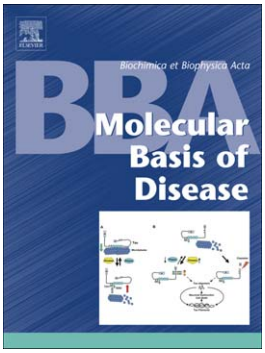

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Induction of hypothyroidism during early postnatal stages triggers a decrease in cognitive performance by decreasing hippocampal synaptic plasticity

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Abstract

Thyroid hormones are vital in the control of multiple body functions, including the correct performance of the brain. Multiple diseases are associated with thyroid gland functioning, including hypothyroidism. To date, little is known regarding the effects of the establishment of this condition at a young age on brain function. Here, we evaluated the effect of hypothyroidism in an early postnatal stage in cognitive abilities with focus on the hippocampus. In our model, hypothyroidism was induced in young rats at 21 days of age using 0.05% 6-propyl-2-thiouracil (PTU) for 4 weeks reaching significantly lower levels of fT_4 (control: 1.337 ng/dL ± 0.115, PTU: 0.050 ng/dL ± 0.001). Following the induction of hypothyroidism, several cognitive tasks were assessed to investigate the effects of hypothyroidism on cognition performance. We determined that hypothyroidism triggers a significant dysfunction in learning and memory processes observed in the Morris Water Maze were the latency times were higher in PTU rats (controls: 37 sec; PTU: 57 sec). The cognitive impairment was correlated with a reduction in hippocampal plasticity with respect to both long-term potentiation (LTP) (control: 1.45, PTU: 1.00) and depression (LTD) (control: 0.71, PTU: 1.01). Furthermore, a decrease in the rate of glucose utilization (control: 223 nmol*mg of protein, PTU:148 nmol*mg of protein) was observed, along with an increase in oxidative stress and a decrease in MAP2 marker in the hippocampus. Our findings suggest that the induction of hypothyroidism in a young rat model alters numerous functions at the level of the hippocampus.

Keywords: Hypothyroidism, hippocampus, cognitive performance and glucose metabolism

1. Introduction

Thyroid hormones are crucial for normal development, including proper brain development, and dysregulation of these hormones leads to severe alterations in nearly all tissues [1,2]. Hypothyroidism is a clinical condition in which the thyroid gland produces insufficient levels of the thyroid hormones triiodothyronine (T_3) and tetraiodothyronine (T_4). This leads to high thyroid stimulating hormone (TSH) values and therefore slower cell metabolism. This condition affects 1-2% of the population, with a 10-fold higher incidence in women compared with men, whereas the prevalence increases to 80% in iodine deficient areas, which is equivalent to one-third of the world's population [2,3]. Hypothyroidism treatment includes the administration of levothyroxine, which, in combination with deiodinases, transforms T_4 to T_3; moreover, the latter hormone produces its main effect at the transcriptional level [4,5,6,7].

Thyroid hormones are implicated in metabolic alterations in several organs, whereby the brain is fundamental. Hypothyroidism may occur during all stages of development from pregnancy through adulthood [7]. In the first stage, subclinical hypothyroidism and congenital hypothyroidism are present, and the latter has a more extensive impact on neurodevelopment impairment via neuronal growth and differentiation, dendritic spine morphology and impaired synaptic transmission at the hippocampus [8,9]. These alterations are accompanied by metabolic impairments, such as poor feeding and deficient weight gain and growth. In adulthood, hypothyroidism provokes memory deficiency and slowness, and it has been related to depression and dementia [10,11,12,13]. Many studies have investigated the alterations in thyroid hormone levels produced during pregnancy or adult states; however, no studies has investigated an intermediate state using a young model. Currently, screening for thyroid hormone levels is performed in all newborns as a preventive measure. This measure has reduced the number of

newborns with hypothyroidism [14]. Nevertheless, this condition remains present in children and teenagers because hypothyroidism may also be triggered in the later stages of birth. For example, in Turkey, the prevalence of hypothyroidism in children of less than 10 years is 21% [15]. Moreover, a long-term study in Toronto, Canada of diagnosed and treated children with congenital hypothyroidism indicated that they presented poor visuomotor and visuospatial abilities, delayed speech and language development, neuromotor deficiencies and poor attention and memory skills until the adolescent stages [16].

In the present work, we analyzed the effects of thyroid hormone deficiency in a young rat model and hypothesized that the condition of early hypothyroidism may impair brain function, specifically at the level of the hippocampus. To conduct this study, we administered propylthiouracil (PTU), a drug widely used to treat patients with hyperthyroidism, to reduce their thyroid hormone levels through inhibition of the enzyme thyroid peroxidase (TPO), which controls T_4 production. PTU at 0.05% was administered to young rats at 21 days of age, and the effects of early hypothyroidism were analyzed as a relevant process related to brain functions, including cognitive performance, synaptic plasticity (LTP and LTD), synaptic and metabolic protein expression and glucose utilization. Our results demonstrated impairments in cognitive aspects, including acquisition, recognition and localization memory; hippocampal plasticity; and metabolic processes. These findings support the significance of thyroid hormone deficiency during early postnatal stages in the function of the brain circuitry involved in learning and memory.

2. Materials and methods

2.1. Animals

Young male Sprague Dawley rats were obtained and housed at the Animal House Facility of the Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, in accordance with the Guide for the Care and Use of Laboratory Animals (NIH-USA Publication 86-23). The animals started the treatment at 21 days of age and were housed two animals per cage. The animal room was maintained on a 12:12 light:dark schedule, and all animals had free access to food and water. The treated group was administered 0.05% PTU in water for 4 weeks. Measurements of animal weight and food and liquid intake were obtained once per week during the treatment. At the end of the treatment, the rats were anesthetized with isoflurane and euthanized by decapitation, and organs, including the liver, kidney and brain, were subsequently removed and weighed [17]. The data represent 16 controls and 16 treated male rats. After treatment the mice were used in the fellow order: 4 animals for electrophysiology experiments (these animals was not used for cognitive tests); 4 animals for immunoblotting and immunofluorescence analysis and 8 animals for the cognitive test and biochemical analysis.

2.2. Biochemical analysis

At the end of the treatment, the thyroid hormone levels were measured from blood samples obtained from 4 rats per condition. Intracardiac blood was collected prior to decapitation, and the serum was separated via centrifugation and stored at -20°C for posterior analysis via immunoassay and chemiluminescence. The samples were analyzed for TSH, T_3, T_4 and free T_4 (fT_4) in the Specialized Clinical Chemistry Laboratory. The glucose levels were

measured according to the hexokinase/G-6-PDH method using Architect Analyzer (Abbott Laboratories, Abbott Park, IL). The insulin and fructosamine levels were measured via chemiluminescence (Beckman Coulter). The cholesterol level was enzymatically assessed using an Architect c8000 analyzer (Abbott Laboratories, Abbott Park, IL).

2.3. Oral glucose tolerance test (OGTT)

An OGTT was performed in the control and PTU treated groups (n=4 per group) once the PTU treatment was concluded. Following an overnight fast (14 h) and baseline sampling, a 2.0 g/kg body weight of glucose was administered via oral gavage. Blood samples were collected from the tails at 15, 30, 60 and 120 min after glucose administration. Plasma glucose was measured using a OneTouch meter with test strips [18,19]. The HOMA-IR was calculated using the following equation: fast glucose (mg/dL) x Fast Insulin (μU/mL)/405 [20].

2.4. Cognitive tests

The Morris Water Maze (MWM) test was performed as previously described [21,22]. The apparatus consists of a circular pool of 1.2 m in diameter (opaque water, 50 cm depth) filled with water at a temperature of 19-21°C. A circular platform with a 10-cm diameter was located 1 cm below the water surface, invisible to the animal. The room where the pool was located had spatial cues on the walls for spatial orientation during the trials, which had a maximum duration of 90 sec, with 10 sec on the platform at the end of each trial. The entire task required 8 days: 5 days of trials, 2 days without trials and 3 days of trials. On the last day, a probe trial was

performed in which the platform was removed, and the time that the animal spent in each quadrant of the pool was recorded. After testing, the rat was removed from the maze, dried and returned to its cage. *Novel object recognition (NOR) and novel object localization (NOL):* The NOR and NOL tasks were performed following a previously described protocol, where one task is followed by the other [23,24,25,26]. The rats were habituated to the experimental room in the experimental cages for 3 consecutive days for 30 min per day and for 1 h on the testing day. The task occurred in a 120×120 cm transparent Plexiglas platform with 35-cm-high transparent walls. For object familiarization, the rats were allowed to explore the platform in the presence of two identical objects placed at specific locations for 10 min. The animals were subsequently returned to their home cages for 1 h, followed by a 5-min exposure to a novel localization of one of the familiar objects (NOL). The rats were again returned to their home cages for 1 h and were subsequently exposed to a novel object (NOR) for 5 min. The rats had no observed baseline preference for the different objects. An object preference index was determined by calculating the time spent near the relocated/novel object divided by the cumulative time spent with both the familiar and relocated/novel objects. The cages were routinely cleaned with ethanol following the testing/habituation of the rats. *Large open-field (LOF) test*: A 120×120 cm transparent Plexiglas platform with 35-cm-high transparent walls was used to study locomotor and stress behavior in our mouse model because the use of a large platform is more effective than that of a small one for the study of behavior. The open field, which measured 40×40 cm, was defined as the "center" area of the field. Data were collected using an automatic tracking system (HVS Imagen, Hampton, UK). Each mouse was placed alone in the center of the open field, and its behavior was tracked for a 20 min session. At the end of the session, the mouse was returned to its home cage. The parameters measured included total time moving, time in the center and

corner, and number of times the mouse crossed the center area of the platform [27,28]. The data represent 8 animals by group, between each test we expect the suggested time.

