Physiology & Behavior 222 (2020) 112892

Contents lists available at ScienceDirect

Physiology & Behavior

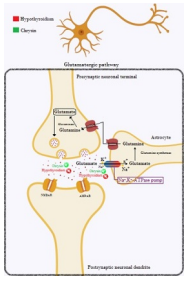
journal homepage: www.elsevier.com/locate/physbeh

Modulation of glutamate levels and Na^+,K^+-ATPase activity contributes to the chrysin memory recovery in hypothyroidism mice

Vandreza Cardoso Bortolotto, Stífani Machado Araujo, Franciane Cabral Pinheiro, Márcia Rósula Poetini, Mariane Trindade de Paula, Luana Barreto Meichtry, Francielli Polet de Almeida, Elize Aparecida Santos Musachio, Gustavo Petri Guerra, Marina Prigol*

Laboratório de Avaliações Farmacológicas e Toxicológicas aplicadas às Moléculas Bioativas – Unipampa, Universidade Federal do Pampa - Campus Itaqui, Itaqui 97650-000, RS, Brazil

GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:
Flavonoid
Chrysin
Hypothyroidism
Methimazole
Glutamate
Na^+
K^+-ATPase
Memory deficit

ABSTRACT

Abnormalities in the thyroid hormones, like in hypothyroidism, are closely related to dementia and Alzheimer's disease demonstrating the main symptom of these disorders: memory deficit. In this study we evaluated the effect of chrysin on deficit spatial and aversive memories and the contribution of glutamatergic, cholinergic pathways and Na^+, K^+-ATPase activity on hippocampus and prefrontal cortex in hypothyroid adult female mice C57BL/6. Hypothyroidism was induced by the continuous exposure to 0.1% methimazole (MTZ) in drinking water for 31 days. The exposure to MTZ was associated to low plasma levels of thyroid hormones (TH) compared to the control group on the 32nd. Subsequently, euthyroid and MTZ-induced hypothyroid mice received (intragastrically) either vehicle or chrysin (20 mg/kg) once a day for 28 consecutive days. After treatments mice performed the following behavioral assessments: open-field test (OFT), morris water maze (MWM) and passive avoidance test. Additionally, plasma TH levels were measured again, as well as glutamate levels, Na^+,K^+-ATPase and acetylcholinesterase (AChE) activities were analyzed in the hippocampus and prefrontal cortex of mice. Mice with hypothyroidism showed a deficit of spatial and aversive memory and chrysin treatment reversed these deficits. It also reduced the levels of glutamate and decreased Na^+,K^+-ATPase activity in both cerebral structures in the hypothyroid mice compared with the euthyroid ones, with the exception of glutamate in the hippocampus, which was a partial reversal. AChE activity was not altered by treatments. Together, our results demonstrate that chrysin normalized hippocampal glutamate levels and Na^+,K^+-ATPase activity, which could be involved in the reversal of memory deficit.

* Corresponding author.
E-mail address: marinaprigol@gmail.com (M. Prigol).

https://doi.org/10.1016/j.physbeh.2020.112892
Received 11 September 2019; Received in revised form 27 February 2020; Accepted 23 March 2020
Available online 15 April 2020
0031-9384/ © 2020 Elsevier Inc. All rights reserved.

1. Introduction

Adult-onset hypothyroidism has been studied recently and there is a close relationship with the main alterations involving several cognitive areas, patients with hypothyroidism have shown dementia-like behaviors and Alzheimer's Disease (AD): psychosis, hallucinations, confusion and significant learning and memory impairment [1]. Thirty-six percent of patients with dementia have shown abnormalities in the thyroid hormones (TH), which is associated to dysfunction in the thyroid gland in AD [1]. However, it is not only the decrease in the TH that can demonstrate these behaviors, the decrease of the TH receptors is also described in patients with AD [2]. The data obtained in these studies using plasma, or the postmortem brain from people with AD, all these studies make an association of this disease with hypothyroidism, showing for example: a decrease in thyroid hormones, an increase in thyroid stimulating hormone (TSH) and a decrease in TH receptors expression, as well as increased β-amyloid expression (found in the brain of AD patients) [1].

The mechanism of action of TH in the brain is through gene expression [3] and from their binding to their receptors, regulating the expression of target genes, from transcription factors [4,5]. The receptors of TH (TRs) are expressed in neurons, oligodendrocytes, and astrocytes, the predominant cell types in the brain [6]. Furthermore, the absence of TH, which results in a TR aporeceptor form often causes the repression of these hormones target genes due to the recruitment of corepressors that contribute to histone deacetylation [7]. In addition to the genomic effects mediated by TRs, TH also exerts non-genomic actions mediated by membrane receptors or by the modulation of signaling pathways [4,5].

