Brain-derived neurotrophic factor and nitric oxide contribute to protective effects of rosiglitazone on learning and memory in hypothyroid rats

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The effects of the well-known peroxisome proliferator-activated receptor gamma (PPAR-γ) agonist rosiglitazone (Rosi) on brain-derived neurotrophic factor (BDNF), nitric oxide (NO), and learning and memory were investigated in hypothyroid rats. Hypothyroidism was induced in immature Wistar rats by administration of propylthiouracil in drinking water. Rats were divided into four groups: control, hypothyroid, and hypothyroid treated with Rosi at doses of 2 mg/kg or 4 mg/kg. Memory was then assessed by the Morris water maze (MWM) and passive avoidance (PA) tests. Following anesthetization, brain samples were collected for biochemical measurements. Hypothyroidism increased the escape latency and traveled path in the learning trials of the MWM and decreased the time spent and the distance traveled in the target quadrant on the probe day. Hypothyroidism also impaired the avoidance behavior of rats in the PA test. Rosi improved the performance of rats in both MWM and PA tasks. Hypothyroidism also decreased hippocampal BDNF levels, increased NO metabolites, and induced oxidative damage in the brain. Treatment of hypothyroid rats with both doses of Rosi increased BDNF levels and decreased NO metabolites and malondialdehyde concentrations. In addition, thiol content and superoxide dismutase and catalase activities were increased in the brain regions of hypothyroid rats receiving Rosi. The administration of 4 mg/kg Rosi also significantly increased serum thyroxin levels. The results of the present study showed that BDNF and NO play a role in the protective effects of Rosi against learning and memory impairment in hypothyroid rats.

Key words: hypothyroidism, learning, memory, nitric oxide, brain-derived neurotrophic factor, rosiglitazone

INTRODUCTION

The brain is well-recognized as a major target organ for thyroid hormones, which are known regulators of cell proliferation during the neonatal and growth periods. Thyroid hormones are also act as regulators of neurotransmitter systems, including the cholinergic and dopaminergic systems, altering brain function (Porter-field, 2000). During brain development and adulthood, thyroid hormone deficiency is associated with brain abnormalities, including biochemical and cellular alterations, which lead to behavioral abnormalities, including learning and memory impairment (Ahmed et al., 2008; Ritchie and Yeap, 2015). These hormones regulate the growth factor expression and synaptogenesis in learning and memory-related brain areas, including

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the hippocampus and cortex (Forrest and Vennström, 2000; Parent et al., 2011). The hippocampus appears to be more sensitive than the cortex to thyroid hormone depletion (Zhang et al., 2009; Parent et al., 2011).

The exact mechanism of cognitive impairment due to hypothyroidism is not fully understood; however, several mechanisms such as tissue oxidative damage due to the excessive production of reactive oxygen and nitrogen species have been suggested. Another mechanism is the loss of neurotrophins (NTs), most importantly brain-derived neurotrophic factor (BDNF), due to reduced NT production (Shafiee et al., 2016; Baghcheghi et al., 2018a).

BDNF is a key protein in the regulation of neuronal growth and survival (Mattson et al., 2004). This neurotrophic factor is important both for cell proliferation, differentiation, and neuronal growth during brain development and the regulation of synaptic transmission and learning and memory throughout life (Yamada et al., 2002; Numakawa et al., 2010). BDNF and its endogenous receptors, TrkB receptors, are widely expressed in the mammalian brain (Lewin and Barde, 1996; Tapia-Arancibia et al., 2004; Wang et al., 2006). In both humans and rodents, BDNF has been detected throughout the brain, including the hippocampus, amygdala, cerebellum, and cerebral cortex, however, the highest level of BDNF is in the hippocampus (Hofer et al., 1990; Timmusk et al., 1993; Miranda et al., 2019). Despite its importance in neuronal and overall brain health, BDNF levels are dysregulated in some disease states, including hypothyroidism. For example, hypothyroidism decreases BDNF levels in the hippocampus (Koibuchi et al., 1999; Koibuchi and Chin, 2000) and cerebellum (Chakraborty et al., 2012) in neonatal rats and mice. Prenatal hypothyroidism in rodents is even capable of decreasing BDNF expression in the brains of offspring (Neveu and Arenas, 1996; Sinha et al., 2009; Liu et al., 2010).

Hypothyroidism-associated learning and memory impairment during neonatal and juvenile growth in rats is accompanied by oxidative damage and excessive nitric oxide (NO) production in hippocampal and cortical tissue (Farrokhi et al., 2014). Increased nNOS expression in the brain has been reported in both maternal and adult-onset hypothyroidism (Cano-Europa et al., 2008; Sinha et al., 2008). Furthermore, our previous work has shown that hypothyroidism increases NO levels in brain tissue (Baghcheghi et al., 2016; 2018b). In the brain, NO contributes to oxidative damage through the formation of the highly reactive metabolite peroxynitrite (Chabrier et al., 1999; Jonnala and Buccafusco, 2001). Moreover, NO has been shown to directly inhibit the production of BDNF in hippocampal cell culture (Canossa et al., 2002). Therefore, excessive NO

production may interfere with several aspects of brain function, such as neuronal plasticity, memory formation, and synaptic transmission.

Peroxisome proliferator-activated receptor gamma (PPAR-γ) is a member of the nuclear hormone receptor superfamily, which is critical for adipocyte differentiation, lipid biosynthesis, glucose homeostasis, and immunomodulation (Jiang et al., 1998; Ricote et al., 1998). PPAR-γ is expressed in the central nervous system (CNS) and can protect the brain against the inflammatory response in CNS disorders (Landreth and Heneka, 2001; Dehmer et al., 2004). Recent studies have shown that the protective effect of PPAR-γ agonists is also mediated by inhibiting the activity of the major reactive oxygen species (ROS), producing xanthine oxidase, reducing the generation of superoxide (O_2^-), increasing superoxide dismutase (SOD)-1 activity, and decreasing of lipid peroxidation (Villegas et al., 2004; Shimazu et al., 2005). It has also been reported that PPAR-γ agonists reduced NO production in the brain through the suppression of inducible nitric oxide synthase (iNOS) expression (Kitamura et al., 1999; Heneka et al., 2000). In animal models of diabetes (Kariharan et al., 2015) and Alzheimer's disease (Prakash and Kumar, 2014), PPAR-γ agonists have significantly improved memory impairment and increased hippocampal BDNF levels. Moreover, in our previous study, we have shown that pioglitazone, a PPAR-γ agonist, effectively reversed the learning and memory impairment induced by hypothyroidism in rats (Baghcheghi et al., 2019).

Here we hypothesized that rosiglitazone (Rosi), a compound in the thiazolidinediones (TZDs) drug class with the highest affinity to PPAR-γ, has a protective effect against the consequences of hypothyroidism in the brain. Therefore, we examined the effects of Rosi on both memory formation, using the Morris water maze (MWM) and passive avoidance (PA) tests, and on biochemical measures, including hippocampal BDNF, brain oxidative damage, and NO production, in hypothyroid rats.

METHODS

Animals

Immature male Wistar rats (n=36) were 21±1 days old at testing and were maintained in a standard environment at a 22 to 24°C temperature and 12 hours' light/dark cycle. Food and water were provided freely. The experimental procedures were approved by the Ethics Committee of Animal Studies at Mashhad University of Medical Sciences (NO: 930952).

Rosi (Selleckchem, Houston, USA) was dissolved in saline supplemented with dimethylsulfoxide (DMSO) (10% final concentration) and injected intraperitoneally (i.p.) (Churi et al., 2008; Hong et al., 2012). To induce hypothyroidism, propylthiouracil (PTU) was added to the drinking water (0.05% PTU). Treatment with Rosi or its vehicle continued for six weeks. In the seventh week, behavioral tests, including Morris water maze (MWM) and passive avoidance, were performed.

The animals were divided into four groups (*n*=9/group): control group: rats received normal drinking water and were injected with vehicle (10% DMSO in saline), hypothyroid group: rats received drinking water containing 0.05% PTU and were injected with the vehicle (10% DMSO in saline), hypothyroid-Rosi 2 and hypothyroid-Rosi 4 groups: rats received drinking water containing 0.05% PTU and were injected with either 2 or 4 mg/kg Rosi.