2.5. Electrophysiological recording

The hippocampus of adults rats were promptly removed and sectioned into 350 μm-thick slices using a vibratome (Leica VT1000S) in ice-cold dissection buffer (5 mM KCl, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$, 212.7 mM sucrose, 10 mM dextrose, 3 mM $MgCl_2$, and 1 mM $CaCl_2$, equilibrated with 95% O_2 and 5% CO_2). The slices were transferred and maintained for 1 h at room temperature in normal artificial cerebrospinal fluid (ACSF), which was similar to the dissection buffer but with sucrose replaced by 124 mM NaCl, $MgCl_2$ lowered to 1 mM, and $CaCl_2$ raised to 2 mM. All recordings were done in a submersion-recording chamber perfused with ACSF (room temperature; 2 mL/min). Field excitatory postsynaptic potentials (fEPSPs) were evoked by stimulating the Schaffer collaterals with 0.2-ms pulses delivered through concentric bipolar stimulating electrodes (FHC) and recorded extracellularly in CA1 stratum radiatum. Basal synaptic transmission was assayed by determining input–output relationships from fEPSPs generated by gradually increasing the stimulus intensity from 10 to 90 μA. Baseline responses were recorded using half-maximum stimulation intensity at 0.033 Hz. Long-term potentiation (LTP) was induced by theta burst stimulation, consisting of four theta epochs delivered at 0.1 Hz. Each epoch in turn consisted of 10 trains of four pulses (at 100 Hz) delivered at 5 Hz. Long-term depression (LTD) was induced by low-frequency stimulation (LFS) which consisted of 900 paired pulses at 1 Hz. Paired-pulse facilitation index was calculated using the equation ((R2-R1)/R1) where R1 and R2 are the peak amplitudes of the first and second fEPSP

in an interval inter-pulse of 50 ms, respectively [29,30,31]. Recordings were filtered at 2.0-3.0 kHz, sampled at 4.0 kHz using an A/D converter, and stored using pClamp 10 software (Molecular Devices). Evoked postsynaptic responses were analyzed off-line using analysis software (pClampfit, Molecular Devices), which allowed events to be visually detected and which computed only those events that exceeded an arbitrary threshold. The data represent 4 controls and 4 treated male rats that were excluded from all the cognitive tests.

2.6. Immunofluorescence

Immunofluorescence was performed in brain slices as previously described [32,33]. Slices of 40 µm were obtained specifically from the hippocampus region. The slices were washed three times in ice-cold phosphate-buffered saline (PBS), permeabilized for 30 min with 0.2% Triton X-100 in PBS and washed with ice-cold PBS. The slices were subsequently incubated in blocking solution (0.2% bovine serum albumin in PBS) for 1 h at room temperature, followed by an overnight incubation at 4°C with primary antibodies. After incubation, the slices were extensively washed with PBS and subsequently incubated with Alexa-conjugated secondary antibodies (Molecular Probes, Carlsbad, CA) for 2 h at 37°C. The primary antibodies included rabbit anti-GFAP (Dako, Denmark, USA), rabbit anti-4-HNE (Abcam, Cambridge, UK) and rabbit MAP2 (Abcam, Cambridge, UK). The nuclear stain was performed by treating the slices with Hoechst (Sigma-Aldrich, St. Louis, MO). The slices were subsequently mounted with mounting medium in gelatin-coated slides and analyzed by fluorescence microscopy. Images were analyzed using NIH Image J software. The data represent 4 controls and 4 treated male rats.

2.7. Immunoblotting

The PTU and control rats were decapitated at the end of the treatment, and the brain was isolated. The hippocampus was dissected and homogenized in RIPA buffer (50 mM, Tris-Cl, pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 1% SDS) supplemented with a protease inhibitor cocktail (Sigma-Aldrich P8340) and phosphatase inhibitors (50 mM NaF, 1 mM Na_3VO_4 and 30 μM $Na_4P_20_7$) using a potter homogenizer. It was subsequently passed sequentially through different caliber syringes. The protein samples were centrifuged twice at 12,500 rpm at 4°C for 15 min. The protein concentration was determined using a Bicinchoninic Acid (BCA) Protein Assay Kit (Pierce Biotechnology, Rockford, IL). Samples of hippocampus (30 and 60 μg) were loaded onto a 10% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE), separated by electrophoresis and subsequently transferred to a polyvinylidenedifluoride (PVDF) membrane. Proteins were detected by incubation with a primary antibody followed by a secondary peroxidase-conjugated antibody (Pierce) and developed using an enhanced chemiluminescence (ECL) kit (Western Lightning Plus ECL, PerkinElmer). The primary antibodies included rabbit anti-PGC1α (Abcam, Cambridge, UK), rabbit anti-p-AMPK (Cell Signaling, Danvers, Massachusetts), rabbit anti-total AMPK (Cell signaling, Denver, Massachusetts), rabbit anti-GFAP (Dako, Denmark), rabbit anti-4-HNE (Abcam, Cambridge, UK) and mouse anti-Actin (Sigma-Aldrich, St. Louis, MO). The data represent 4 controls and 4 treated male rats.

2.8. Glucose uptake in hippocampal slices

Hippocampal slices were prepared from rats as previously described. The slices were washed with incubation buffer (15 mM HEPES, 135 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$ and

0.8 mM $MgCl_2$) supplemented with 2 mM of glucose. The slices were subsequently treated with 1-1.2 μCi_2-deoxy-D-[1,2-(N)^{3}H]glucose (2-DG) (26.2 Ci/mmol; Dupont NEN, Boston, MA), an analog of glucose, to a final concentration of 100-500 μM several times (0-60 min). Uptake was terminated by washing the cells with ice cold PBS. The cells were lysed in 0.5 mL lysis buffer (10 mM Tris- HCl, pH 8.0 and 0.2% sodium dodecyl sulfate), and the incorporated radioactivity was assayed via liquid scintillation. For the pharmacological experiments, 20 μM cytochalasin B and 50 μM H_2O_2 were pre-incubated for 30. In the slice experiments, the incubation time with 2-DG was 60 min, followed by previously described methodology [34]. The data represent 4 controls and 4 treated male rats.

3. Results

3.1. Hypothyroidism induction in young rats

The protocol designed to induce hypothyroidism consisted of the addition of 0.05% PTU in tap water for 4 weeks (Fig. 1A). PTU treatment induced an approximately 5 cm difference in the length of the rats, whereby the growth of the rats with PTU was reduced (Fig. 1B, i). Furthermore, hypertrophy of the thyroid gland was identified in the PTU group of approximately twice the size compared with the controls (Fig. 1B, ii). Throughout the treatment, the weight, fluid and food intake were measured. These results exhibited clear alterations in all parameters, and the weight was significantly lower from week 4. In both cases, the PTU group had 50% lower weight compared with the controls at the end of the treatment (control: 310 g ± 16.78, PTU: 148 g ± 10) (Fig. 1C, i). These results correlated with a decrease in fluid intake from week

2 (control: 238 mL ± 8, PTU: 100 mL ± 12) (Fig. 1C, ii) and food intake (represented in kcal) from week 3 (control: 562.4 kcal ± 5.82, PTU: 277 kcal ± 2.38) (Fig. 1C,iii). Following euthanasia, the organs (kidney, liver and brain) were weighed, and the values were normalized to the weight of each rat; there was a difference in the brain weight of the rats treated with PTU, which was increased compared with the controls (control: 3.59 mg/g ± 0.19, PTU: 10 mg/g ± 0.75) (Fig. 1C, iv).

Once the treatment was completed, a reduction in thyroid hormones was identified in the PTU treated rats compared with the control rats. The serum samples were analyzed for tT3, fT_4 and TSH. The results indicated significantly higher levels of TSH (control: 0.015 µIU/mL ± 0.008, PTU: 0.025 µIU/mL ± 0.008) and significantly lower levels of fT_4 (control: 1.337 ng/dL ± 0.115, PTU: 0.050 ng/dL ± 0.001) and T_3 (control: 0.512 ng/mL± 0.002, PTU: 0.255 ng/mL ± 0.056) compared with the control rats, which confirms the induction of a hypothyroid state (Table 1).

The serum samples were also analyzed to detect the levels of cholesterol, insulin and fructosamine to obtain a general view of the metabolism. The results indicated that the cholesterol levels were normal, whereas fructosamine increased with PTU treatment (control: 236 µmol/L ± 2.45, PTU: 329 ± 8.32) (Table 1). The insulin levels were also measured, and a reduction was identified in the PTU rats; however, this change was not significant (Fig. 2, i). Moreover, OGTT was performed, which initially showed that the basal glucose levels were weakly increased compared with the controls (control: 92.5 mg/dL ± 1.32, PTU: 108 mg/dL ± 3.05) (Fig. 2, ii). Following glucose administration, the glucose levels increased and reached a peak at 15 min in both conditions (control: 201 mg/dL ± 24.5, PTU: 189 mg/dL ± 6.59). Finally, both groups exhibited decreased glucose levels at 120 min, similar to the basal conditions

(control: 118 mg/dL ± 10.2, PTU: 152 mg/dL ± 8.81) (Fig. 2, iii). From the insulin and basal glucose, the insulin resistance index (HOMA-IR) was calculated (control: 0.67 mg/dL ± 0.23, PTU: 0.19 ± 0.02); however, in both conditions, the value was less than 2.5, which rules out the possibility that hypothyroidism induces insulin resistance in young rats (Fig. 2, iv). These findings indicate a weak alteration in general metabolism following the induction of hypothyroidism in young rats.

3.2. The hypothyroid condition affects the metabolic status of animals

Thyroid hormones are critical for general metabolism. Thus, we analyzed whether the hypothyroid condition leads to metabolic alterations in the brain, specifically in the hippocampus. We analyzed the protein expression of AMP activated protein kinase (AMPK) and the peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α), two proteins related to the metabolic status. Using Western blot, we did not identify changes in the expression of either protein following the induction of hypothyroidism (Fig. 3A, i and ii).