The TH act at molecular levels, by genomic via, modulating the glutamate-transporter gene, increasing the gene expression [8], and by non-genomic was mediated by membrane receptors or by the modulation of signaling pathways [5,9], reaching neuroprotective and vasodilatorory effects in the brain and peripheral tissues [8]. The effects of glutamate deregulation at the cellular and circuit levels may be due the glutamate transport abnormalities leading to irregular distribution of glutamate [8]. The reduction of the TH level is responsible for the decrease of Na^+,K^+-ATPase activity in a selective way. The Na^+,K^+-ATPase has two subunits named alpha and beta, TH regulates the concentration of mRNA expression of the alpha and beta subunit by binding T3 to nuclear receptors [3]. The Na^+,K^+-ATPase is responsible for maintaining resting cell membrane potential, is abundant in the brain and plays a major role in neuronal activity [3]. Some authors have reported that TH has a direct effect on the total activity of Acetylcholinesterase (AChE) and, consequently, on the metabolism of acetylcholine [10]. AChE can improve cognitive function by inducing long-term potentiation of pyramidal neurons of the hippocampus, which suggests that AChE per se might enhance cognitive functions [11].

Therefore, the flavonoid chrysin (5,7-Dihydroxyflavone) has presented itself as a promising natural compound, and various beneficial effects have been reported, known for its antioxidant activity [12], and potential aromatase enzyme inhibitor [13]. Chrysin acts in central nervous system (CNS), having anxiolytic activity [14,15], once it possesses antidepressant properties that can be helpful in treatments for different diseases [16-20], and also, promotes improvement of cognitive and memory deficits in different memory loss induction models [21–27], acting as a neuroprotective agent, [21], neuroprotective effect in chronic cerebral hypoperfusion [23], neuroprotective effect in Parkinson disease [22,24,25], acting in general involved the decreased of neuroinflamation and increased of neurotrofic factor levels. Chrysin has recently been shown to have potential to improve cerebral ischemia, it acts preventing cognitive and long-term potentiation impairments due to cerebral ischemia [26] and regulating the PI3K/Akt/mTOR pathway [27].

Considering that the dysfunctions caused by hypothyroidism and knowing that THs have been considered as decisive factors involved in the development of dementia [1,2,28], our objective was to explore if chrysin restores spatial and aversive memory deficits as well as glutamatergic and cholinergic alterations associated to hypothyroidism.

2. Materials and methods

2.1. Chemicals

Chrysin, methimazole (MTZ), adenosine triphosphate (ATP), ouabain and acetylthiocholine (AcSCh) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Chrysin was dissolved in 20% polyethylene glycol and a saline solution (pH 7.4, 80%), while MTZ was dissolved in distilled water. All the other chemicals were obtained from the analytical grade of our laboratory or from standard commercial suppliers.

2.2. Animals

Female C57BL/6 mice (total of 35 adult), aged between 3 and 4 months, were used for all the studies, assigned by the Federal University of Santa Maria. We use females because the prevalence of hypothyroidism is higher in women [17]. Mice were maintained under a 12:12 h light/dark cycle at 23 ± 2 °C and 50 ± 5% humidity. Standard laboratory diet and water were available ad libitum before and during the treatments. The manipulations were carried out between 08.00 a.m. and 04.00 p.m. All efforts were made to minimize animal suffering and to reduce the number of animals used in the experiments. The present experimental study was approved by the Institutional Ethics Committee on Care and Use of Experimental Animal Resources from the Federal University of Pampa, Brazil, and it was registered under the protocol number 024/2018.

2.3. Experimental design

2.3.1. Hypothyroidism induction

On the first 31 days, the animals were divided into two groups: control group (n = 17) and MTZ (n = 18) (Fig. 1). Hypothyroidism was

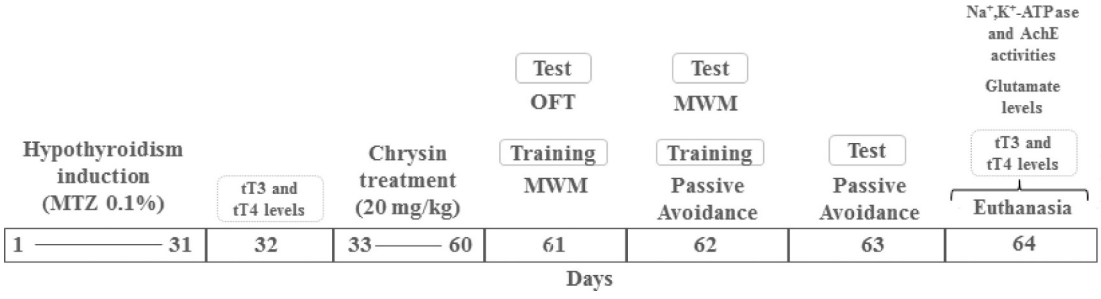


Fig. 1. Experimental protocol.

induced by continuous exposure to the anti-thyroid drug MTZ 0.1% + 0.475% sucralose in the drinking water [17,29], sucralose has been added to improve the taste of MTZ. The control group received 0.475% sucralose in the drinking water. On day 32, the exposure of MTZ and sucralose were stopped, and the levels of total thyroid hormones tT3 and tT4 were measured using blood extracted from the mice tail vein.

