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Prenatal thyroxine treatment promotes anxiolysis in male Swiss mice offspring

Roberto Laureano-Melo[a,*], Janaina Sena de Souza[b], Rodrigo Rodrigues da Conceição[b], Josie Marcelle Lira Albuquerque[c], Nayana Coutinho Rodrigues[a], Bruno Guimarães Marinho[a], Emerson Lopes Olivares[a], Gisele Giannocco[b], Wellington da Silva Côrtes[a]

[a] Multicenter Graduate Program in Physiological Sciences, Department of Physiological Sciences, Institute of Biological and Health Sciences, Federal Rural University of Rio de Janeiro, Seropédica, Brazil
[b] Molecular and Translational Endocrinology Laboratory, Department of Medicine, Federal University of São Paulo, São Paulo, SP, Brazil
[c] Federal Fuminense University, Niterói, RJ, Brazil

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ABSTRACT

The proper functioning of the maternal thyroid plays a crucial role in fetal development. Thus, the aim of our study was to verify how maternal hyperthyroidism is able to change behavioral parameters in mice offspring during adulthood. For this purpose, pregnant Swiss mice (n = 24 and ~35 g) were randomly assigned into two groups: a control and a thyroxine (T4)-treatment group. The control was treated with 0.9% saline, while the treatment group received T4 (200 μg/kg, *s.c.*) once daily during the entire pregnancy period. After completing 70 days of life, a part of male offspring underwent a battery of tests, including open field, dark-light box, elevated plus maze, marble burying, rotarod and tail suspension tests. The other male pups were euthanized, being hippocampus and serum collected for RNA analysis and hormones measurement, respectively. Statistical analysis was performed using Student's *t*-test, and the means were considered significantly different when $p < 0.05$. In adult offspring, a significant decrease was observed for serum T3 in treated group. It was demonstrated that the T4 group had an increase in total distance traveled in an open field test. In the elevated plus maze test, we observed a higher time in opened arms as well as an increased in percentage of entries in these arms. In the hippocampus, T4 offspring had a higher expression of tryptophan hydroxylase 2 (TPH2), serotonin transporter (SERT) and glutamate decarboxylase 67 (GAD 67) in comparison to controls. These findings suggest that prenatal T4 treatment alters hippocampal serotonergic and GABAergic systems, promoting anxiolysis in male adult offspring.

1. Introduction

The prevalence of some diseases in adulthood presents a significant correlation with homeostatic disturbances during the fetal stage (Barker, 1990). This process is known as fetal programming (Godfrey and Barker, 2001). One of the main physiological factors that can affect the ontogeny of different physiological systems is maternal endocrine function (Fowden and Forhead, 2009). Therefore, the proper functioning of the maternal thyroid plays a crucial role in fetal development (Stricker et al., 2007). To reinforce this fact, thyroid function in humans is established only from the 16th week of pregnancy (Obregon et al., 2007). Until this period, the maternal thyroid is the major source of thyroid hormones (THs) to the fetus, being responsible for approximately 30% fetal bioavailability of these hormones (Calvo et al., 2002).

The prevalence of overt hyperthyroidism ranges from 0.2% to 1.3% in iodine-sufficient parts of the world (Madariaga et al., 2014). A 20-year follow-up of the Whickham cohort reported an incidence of 80 cases/100,000 women/year (Vanderpump et al., 1995; Hollowell et al., 2002). A meta-analysis of European studies showed a mean prevalence of 0.75% and an incidence of 51 cases/100,000 women/year (Madariaga et al., 2014). In the United States, the National Health and Nutrition Examination Survey reported overt hyperthyroidism in 0.5% of the population while 0.7% had subclinical hyperthyroidism (Hollowell et al., 2002) with an overall prevalence of 1.3%. Studies from many other countries have all reported comparable incidence and

* Corresponding author at: Departamento de Ciências Fisiológicas, Instituto de Biologia, Universidade Federal Rural do Rio de Janeiro, BR 465, Km 7, 23897-000 Seropédica, RJ, Brazil.
E-mail addresses: laureanomelor@gmail.com, laureanomelor@ufrrj.br (R. Laureano-Melo).

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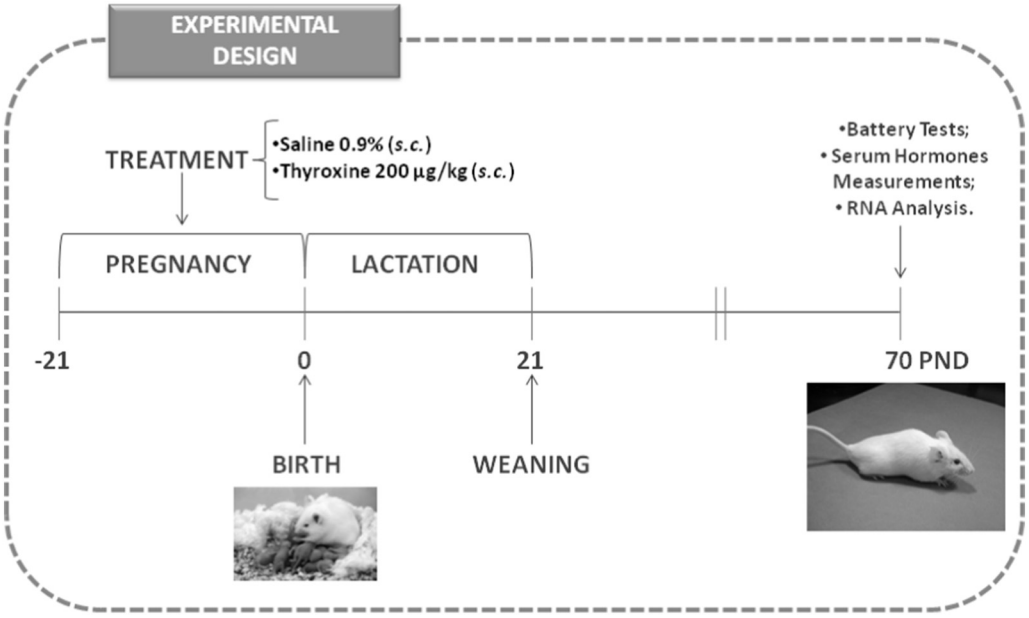

Fig. 1. Schematic representation of the experimental design. Pregnant females were treated with saline or thyroxine during all pregnancy. At 21 postnatal days of life (PND), the animals were weaned. After completing 70 days of life, a part of the male offspring underwent to a behavioral analysis (n = 12). On the other hand, male offspring that were not exposed to behavioral tests were euthanized (n = 6). In these animals, serum hormones measurements and RNA analysis were performed.

prevalence rates.