To further analyze the metabolic state, we investigated whether the hypothyroidism condition affects glucose metabolism. Glucose is the main energy supply of the brain; thus, to identify a potential energy imbalance, we measured the uptake of 2-DG in the hippocampal slices of the control and PTU-treated rats. We did not identify changes after 30 min; however, significant differences were identified following 45 and 60 min of incubation, in which the 2-DG accumulation was lower in the PTU group (Fig. 3B, i). The same experiment was performed at 60 min of incubation in the presence of the GLUT transporter inhibitor Cyt B and H_2O_2. The results demonstrated that uptake was reduced in the PTU group; moreover, in the presence of the

inhibitors, the utilization of 2-DG was blocked (Fig. 3B, ii). To further analyze these changes, incubation with different concentrations of glucose was performed for 60 min. The PTU group had significantly lower levels of 2-DG, 148 ± 25 nmol*mg of protein, compared with the control condition of 223 ± 32 nmol*mg of protein in the consumption of 2-DG in the range of concentrations (Fig. 3B, iii). These findings indicated that, despite the lack of identified changes in the metabolic regulators AMPK and PGC1α, the capacity of glucose accumulation in the brain was reduced.

3.3. Hypothyroidism decreases cognitive performance

To assess whether the hypothyroid condition may cause cognitive alterations in spatial memory, a Morris Water Maze (MWM) test was performed in the PTU and control rats. During the 8 days of the trial, the rats were placed in the water, and the time required to reach the hidden platform (latency time) between both groups was measured. The results indicated significant differences in the first five days of the trial, in which the average latency times for the males were 37 ± 8.2 sec for the controls and 57 ± 6.1 sec for the PTU animals. However, in the last 3 days of the test, we identified a decrease in the difference between both groups (6 ± 0.8 sec for the controls and 13 ± 2.1 sec for the PTU animals) (Fig. 4A, i). This latter result was correlated with the absence of differences in the probe trial, in which the platform was removed, and the time that the animal spent in each quadrant of the pool was measured (Fig. 4A, ii). These findings suggest that PTU rats require more time to learn the spatial location of the hidden platform compared with the controls. This delay in escape latency was not attributed to the

swimming speed or visual defects because no differences or defects in these aspects were identified between the two groups (Fig. 4A, iii).

To further analyze memory impairments, Novel Object and Novel Localization recognition (NOR and NOL) tests were performed. These tests rely on the innate exploratory behavior of rats in the absence of external reinforcement [35]. The results indicated significant differences in the preference index between the PTU and control groups in the NOR (control: 0.72 ± 0.02, PTU: 0.56 ± 0.01) (Fig. 4B, i) and NOL (control: 0.64 ± 0.02, PTU: 0.41 ± 0.02) (Fig. 4B, ii) tasks, in which the PTU rats were impaired in the recognition of the object that changed with respect to object or location. These findings indicated that the induction of hypothyroidism leads to a decrease in cognitive performance.

The LOF test was used to evaluate general locomotor activity and exploration. We evaluated parameters related to spontaneous behavior in mice including the time of moving and the number of lines that crossed the center. No significant difference was observed between the two groups, which suggest that the hypothyroidism does not influence the general status of animals (Fig. 4C, i and ii).

3.4. PTU-induced hypothyroidism impairs synaptic transmission and plasticity in the CA3-CA1 synapses of the adult rat hippocampus.

To determine whether hypothyroidism triggers alterations in the functional integrity of the hippocampus, electrophysiological recordings were used to assess synaptic plasticity. Among the types of plasticity, LTP and LTD are present and are expressed as a persistent increase or

decrease in synaptic efficiency following a high or low frequency stimulation, respectively [36,37].

The first register was the baseline synaptic transmission; this variable was assessed by the fEPSP slope in response to the electrical stimulation of varying current amplitudes and was registered by placing a recording electrode in the CA1 area of the stratum radiatum to assess dendritic activation. The results indicated an increase in the magnitudes of the fEPSP recordings in the hippocampus of the rats treated with PTU compared with the controls (Fig. 5A, i). The results in Figure 5A, ii indicate a repeated measures ANOVA of the baseline I/O functions, which confirms that the fEPSP slope increased as a function of the stimulus intensity ($F(8, 32) = 339.2$). The paired pulse facilitation (PPF) index was assessed by the ratio between two pulses separated by 50 ms. PTU treatment induced an impairment in the PPF at this interval, whereas PTU treatment reduced the facilitation of the second pulse compared with the control animals (Fig. 5B, i and ii).

We subsequently assessed whether synaptic plasticity was affected at the CA3–CA1 synapses of the PTU-treated rats by measuring the LTP and LTD of synaptic strength. In the control animals, TBS induced a persistent increase in the fEPSP slope, which was maintained up to 60 min, a result expected in young-adults rats, whereas the PTU treated group failed to induce LTP (control: 1.45 ± 0.11, PTU: 1.00 ± 0.01) (Fig. 6A, i-iii). Similarly, for LTD, the LFS induced a persistent depression in the control rat hippocampus, whereas it failed to induce such a response in the PTU-treated rats (control: 0.71 ± 0.04, PTU: 1.01 ± 0.04) (Fig. 5D, i-iii). These findings suggest impairment in the synaptic plasticity of PTU-treated animals.

3.5. Hypothyroidism alters neural and oxidative stress markers in the hippocampus

To analyze a neural structural component, microtubule-associated protein 2 (MAP2), a cytoskeleton member that labels the dendrites of neurons, was investigated [38]. We analyzed the expression of MAP2 in the cortex and hippocampus. In the control group, we identified strong expression of this marker in both regions (Fig. 7A, i-viii), whereas the expression was significantly decreased by 70% in the PTU group (Fig. 7A, ix-xvi) in the hippocampus CA1 and CA3 (Fig. 7A, xvii). Our results indicate a decrease in MAP2 when insufficient levels of thyroid hormones are produced. This finding indicates that neural structures are altered in the hypothyroidism condition.

GFAP, a glial marker, was also analyzed; however, there were no differences between the conditions (Fig. 8A, i-ix) as demonstrated via quantification (Fig. 8A, ix). Furthermore, this result was confirmed by the protein expression levels of GFAP, as indicated by western blot (Fig. 8B, i) and the respective quantification (control: 1 ± 0.02, PTU: 0.99 ± 0.03) (Fig. 8B, ii).

To determine whether hypothyroidism has an effect on oxidative stress and therefore a neurotoxic role, we investigated a harmful product of lipid peroxidation, 4-hydroxy-2-nonenal (4-HNE) [39,40]. The results indicated a low expression of 4-HNE in the control group in both the cortex and hippocampus (Fig. 9, i-viii). In a comparison of PTU and controls, the expression of 4-HNE significantly increased in a 30% in CA3 of the hippocampus (control: 1 ± 0.28, PTU: 3.67 ± 0.16) and 60% in the cortex (control: 1 ± 0.20, PTU: 7 ± 1.05) (Fig. 9, ix-xvi). These findings suggest that low levels of thyroid hormones promote the formation of oxidative compounds.

4. Discussion

The present study presents evidence of the negative effects triggered by PTU-induced hypothyroidism in young rats, with a specific focus on the hippocampal region because of its relevance to memory retrieval. Our findings indicated a clear impact on global metabolism, which was demonstrated in the weight and diet. Moreover, our results demonstrated a decrease in cognitive performance in tasks that involved acquisition, recognition and localization memory. Furthermore, these findings were supported by hippocampal plasticity impairments and a decrease in glucose uptake.

Deficits in the thyroid hormones triiodothyronine (T_3) and thyroxine (T_4) occur in the hypothyroid condition, which are predominately generated by iodine deficiency or an autoimmune response [7,41,42,43]. Hypothyroidism may present in all stages of development, ranging from pregnancy through adulthood. During pregnancy, this manifests as subclinical hypothyroidism and congenital hypothyroidism, in which the latter has a more substantial impact on neurodevelopmental retardation and metabolic impairments, such as poor feeding and deficits in weight gain and growth. In contrast, during adulthood, subclinical hypothyroidism has been related to depression and dementia [10,11,12]. Depression and cognitive impairments have been related to poor neurogenesis; during adulthood, this process is mainly triggered in the subventricular and subgranular zone, where thyroid hormones are present, and T_3 increases the differentiation in neuronal precursors [44].

Thyroid hormones are also implicated in metabolic alterations in several organs, in which the brain is fundamental. This condition has various adverse effects in the brain, which have primarily been investigated in the previously described stages and include dysregulations in

memory and attention, maintenance of the metabolic rate, equilibrium of reactive oxygen species (ROS), thermogenesis and feeding [45,46,47]. More specifically, studies have demonstrated alterations in neural growth and differentiation, hippocampal excitability and plasticity, gliosis and apoptosis [46,48,49,50]. During pregnancy, the fetus relies on the mother's thyroid hormones until 12-14 weeks of gestation. Hypothyroidism during pregnancy affects 2.5% of women, and congenital hypothyroidism has a 3500-4000 incidence in newborns. The impact of a deficiency during the developmental period results in numerous alterations in dendritic spine morphology, cell migration and proliferation and synaptic transmission in the hippocampus and other issues [8,9]. Furthermore, a relationship between suboptimal thyroid function during pregnancy and low child IQ and attention-deficit/hyperactivity disorder (ADHD) has been established. Moreover, postnatal studies that indicate alterations in thyroid hormones have demonstrated perturbations in glucose metabolism, a hypothalamic energy imbalance and hippocampus structure and plasticity [46,50,51].

In the present work, hypothyroidism was induced as indicated by the thyroid hormone levels, weight and fluid and food intake. Impairments in cognitive aspects, including acquisition, recognition and localization memory, were identified in the rats treated with PTU. In addition to these changes, clear differences were identified in the size between the control and PTU groups, an effect that has previously been demonstrated with this drug [52]. These changes may be a result of a slowdown in development because thyroid hormones affect the metabolism of proteins, carbohydrates and lipids, and the affected targets are broad, including metabolic pathways, growth and inflammatory processes [53,54]. Other studies have also demonstrated that hypothalamic tanycytes, which control feeding behavior, are affected by thyroid hormones;

therefore, the effects on intake may be related to dysregulation of these cells at the hypothalamic level [55].