2.3.2. Chrysin treatment

After the induction of hypothyroidism, the euthyroid (control) and hypothyroid (MTZ) mice were subdivided into four groups (n = 8–9): euthyroid; euthyroid chrysin; hypothyroid; and hypothyroid chrysin. Chrysin (20 mg/kg, 10 ml/kg) was administered intragastrically starting at the 33nd day, once a day, for 28 consecutive days, in which chrysin has been shown to have a neuroprotective effect at this time and dosage [16,17]. The groups euthyroid and hypothyroid, received the vehicle, administered intragastrically.

2.4. tT3 and tT4 levels

The total thyroid hormones (tT4 and tT3) from the blood plasma were measured on the 32nd and 64th (n = 3–4), through a microparticle enzyme immunoassay (MEIA), using the AxSYM® system (Abbott Laboratories, Abbott Park, Illinois, USA), according to the manufacturer's instructions; tT3 was expressed in ng/mL and tT4 in pg/mL.

2.5. Behavioral assessments

At the end of chrysin treatment, mice performed behavioral tests: Open-Field Test (OFT) and training of Morris Water Maze (MWM) on the 61st; on the next day the MWM test and training of Passive Avoidance; and on 63rd day the passive avoidance test was performed.

2.5.1. Open-field test (OFT)

Locomotor activity was assessed through the distance traveled in meters on the OFT (details in S1) [30]. This test was the first performed, with the purpose of evaluating the locomotor activity and validating the memory tests.

2.5.2. Morris water maze (MWM) test

Spatial learning and memory were assessed using the Morris water maze test according to the method of Morris [31], with some modifications of Souza [21](details in S1).

2.5.3. Passive avoidance test

Non-spatial long-term aversive memory was measured using the passive avoidance test, which utilizes the innate preference of rodents for dark environments due to their photophobia, in other words, emotional memory based on contextual fear conditioning [33] (details in S1).

2.6. Tissue preparation

On the 64 day, after the behavioral tests, the animals were anesthetized by isoflurane inhalation and blood samples were collected directly from the right ventricle of their heart, using heparin as the anticoagulant. The plasma was used to analyze tT3 and tT4 levels, the plasma was separated by centrifugation (2400× g) for 15 min and used to determine tT3 and tT4 levels.

After euthanasia, the hippocampus and prefrontal cortex were removed and homogenized in 50 mM Tris–HCl, pH 7.4 (1:5, w:v). The homogenates were subsequently centrifuged at 2400× g at 4 °C for 15 min. A low-speed supernatant fraction (S1) was used to measure the levels of glutamate, Na^+, K^+-ATPase and acetylcholinesterase activities.

2.7. Glutamate levels

The levels of glutamate were measured in the hippocampus and the prefrontal cortex using reverse-phase high-performance liquid chromatography with electrochemical detection as it was previously described [36]. The concentrations of glutamate were measured and expressed as ng/g of tissue.

2.8. Na^+, K^+-ATPase activity

The amount of inorganic phosphate (Pi) released was quantified by the colorimetric method [37] and expressed in nmol Pi/mg protein/min (details in S1).

2.9. Acetylcholinesterase (AChE) activity

AChE activity was determined according to the method of Ellman et al., [38] using acetylthiocholine (AcSCh) as the substrate. The enzymatic activity was expressed as Units AChE (UAChE)/mg protein (details in S1).

2.10. Rotein determination

Protein determination was measured by the method of Bradford [39] using bovine serum albumin as the standard.

2.11. Statistical analysis

Data were checked for normality of distribution using the Shapiro–Wilk test. The OFT, MWM and passive avoidance tests results were analyzed using an ANOVA followed by Newman–Keuls test, and the biochemical results were compared using two-way ANOVA using Bonferroni correction for multiple comparisons. Descriptive data were expressed as the mean ± standard error of the mean (S.E.M.). Probability values less than 0.05 ($p < 0.05$) were considered statistically significant.

3. Results

3.1. Thyroid hormone levels

The effect of MTZ on the plasma levels of tT3 and tT4 in mice on the 32st day, are show in Table 1. These dosages aimed to know if the animals were really hypothyroid, so we can continue with the study. Student's unpaired t-test indicated, showed that mice exposed to MTZ had to lower tT3 and tT4 plasma levels than the control group on the 32nd. The results indicated a significant main effect of MTZ on the levels of tT3 (t = 28.17, df = 4, $P < 0.0001$) and tT4 (t = 94.31, df = 4, $P < 0.0001$).