Regarding hyperthyroidism during pregnancy, the prevalence of Graves disease, with ranges between 0.1% and 1%, and transient gestational hyperthyroidism syndrome, with ranges between 1 and 3%, have been reported (Krassas et al., 2015). This endocrine disorder during pregnancy is related to recurrent miscarriages, reduced intrauterine growth, and cardiovascular and neurological abnormalities (Drews and Seremak-Mrozikiewicz, 2011). Hyperthyroidism is also known to directly or indirectly affect cell migration, dendrite and axon outgrowth, synapse formation, myelination and gliogenesis during the prenatal period (Lauder, 1983; Oppenheimer and Schwartz, 1997; Bernal, 2005).

Scientific articles have appeared looking at the relationship between psychiatric diseases and thyroid hormones (Kirkegaard and Faber, 1998; Hage and Azar, 2012). Recently, Andersen and coworkers observed that children born to mothers with hyperthyroidism had an increased risk of neurologic and psychiatric diseases later in life, including seizures, attention-deficit hyperactivity disorders and schizophrenia (Andersen et al., 2015). However, the causal relationship, which may later predispose the offspring to developmental neurobehavioral disease, is not well known. According to an animal study published by Ahmed and coworkers, this pathophysiological state may be explained by neurochemical disturbances in different brain regions of the offspring (Ahmed et al., 2010).

In this context, the hippocampus is a thyroid hormone receptor–rich region of the brain, being also sensitive to developmental thyroid hormone disruption. Consequently, alterations in thyroid hormone levels have been reported to impair hippocampal-associated memory, neurogenesis and affective behavior (Alzoubi et al., 2009; Da Conceição et al., 2016; Gilbert et al., 2017). Previous studies have demonstrated changes in expression of hippocampal brain-derived neurotrophic factor (BDNF) in response to thyroid disturbances in developing and adult rats (Sui et al., 2010; Chakraborty et al., 2012; Yu et al., 2015). Moreover, other studies described that thyroid hormones promotes genomic and nongenomic actions in hippocampal neurotransmitter release, an effect that could have an important role in their modulation of brain function in physiological and pathological states (Vara et al., 2002; Losi et al., 2008; Puia and Losi, 2011).

Based on this assumption, as thyroid hormones are involved in a number of events during early brain development, we also believe that maternal hyperthyroidism may promote fetal programming of affective systems. Thus, the aim of our study was to verify how maternal hyperthyroxinemia is able to change behavioral parameters in the offspring of mice during adulthood. A molecular biology study was also performed to investigate the expression of genes related to neuroplasticity and different neurotransmitter systems in hippocampus.

2. Materials and methods

2.1. Experimental design

Swiss Webster mice of 60 days of age (~35 g) derived from the Federal Rural University of Rio de Janeiro colony were used in this protocol. After an acclimatization period of 15 days, the mice were housed in plastic cages (35 cm × 50 cm × 20 cm) and mated together with a ratio of two females to one male. Day 1 of pregnancy was determined by the presence of spermatozoa in a vaginal smear. Following confirmation that mating had occurred, females were randomly assigned into two groups (n = 6 per group): control and thyroxine-treated groups. The control was treated with 0.9% saline, while the treatment group received thyroxine (Sigma-Aldrich®) at a dose of 200 µg/kg b.w., both by subcutaneous routes. This dose was chosen to induce hyperthyroidism in accordance with data contained in the literature (Ahmed et al., 2012) and the administration route was used to prevent low drug absorption caused by food consumption. Thyroxine solution are prepared in 40 mM sodium hydroxide and diluted in saline for injections. The injections were performed at 8 a.m. once a day and the treatment was performed throughout the pregnancy period.

To demonstrate the accuracy of our protocol, half of dams were euthanized at the end of pregnancy for hormonal measurements. The other half continued in the experiment. After birth, the offspring were standardized into group maximums of 10 pups (5 males and 5 females) per female. At postnatal day (PND) 21, the female pups were euthanized and the male ones were weaned to eight or ten animals per plastic cage (35 cm × 50 cm × 20 cm). After completing 70 days of life, a part of the male offspring underwent a behavioral analysis (n = 12). On the other hand, male offspring that were not exposed to behavioral tests were euthanized (n = 6). In these animals, serum was collected to assess T3, T4 and corticosterone levels while the hippocampus was dissected from the whole brain under cold plate and kept at −70 °C for RNA analysis (Fig. 1). All animals used in this work were housed at a controlled temperature (20 ± 2 °C) with daily exposure to a 12 h light-dark cycle and free access to water and commercial rodent diet.

2.2. Ethics committee

This investigation was carried out according to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No.85-23, revised 1996) and was approved by the institutional animal welfare committee in accordance with pertinent Brazilian legislation under Protocols number: 12935-007/2017.

2.3. Behavioral tests

Between 70 and 80 days of age, the offspring underwent a battery of tests, including open field, dark-light box, elevated plus maze, marble burying, rotarod and tail suspension tests. The tests were performed at two-day intervals, and the order of tests within the battery was determined according to the progressive degree of invasiveness.

All testing was performed between 8 and 11 a.m. During each test, the experimenter remained outside the testing room. Each test was recorded, and behavior parameters were analyzed by at least two observers. However, the open field test was analyzed using the ANY-Maze data collection program (Stoelting Co., Wheat Dale, IL, USA).

2.3.1. Open field test

Each mouse was placed individually in the center of a white acrylic cage (30 cm × 30 cm × 15 cm) and allowed to explore the cage for 5 min. During this time, total distance traveled, number of rearings (standing on hind legs with paws pressed against the wall of the arena), time of grooming, time in center zone, center distance (the distance traveled in the center of the arena), center ratio (center distance to total distance ratio) and freezing time were assessed. At the end of testing, the number of fecal pellets was also counted and the arena was cleaned with a 10% ethanol solution. In this test, locomotor activity is indicated by the total distance traveled in the apparatus, while the vertical activity is assigned by number of rearings. In relation to defecation, this parameter appeared, under some circumstances, to represent not an emotional response but a form of scent marking in male mice (Archer, 1973; Walsh and Cummins, 1976). Lastly, anxiety-like responses were linked to time in the center zone, center ratio and freezing time.

2.3.2. Rotarod test

The rotarod test was performed by placing a mouse on a rotating drum and measuring the time each animal was able to maintain its balance while walking on top of the rod. Mice underwent 4 trials of up to 5 min, and the inter-trial interval was 30–40 min. The speed of the rotarod was 10 rpm and the height from the ground was 50 cm. Some animals were attached to the rotarod axis. The latency to the first fall was recorded for each trial, and the animals were allowed to fall three times. Some mice were attached to the rotating axis as they begin to fall and rode completely around the rod. For these animals, the latency to the first fall was still considered. In this protocol, coordination and motor skill learning was evaluated (Shiotsuki et al., 2010).