Evaluation of learning and memory MWM

A pool (1.36 m in diameter and 0.6 m in total depth) was filled with water with a 24°C – 25°C temperature until the liquid reached a depth of 0.3 m. A camera mounted above the pool recorded the behaviors of the animals. The pool included four quadrants, and a water-submerged escape platform was located in the target quadrant. The task occurred in two phases. First, the learning phase was completed for five days in which rats learned the location of the water-submerged escape platform. On each day of the five days of the learning phase, each animal completed four trials where the rat was placed inside the pool and allowed to search the platform for 60 s. If the rat did not find the platform in the 60 s period, it was placed on the platform for 20 s. The probe test was done on the sixth day. In this phase, the rat was tested for memory of the location of the water-submerged escape platform. Each rat was placed inside the pool without a platform and allowed to swim for 60 s. The video recordings of the testing were transferred to a computer for comparison between experimental groups. For analysis of the learning phase, experimenters recorded both the time and distance traveled until the rat reached the escape platform during all five days. For analysis of the probe phase, experimenters recorded the time and distance traveled in the target area.

Evaluation of learning and memory using PA task

A box with both dark and light (20 × 30 × 20 cm each) segments separated by a small removable door was used. In the first phase of testing, rats were familiarized with the equipment with the removable door open and were allowed to freely explore the dark and light segments of the apparatus. In the second phase, rats were placed inside the lightbox, and the adjoining door was gently opened to allow the rats to access the dark side of the testing box. Upon entry, the door was closed, and the rat received a 2mA shock to the feet (2 s). The third phase was done at 3, 24, and 48 h after the shock (phase 2). In phase 3, rats were placed inside the light segment of the box. After the door was removed, the latency to enter the dark segments, the total time spent in the dark segment, the frequency of entering into the dark segment, and the total time spent in the light segment were recorded to compare between the groups.

Biochemical tests

After the behavioral tests, the animals were deeply anesthetized (1.6 g/kg urethane), and blood sampling from the heart was performed by cardiac puncture. Blood serum was separated, and thyroxin level was measured using commercial methods. Brains were then removed, and the hippocampal and cortical tissues were collected and stored at -80°C to be used for biochemical measurements. For biochemical tests, 0.1 g of sample tissue was homogenized in 1 ml phosphate-buffered saline (PBS) to make a 10% (w/v) sample concentration. Homogenized samples were centrifuged, and the supernatants were separated to be used for biochemical measurements. BDNF concentration was evaluated in the hippocampus. Malondialdehyde (MDA), nitric oxide (NO) metabolites, thiol content, and superoxide dismutase (SOD) and catalase (CAT) activities were measured in the cortex and hippocampus. Chemicals used for the measurement of MDA levels, thiol content, and SOD and CAT activity were purchased from the Merck Company (Darmstadt, Germany).

BDNF measurement in the hippocampus

BDNF levels were measured using a rat ELISA Kit (MyBioSource Company, San Diego, CA, USA) with sandwich enzyme-linked immune-sorbent assay technology. Briefly, anti-BDNF polyclonal antibody was pre-coated onto 96-well plates. The biotin-conjugated anti-BDNF polyclonal antibody was used as the detection antibody. The standards, test samples, and biotin-conjugated detection antibody were added to the wells. The plate was sealed and incubated at 37°C for 60 min. After washing the plate with buffer, Avidin-Biotin-Peroxidase Com-

plex was added, and the plate was incubated at 37°C for 30 min. Unbound conjugates were washed away with buffer. TMB substrates were used to visualize the HRP enzymatic reaction, and the plate was incubated at 37°C in the dark for 30 min. TMB is catalyzed by HRP to produce a blue color product that changes to yellow after adding the acidic stop solution. The density of yellow color is proportional to the amount of BDNF in the well. Absorbance was read at 450 nm using a microplate reader (EL800, BioTek Instrument, Winooski, VT, USA), and then the concentration of BDNF was calculated using known standards. Each standard and sample was measured in duplicate.

Measurement of lipid peroxidation and NO metabolites in the hippocampus and cortex

To estimate lipid peroxidation in the hippocampus and cortex, thiobarbituric acid-reactive substances (TBARS) formation during an acid-heating reaction, where MDA is the main biproduct, was measured. Supernatants of cortex and hippocampus samples (homogenized in PBS) were added to the TBA reagent (15 g trichloroacetic acid (TCA), 0.375 mg thiobarbituric acid (TBA) and 2.5 mL hydrochloric acid (HCL) in 100 ml H_2O), heated in a boiling water bath for 40 min, and cooled to room temperature. Samples were centrifuged (1000 × g for 10 min), and the absorbance of the supernatants was read at 535 nm at room temperature. Each sample was measured in duplicate. This procedure was based on a method that was previously described (Baghcheghi et al., 2018a). For the measurement of NO metabolites, the Griess colorimetric method was used according to the manufacturer's instructions (Promega Company, Madison, Wisconsin, USA). In brief, after adding 100 μL supernatant to the Griess reagent, samples were transferred to a 96-well flat-bottomed microplate, and the absorbance was read at 520 nm using a microplate reader (EL800, BioTek Instrument, Winooski, VT, USA). Final values were calculated from standard calibration plots (Kleinbongard et al., 2002; Giustarini et al., 2008; Baghcheghi et al., 2018b). Each standard and sample was measured in duplicate.

Measurement of total thiol content and the activity of SOD and CAT in the hippocampus and cortex

Thiol content in hippocampal and cortical tissues was estimated by measuring the yellow color produced in the reaction of DTNB (2, 2'-dinitro-5, 5'-dithiodibenzoic acid) with thiol groups as previously described

(Baghcheghi et al., 2016). Briefly, 50 μl of brain tissue homogenates were mixed with Tris-EDTA (ethylenediaminetetraacetic acid), and the absorbance of the solution was detected at 412 nm using a microplate reader (EL800, BioTek Instrument, Winooski, VT, USA). Next, DTNB (20 μl) was added to the solution and kept at room temperature for 15 min after which the absorbance of the solution was read again. The absorbance of DTNB was also read and used as a blank. Total thiol concentration was calculated as previously reported (Baghcheghi et al., 2016). The activity of the CAT enzyme was evaluated using a kit purchased from Cayman Company (Michigan, USA) according to the manufacturer's instructions. SOD enzyme activity was measured using the previously reported method (Baghcheghi et al., 2018a). Each sample was measured in duplicate.

Statistical analysis

All data are expressed as mean ± standard error of the mean (SEM). The data were analyzed using the SPSS 16.0 software (SPSS Inc., Chicago, IL, USA). The data from the acquisition part of the MWM were analyzed using a two-way repeated measure ANOVA (group (4) × day (5)) with repeated measures on the last factor (day)) followed by Tukey's *post hoc* comparison test. The data of the passive avoidance test, the probe trial data of the MWM, and the biochemical results were compared by one-way ANOVA followed by Tukey's *post hoc* comparison test. The differences were considered statistically significant when P<0.05.

RESULTS

The effects of Rosi on the performance of hypothyroid rats in the MWM

Fig. 1 shows the performance of rats during the five days of learning sessions on the MWM. In these sessions, rats with impaired learning spent more time and traveled a longer distance to reach the platform. Two-way ANOVA ((4 group × 5 day) with repeated measures on the last factor (day)) confirmed the main effect of group ($F_{(3,105)}$=25.8; P<0.001) and day ($F_{(4,140)}$=34.79; P<0.001) on traveling time and main effect of group ($F_{(3,105)}$=15.78; P<0.001) and day ($F_{(4,140)}$=28.6; P<0.001) on the traveled distance during the five days of learning trials. *Post-hoc* comparisons for groups showed that hypothyroidism induced by PTU increased the traveling time and distance for reaching the water-submerged escape platform during the five days of the learning

phase (P<0.01 – P<0.01). Compared to the hypothyroid group, treatment with both doses of Rosi reduced the traveling time and distance for reaching the water-submerged escape platform during the five days of the learning phase (P<0.05- P<0.001). There were no significant differences in traveling time and distance between the two doses of Rosi (Fig. 1A, B) and also between the Rosi-treated groups with the control group. Fig. 1C shows the traveling time to reach the submerged escape platform in the first trial on the first day of the MWM test. There was no significant difference in the traveling time to reach the escape platform between the groups.

