsive genes may undergo epigenetic modification of DNA methylation at the very early developmental stage. Moreover, other studies indicated that the level of methylation was reversible as the thyroid state was altered [64, 65], consistent with our results.

However, the time window of the methylation of *BDNF* gene and the alteration of BDNF protein levels was not matched in all hypothyroid groups. These results suggest that epigenetic modulation of DNA

methylation might partly contribute to the alterations of thyroid hormone-responsive gene expression, some other factors may influence their transcriptional activities.

With respect to the OS state, hypothyroidism state was accompanied by significantly increased MDA and significantly decreased TAC levels in the offspring hippocampi. This difference was demonstrated only in the early life stage (3 and 7 day groups. Petrulea *et al*. [66] highlighted that THs have well-known effects on mitochondrial oxygen consumption, but data about how hypothyroidism affects OS are controversial. Furthermore, Villanueva *et al*. [67] reported that THs are related to OS not only by their stimulation of metabolism but also by their effects on antioxidant mechanisms. Besides, Lakshmi *et al*. [68], explained the relation between hypothyroidism and OS by the associated dyslipidemia which induces OS. Bhimte *et al*. [69], further added the finding of reduced TAC in hypothyroid patients which reflects increase OS in hypothyroidism.

On the other hand, *BDNF* gene methylation had no significant effect on any of MDA and TAC which further confirms the role of other factors in regulating BDNF protein level and functions. The previous findings could be explained by: the potential protective effect of BDNF, being a major neurotropin, on the OS state and/or the potential role of oxidants and antioxidants in regulating BDNF protein levels.

In accordance to our proposed theories, Kapczinski *et al*. [70] reported that BDNF levels were decreased in situations of increased OS. That was suggested by decreased cAMP response element binding (CREB), increased nuclear factor-kB (NF-kB) DNA-binding activity or energy depletion due to OS state. Additionally Abedel Haffez *et al*. [22], showed that BDNF level significantly decreased while OS index significantly increased in both the hippocampus and cerebellum in offspring born to hypothyroid dams. Moreover, selenium, a well-known trace element, caused upregulation of BDNF protein and had a potent antioxidant effect.

CONCLUSION

In summary, the current results support the view that used dose of PTU throughout pregnancy represent a valuable model to evaluate the effects of fetal hypothyroidism. Besides that, measurements of neurotrophin levels as well as OS markers could be useful in assessing the potential effects of prenatal exposure to drugs and chemicals that interfere with thyroid function.

The long-term memory deficits of offspring born to maternal hypothyroidism dams likely related with the decrease in BDNF protein level in hippocampi at the early developmental period. These deficits persist even with the return of BDNF protein level to normal late in life. DNA methylation may partly play a role in the perinatal hypothyroidism-induced regulation of BDNF expression in the developmental rat hippocampus. Additionally, the highlighted interplay between the BDNF protein level, the excess OS and deteriorated antioxidant defence system in the brain hippocampus might be another mechanism underlying the adverse neurological cognitive deficits observed in hypothyroidism.

List of abbreviations

Brain-derived neurotrophic factor (BDNF), Central nervous system (CNS), Enzyme-linked immunosorbent Assay (ELISA), Malondialdehyde (MDA), Methylation specific polymerase chain reaction (MSP), Morris Water Maze (MWM), Oxidative stress (OS), Propylthiouracil (PTU), Triiodothyronine (T3), Thyroxine (T4), Thyroid hormones (THs), Thyroid stimulating hormone (TSH), Total anioxidant capacity (TAC).

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