2.3.3. Light-dark box test

The light/dark test is based on the innate aversion of rodents to brightly illuminated areas and on the spontaneous exploratory behavior of rodents in response to mild stressors such as novel environment and light (Crawley and Goodwin, 1980). The animals were individually placed in an acrylic cage (45 cm × 27 cm × 27 cm) unequally divided into two chambers by a black partition containing a small opening. Two thirds of this chamber was illuminated (200 lx) and the remaining section was closed and dark. Mice were placed inside the dark side and allowed to freely move between the two chambers for 5 min. During this time, the time spent in light side, number of transitions and latency to first entry into the light side were recorded. In this test, these parameters are associated to anxiety-like behavior.

2.3.4. Elevated plus maze test

The elevated plus maze test reflects a conflict between the rodent's preference for protected areas and their innate motivation to explore novel environments (Pellow et al., 1985). The apparatus, consisting of four arms (30 cm × 5 cm), was placed 50 cm above the ground. Two opposite arms were delimited by acrylic, vertical walls, whereas the other two opposite arms had unprotected edges (open arms). Mice were placed in the center of the maze and allowed to move about freely for 5 min. During this period, the cumulative time and frequency of entries into open and closed arms were registered. An arm entry was defined as the entry of four paws into an arm. From the obtained results, the percentage of entries into opens arms and time in the central platform were calculated. In this protocol, anxiety-like behavior was associated to a higher time and percentage of entries in opened arms.

2.3.5. Marble burying test

In both natural and laboratory conditions, rodents spontaneously use available bedding material to bury unpleasant sources of discomfort present in their home environment (Archer et al., 1987). In this protocol, twenty-five glass marbles were evenly spaced in the plastic cage (35 cm × 50 cm × 35 cm) in the presence of the mouse. After 30 min, marbles that were up to two-thirds covered were counted. The number of marble buried is directly related to anxiety response.

2.3.6. Tail suspension test

This test is based on the observation that rodents, after initial execution of escape-oriented movements, develop an immobile posture when placed in an inescapable and stressful situation. In the tail suspension test, the plight involves hemodynamic stress caused by being hung by the tail (Porsolt et al., 1977; Thierry et al., 1986). In this protocol, the mice were suspended 100 cm above the stand by adhesive tape placed approximately 1 cm from the tip of the tail. The test was videotaped for 5 min. During this period, the time of immobility and latency to the first immobility episode were evaluated. The immobility assumes a low resilience and consequently a high level of depression-like behavior.

2.4. Serum hormone measurements

Blood samples were centrifuged (3000 rpm for 20 min), and the serum was used for corticosterone, T4 and triiodotironine (T3) determination by commercial kits for radioimmunoassay following the manufacture's recommendations (MP Biomedicals, LLC. USA).

2.5. RNA analysis

Total RNA was extracted using a standard method (TRIzol reagent; Invitrogen, Carlsbad, CA, USA). The RT-PCR analyses were carried out using 1 μg of total RNA extracted from the hippocampi of 70 PND male pups using a Superscript III kit (Invitrogen).

Real-time RT-PCR analyses were performed in a fluorescent temperature cycler (Applied Biosystems 7500; Life Technologies Co., Carlsbad, CA, USA) according to the recommendations of the manufacturer. Briefly, after initial incubation at 50 °C for 2 min and 95 °C for 10 min, reactions were cycled 40 times using the following parameters for all genes studied: 95 °C for 15 s, 60 °C for 30 s and 72 °C for 45 s. SYBR Green (Applied Biosystems, Foster City, CA, USA) fluorescence was detected at the end of each cycle to monitor the amount of PCR product formed during that cycle. We used genes that coded proteins related to GABAergic system (Gad 65, Gad 67, α3 and γ2 subunits of $GABA_A$ receptors), serotonergic system (Tph2, Sert, 5HT1a receptor) and neuroplasticity (Bdnf and Trkb). Primers used for the amplification of cDNAs of interest were synthesized by Extend Biotecnologia Ltda. The forward and reverse primers' sequences are listed in Table 1.

We determined relative mRNA levels ($2^{-\Delta\Delta Ct}$) by comparing the PCR cycle threshold (Ct) between groups, after correcting for the

Table 1
List of primers used for qRT-PCR.

GenBank	Coded protein	Function	Primers
GAD1 (NM_008077.5)	Glutamate decarboxylase 1 (GAD67)	GABA synthesis	F: 5′-CTCAGGCTGTATGTCAGATGTTC-3′ R: 5′-AAGCGAGTCACAGAGATTGGTC-3′
GAD2 (NM_008078.2)	Glutamate Decarboxylase 2 (GAD65)	GABA synthesis	F: 5′-TCAACTAAGTCCCACCCTAAG-3′ R: 5′-CCCTGTAGAGTCAATACCTGC-3′
Gabra 3 (NM_008067.4)	$GABA_A$ receptor α3 subunit	Binding site of the benzodiazepines	F: 5′-CCGCACAGTCTTTGGTGTCA-3′ R: 5′-GAAGAAGCACTGGGA GCAGC-3′
Gabrg 2 (NM_008073.3)	$GABA_A$ receptor γ2 subunit	Binding site of the benzodiazepines	F: 5′-GGTGGAGTATGG CACCCTGCATT-3′ R: 5′-AGGCGGTAG GGAAGAAGATCCGA-3′
TPH2 (NM_173391.3)	Tryptophan hydroxylase (TPH2)	Serotonin synthesis	F: 5′-AGTCTACATCCATCCCAACTGCTG-3′ R: 5′-CATTCCTCGCACAATTCCAGTCG-3′
HT1RA (NM_008308.4)	5HT1a receptor	Serotonin Gi protein-coupled receptor	F: 5′-GTGAGAGGAAGACAGTGAAGAC-3′ R: 5′-CCGTGAGAGGAAGACAGTGAAGAC-3′
SLC6A4 (NM_010484.2)	Serotonin transporter (SERT)	Serotonin Reuptake	F: 5′-CTCACCAGCAGG ACAGAAAG-3′ R: 5′-CTCATCTTCACCATTATCTACTTCAG-3′
Bdnf (NM_001048139.1)	Brain-derived neurotrophic factor (BDNF)	Neuroplasticity	F: 5′-AGCAGAGTCCATTCAGCACC-3′ R: 5′-TGGCTTGACAGCGAGGAAAA-3′
Ntrk2 (NM_001025074.2)	Tropomyosin receptor kinase B (TRKB)	BDNF receptor	F: 5′-CCAAGTTTGGCATGAAAGGC-3′ R: 5′-TGCCAAAGTACTGGGGGTTT-3′
Ppia (NM_008907.1)	Cyclophilin A	Housekeeping (internal control)	F: 5′-GCCGATGACGAGCCCTTG-3′ R: 5′-TGCCGCCAGTGCCATTATG-3′

internal control cyclophilin A (Pfaffl, 2001). Assays were repeated two or three times and the data were merged after normalization.