The effect of MTZ and chrysin on the plasma levels of tT3 and tT4 in mice on the 64th of the treatment, are shown in Table 1. Two-way ANOVA followed by the *post-hoc* comparisons showed a decrease in the levels of both hormones in hypothyroid mice compared to euthyroid mice. A significant main effect of hypothyroidism on tT3 ($F_{1,8}$ = 11.52, P = 0.0094) and tT4 ($F_{1,12}$ = 20.84, P = 0.0006) was observed. Levels persisted low after chrysin administration, in the hypothyroidism chrysin group compared with euthyroid group.

3.2. Behavioral tests

3.2.1. OFT

Two-way ANOVA followed by the *post-hoc* comparisons showed that neither MTZ nor chrysin affected spontaneous locomotor activity in female mice ($F_{1,\ 31}$ = 0.38, P = 0.5421; Table 2).

Table 1
Plasma tT3 and tT4 total levels in mice treated with MTZ and chrysin on the 32nd and 64th day.

	tT3 (ng/mL)	tT4 (pg/mL)
32 days		
Euthyroid	3.50 ± 0.10	8.60 ± 0.10
Hypothyroid	1.2 ± 0.10*	0.90 ± 0.10*
64 days		
Euthyroid	3.8 ± 0.31	7.85 ± 1.58
Euthyroid chrysin	3.7 ± 0.42	9.03 ± 0.76
Hypothyroid	1.6 ± 1.25* #	3.54 ± 3.02* #
Hypothyroid chrysin	2.7 ± 0.92* #	4.12 ± 2.03* #

Values are expressed as means ± S.E.M. of 3 or 4 mice/group.
* Significant difference in relation to the Euthyroid groups;.
# Significant difference in relation to the Euthyroid Chrysin group. $p < 0.05$ is considered significant.

3.2.2. MWM test

The latency to find the aim quadrant was demonstrated in Fig. 2A, two-way ANOVA followed by the *post-hoc* comparisons showed a significant hypothyroidism × chrysin interaction ($F_{1, 21} = 7.74$, $P = 0.0112$). The hypothyroid group showed an increase of latency in comparison to the euthyroid group. The treatment with chrysin was able to reverse this deficit.

Two-way ANOVA followed by the *post-hoc* comparisons showed a significant hypothyroidism × chrysin interaction ($F_{1, 21} = 4.52$, $P = 0.0455$). The data of the time that animals spend in the aim quadrant (Fig. 2B) shows a decrease of the hypothyroidism group compared to the euthyroid group and treatment with chrysin was able to reverse this effect.

Neither MTZ nor chrysin affected the time in the opposite quadrant in female mice. Two-way ANOVA followed by the *post-hoc* comparisons showed no significant hypothyroidism × chrysin interaction (Fig. 2C, $F_{1, 21} = 0.50$, $P = 0.4852$).

3.2.4. Passive avoidance test

Fig. 3 shows the latency to go to the dark side in the passive avoidance test. The two-way ANOVA followed by the *post-hoc* comparisons indicated a hypothyroidism × chrysin interaction ($F_{1, 25} = 3.20$, $P = 0.0856$). The data showed a significant decrease in latency in the hypothyroid group compared to the euthyroid group, the treatment with chrysin was able to reverse this deficit.

3.3. Glutamate levels

Fig. 4 illustrates the effects of chrysin and hypothyroidism when analyzing the glutamate levels in the hippocampus (Fig. 4A) and prefrontal cortex (Fig. 4B). The two-way ANOVA followed by the *post-hoc* analysis indicated that hypothyroidism decreased glutamate levels in both structures, when compared to the euthyroid mice. The treatment with chrysin recovered this level partially in the hippocampus ($F_{1,12} = 0.62$, $P = 0.4449$) and totally in the prefrontal cortex ($F_{1,12} = 7.23$, $P = 0.0197$).

3.4. Na^+,K^+-ATPase activity

Fig. 5A and B demonstrate the effect of hypothyroidism and chrysin on Na^+,K^+-ATPase activity in the hippocampus and the prefrontal cortex of mice, respectively. The two-way ANOVA demonstrated a hypothyroidism × chrysin interaction in the hippocampus ($F_{1,16} = 0.58$, $P = 0.4589$) and the prefrontal cortex ($F_{1,12} = 5.61$, $P = 0.0354$). The *post-hoc* analysis indicated that hypothyroidism decreased Na^+,K^+-ATPase activity in the hippocampus when compared to the euthyroid mice. The treatment with chrysin recovered this activity; in the prefrontal cortex it is showed that hypothyroidism decreased Na^+,K^+-ATPase activity but not significantly, when associated with chrysin there was an increase compared to hypothyroidism group.