After the learning sessions, the probe phase was used to measure memory of the escape platform location in the rats. In this phase, the platform was re-moved, and rats were allowed to swim in the pool. Animals that did not remember the location of the escape platform spent a shorter time and traveled a shorter distance in the target zone. The results showed that group had a significant effect on both traveling time ($F_{(3,140)}$=6.76; P<0.001) and traveling distance in the target quadrant ($F_{(3,140)}$=7.39; P<0.001). *Post-hoc* comparisons for groups showed that hypothyroid rats had shorter traveling time and distance in the target quadrant than the control group (P<0.001). Compared to the hypothyroid group, treatment by both doses of Rosi was followed by a longer traveling time (P<0.01 – P<0.001) and distance (P<0.01) in the target quadrant. There was no significant difference between the two doses of Rosi nor between the control and Rosi-treated groups (Fig. 2A, B).
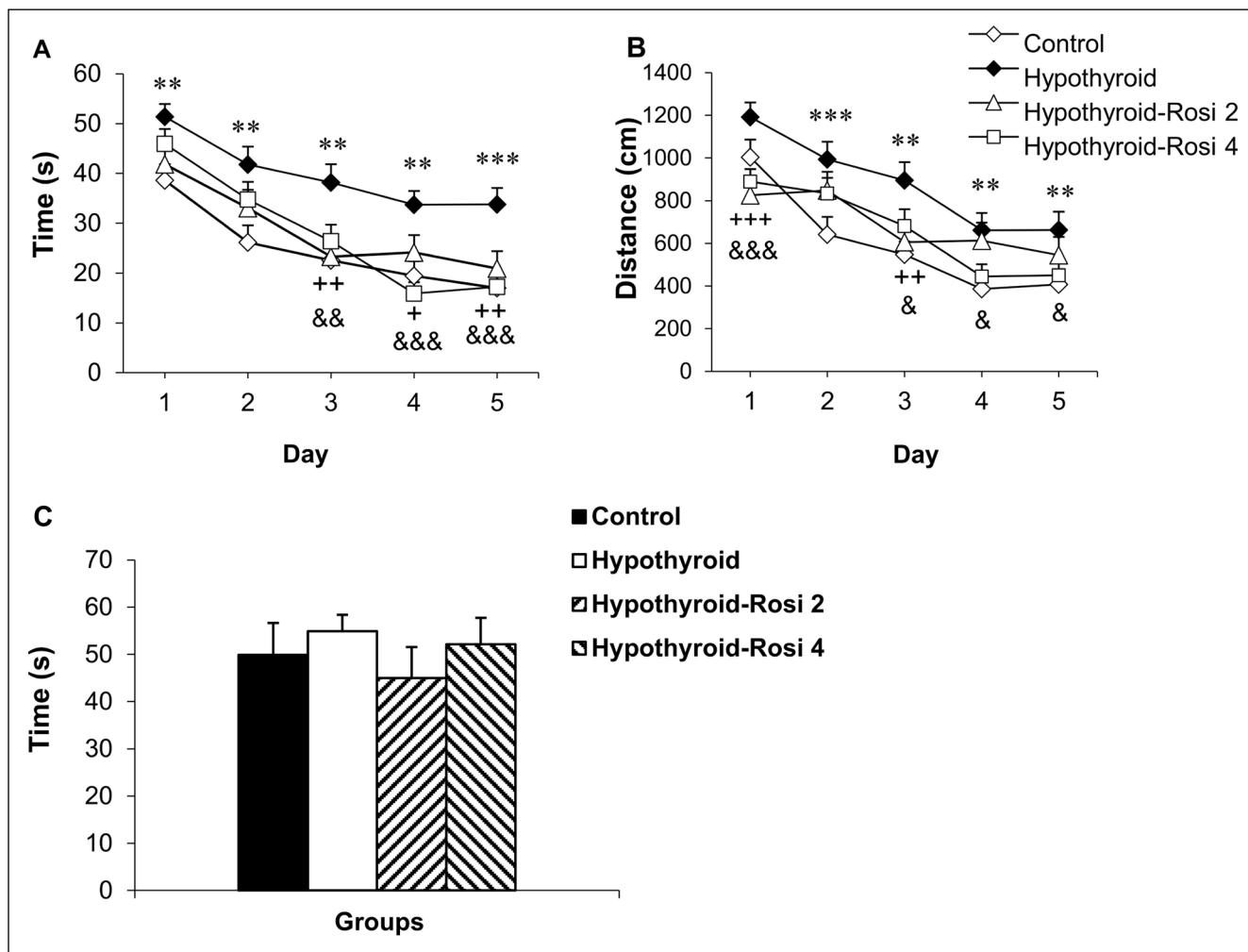

Fig. 1. The results of the elapsed time (A) and the traveled distance (B) to find the hidden platform during the five days of learning and (C) the elapsed time to reach the platform during the first trial of the first day in the Morris water maze test. The data were expressed as mean ± standard error of the mean (SEM). **P<0.01 and ***P<0.001 show the difference between the hypothyroid and control groups, +P<0.05, ++P<0.01 and +++P<0.001 show the difference between the hypothyroid-Rosi 2 and hypothyroid groups, &P<0.05, &&P<0.01 and &&&P<0.001 show the difference between the hypothyroid-Rosi 4 and hypothyroid groups.
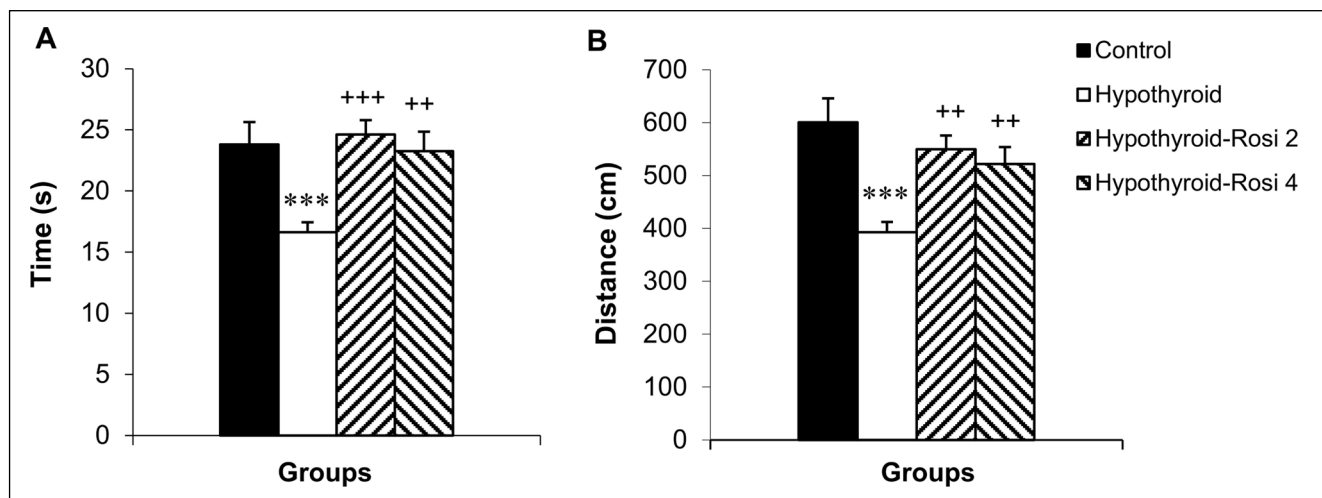
Fig. 2. The results of the time (A) and distance (B) in the target area during the 6th day of the Morris water maze test. On this day, the platform was removed from the pool, and the rats were assessed for their ability to remember the location of the platform. The data were expressed as mean ± standard error of the mean (SEM). ***P<0.001 shows the difference between the hypothyroid with the control groups, [++]P<0.01 and [+++]P<0.001 show the difference between the hypothyroid-Rosi 2 or hypothyroid-Rosi 4 groups with the hypothyroid group.