2.6. Statistical analysis

All results are presented as the means ± SE. The assumption of a normal data distribution was assessed with the Shapiro-Wilk test. If the data did pass the normality test, parametric comparisons were performed. In this case, between-group comparisons were analyzed with the Student's unpaired *t*-test. In the other hand, the Mann-Whitney test was used to compare data without normal distribution. Grubbs' test was used for detecting outliers. Cohen's d analysis was used to evaluate the effect sizes between the groups, which is the difference between means divided by standard deviation. In this measure, effect sizes were interpreted as small ($0.2 < d < 0.5$), moderate ($0.5 < d < 0.8$) and large ($d > 0.8$). Differences were considered statistically significant when $p < 0.05$. GraphPad Prism 5 statistical software (La Jolla, CA, USA) was used for all statistical analysis.

3. Results

3.1. Behavioral analysis

In behavioral analysis, according Grubb's test, we excluded one outlier animal from each group in the open field and the elevated plus maze tests. Moreover, there was one outlier from treated group in tail suspension test and other one from control group in light-dark box protocol.

In the open field test, it was demonstrated that the T4 group had an increase in total distance traveled (16.31 ± 1.04 m *vs.* 10.7 ± 0.68 m, $p < 0.001$) and a reduction of freezing time (43.78 ± 3.11 s *vs.* 31.83 ± 3.08 s, $p = 0.01$). However, differences in rearing (26.7 ± 1.66 *vs.* 29.4 ± 1.54, $p = 0.25$) and time of grooming (3.95 ± 1.03 s *vs.* 5.92 ± 1.38 s, $p = 0.26$) were not observed. In anxiety-related parameters, no difference was found for center zone time (8.60 ± 1.26 s *vs.* 7.82 ± 1.67 s, $p = 0.71$) and in center ratio (0.05 ± 0.005 *vs.* 0.07 ± 0.009, $p = 0.20$). The number of fecal pellets (2.08 ± 0.59 *vs.* 3.08 ± 0.45, $p = 0.19$) was also not significantly different (Fig. 2).

Regarding anxiety-related tests, we observed interesting results. In the light-dark box paradigm (Fig. 3), there was a slight increase in light time side in the T4 group *vs.* control (126.8 ± 3.10 s *vs.* 111.6 ± 3.59 s, $p = 0.004$). However, there were no differences between the groups in transitions (23.9 ± 0.91 *vs.* 24.4 ± 1.58, $p = 0.78$) and latency to light side (17.1 ± 1.76 s *vs.* 22.4 ± 2.96 s, $p = 0.13$). In the elevated plus maze test (Fig. 4), T4 offspring had higher time (42.09 ± 4.29 s *vs.* 24.64 ± 6.00 s, $p = 0.02$) and percentages of entries (27.88 ± 3.33 *vs.* 15.56 ± 2.47, $p = 0.008$) in opened arms compared to controls. There were no differences in time (146.2 ± 7.81 s *vs.* 164.5 ± 6.63 s, $p = 0.08$) and entries (14.1 ± 1.52 *vs.* 12.7 ± 0.88, $p = 0.16$) in closed arms, as well as in central platform time (111.3 ± 3.40 s *vs.* 112.9 ± 4.80 s, $p = 0.78$) between the groups.

In Table 2, according to Cohen's d analysis, prenatal treatment with T4 induced a strong effect on some behavioral parameters, such as: total distance traveled and freezing time in the open field test ($d = 1.82$ and $d = 1.11$, respectively), time and percentage of entries in opened arms in the elevated plus maze test ($d = 1.01$ and $d = 1.22$, respectively), as well as time in light side in light-dark box test ($d = 1.31$).

In the remaining tests, there were no verified differences in time spent walking on the rotarod (96.3 ± 29.5 s *vs.* 87.8 ± 29.1 s, $p = 0.83$, data not shown), in marble burying (6.18 ± 1.91 s *vs.* 5.91 ± 1.13, $p = 0.90$) or in immobility time during the tail suspension test (121.8 ± 12.9 s *vs.* 107.8 ± 6.72, $p = 0.35$) between the groups (Figs. 5 and 6, respectively). In these tests, the prenatal T4 treatment had a very small effect (Table 2).

3.2. Serum hormones measurements

To demonstrate the accuracy of our protocol, we decided to measure maternal THs. The treatment promoted a significant increase and a huge effect in serum total T4 (10.02 ± 0.44 μg/dl *vs.* 2.43 ± 0.44 μg g/dl, $p < 0.001$; $d = 8.47$) and serum total T3 (673.7 ± 39.24 ng/dl *vs.* 80.44 ± 4.15 ng/dl, $p < 0.001$; $d = 7.52$) when compared to the control group (Table 3). In adult offspring, there were also a significant decrease and a strong effect in serum total T3 (72.67 ± 4.55 ng/dl *vs.* 95.49 ± 3.06 ng g/dl, $p = 0.003$; $d = 2.27$) but not in total T4 ($p = 0.47$; $d = 0.40$) and corticosterone ($p = 0.5$; $d = 0.43$) serum levels (Table 4).

3.3. RNA analysis

In the hippocampus (Figs. 7 to 9), programmed offspring had a higher expression of TPH2 (4.26 ± 0.71 *vs.* 1.33 ± 0.49, $p = 0.01$), SERT (3.92 ± 0.78 *vs.* 0.56 ± 0.18, $p = 0.005$), GAD 67 (2.62 ± 0.40 *vs.* 1.18 ± 0.35, $p = 0.02$) and TRKB (0.03 ± 0.01 *vs.*