3.5. AChE activity

Fig. 6A and B display the effects of hypothyroidism and chrysin on AChE activity in the hippocampus and the prefrontal cortex of mice, respectively. The two-way ANOVA indicated a significant hypothyroidism × chrysin interaction in the hippocampus ($F_{1, 16} = 4.78$, $P = 0.0440$) but not in the prefrontal cortex ($F_{1, 12} = 1.08$, $P = 0.3188$). The *post-hoc* analysis showed neither MTZ nor chrysin affected the AChE activity.

4. Discussion

The hypothyroidism is known to exert a wide spectrum in cognitive dysfunction and biochemical alteration. Our results demonstrate a deficit of spatial and aversive memories in hypothyroid animals, as well as a decrease in the glutamate levels and Na^+,K^+-ATPase activity, in hippocampus and prefrontal cortex. The treatment with chrysin showed an improvement in these memories deficits, and restores some of the neurochemical markers in hypothyroid female mice. As in our previous study [17], the effectiveness of chrysin was obtained independently of the thyroid hormone levels, which remained low even after the treatment.

According to the OFT results, locomotor abnormalities were not detected in our study. This result is in agreement with our previous study, demonstrating that in the OFT the locomotor function remained the same in all groups tested [17]. Additionally, this data validate the results obtained from the memory tests.

In this study, the deficit of memory evaluation was performed by two different tests: MWM (long-term spatial memory) and Passive Avoidance test (long-term aversive memory). The MWM test is usually accepted as an indicator of spatial and reference memory [31], the hypothyroidism mice took more time to find the aim quadrant in comparison to the euthyroid mice, and the treatment with chrysin was efficient in reversing this spatial memory deficit, even when the hypothyroidism mice spent less time in the quadrant where the platform was 24 h earlier, chrysin was able to make the animals remember which quadrant was the platform. Taking these data into consideration, we evaluated the time in which the animals remained in the opposite quadrant of the aim quadrant and we did not obtain significantly differences between the groups, with what we can conclude in this test that when the animals were not in the platform quadrant, they were dispersed swimming through the maze. Our results showed a deficit in the spatial memory of animals with hypothyroidism due to the decrease in TH and/or TR, subsequent to some biochemical alteration, the same way other authors stated [40–43] and the treatment with chrysin was able to ameliorate this long-term spatial deficit, as demonstrated by [21,23]. It has already been demonstrated that deletion of all TRα is associated to behavioral inhibition, memory deficits and spatial learning [44], then the TH can increase behavior and spatial learning, that is, when hormones bind to the receptor, it activates the behavior of spatial memory. This deficit is caused by the absence of either the receptor or the hormone, so we suggest treatment with chrysin may

Table 2
Effects of hypothyroidism and chrysin on mice subjected to the OFT.

	Distance (m)
Euthyroid	29.42 ± 22.74
Euthyroid chrysin	28.65 ± 10.52
Hypothyroid	35.22 ± 19.23
Hypothyroid chrysin	27.01 ± 15.75

Values are expressed as means ± S.E.M. of 8 or 9 mice/group. $p < 0.05$ is considered significant.