The effects of Rosi on the performance of hypothyroid rats in the passive avoidance test

The results showed a significant effect of group on delay time to enter the dark segment at 3 ($F_{(3,32)}$=6.42; P<0.001), 24 ($F_{(3,32)}$=27.76; P<0.001) and 48 h ($F_{(3,32)}$=5.25; P<0.01) after shock delivery. Hypothyroidism induced by PTU shortened the delay time to enter the dark segment at 3 (P<0.001), 24 (P<0.001), and 48 h (P<0.05) (Fig. 3A). Both the 2 and 4 mg/kg Rosi doses increased the delay time for entering the dark segment at 3 (P<0.001 for both doses), 24 (P<0.001 for both doses), and 48 h (P<0.05 and P<0.01, respectively) when compared to the hypothyroid group (Fig. 3A). No significant differences were shown between the two doses of Rosi and also between the control and Rosi-treated groups.

The results also showed a significant effect of group on time spent in the dark segment at 3 ($F_{(3,32)}$=61.12; P<0.001), 24 ($F_{(3,32)}$=15.87; P<0.001) and 48 h ($F_{(3,32)}$=9.02; P<0.001) after shock delivery. Hypothyroidism induced by PTU increased the time spent in the dark segment at 3 (P<0.001), 24 (P<0.001), and 48 h (P<0.001) (Fig. 3B). In addition, both doses of Rosi decreased the time spent in the dark segment at 3 (P<0.001 for both doses), 24 (P<0.001 for both doses), and 48 h (P<0.01 and P<0.05 for both doses) (Fig. 3B). There were no significant differences between the two doses of Rosi nor between the control and Rosi-treated groups.

The results also showed a significant effect of group on the number of entries into the dark segment at 3 ($F_{(3,32)}$=16.20; P<0.001), 24 ($F_{(3,32)}$=23.52; P<0.001), and 48 h ($F_{(3,32)}$=6.29; P<0.01) after shock delivery. Hypothyroidism increased the number of entries into the dark

segment at 3 (P<0.01), 24 (P<0.001), and 48 h (P<0.05) (Fig. 4A). Both doses of Rosi decreased the number of entries into the dark segment at 3 (P<0.001 for both doses), 24 (P<0.001 for both doses), and 48 h (P<0.01 for both doses) compared to the hypothyroid group (Fig. 4A).

The results showed a significant effect of group on the time spent in the light segment at 3 ($F_{(3,32)}$=61.12; P<0.001), 24 ($F_{(3,32)}$=15.87; P<0.001), and 48 h ($F_{(3,32)}$=9.29; P<0.01) after shock delivery. Hypothyroidism decreased the time spent in the light segment at 3 (P<0.001), 24 (P<0.001), and 48 h (P<0.001) (Fig. 4B). In addition, Rosi at both 2 and 4 mg/kg increased the time spent in the light segment at 3 (P<0.001 for both doses), 24 (P<0.001 for both doses), and 48 h (P<0.01 for 2 mg/kg and P<0.05 for 4 mg/kg) (Fig. 4B). There were no significant differences between the two doses of Rosi. Additionally, there was no significant differences between the control group and Rosi groups.

The effects of Rosi on serum thyroxin level

The results showed a significant effect of group on serum thyroxin level ($F_{(3,32)}$=57.61; P<0.001). Hypothyroidism induced by PTU showed lower levels of serum thyroxin compared to the control group (P<0.001). Administration of 2 mg/kg of Rosi did not change serum thyroxin levels, and there was a significant difference between the hypothyroid-Rosi 2 and the control group (P<0.001). Serum thyroxin levels in the hypothyroid-Rosi 4 group was higher than that in the hypothyroid (P<0.001) and hypothyroid-Rosi 2 (P<0.001) groups (Fig. 5).
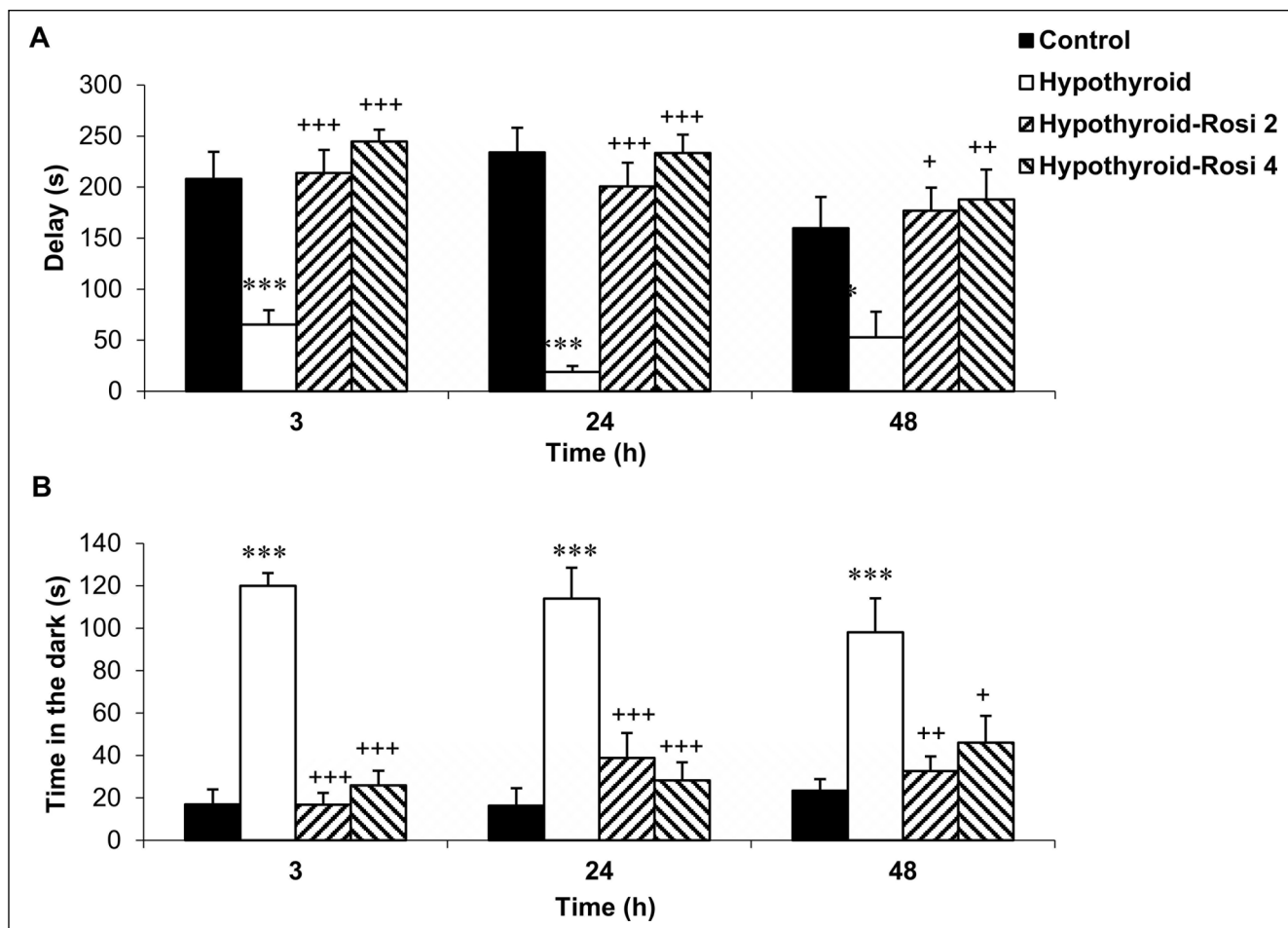
Fig. 3. The results of the delay in entering the dark chamber (A) and the time spent in the dark chamber (B) in different time points (3, 24, and 48 h) after the shock in the passive avoidance test. The data were expressed as mean ± standard error of the mean (SEM). *P<0.05 and ***P<0.001 show the difference between the hypothyroid and control groups, +P<0.05, ++P<0.01 and +++P<0.001 show the difference between the hypothyroid-Rosi 2 or hypothyroid-Rosi 4 groups with the hypothyroid group.

The effects of Rosi on hippocampal BDNF levels

The results showed a significant effect of group on hippocampal BDNF levels ($F_{(3,32)}$=70.02; P<0.001). Hypothyroid rats showed lower hippocampal BDNF levels compared to the control group (P<0.05). Both the 2 mg/kg and 4 mg/kg Rosi treatment groups showed increased hippocampal BDNF levels compared to the hypothyroid group (P<0.001 for both). There was no significant difference between the hypothyroid-Rosi 2 and hypothyroid-Rosi 4 groups (Fig. 6).