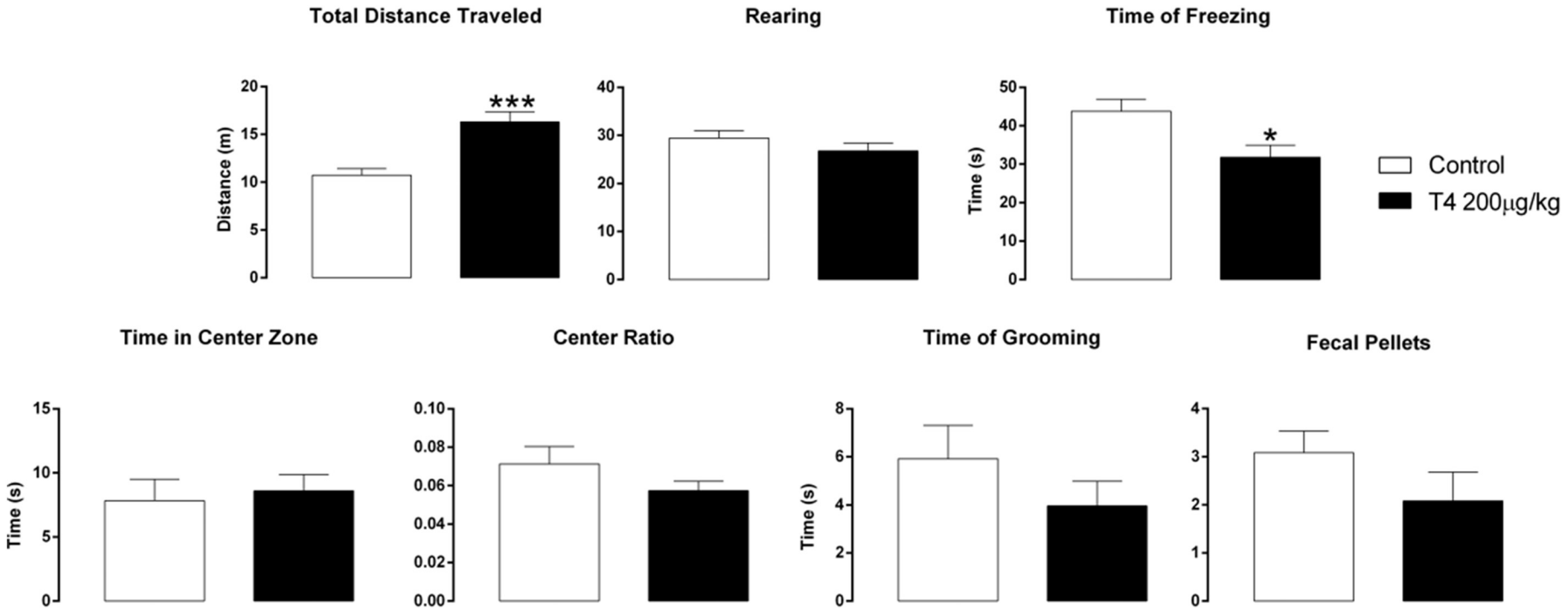

Fig. 2. The results represented above shows the behavioral parameters of the open field test in 70 PND offspring of females treated with T4 200 μg/kg during pregnancy. In this protocol, it was demonstrated difference in locomotor activity. The significance between groups was * $p < 0.05$ and *** $p < 0.001$, n = 12.

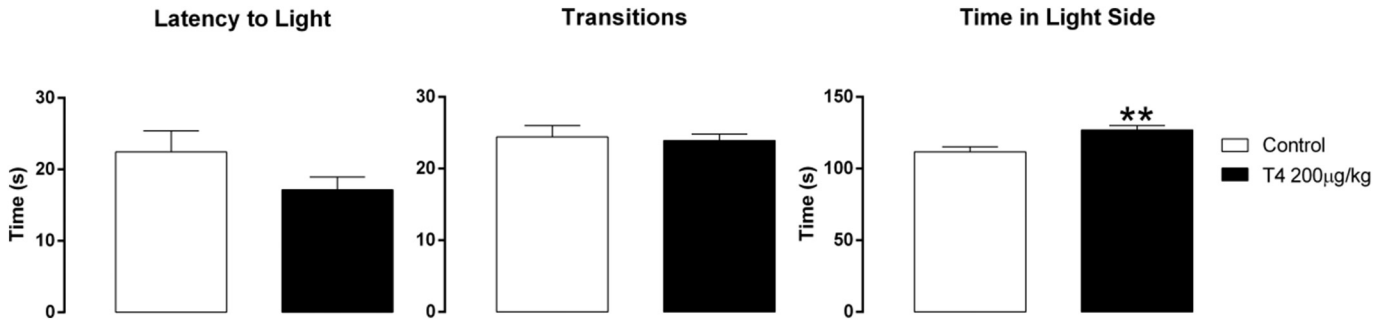

Fig. 3. This figure shows the behavioral parameters of the light-dark box test in 72 PND offspring of females treated with T4 200 μg/kg during pregnancy. In this protocol, it was demonstrated difference in anxiety-like behavior. The significance between groups was ** $p < 0.01$, n = 12.

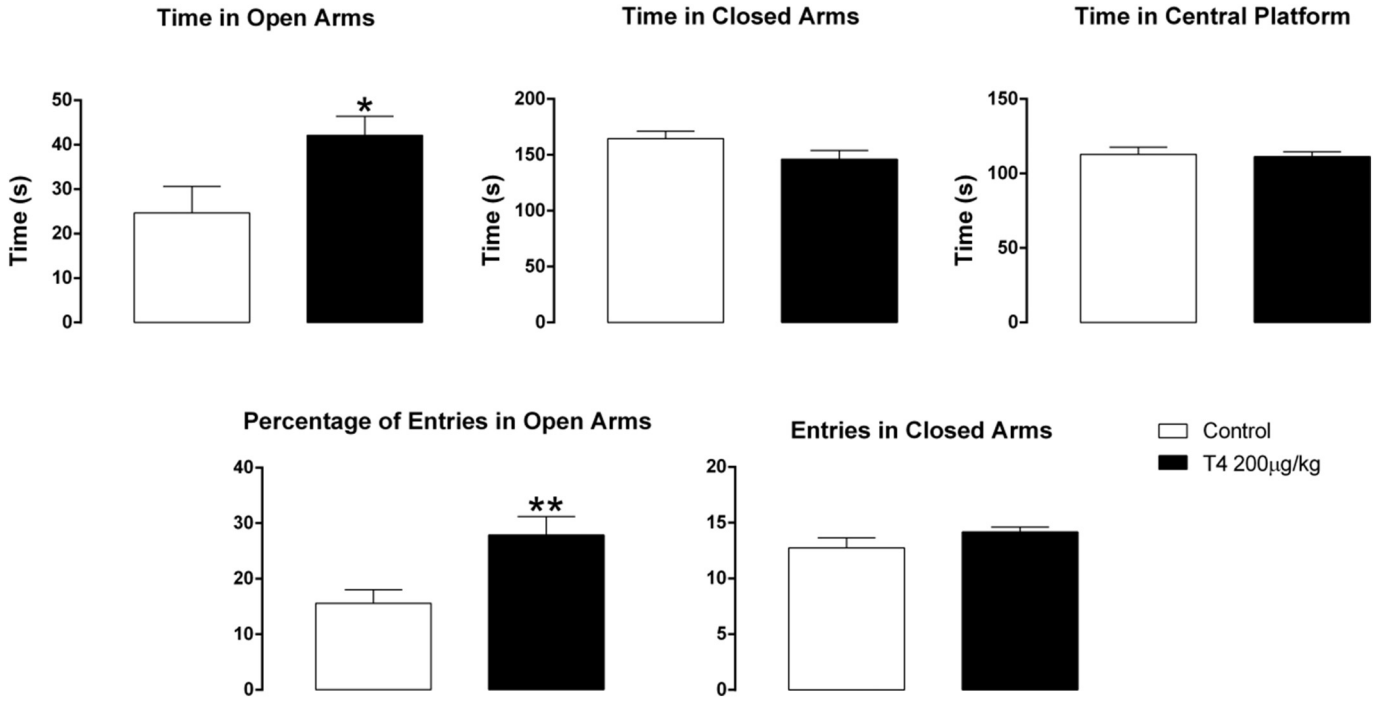

Fig. 4. The results represented above shows the behavioral parameters of the elevated plus maze test in 74 PND offspring of females treated with T4 200 μg/kg during pregnancy. In this protocol, it was demonstrated difference in anxiety-like behavior. The significance between groups was *** $p < 0.001$, n = 12.