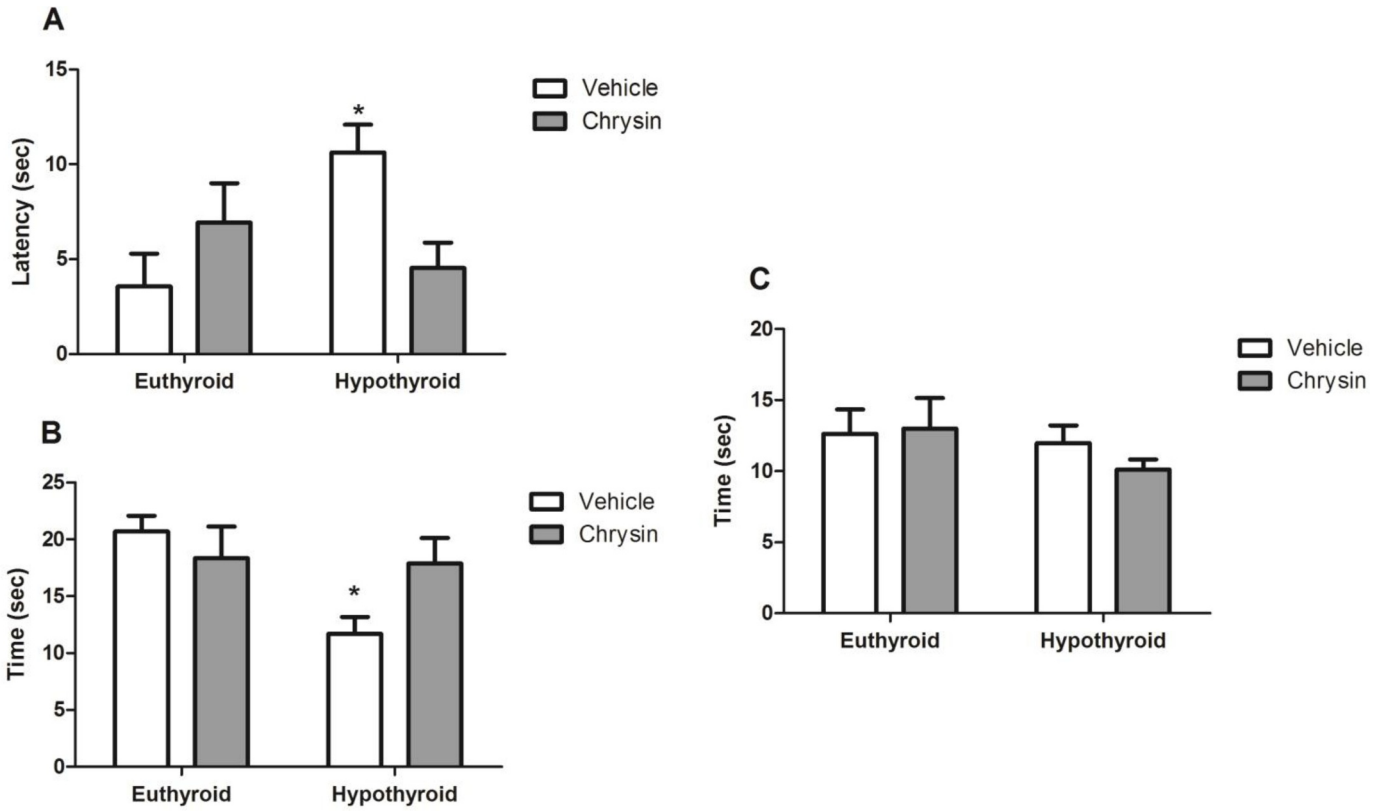


Fig. 2. Effects of hypothyroidism and chrysin on the MWM test. (A) Latency to find the quadrant of platform. (B) Time spent in the aim quadrant. (C) Time spent in the opposite quadrant. *Significant difference in relation to the euthyroid group. Data are shown as mean ± standard error of the mean (n = 5–8 per group).

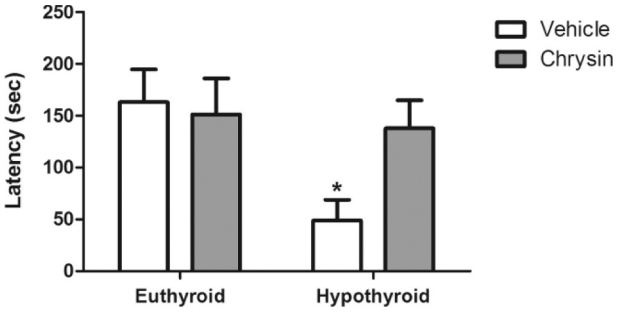


Fig. 3. Effects of hypothyroidism and chrysin on the Passive avoidance test (light and dark). The graphic shows the latency of animals to pass to the dark-side. *Significant difference in relation to the euthyroid group, euthyroid + chrysin and hypothyroid + chrysin group. Data are shown as mean ± standard error of the mean (n = 6–8 per group).

modulate these connections.

Another test that we used to examine the memory deficit was the Passive Avoidance test, this is based on an adaptive response to a stressful experience. In this test, our results were similar to the MWM, in which we proved, once again, the memory deficit induced by this model of disease. Hypothyroidism makes animals more susceptible a decreased in aversive memory, causing a memory deficit in this specific test [10,40–42,45], and the animals treated with chrysin showed aversion to shock. This fact is important, since it is the first time that chrysin presents efficiency in the treatment of aversive memory deficit.

The decrease of these hormones, as in the case of hypothyroidism, causes an imbalance in the glutamatergic system. Our data demonstrated, for the first time, a decrease in the glutamate levels in the hypothyroid groups in hippocampus and prefrontal cortex, and the treatment with chrysin was able to perform a complete restoration in