The effects of Rosi on hippocampal MDA and NO metabolites

Analysis with a one-way ANOVA show a significant effect of group on both NO metabolites ($F_{(3, 32)}$=47.81;

P<0.001) and MDA concentration ($F_{(3, 32)}$=450.86; P<0.001) in the hippocampus. Hypothyroidism induced by PTU increased the levels of NO metabolites and MDA in the hippocampal tissue of the hypothyroid group compared to the control group (P<0.001 for both). Treatment with Rosi at both doses decreased NO metabolites in the hippocampus of both the hypothyroid-Rosi 2 and hypothyroid-Rosi 4 groups compared to the hypothyroid group (P<0.001 for both). There was no significant difference between hypothyroid-Rosi 2 and hypothyroid-Rosi 4 groups (Fig. 7A). Both doses of Rosi decreased hippocampal MDA levels compared to the hypothyroid group (P<0.001 for both), but hippocampal MDA levels in the hypothyroid-Rosi 2 and hypothyroid-Rosi 4 groups were higher than that of the control group (P<0.001 for both). Additionally, hippocampal MDA level in the hypothyroid-Rosi 4 group was lower than that in the hypothyroid-Rosi 2 group (P<0.001) (Fig. 7B).
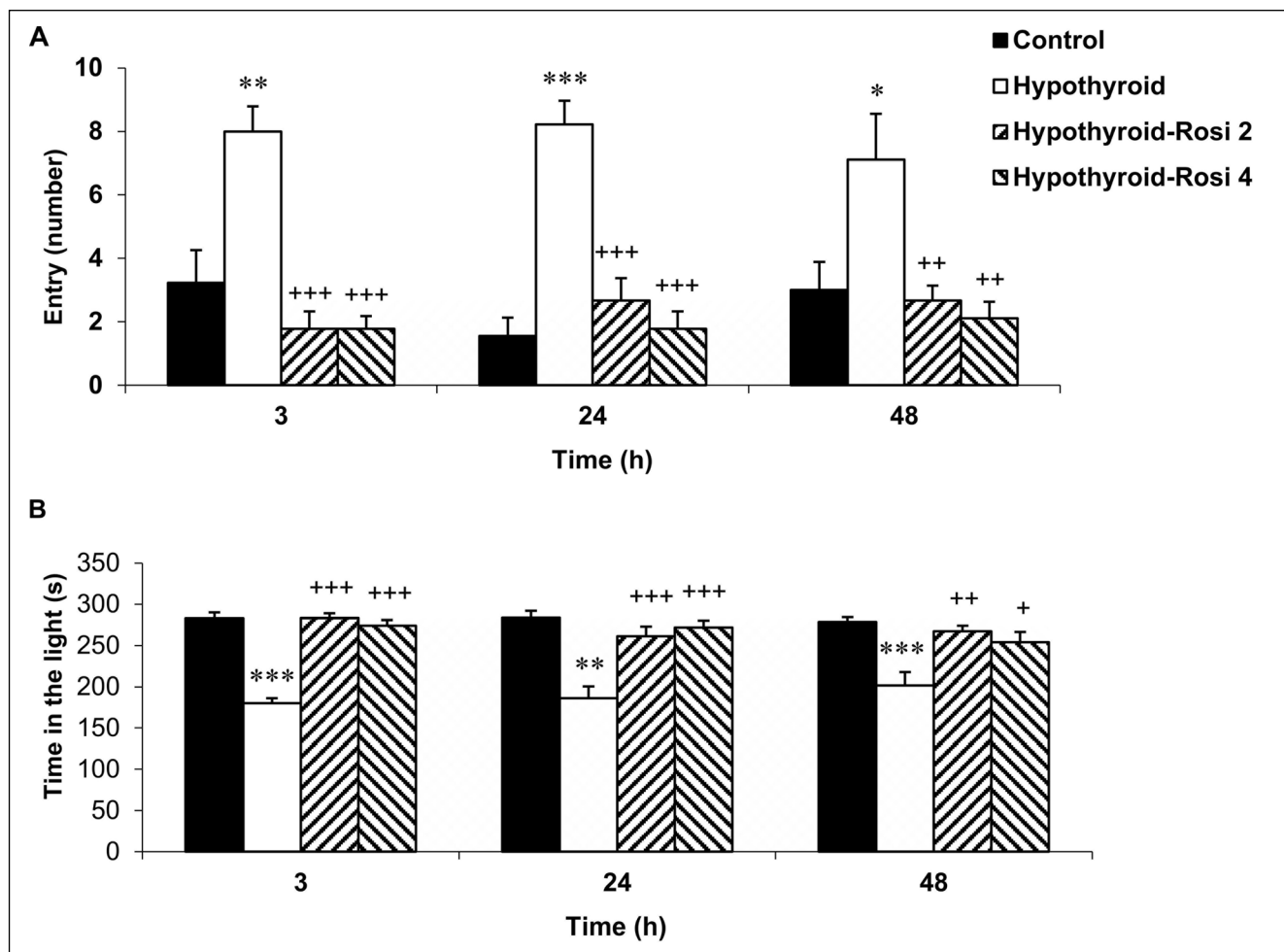
Fig. 4. The number of entries to the dark chamber (A) and the time spent in the light chamber (B) at different time points (3, 24, and 48 h) after the shock in the passive avoidance test. The data were expressed as mean ± standard error of the mean (SEM). *P<0.05, **P<0.01 and ***P<0.001 show the difference between the hypothyroid and control groups, +P<0.05, ++P<0.01 and +++P<0.001 show the difference between the hypothyroid-Rosi 2 or hypothyroid-Rosi 4 groups with the hypothyroid group.

The effects of Rosi on hippocampal thiols, SOD, and CAT

The results showed a significant effect of group on thiol content ($F_{(3,32)}$=420.14; P<0.001) and SOD ($F_{(3,32)}$=296.95; P<0.001) and CAT activities ($F_{(3,32)}$=14.83; P<0.001) in the hippocampus. Hypothyroidism significantly decreased thiol content (P<0.001) and SOD (P<0.001) and CAT (P<0.05) activities in the hippocampus of the hypothyroid group compared to the control group (Fig. 8A-C). Thiol content and SOD and CAT activities in the hippocampus of both hypothyroid-Rosi 2 and hypothyroid-Rosi 4 groups were higher than those in the hypothyroid group (P<0.001 for all). Additionally, the thiol content and SOD activity in the hippocampus of the hypothyroid-Rosi 4 group was higher than that in the hypothyroid-Rosi 2 group (P<0.001 for both). There

was no significant difference between the effects of the two doses of Rosi on hippocampal CAT activity. In addition, thiol content and SOD activity in the hippocampus of the hypothyroid-Rosi 2 and hypothyroid-Rosi 4 groups were still lower than those in the control group (P<0.001 for all). Interestingly, the hippocampal CAT activity in the hypothyroid-Rosi 2 and hypothyroid-Rosi 4 groups were higher than those in the control group (P<0.05 for both) (Fig. 8A-C).