Table 2
Calculation of Cohen's *d* effect size between groups for all behavioral parameters.

Behavioral parameters		Cohen's *d*
Open field test	Total distance traveled	**1.82**
	Rearing	0.48
	Time of freezing	**1.11**
	Time in center zone	0.15
	Center ratio	*0.55*
	Time of grooming	0.46
	Fecal pellets	*0.54*
Light-dark box test	Latency	*0.64*
	Transitions	0.11
	Time in light side	**1.30**
Elevated plus maze test	Time in opened arms	**1.00**
	Time in closed arms	*0.72*
	Time in central platform	0.11
	Percentage of entries in opened arms	**1.22**
	Entries in closed arms	*0.58*
Marble burying test	Marble buried	0.05
Tail suspension test	Latency to immobility	0.006
	Immobility time	0.39

Numbers in italic represent medium magnitude of the effect, whereas bold numbers represent large magnitude of the effect.

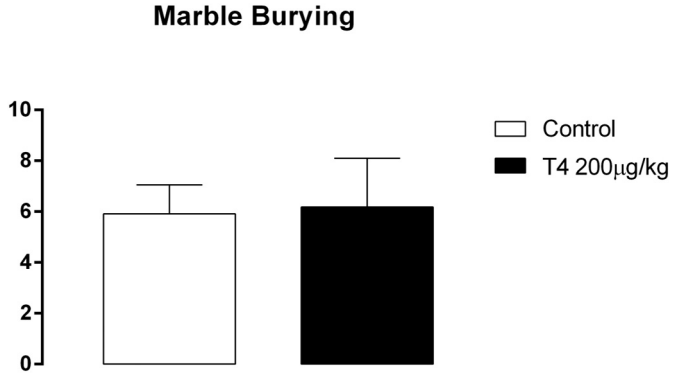

Fig. 5. This figure shows the behavioral parameters of the marble burying test in 76 PND offspring of females treated with T4 200 μg/kg during pregnancy. In this protocol, it was not demonstrated difference in anxiety-like behavior. n = 12.

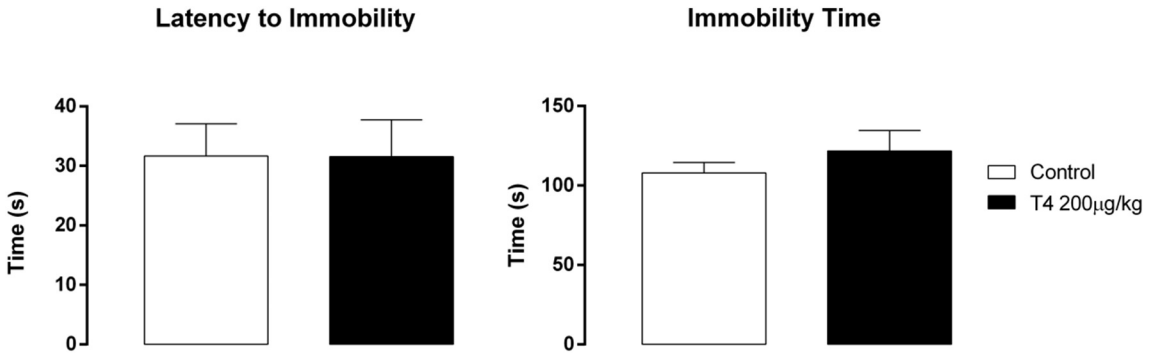

Fig. 6. This figure shows the behavioral parameters of the tail suspension test in 80 PND offspring of females treated with T4 200 µg/kg during pregnancy. In this protocol, it was not demonstrated difference in depression-like behavior. n = 12.

Table 3
Serum thyroid hormone levels in dams treated with T4 during pregnancy.

	Total serum T4 (µg/dl)	Total serum T3 (ng/dl)
Control	2.43 ± 0.44[a]	80.44 ± 4.15[a]
T4-Treated	10.02 ± 0.44[b]	673.7 ± 39.24[b]

Values are mean ± SEM. n = 6 animals/group. Different letters in the same column indicate $p < 0.05$ by Student's *t*-test.

Table 4
Serum hormones levels in adult mice treated with T4 prenatally.

	Total serum T4 (µg/dl)	Total serum T3 (ng/dl)	Corticosterone (ng/ml)
Control	2.34 ± 0.10[a]	95.49 ± 3.06[a]	78.11 ± 9.11[a]
T4-treated	2.23 ± 0.09[a]	72.67 ± 4.55[b]	65.12 ± 14.5[a]

Values are mean ± SEM. n = 6 animals/group. Different letters in the same column indicate $p < 0.05$ by Student's *t*-test.

0.97 ± 0.37, p = 0.01) compared to controls. A strong tendency was observed in GAD 65 expression (1.06 ± 0.16 *vs.* 1.57 ± 0.18, p = 0.06). No differences were observed in expression of BDNF (1.46 ± 0.09 *vs.* 1.11 ± 0.21, p = 0.14), 5HT1aR (1.57 ± 0.27 *vs.* 1.10 ± 0.19, p = 0.19) as well as in α3 (0.75 ± 0.04 *vs.* 1.05 ± 0.16, p = 0.14) and γ2 (1.21 ± 0.08 *vs.* 1.02 ± 0.09, p = 0.14) subunits of $GABA_A$ receptors. We also excluded one outlier from each group in this protocol. In Table 5, according to Cohen's d analysis, prenatal treatment with T4 induced a strong effect in all genes studied.

4. Discussion

As far as we know, this is the first work to investigate the neurobehavioral effects of maternal hyperthyroxinemia in mice offspring. According to our results, maternal hyperthyroxinemia during pregnancy causes long-term behavioral alterations in offspring, characterized by low anxiety and hyperlocomotion. In our study, we consider that this hyperlocomotion may be also related to an anxiety reduction. To reinforce this fact, it is noteworthy that diazepam induced-anxiolysis typically results in hyperlocomotion, which is considered an increase in exploratory activity on reduced anxiety (Seale et al., 1996). Moreover, this behavioral profile is accompanied by changes in the expression of genes related to hippocampal serotonergic and GABAergic systems.