Considering that glucose is one of the main energy supplies of the body, the regulation and maintenance of glycemic levels are essential for the correct functioning of organisms. Thyroid hormones have a role in the regulation of carbohydrates, and it has been reported that hypothyroidism may reduce glucose availability, alter the precise absorption of glucose and delay gluconeogenesis, which reduces insulin synthesis. Moreover, hypothyroidism has been associated with metabolic diseases, such as diabetes, and thyroid dysfunction has been reported in 11% of diabetic patients [56]. In the present work, it was demonstrated that the induction of hypothyroidism leads to a mild deregulation of glycemic control because of the increase in fructosamine levels in the PTU-treated rats, which indicated that the glucose levels were increased during the treatment compared with the control condition. This finding was confirmed by the basal glucose levels after fasting because of the increased glucose levels in the PTU rats. Moreover, in the glucose tolerance test, the PTU group required a longer time to return to basal glucose levels. Nevertheless, the PTU group reached its normal glycemic levels, which may imply a slower release of insulin in the hypothyroid group.

A deregulation in glucose metabolism was also identified when the radioactive glucose accumulation was assessed in hippocampal slices. The effect of hypothyroidism on brain glucose consumption is relevant because glucose is the main source of energy for this organ and is mainly consumed by neurons; thus, cognitive performance depends on it, and an energy imbalance may affect major functions in the brain [57]. PET studies of patients with hypothyroidism have demonstrated a reduction in brain regional activity, including the hippocampus [51]. PTU treatment clearly generates a metabolic imbalance, as demonstrated by

the difference in weight, consumption of food and liquid as well as a reduction in glucose accumulation in hippocampal slices. AMPK controls feeding behavior, cellular energy status, cellular survival and apoptosis. Studies have demonstrated that AMPK responds to nutritional status specifically in the hippocampus and affects cognitive ability by altering neural cell fate [58,59,60]. Furthermore, studies have demonstrated that AMPK couples energy metabolism to LTP expression [61]. In the present work, the results did not indicate a difference in the expression of AMPK. We also analyzed PGC-1α, a coactivator of PPAR-dependent transcription, which co-activates the thyroid hormone transcription factor that is typically activated by T_3 and explains why we analyzed a potential deregulation of PGC-1α. However, no changes were identified in the hippocampus, which may have been a result of compensatory mechanisms [53]. This finding does not rule out further metabolic alterations that explain the glucose alterations because other metabolic sensors may be dysregulated, such as Akt and SIRT1. Thus, additional studies are necessary to elucidate the implications in cell signaling [62].

In our model, we demonstrated that hypothyroidism causes a disruption in spatial learning during the acquisition process of the MWM task, as well as in object recognition and object location memory. The MWM results indicated an impaired performance, as demonstrated by the significant time of latency to reach the platform in the second, third and fourth days. Nevertheless, from the fifth day, the difference between the groups was not significant, which suggests that the hypothyroid rats must repeat the test more times compared with controls to establish precise spatial learning. The study of Gilbert and Sui [63] also demonstrated a deficit in the MWM; however, the effect was maintained during all days. Nevertheless, we attribute this behavior to the animal model, in which PTU was administered during pregnancy, and the effect may have been more aggressive. In the NOR and NOL tests, the failure to interact more with the

novel or relocated object has been attributed to an impairment in the retrieval of memory for the familiar object [64,65]. These behavioral alterations, in addition to plasticity impairments, have been identified in neurodegenerative diseases associated with the hypothyroid condition, including autism spectrum disorders, which have been related to gestational hypothyroidism, and Alzheimer's disease (AD), a neurodegenerative disease characterized by a decline in cognitive functions. In AD, a thyroid hormone imbalance has been identified in previous years [66,67,68,69,70]. The effects identified in our young model may represent a less aggressive form of disruptions that this condition may produce. A comparable study of the effects of thyroid hormone deficiencies during gestational stages, young and adult development may contribute to an analysis of the severity of the lack of these hormones.

In agreement with the behavioral alterations, the results of electrophysiological recordings showed that excitatory basal transmission and synaptic plasticity were affected in PTU-treated rats. The basal excitatory transmission, measured as I/O relationship, was significantly increased in slices from PTU-exposed animals. This could be related to the reduction in the PPF index observed in the PTU-treated animals, which suggest an increase in the probability of neurotransmitter release [71,72,73]. In hippocampal neurons, the release probability is low, for that reason first spike will cause a small fEPSP, but the build-up of calcium in the presynaptic terminal will lead to an increased release probability on the second spike and as a result, greater transmitter release and a greater postsynaptic response, and a higher PPF. The fact that PTU treatment generates a reduction in the PPF index, suggest an increase in the release probability, because now both spikes are more similar indicating that the first pulse will deplete the available transmitter, and the second pulse will cause less transmitter to be release [72,73]. Previously reported findings have also demonstrated disruptions in

neurotransmitter release in the hippocampus of hypothyroid-induced animals [74]. To evaluate if synaptic plasticity was affected at the CA3–CA1 synapses of the PTU-treated rats, we assessed two electrophysiological paradigm of learning and memory, LTP and LTD. Our results indicate that LTP and LTD induction was defective in PTU-treated rats and this was consistent with the poor performance of those animals in behavioral task. This increase in basal synaptic transmission and decrease in plasticity induction is similar to the observations made by Sui and Gilbert in a neonatal rat model of hypothyroidism exposed *in utero* and postnatal to PTU with the exception that they do not report changes in the induction of LTD [75]. This is interesting, because like in our study, the T3 and T4 levels of the treated animals are severely reduced at the time of performing the measurements [75]. In other studies of hypothyroidism, the recording of behavioral task and synaptic physiology was performed at time points in which the hormone levels have returned to basal levels. In consequence, they observe opposite results including an increase in the induction of LTP [76], which could be due to the restoration of the hormone levels. Additionally, our observations are also consistent with previous reports that described the relations between initial PPF conditions and the direction and amplitude of synaptic modifications. The direction of synaptic modifications depended on the initial state of presynaptic release mechanisms, that means that inputs with a high initial PPF ratio tended to be potentiated (control conditions), while inputs with a low initial PPF ratio usually underwent depression or did not change (PTU treated animals) [77]. Those evidences indicate that hormone deprivation interferes with the mechanisms of information storage and processing in the hippocampus of young male rats.

MAP2 is a cytoskeleton protein that regulates microtubule assembly in dendrites, and if it is altered, a destabilization of dendrites and synaptic signal transduction may occur. Thus, we

also analyzed the expression of MAP2 protein. Our findings of MAP2 clearly indicate a decrease in the dendritic arbors of neurons in the hippocampus and cortex of PTU-treated rats. Moreover, we also investigated whether the glial population was altered because previous studies in neonatal hypothyroidism have demonstrated that GFAP mRNA concentrations are reduced up to 55% in the hippocampus. However, GFAP staining did not exhibit significant changes; therefore, the alterations may be attributed to a change in the plasticity of neurons and not gliosis [38,78]. In addition to the described alterations, we also identified an increase in the oxidative marker 4-HNE, a product of lipid peroxidation, which has been implicated in pathophysiological conditions that may result in cellular death [39]. This increase was identified in all investigated regions; however, it was significantly increased in the cortex and in CA3 regions.

5. Conclusions

Hypothyroidism is a disease that affects the function of several tissues, including the brain. In the present work, we demonstrated that the early induction of this pathology triggers a general dysregulation in important brain functions, including the processes of memory, learning and neuronal plasticity; a decrease in the expression of structural markers, an increase in oxidative stress; and a strong decrease in the utilization of glucose. Together, our data emphasize the importance of the prevention and early diagnosis of this pathology to avoid the onset and progression of this general brain dysfunction (Fig. 10).

Conflict of interest

The authors declare that they have no conflict of interests.

Author contributions

Conceived and designed the experiments: P.S, P.C. and N.C.I.

Performed the experiments: P.S, J.F.C. and P.C.

Analyzed the data: P.S, J.F.C. and P.C.

Contributed reagents/materials/analysis tools: N.C.I.

Wrote the manuscript: P.S, P.C. and N.C.I.

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Figure Legends

Table 1. Effect of PTU on hormone plasma levels in male rats. Plasma levels of TSH, tT_3, tT_4, fT_4, cholesterol and fructosamine in control or PTU-treated rats. Samples were analyzed in the Specialized Clinical Chemistry Laboratory. Values are expressed as the means ± SEM of 8 animals per group, statistically analyzed with one way ANOVA, followed by Bonferroni post test ($p \leq 0.05$*, $p \leq 0.001$**, $p \leq 0.0001$***).

Figure 1. PTU treatment induces hypothyroidism in young rats. (**A**) Representative model of the induction of hypothyroidism in young rats with the specific order in which experiments were conducted. (**B**) (**i**) Representative image of the body size and (**ii**) thyroid gland of control and PTU-treated rats. (**C**) (**i**) Weight of the control or PTU-treated rats during the time of hypothyroidism induction. (**ii**) Fluid intake of both groups. (**iii**) Food intake of both groups. (**iv**) Relative organ weight expressed as an absolute organ weight/100 g body weight of the liver, kidney and brain of controls or PTU rats. Values are expressed as the means ± SEM of 16 animals per group, statistically analyzed with one way ANOVA, followed by Bonferroni post test ($p \leq 0.0001$***).

Figure 2. Effect of hypothyroidism on biochemical parameters and glycemic regulation. (**i**) Insulin and (**ii**) glucose levels in both groups. (**iii**) Time glucose curve (mg/dL) after the glucose

tolerance test in both conditions. (**iv**) HOMA-IR values calculated from glucose and insulin values. Normal values correspond to ≤ 2.5, whereas values ≥ 2.5 are considered insulin resistance (IR). Values are expressed as the means ± SEM of 8 animals per group, statistically analyzed with one way ANOVA followed by Bonferroni post test ($p \leq 0.05$*, $p \leq 0.0001$***).