The effects of Rosi on cortical MDA concentration and NO metabolites

There was a significant effect of group on NO metabolites ($F_{(3,32)}$=37.13; P<0.001) and MDA concentration ($F_{(3,32)}$=134.12; P<0.001) in the cortex. Hypothyroidism
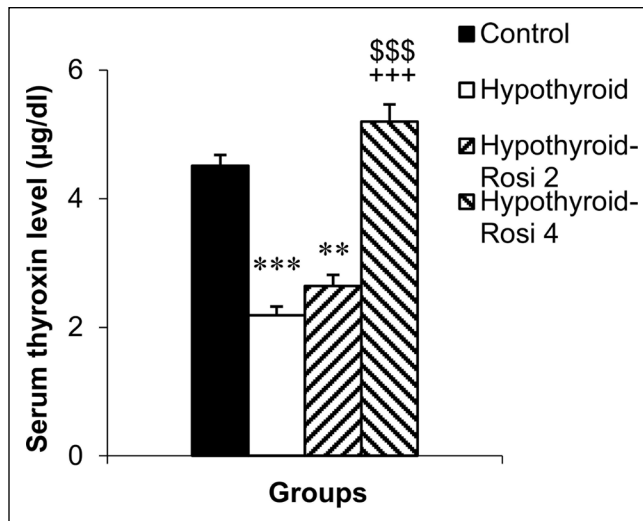
Fig. 5. The results of measurements of serum thyroxin level. The data were expressed as mean ± standard error of the mean (SEM). ***P<0.001 shows the difference between the hypothyroid and hypothyroid-Rosi 2 groups with the control group, +++P<0.001 shows the difference between the hypothyroid-Rosi 4 group with the hypothyroid group, $$$P<0.001 shows the difference between the hypothyroid-Rosi 4 group with the hypothyroid-Rosi 2 group.
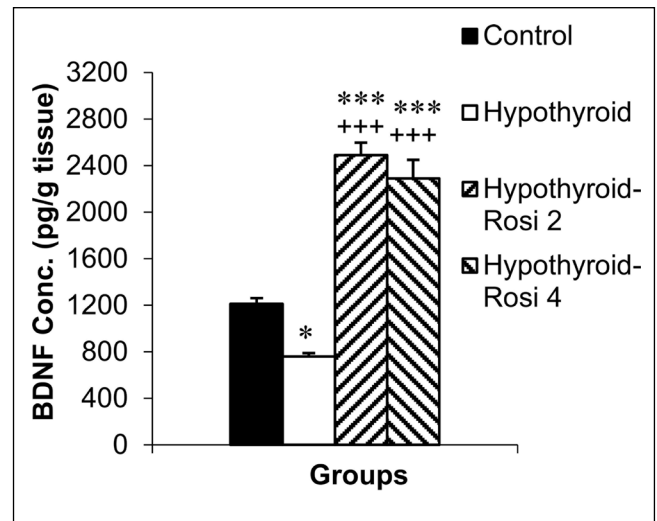
Fig. 6. The results of measurements of the hippocampal brain-derived neurotrophic factor (BDNF) level. The data were expressed as mean ± standard error of the mean (SEM). *P<0.05, ***P<0.001 shows the difference between the hypothyroid and control groups, +++P<0.001 shows the difference between the hypothyroid-Rosi 2 or hypothyroid-Rosi 4 groups with the hypothyroid group.

induced by PTU increased NO metabolite levels and MDA concentrations in the cortex (P<0.001 for both) (Fig. 9A, B). Administration of both doses of Rosi doses decreased NO metabolites in the cortex (P<0.001 for both) to levels similar to the control group. Treatment with both the 2 mg/kg and 4 mg/kg doses of Rosi also resulted in reduced cortical MDA compared to the hypothyroid group (P<0.01 and P<0.001, respectively), but these MDA levels were still higher than control levels (P<0.001 for both). MDA level in the cortex of the hypothyroid-Rosi 4 group was lower than that in the hypothyroid-Rosi 2 group (P<0.001) (Fig. 9A, B).
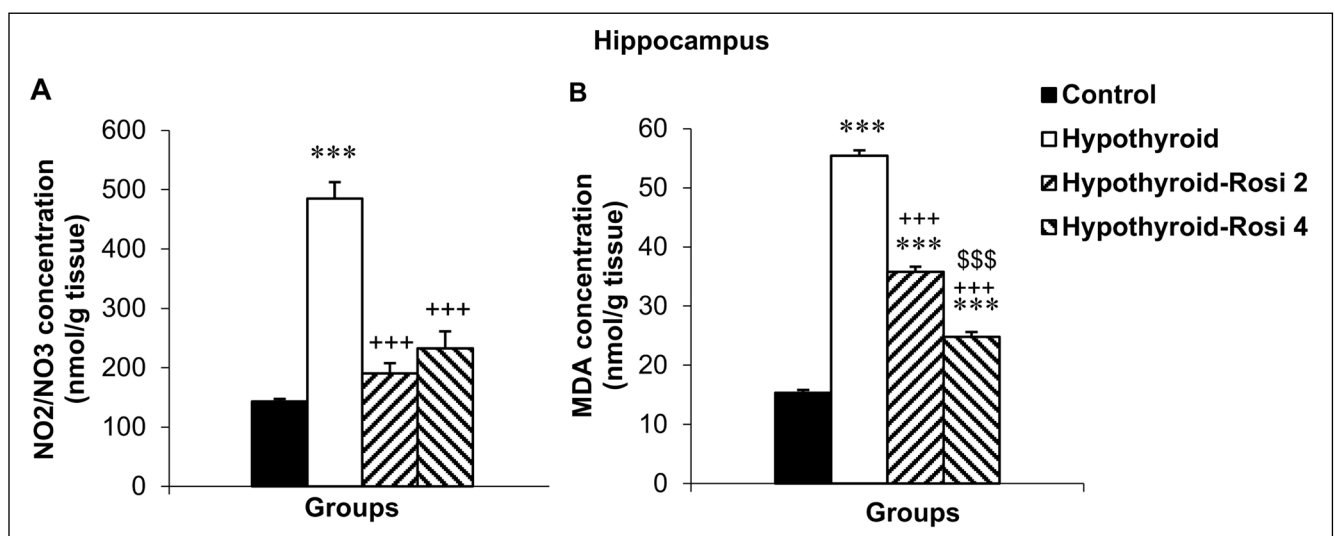

Fig. 7. The results of measurements of hippocampal nitric oxide (NO) metabolites (A) and malondialdehyde (MDA) level (B). The data were expressed as mean ± standard error of the mean (SEM). ***P<0.001 shows the difference between the hypothyroid and control groups, +++P<0.001 shows the difference between the hypothyroid-Rosi 2 or hypothyroid-Rosi 4 groups with the hypothyroid group, $$$P<0.001 shows the difference between the hypothyroid-Rosi 4 group with the hypothyroid-Rosi 2 group.
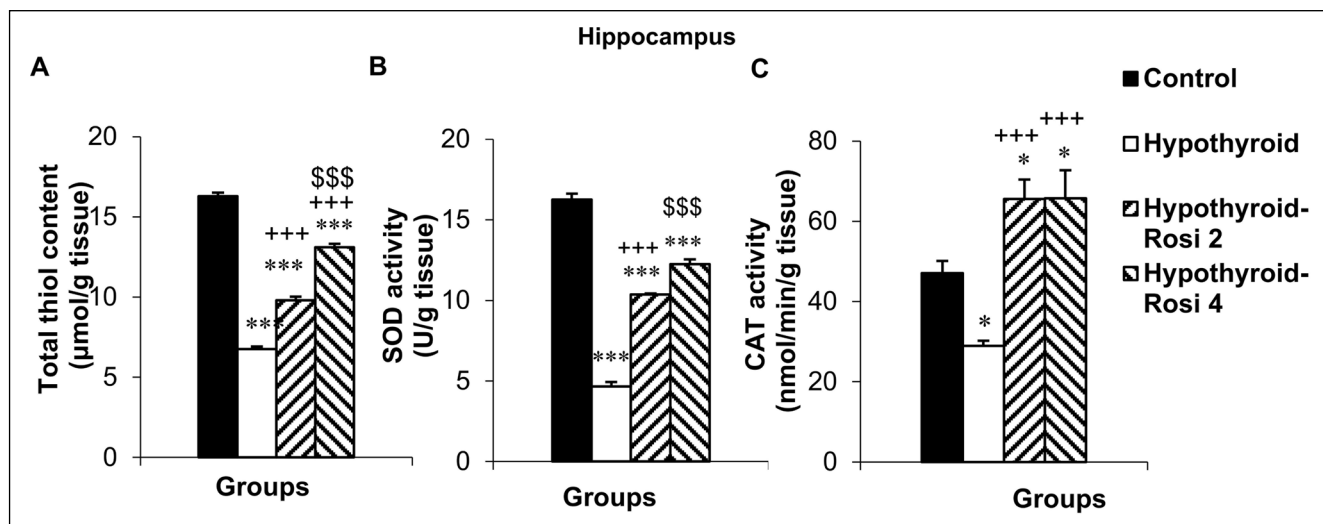
Fig. 8. The results of measurements of hippocampal thiol (A), superoxide dismutase (SOD) (B), and catalase (CAT) (C). The data were expressed as mean ± standard error of the mean (SEM). *P<0.05 and ***P<0.001 shows the difference between the hypothyroid, hypothyroid-Rosi 2 or hypothyroid-Rosi 4 groups with the control group, +++P<0.001 shows the difference between the hypothyroid-Rosi 2 or hypothyroid-Rosi 4 groups with the hypothyroid group, $$$P<0.001 shows the difference between the hypothyroid-Rosi 4 group with the hypothyroid-Rosi 2 group.