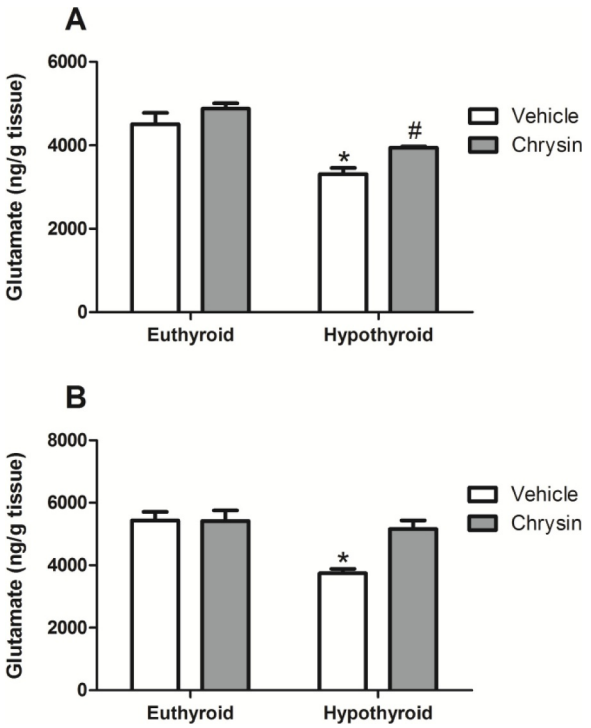


Fig. 4. Effects of hypothyroidism and chrysin on the glutamate level. (A) Hippocampus (B) Prefrontal cortex. *Significant difference in relation to the euthyroid, euthyroid + chrysin and hypothyroid + chrysin groups. #Significant difference in relation to the euthyroid and euthyroid + chrysin groups. Data are shown as mean ± standard error of the mean (n = 4 per group).

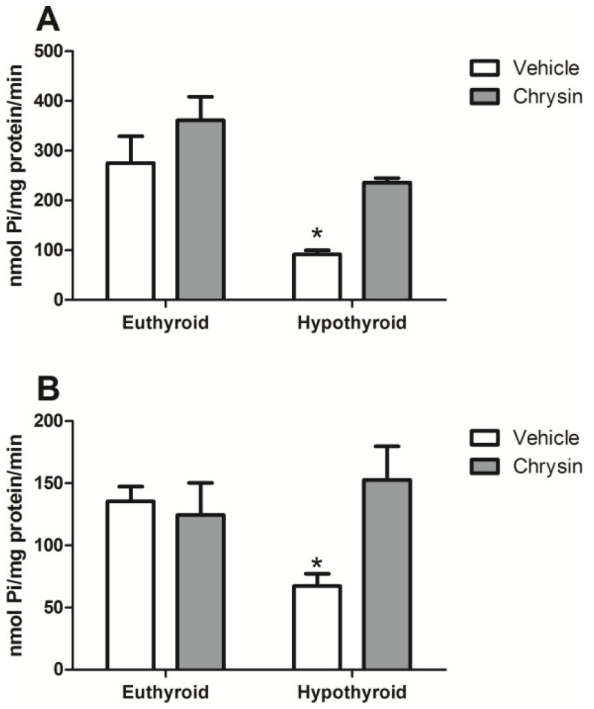


Fig. 5. Effects of hypothyroidism and chrysin on Na^+, K^+-ATPase activity. (A) Hippocampus. *Significant difference in relation to the euthyroid, euthyroid + chrysin and hypothyroid + chrysin groups. (B) Prefrontal cortex. *Significant difference in relation to the hypothyroid + chrysin group. Data are shown as mean ± standard error of the mean (n = 4 per group).

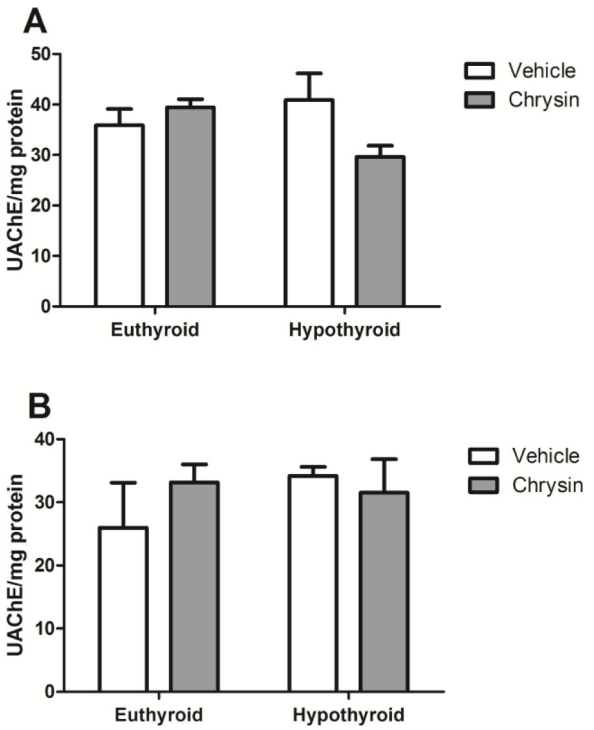


Fig. 6. Effects of hypothyroidism and chrysin on AChE activity in the hippocampus (A) and the prefrontal cortex in (B). Data are shown as mean ± standard error of the mean (n = 4–5 per group).