Figure 3. Metabolic status with respect to protein levels and glucose accumulation following the induction of hypothyroidism. (**A**) (**i**) Western blot analysis of the metabolic proteins AMPK and PGC1α in the hippocampus of control and PTU-treated rats. (**ii**) Quantification of protein levels in both groups. (**B**) (**i**) Time course of 2-DG uptake was measured in slices from PTU and control rats up to 60 min of incubation. (**ii**) Uptake of 2-DG at 60 min, at which point slices were treated with Cyt B and H_2O_2 as negative controls. (**iii**) Uptake of 2-DG at 60 min in the presence of increasing concentrations of unlabeled glucose (0-20 mM). Values are expressed as the means ± SEM of 4 animals per group or 9 slices per group, analyzed statistically via one way ANOVA followed by a Bonferroni post hoc analysis ($p \leq 0.05$*, $p \leq 0.001$**).

Figure 4. **Hypothyroidism affects memory and learning cognitive functions.** (**A**) Rats with hypothyroidism (PTU) and control rats were subjected to the Morris water maze for 8 days. (**i**). The mean time required to reach the hidden platform (latency time) is represented in the y-axis for males. (**ii**) Time spent in each quadrant in the absence of the platform (probe trial). (**iii**) Swim speed of both groups in a comparison of day 1 and day 4. (**B**) NOR and NOL tasks were performed by PTU and treated rats. (**i**) Preference index for the novel object (NOR). (**ii**)

Preference index for the new localization of the object (NOL). (**C**) Large open field test in control and treated group. (i) Time of moving and (ii) number of lines thath the animal cross the center of the cage. Values are expressed as the means ± SEM of 8 animals per group, analyzed statistically via one way ANOVA followed by Bonferroni post hoc analysis ($p \leq 0.05$*, $p \leq 0.0001$***).

Figure 5. Baseline synaptic transmission is impaired by PTU treatment. (**A**) Representative traces of fEPSP elicited by different stimuli strengths (10-90 µA) in hippocampal slices of control and PTU-treated animals. (**ii**) Input-output curves obtained from (**i**) indicated that the EPSP slope was increased in the slices obtained from PTU-treated animals. (**B**) Representative traces of pair pulse fEPSP (R1 and R2) obtained from hippocampal slices of control and PTU-treated animals. (**ii**) Pair pulse ratio (R1/R2) obtained from (**i**). PTU treatment reduced the facilitation of the second pulse typically observed in the CA1 region of the hippocampus. Values are expressed as the means ± SEM of 4 animals per group.

Figure 6. Hypothyroidism impairs synaptic plasticity. (**A**) (**i**) Representative traces of fEPSP recorded before (1) and after (2) theta burst stimulation in hippocampal slices of control and PTU-treated animals. (**ii**) Long-term potentiation (LTP) is impaired in animals treated with PTU (black dots) with respect to controls (white dots). (**iii**) Mean fEPSP at 1 h after LTP induction. (**B)** (**i**) Representative traces of fEPSP recorded before (1) and after (2) low frequency stimulation in hippocampal slices of control and PTU-treated animals. **(ii)** Long-term depression (LTD) is impaired in animals treated with PTU (black dots) with respect to controls (white dots).

(**iii**) Mean fEPSP at 1 h after LTD induction (Mann Whitney test, * $p \leq 0.05$). Values are expressed as the means ± SEM of 4 animals per group.

Figure 7. Hypothyroidism reduces the expression of the dendritic marker MAP2 in the cortex and hippocampus. (**A**) Representative immunofluorescence of MAP2 in control and PTU rats. MAP2, red; nuclear stain Hoechst, blue. MAP2 in the cortex in control (**i**) and PTU rats (**ix**). MAP2 mark in the hippocampus CA1 (**iii**), CA3 (**v**) and dentate gyrus (**vii**) in control and CA1 (**xi**), CA3 (**xiii**) and dentate gyrus (**xv**) in PTU rats. (**xvii**) Graphs indicate the quantification of the immunofluorescence signal normalized to the control conditions. Scale bar: 100 µm. Magnification: 50 µm. Values are expressed as the means ± SEM of 4 animals per group, analyzed statistically via one way ANOVA followed by Bonferroni post hoc analysis ($p \leq 0.001$**).

Figure 8. Hypothyroidism does not alter the expression of GFAP in the cortex or hippocampus. (**A**) Representative immunofluorescence of GFAP in control and PTU rats. GFAP, red; nuclear stain Hoechst, blue. GFAP in the cortex in control (**i**) and PTU rats (**v**) and the dentate gyrus in control (**iii**) and PTU rats (**vii**). Each image has a corresponding magnification. (**ix**) Graphs indicate the quantification of the number of GFAP-positive cells. (**B**). **(i)** Western blot analysis of GFAP protein expression in the hippocampus of control and PTU-treated rats. (**ii**) Quantification of the levels of each protein in both groups. Values are expressed as the means ± SEM of 4 animals per group, analyzed statistically via one way ANOVA followed by Bonferroni post hoc analysis. Scale bar: 100 µm. Magnification: 50 µm.

Figure 9. Hypothyroidism increases the expression of the lipid peroxidation marker 4-HNE in the cortex and hippocampus. Representative immunofluorescence of 4-HNE in control and PTU rats. 4-HNE, red; nuclear stain Hoechst, blue. 4-HNE in the cortex in control (**i**) and PTU rats (**ix**). 4-HNE mark in the hippocampus CA1 (**iii**), CA3 (**v**) and dentate gyrus (**vii**) in control and the CA1 (**xi**), CA3 (**xiii**) and dentate gyrus (**xv**) in PTU. Each image has a corresponding magnification. (**B**) Graphs indicate the quantification of the immunofluorescence signal normalized to the control conditions. Values are expressed as the means ± SEM of 4 animals per group, analyzed statistically via one way ANOVA followed by Bonferroni post hoc analysis ($p \leq 0.001$**, $p \leq 0.0001$***). Scale bar: 100 µm. Magnification: 50 µm.

Figure 10. Alterations caused by the induction of hypothyroidism in a young rat model. The induction of hypothyroidism in rats at 21 days of age via the administration of 0.05% PTU during a one-month period triggered deficits in synaptic and cognitive functions at the hippocampal level, as demonstrated via behavior and cognitive tasks that involve memory and acquisition processes, as well as a reduction in LTP induction. Moreover, hypothyroidism generates a metabolic imbalance in the energetic state as demonstrated by a decrease in the accumulation of glucose in hippocampus slices. Finally, an increase in oxidative stress was identified via the expression of 4-HNE in the hippocampus.