The effects of Rosi on cortical thiols, SOD, and CAT

There was a significant effect of group on thiol content ($F_{(3,32)}$=151.48; P<0.001) and SOD ($F_{(3,32)}$=76.41; P<0.001) and CAT activities ($F_{(3,32)}$=44.08; P<0.001) in the cortex. Hypothyroidism induced by PTU showed decreased thiol content (P<0.001) and SOD (P<0.001) and CAT (P<0.01) activities in the cortex (Fig. 10A-C). Com-pared with the hypothyroid group, administration of Rosi dose-dependently increased thiol content (P<0.001 for both doses) and SOD activity (P<0.001 for both doses). In the hypothyroid-Rosi 4 group, cortical thiol content (P<0.001) and SOD activity (P<0.01) was higher than those in the hypothyroid-Rosi 2 group. The effect of Rosi on cortical CAT was not dose-dependent, and there was no significant difference between the effects of the two doses of Rosi on CAT activity. In the cortex of
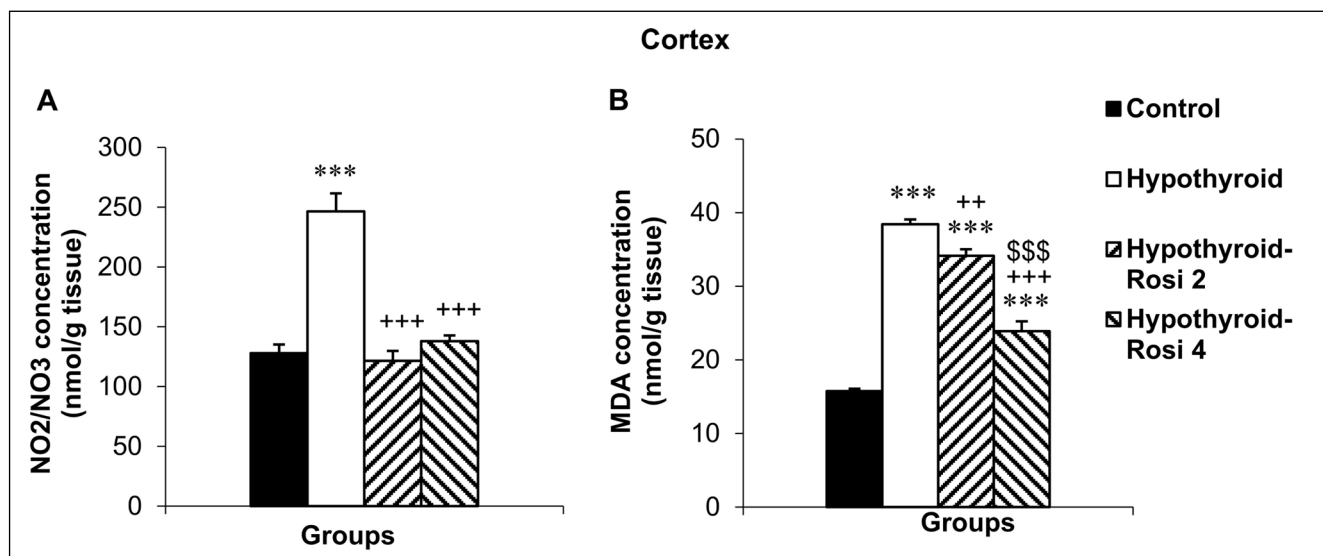

Fig. 9. The results of measurements of cortical nitric oxide (NO) metabolites (A) and malondialdehyde (MDA) level (B). The data were expressed as mean ± standard error of the mean (SEM). ***P<0.001 shows the difference between the hypothyroid, hypothyroid-Rosi 2 or hypothyroid-Rosi 4 groups with the control group, ++P<0.01, +++P<0.001 show the difference between the hypothyroid-Rosi 2 or hypothyroid-Rosi 4 groups with the hypothyroid group, $$$P<0.001 shows the difference between the hypothyroid-Rosi 4 group with the hypothyroid-Rosi 2 group.
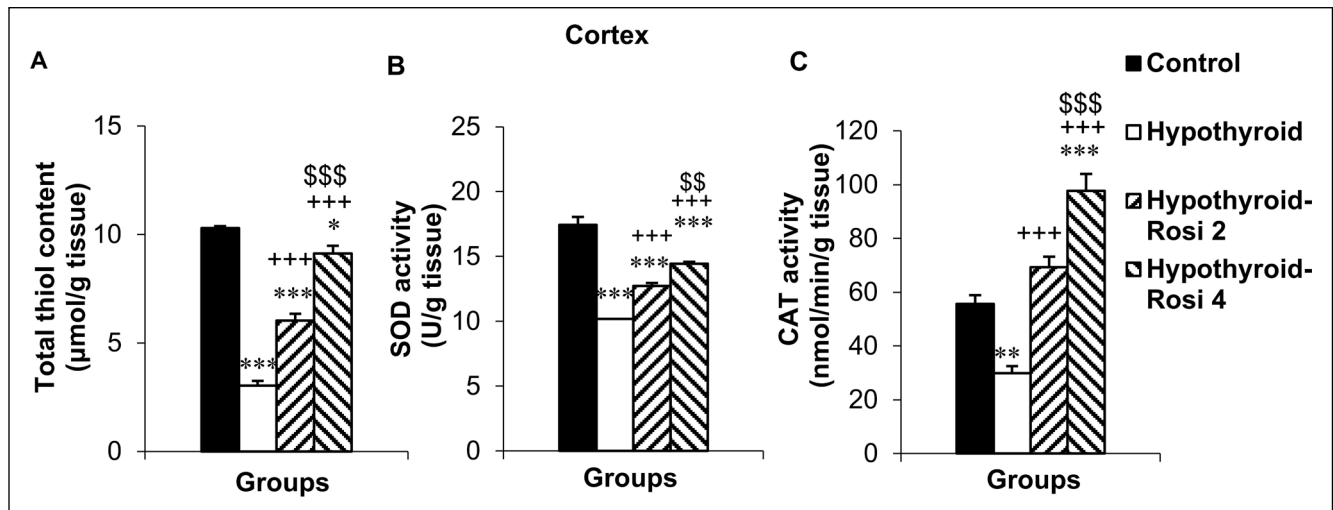
Fig. 10. The results of measurements of the cortical thiol content (A) and superoxide dismutase (SOD) (B) and catalase (CAT) activities (C). The data were expressed as mean ± standard error of the mean (SEM). **P<0.01, **P<0.01 and ***P<0.001 shows the difference between the hypothyroid, hypothyroid-Rosi 2 or hypothyroid-Rosi 4 groups with the control group, +++P<0.001 shows the difference between the hypothyroid-Rosi 2 or hypothyroid-Rosi 4 groups with the hypothyroid group, $$ P<0.01 and $$$P<0.001 show the difference between the hypothyroid-Rosi 4 group with the hypothyroid-Rosi 2 group.

the hypothyroid-Rosi 2 and hypothyroid-Rosi 4 groups, thiol content and SOD activity were still lower than those in the control group (P<0.05- P<0.001). There was no significant difference between the hypothyroid-Rosi 2 and control groups in cortical CAT activity, but cortical CAT was higher in the hypothyroid-Rosi 4 group compared to the control group (P<0.001) (Fig. 10A-C).

DISCUSSION

In the present study, we demonstrated that hypothyroidism-induced learning and memory impairment in rats as tested in the MWM and passive avoidance tests. Hypothyroidism-induced memory impairment was accompanied by a marked increase in oxidative stress markers and NO production in the brain and a significant decrease in BDNF levels in the hippocampus. Additionally, this study provided compelling evidence that the PPAR-γ agonist Rosi improved hypothyroid rats' performance in the MWM and passive avoidance tasks. This neuroprotective effect was manifested by reduced oxidative damage, reduced NO production, and increased BDNF levels in the hippocampus.