We observed increased thyroid hormone levels in dams treated with T4 during pregnancy. These results reinforce the accuracy of our gestational hyperthyroxinemia model and corroborate some data previously reported (Varas et al., 2001; Navas et al., 2011). In adult offspring from hyperthyroxemic mothers, we verified a reduction in serum T3 levels. Accordingly, Shukla and coworkers observed similar results in rats. They suggested that this hormonal profile could be explained by a set-point reprogramming for adult thyroid function in response to maternal hyperthyroxinemia during the fetal development period (Shukla et al., 2010). However, we also need to take into account the extra hypothalamus-pituitary-thyroid axis reset and look for the effects of this experimental model on the deiodinases expression and activity in peripheral tissues of adult offspring.

To discuss neurochemical effects induced by maternal hyperthyroidism in the offspring, we need to highlight a well-designed study published by the Ahmed group. This group described hyperthyroid status during pregnancy and lactation as positively modulating monoamine levels as well as acetylcholinesterase and ATPases activities while negatively alters GABA levels in different brain regions of young rat offspring. This neurochemical profile is accompanied by elevated serum T3, T4, growth hormone levels, increased type 1 iodothyronine deiodinase activity, and histopathological changes in the thyroid gland (Ahmed et al., 2010). In another work, this same group verified that maternal hyperthyroidism caused severe growth retardation in the neurons of the cerebellum and cerebral cortex of their offspring in the first three weeks of life. Moreover, puppies of hyperthyroid dams showed increased oxidative damage, decreased levels of non-enzymatic antioxidants and lower antioxidant enzyme activity in different encephalic regions (Ahmed et al., 2012). Therefore, oxidative stress induced by maternal hyperthyroidism could explain, at least in part, the neurobiological changes observed here and in these cited works.

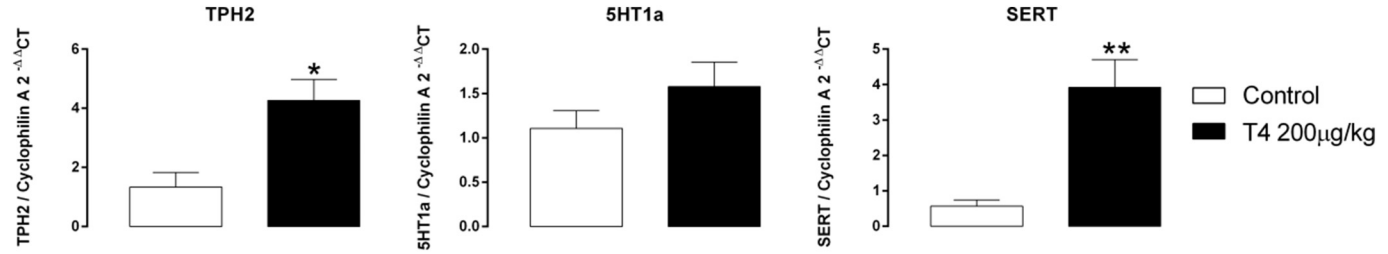

Fig. 7. Hippocampal 5-HT-related genes expression in 70 PND offspring of females treated with saline 0.9% or T4 200 µg/kg during pregnancy. The significance between groups was * $p < 0.05$ and ** $p < 0.01$, n = 6.

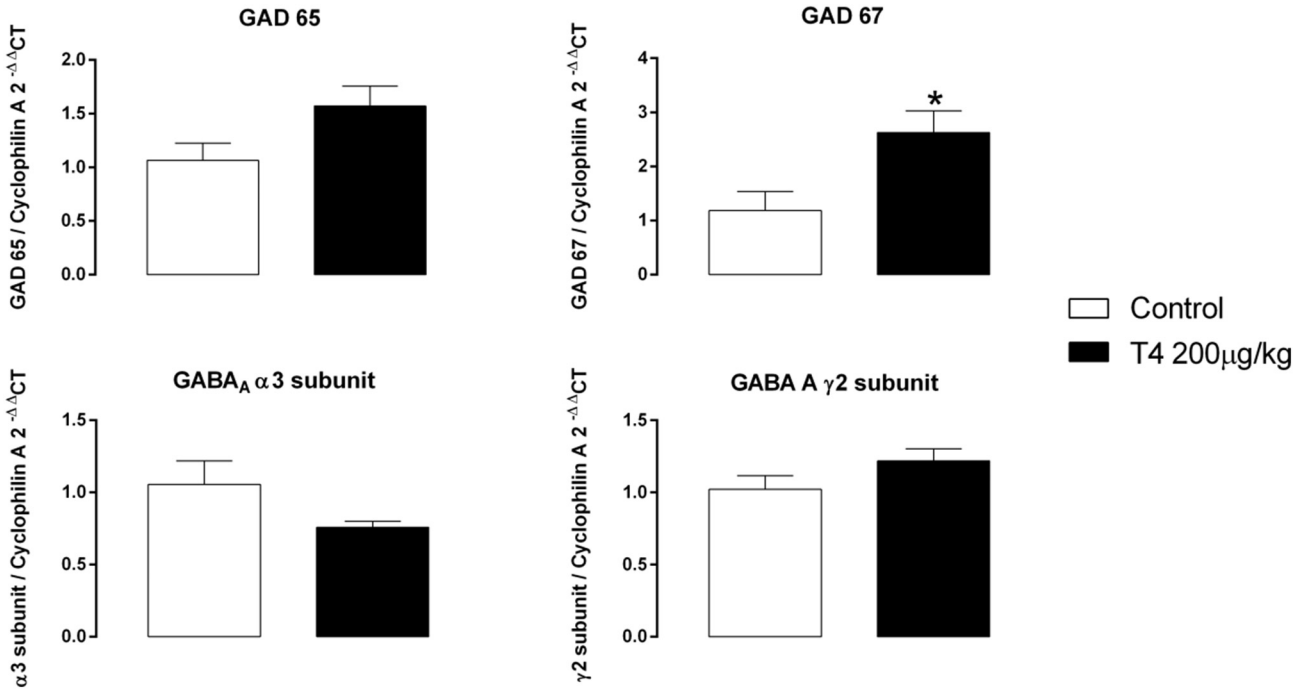

Fig. 8. Hippocampal GABA-related genes expression in 70 PND offspring of females treated with saline 0.9% or T4 200 μg/kg during pregnancy. The significance between groups was * $p < 0.05$, $n = 6$.