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Figure 1

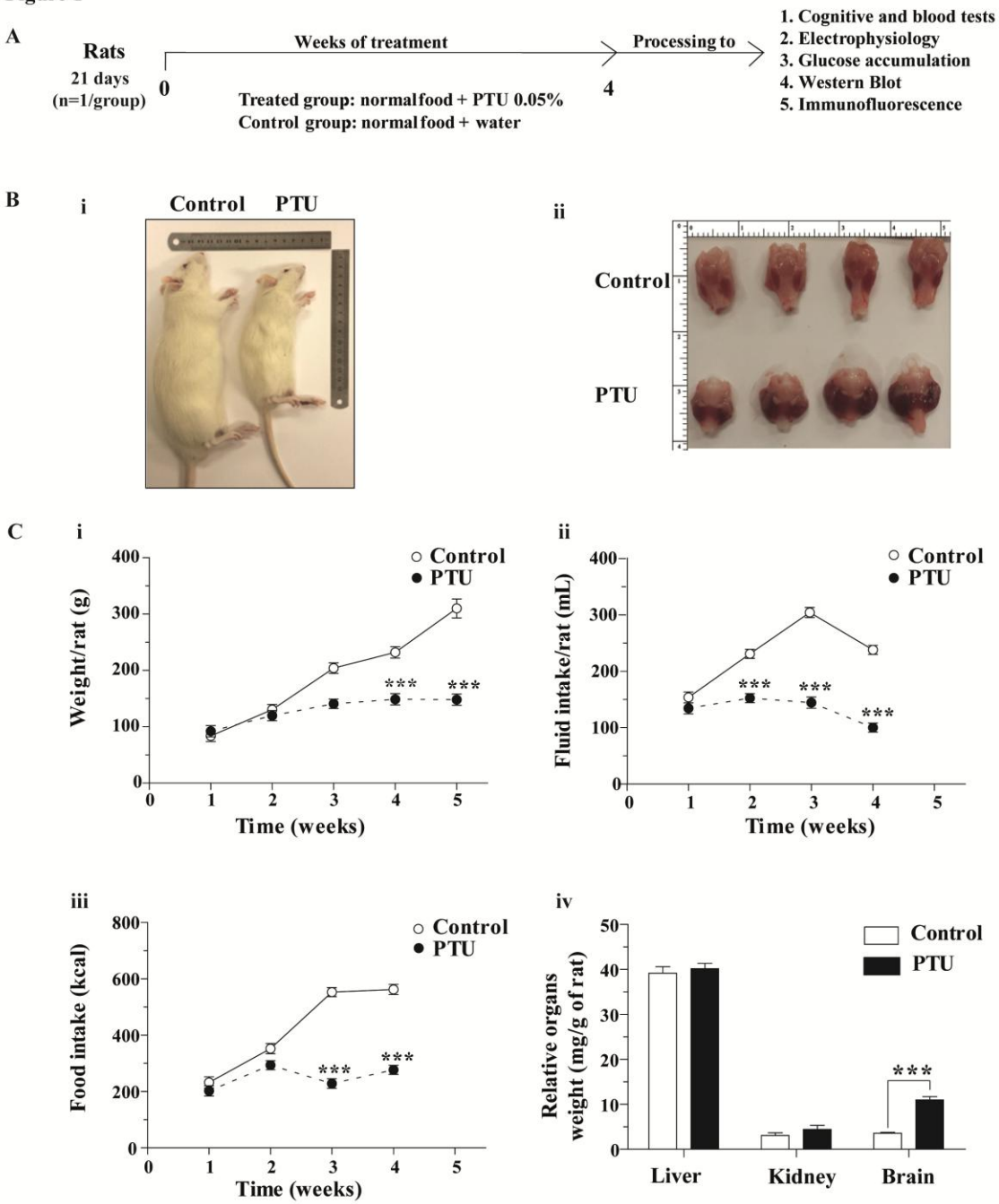


Figure 2

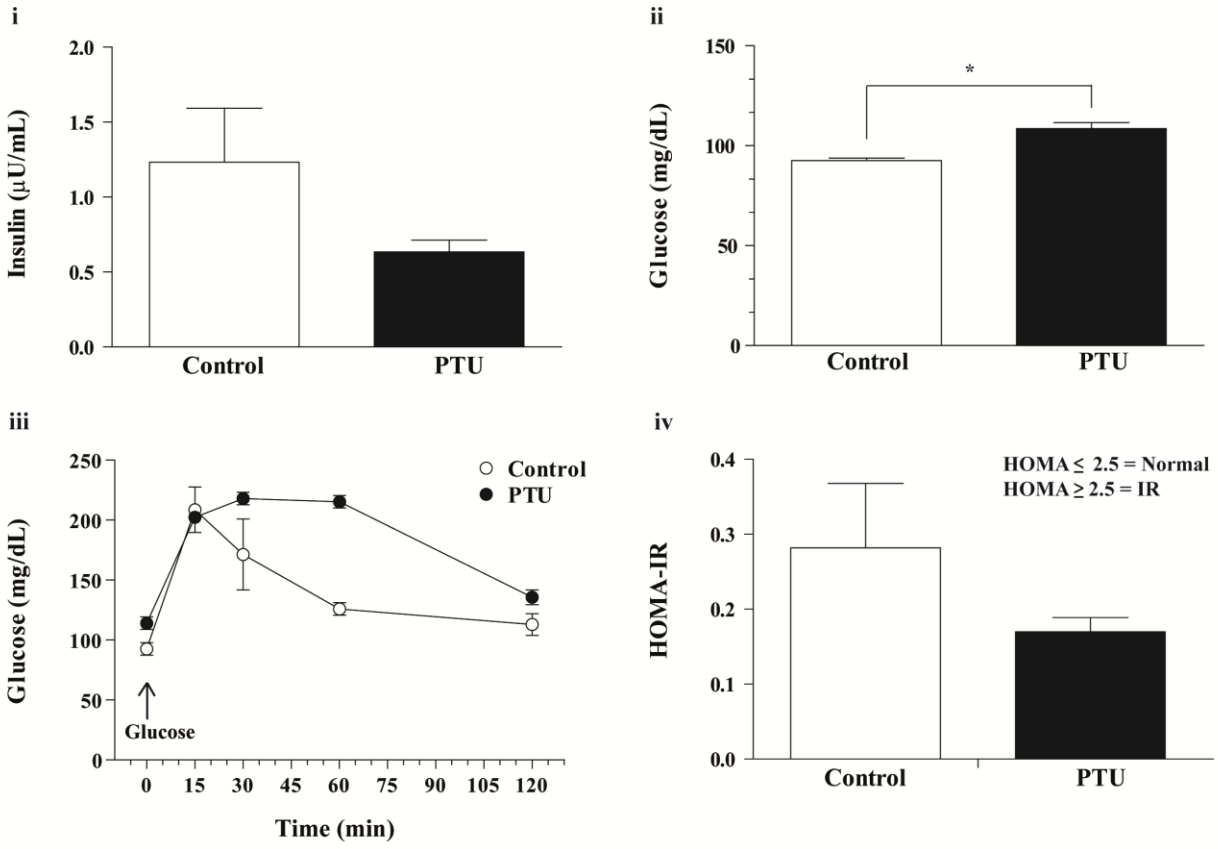


Figure 3

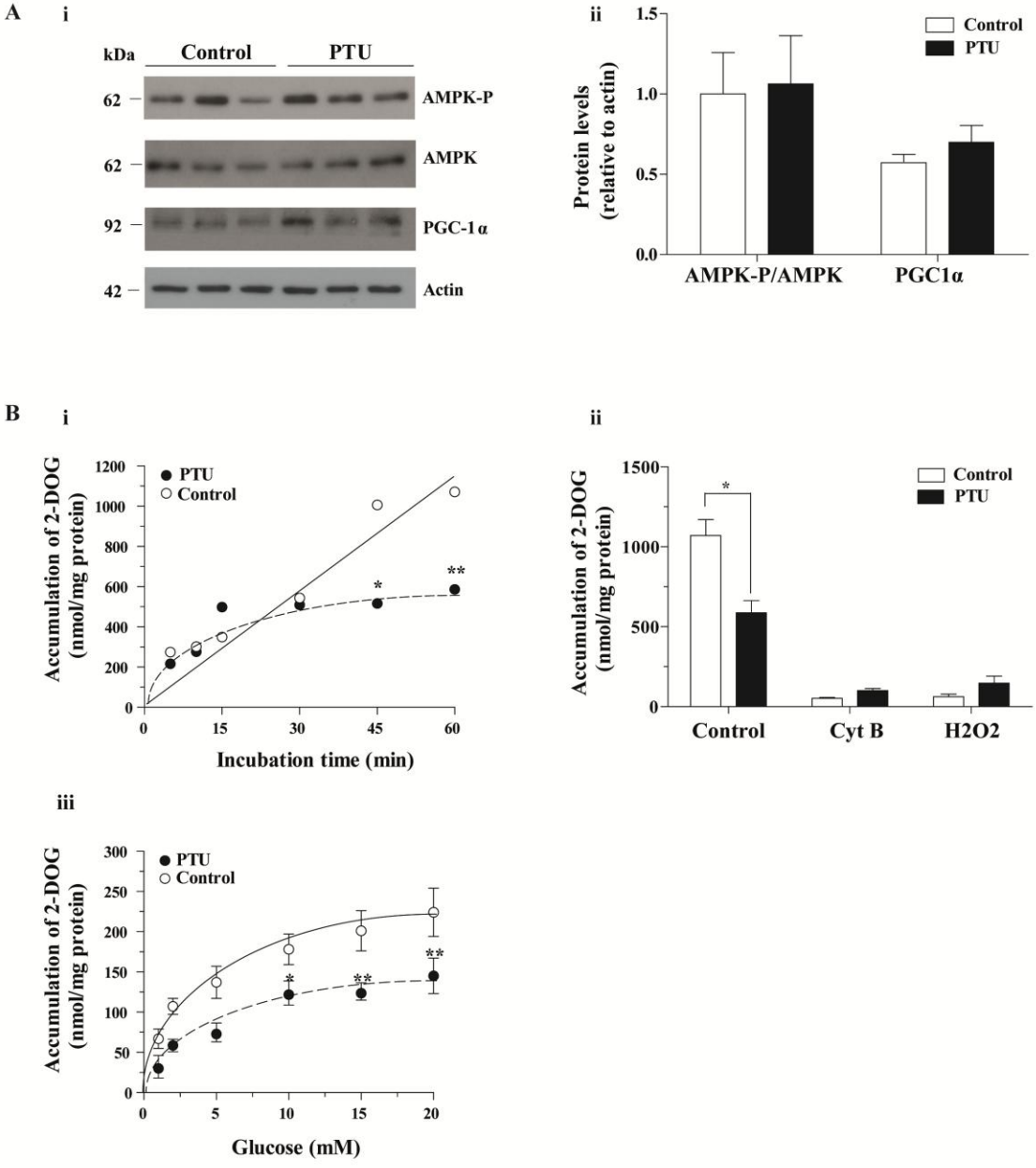
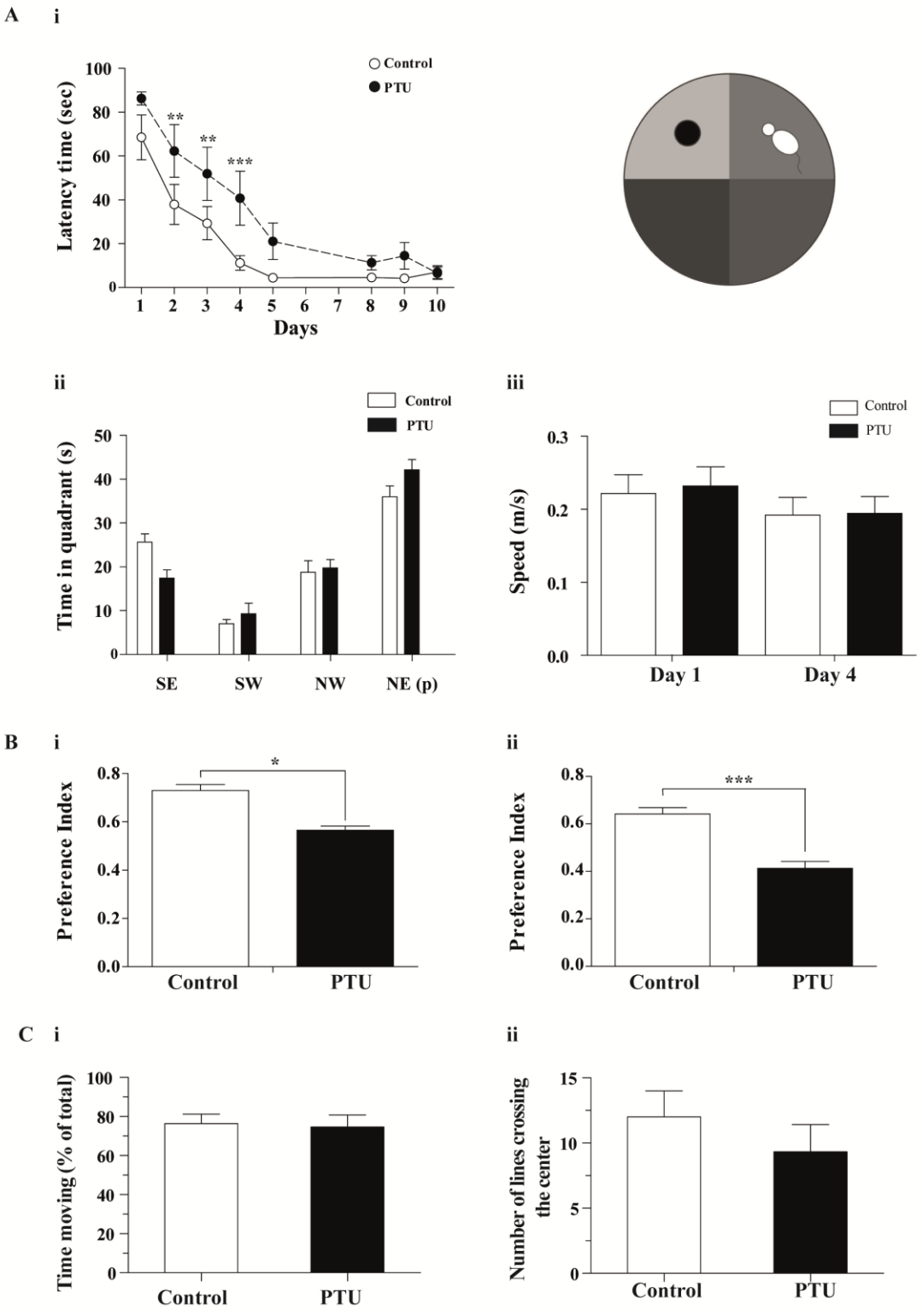