BDNF is involved in important brain functions such as memory processing and synaptic plasticity (Bekinschtein et al., 2014). It is well documented that decreased BDNF levels or reduced activity of its downstream signaling pathways are associated with learning and memory impairment (Yamada et al., 2002). In line with our findings, previous studies have con-

firmed decreased hippocampal BDNF levels in hypothyroid rats (Zhang et al., 2009; Sui and Li, 2010; Lasley and Gilbert, 2011; Chakraborty et al., 2012). The hypothyroidism-induced learning and memory impairment observed in the present study could be attributed to the decreased level of hippocampal BDNF levels. The present study also indicated that Rosi rescued BDNF levels in hippocampal tissue of hypothyroid rats. Previously, administration of PPAR-γ agonists have been shown to increase hippocampal BDNF levels in diabetic rats (Kariharan et al., 2015). Considering these results, the beneficial effects of PPAR-γ agonists on the learning and memory impairment of hypothyroid rats, at least in part, may also be elucidated by this mechanism.

In both humans and rodents, BDNF has been detected in different brain areas, including the hippocampus, amygdala, cerebellum, and cerebral cortex; however, it has been well documented that the highest level of BDNF is in the hippocampus (Hofer et al., 1990; Timmusk et al., 1993; Miranda et al., 2019). Considering this evidence, the current study measured BDNF levels in the hippocampus. It has been previously reported that maternal thyroidectomy in rats is followed by a decreased BDNF expression in the brains of developing pups (Koibuchi et al., 1999; Koibuchi and Chin, 2000; Liu et al., 2010). Hypothyroidism in neonatal rodents also reduces BDNF levels in the cerebellum (Sinha et al., 2008; 2009). It has been reported that the cerebellum plays a role in learning and memory, and amyloid-β (Aβ) deposition in the cerebellum has been reported in learning and memory impairment models

(Rochefort et al., 2013; Ali et al., 2016). Considering this evidence, measurements of BDNF in other brain regions, including the cerebellum, should be considered in future studies. Interestingly, BDNF concentration in the hippocampus of both hypothyroid-Rosi 2 and hypothyroid-Rosi 4 groups was higher than that of the control group. These results suggest that PPAR-γ agonists improve BDNF levels in non-hypothyroid conditions. Previous studies have also shown that Rosi increased BDNF in the hippocampus of normal rats (Kariharan et al., 2015). Additionally, telmisartan, an angiotensin receptor blocker and a partial PPAR-γ agonist, increased BDNF levels in the brain of normal rats *via* PPAR-γ activation (Kishi et al., 2012).

In line with previous reports, our results indicated that hypothyroidism increased NO production in hippocampal tissue (Hosseini et al., 2010; Baghcheghi et al., 2018b). NO has been shown to rapidly downregulate BDNF production in cultured hippocampal neurons through the cGMP-activated protein kinase G signaling pathway (Canossa et al., 2002). Moreover, under pathological conditions, NO has been shown to increase oxidative damage through the generation of the reactive metabolite peroxynitrite (Chabrier et al., 1999; Jonnala and Buccafusco, 2001). During cases of ischemia/reperfusion, NO has been shown to react with reactive oxygen species to produce peroxynitrites, which have deleterious effects on neuronal survival (Warner et al., 2004). Therefore, NO could also reduce BDNF production through oxidative stress mechanisms in this study. There is evidence that excessive oxidative stress can reduce BDNF, leading to a decline in cognition and neuroplasticity (Wu et al., 2004). It has been suggested that the role of BDNF in maintaining synaptic plasticity is closely related to cellular energy metabolism, such that a disruption in energy homeostasis can affect synaptic plasticity and cognitive function (Vaynman et al., 2006; Gómez-Pinilla, 2008). Our findings also indicate that Rosi reduces NO production in the hippocampus of hypothyroid rats. It has been previously reported that PPAR-γ agonists reduced iNOS expression and subsequent cell death in cerebellar granule cells of rats following lipopolysaccharide and interferon-γ microinjection in the cerebellum (Heneka et al., 2000). Moreover, Rosi has been reported to reduce hippocampal NO levels and improve the long-term potentiation (LTP) in aged rats (Loane et al., 2009). A decrease in excessive NO production is another possible mechanism by which Rosi was able to improve learning and memory impairment in hypothyroid rats.

Increased MDA levels suggest that lipid peroxidation is a possible mechanism for learning and memory impairment in hypothyroid rats. Similarly, hypothy-roidism decreased thiol content and SOD and CAT activities in the brain, all of which indicate impairment of the antioxidant machinery in the brain by hypothyroidism. However, increased lipid peroxidation implies that the existing antioxidant capacity is not enough to protect brain cells from oxidative stress. In addition, the results of the present study are consistent with our previous reports demonstrating that there is a connection between oxidative stress in the brain and learning and memory impairments due to hypothyroidism (Baghcheghi et al., 2016; Baghcheghi et al., 2018a,b). The results of this study showed that Rosi could improve antioxidant capacity and decrease the lipid peroxidation in the brain of hypothyroid rats. Our results also showed that Rosi protects against hypothyroidism-induced reductions in SOD and CAT activities and thiol content. However, the precise neuroprotective mechanisms of PPAR-γ agonists have not yet been fully clarified. These data suggest that the protective effects of Rosi against hypothyroid injury are partially due to its ability to reduce oxidative stress. This finding extends the protection profile of Rosi beyond previously described beneficial effects in other models such as ischemia/reperfusion (Ito et al., 2004; Villegas et al., 2004; Collino et al., 2006), diabetes (Ishida et al., 2004), burn (Şener et al., 2007), and cardiomyocytes (Ding et al., 2007). Recent studies have also reported that several antioxidative enzymes, such as SOD-1 (Hwang et al., 2005), SOD-2 (Ding et al., 2007), and CAT (Girnun et al., 2002) are the target genes of PPAR-γ. To our knowledge, this is the first study showing that Rosi improves learning and memory functioning in MWM and passive avoidance tasks in hypothyroid rats by decreasing NO production, improving BDNF levels, and decreasing oxidative stress indexes.

Interestingly, we observed that Rosi at the dose of 4 mg/kg could improve thyroid gland function and prevent hypothyroidism induction in juvenile rats. The mechanism of this effect remains unknown, but studies have reported that Rosi treatment increases iodine (I) uptake in the thyroid gland (Kebebew et al., 2006; Tepmongkol et al., 2008). Therefore, in our study, Rosi, probably *via* the increase of I uptake, was able to increase thyroxin level. The homeostatic effects of Rosi on thyroid hormones may be a mechanism that indirectly contributes to the protective effects of the drug in the present study. The exact mechanism(s) responsible for these effects of Rosi on thyroid function needs further investigation. Furthermore, the administration of PTU and Rosi was done simultaneously in the present study, and, therefore, it is impossible to judge the clinical application of the results. Future studies should be completed to provide new insights about clinical relevance.

Finally, it is necessary to mention that hypothyroidism status may negatively affect the motor ability of rodents and humans (Salazar et al., 2019). On the other hand, PPAR-γ agonists, including Rosi, has been shown to improve motor ability in some animal models such as those for Parkinson's disease (Schintu et al., 2009). Positive effects of another PPAR-γ agonist, pioglitazone, on animal models of amyotrophic lateral sclerosis and Huntington's disease have also been reported (Kiaei, 2008). Considering this evidence, the behavioral performances of the rats in the present study may be due to drug effects on motor performance. To assess this issue, the time to reach the platform in the first trial of the first day was compared between the groups (Hosseini et al., 2011), and no significant difference was observed (Fig. 1C). Therefore, it seems that the results of the present study were not due to the motor performance of the rats; however, this contribution could be investigated further.

In summary, our results indicated that Rosi causes a substantial reduction of hypothyroid injury in the brain by increasing hippocampal BDNF level and by decreasing excessive NO production and oxidative stress; however, further studies are needed to determine additional molecular mechanisms.

ACKNOWLEDGMENTS

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