Regarding the serotonergic system, thyroid hormone replacement therapy reverts low serotonin responsiveness induced by hypothyroidism status in humans (Cleare et al., 1996). Experimentally in rats, a single T3 injection does not affect levels of 5-HT in the hippocampus and frontal cortex, but daily administrations for 1 week resulted in increased levels of this neurotransmitter only in the latter region (Gur et al., 1999). Similarly, Heal and Smith also verified that repeated injections of T3 for 10 days increased 5-HT synthesis and turnover in mice brains. Moreover, these same researchers observed that transient hyperthyroid status attenuates the 5-HT 1a receptor agonist-induced responses, however it potentiates effects mediated by the 5-HT 2a receptor (Heal and Smith, 1988). Altogether, thyroid hormones could modulate serotonergic neurotransmission by regulating the expression of their receptors. Although this idea is quite plausible, we did not observe significant differences in 5-HT 1a receptor expression in both the hippocampus. Therefore, to explain the TPH2 and SERT upregulation, we need to highlight a paper published by Wang and coworkers. In this study, they showed that developmental hypothyroxinemia promotes downregulation of the sonic hedgehog signaling pathway (Wang et al., 2014), which is essential for serotonergic system embryogenesis (Gaspar et al., 2003). Based on this assumption, maternal hyperthyroxinemia could modulate sonic hedgehog and other transcription factors involved with phenotypic characterization of serotonergic neurons, upregulating TPH2 and SERT expression. Previous studies also suggest that 5-HT increasing in ventral hippocampus would be critical to promote reduction in anxiety-like behavior (Barr and Forster, 2011; Tu et al., 2014). Thus, the 5-HT related genes upregulation and, consequently, a possible increase in 5-HT neurotransmission could be involved at least partially to the low anxiety observed in our study.

In addition, previous studies have shown that perinatal thyroid dysfunctions can especially affect the development of the embryogenesis of GABAergic system. An *in vitro* study using fetal rat brain cells showed that the addition of T3 in the culture medium significantly increased GAD activity (Honegger and Lenoir, 1980). This T3 effect was also observed in cortical neuronal cultures, however this action required the presence of insulin (Aizenman and de Vellis, 1987). Supporting these *in vitro* studies, T4 rescues the low GAD activity in 10-day-old rats with hypothyroidism by perinatal treatment with propylthiouracil (Patel et al., 1988). To reinforce this fact, neonatal thyroidectomy-induced hypothyroidism has been shown to promote reduction of parvalbumin, a calcium-binding protein present in

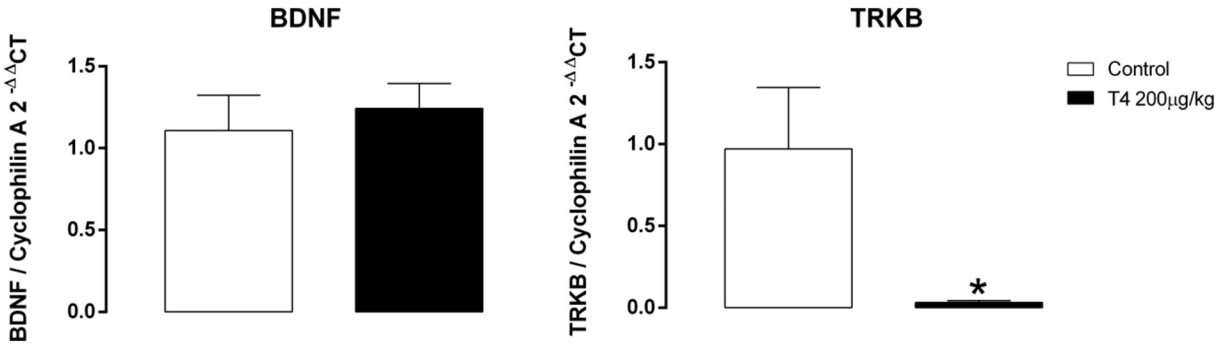

Fig. 9. Hippocampal BDNF and TRKB expression in 70 PND offspring of females treated with saline 0.9% or T4 200 μg/kg during pregnancy. The significance between groups was * $p < 0.05$, $n = 6$.

Table 5
Calculation of Cohen's *d* effect size between groups for RNA analysis.

Coded protein	Cohen's *d*
GAD 65	**1.18**
GAD 67	**1.55**
$GABA_A$ α3 subunit	**1.01**
$GABA_A$ γ2 subunit	**0.87**
TPH2	**1.89**
SERT	**2.25**
5HT1a receptor	**0.80**
BDNF	**0.86**
TRKB	**1.58**

Bold numbers represent large magnitude of the effect.

GABAergic interneuron subpopulations, in the immunostaining of the neocortex of rats (Berbel et al., 1996). Moreover, hypothyroid rats, whose dams were treated with methimazole in drinking water, had a reduction in the GAD65 protein and in the number of GAD65-positive immunostained cells in the hippocampus. This phenotype is recovered to control levels by daily thyroxine-replacement after birth (Sawano et al., 2013). Thus, these works corroborate the finding of a higher expression of GAD65 and GAD67 observed in the T4-programmed mice of this study. The upregulation of these genes could also explain the low anxiety of treated group. In different animal models of the GAD 67 deficiency, it was shown increased anxiety in social behavior paradigms (Tremolizzo et al., 2005; Sandhu et al., 2014). Anxiety-like behavior and diminished responses to low doses of diazepam are also observed in GAD 65 knockout mice (Kash et al., 1999). Based on these results, we propose that GABA generated by GAD 65 and GAD 67 could be involved in modulating responses to anxiolytic stimuli.

On the other hand, we observed that maternal hyperthyroxinemia induces a strong downregulation of TRKB in adult offspring. This result is consistent with a previous study published in the literature. According Pombo and colleagues, T3 decreases TRKB expression in developing rat brains. They showed that this repression requires a binding of thyroid hormone receptors to a specific region where these receptors preferentially form heterodimers with retinoid X receptors (Pombo et al., 2000). Curiously, transgenic mice with TRKB-receptor disruption in the forebrain showed a stereotyped hyperlocomotion, however without depression-like behavior (Zörner et al., 2003). Such results are similar to ours and could explain the results obtained in open field and tail suspension tests.

Additionally, there are two studies that have been performed with rats that help us to explain our results. In the first one, Yilmazer-Hanke and coworkers showed that neonatal T4 treatment promotes hyperactivity in the motility box and anxiolysis in the elevated plus maze. They also observed that these results are strongly correlated with a reduction in the number of anxiogenic peptide corticotropin releasing factor neurons and an increase in anxiolytic neuropeptide Y ones in amygdala (Yilmazer-Hanke et al., 2004). Our behavioral results show high accordance with this cited work. In another model, Shukla and colleagues demonstrated that prenatal T4 treatment induces anxiogenic effects in the defensive burying test. However, as is also shown in our work, there was no difference in depression-like behavior (Shukla et al., 2010). Possibly, these differences may be related to species, strains and the methodology adopted.

In summary, we demonstrated that maternal hyperthyroxinemia alters hippocampal serotonergic and GABAergic systems of adult male offspring, promoting anxiolysis. However, further studies will be necessary to understand the epigenetic mechanisms by which maternal hyperthyroxinemia reprograms offspring transcriptional profiles, even in adulthood. Moreover, we need to study not only the relationship between fetal thyroid dysfunction and affective disorders but also other psychiatric diseases, such as autism, schizophrenia and attention deficit hyperactivity disorder.

Conflict of interest statement

We wish to confirm that there are no conflicts of interest associated with this publication, and there has been no significant financial support for this work that could have influenced its outcome.

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