Figure 4



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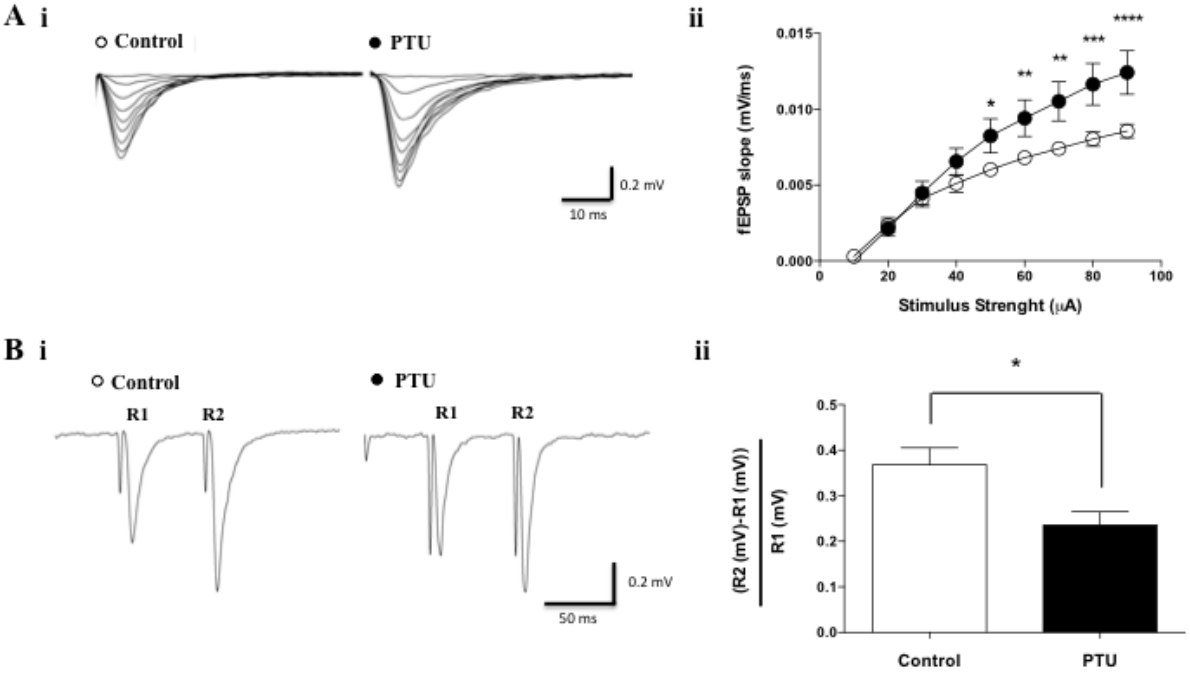

Figure 6

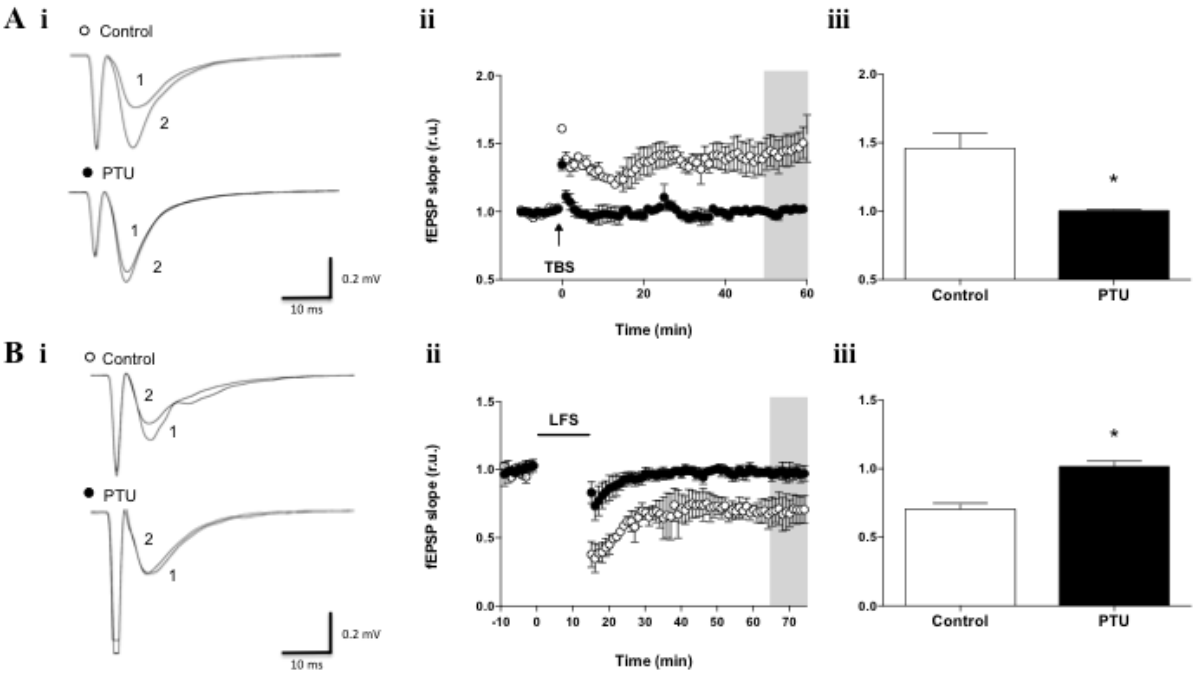

Figure 7

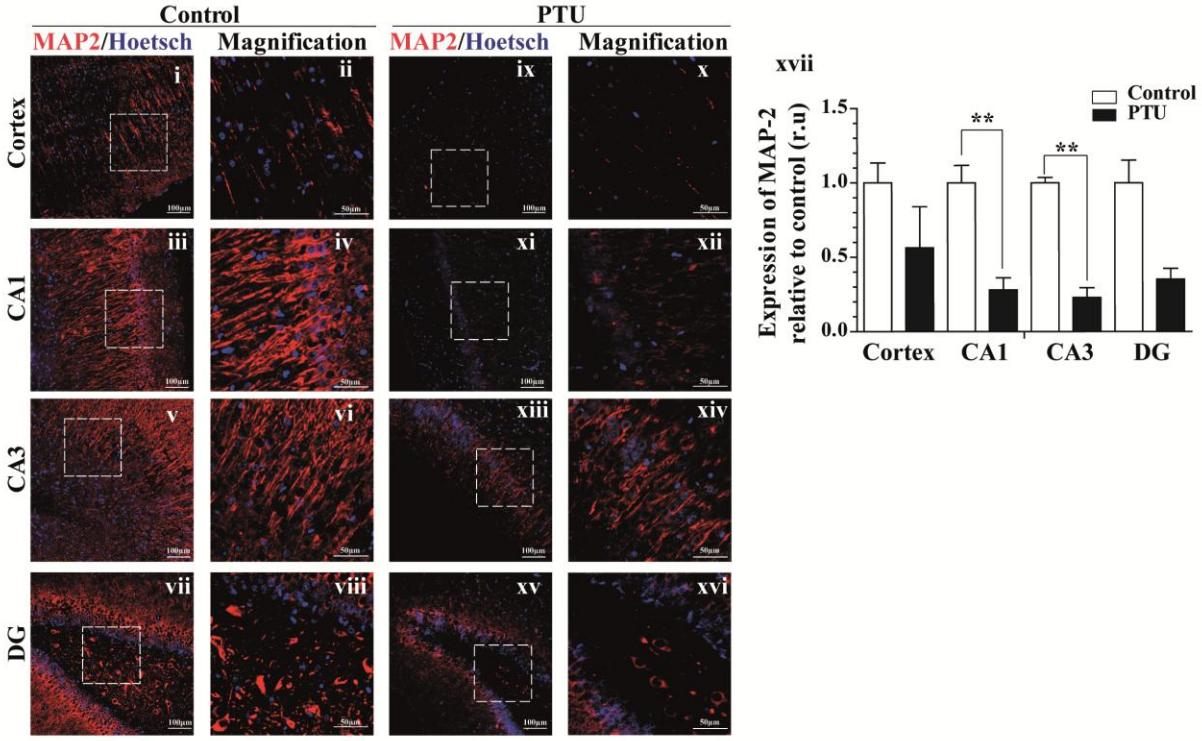

Figure 8

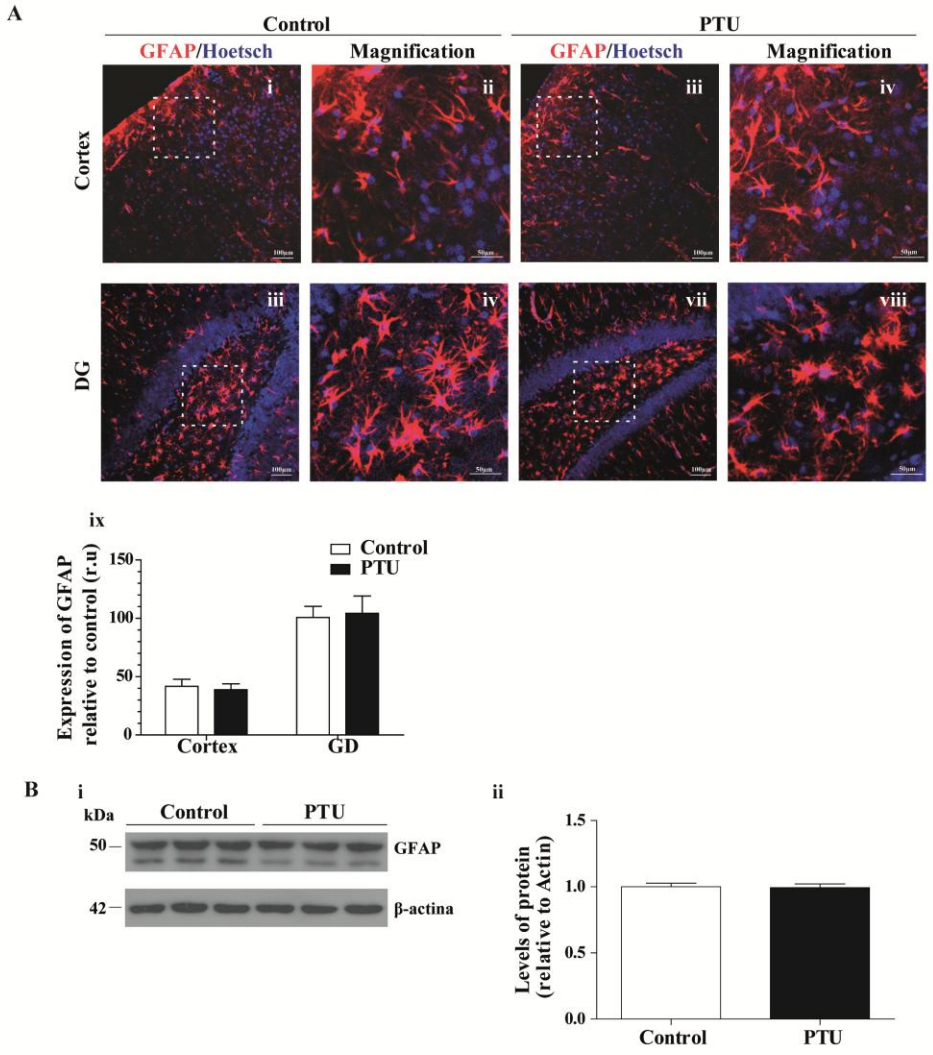

Figure 9

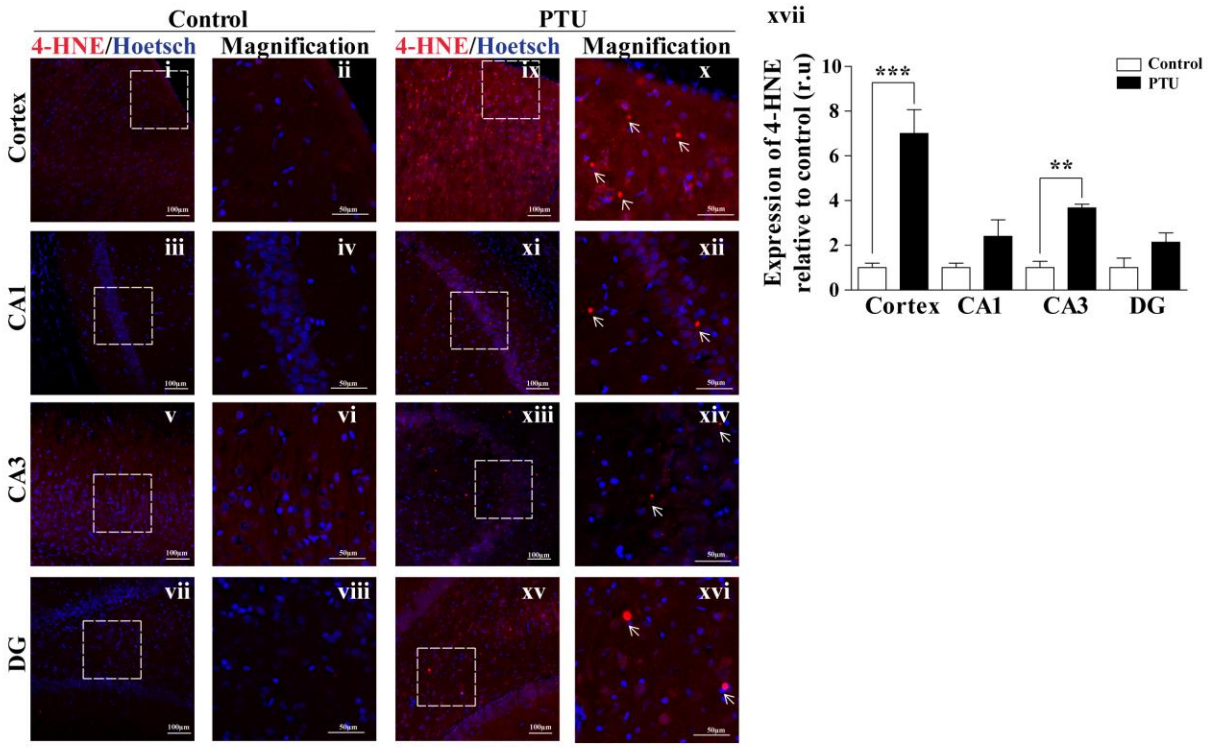

Figure 10

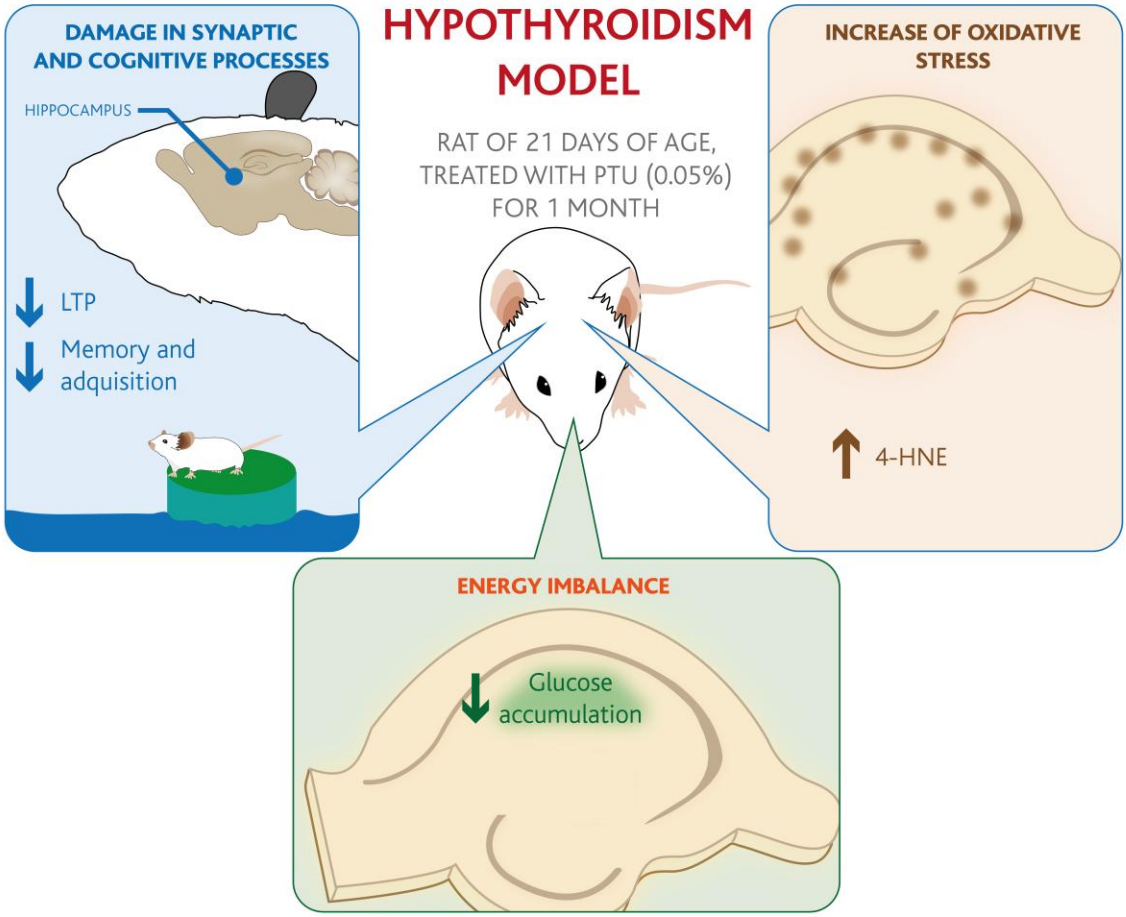

Table 1

TSH (μIU/mL)	0.015 ± 0.008	0.025 ± 0.008*
T3 (ng/mL)	0.512 ± 0.002	0.255 ± 0.056*
T4 (μg/dL)	4.125 ± 0.298	1.005 ± 0.037***
T4L (ng/dL)	1.337 ± 0.115	0.050 ± 0.001***
Cholesterol (mg/dL)	62.00 ± 5.447	81.70 ± 2.394
Fructosamine (ng/dL)	236.2 ± 2.454	329.0 ± 8.317***

Highlights

- The induction of hypothyroidism in a young rat model triggers a decrease in the cognitive performance.
- The hypothyroidism induction lead to a strong decrease of the neuronal plasticity in the hippocampus.
- The hypothyroidism condition induces a deregulation in the hippocampus area.
- The establishment of the hypothyroidism condition induces a decrease in the glucose metabolism of brain.