the prefrontal cortex but partially in the hippocampus, we believe that a longer treatment time would make this restoration complete. We can still infer from earlier studies [16,19,21,22] that chrysin may be acting on synaptic plasticity and positively regulating glutamate, still, in the literature there are reports that T3 influences glutamate levels through its effect on glutamate transporters that carry glutamate into astrocytes [8]. A study in rats showed that T3-treated astrocytes increased mRNA levels, protein expression of glutamate transporters, glutamate aspartate transporter (GLAST) and glial-1 glutamate transporter (GLT1). This eventually regulated the extracellular glutamate content [8], considering this, we can say that there are TRs in the brain and that the binding of hormones (mainly T3) to their receptors leads to a series of signs, where with the presence of hypothyroidism, the absence or reduction of these signals can occur. However a limitation of our study is the absence of glutamatergic transporters, which are a prospect for future study, with emphasis on the glutamatergic system.

Although total glutamate levels were measured in different regions (hippocampus and prefrontal cortex) by the technique used in this study, it may not directly reflect neurotransmitter levels within the synapse, because neurotransmitters in the synaptic cleft usually overflow, causing a change in interstitial neurotransmitter levels near the synapse [46], so our dosage reflects both that are in the astrocyte, in the synaptic cleft, and other places where the neurotransmitter is present. We can infer that our data reflect on the interference of the TH in the total synthesis of the neurotransmitter glutamate, and this reflect the spatial and aversive memory deficit in the hypothyroid animals, as it was already demonstrated by [46]. The decrease of glutamate levels, in our study, can be explain because the hypothyroidism may affect the release mechanisms in the presynaptic receptors [47], and also by a possible decrease in the number of NMDA receptors, which is caused by hypothyroidism, causing a decreased performance on the hippocampal-dependent cognitive tests [48].

Glutamate is released by the high affinity sodium dependent carrier system (GluTs), which is responsible for maintaining low extracellular concentrations of the potentially excitotoxic amino acid, which is released from the glutamatergic nerve terminals [49]. Considering GluTs depend on the transmembrane Na^+ gradient [50], which is maintained by the Na^+, K^+-ATPase activity [51], and that the hypothyroid condition leads to a decrease in TH, which decreases the activity of Na^+, K^+-ATPase, and knowing that the binding of T3 to nuclear receptors regulates the expression of mRNA [3], we can state that when TH is low, the synthesis of Na^+, K^+-ATPase is impaired [3,52], and that the inhibition of the activity of this enzyme is due to the distribution of TRs in the brain areas [3]. There are data in the literature that the inhibition of the brain Na^+, K^+-ATPase activity causes edema and cell death in the central nervous system and interferes in the learning and memory processes [3], and that the activity of this enzyme is selectively reduced in patients with cognitive deficits, such as AD [3,53]. Our work corroborates these data, once the animals with hypothyroidism had a decrease in the activity of Na^+, K^+-ATPase in both of the brain structures studied, as already evidenced [54]. The treatment with chrysin was efficient in restoring the activity of Na^+, K^+-ATPase in both brain structures, as evidenced previously [16,21], a hypothesis of what may have occurred for the explanation of this result is the distribution and density and possible increase of TRs in these areas of the brain, in which it restored the activity of that enzyme. These data may be correlated to the data of glutamate levels, demonstrating a decrease in these two markers in the hypothyroidism group [49,51].

The enzyme AChE plays a critical role in controlling the hydrolysis of acetylcholine during synaptic transmission in cholinergic synapses, dividing acetylcholine (Ach) into acetate and choline [3]. It is described that, the decrease in the supply of choline in the membrane is reflected by the reduction in the biosynthesis and concomitant release of Ach from biologically active neurons and, therefore, it increases the activity of AChE [3]. There are studies that show a change in AChE activity, but it was already demonstrated that choline levels in humans have no correlation to TH [8], this data may be in agreement with our study, in which no such increment in AChE activity was noted, since the AChE activity was in equilibrium, the amount of choline may also have been in equilibrium.

Furthermore, with the correlation data (additional data – Table 1S), it is clearly demonstrated that the relationship between the deficit of memory evaluated by the tests on the molecular targets evaluated: glutamate, Na^+, K^+-ATPase and AChE, showed a relation with memory, as well as reinforced the neuroprotective effect of chrysin related to the two types of memory evaluated: spatial and aversive, and the mechanisms in association are involved in hypothyroidism, which has already been shown to act in the Na^+, K^+-ATPase [16]. It is already known that the hippocampus is a brain structure highly vulnerable to the action of thyroid hormones and well known to be involved in memory and learning processes [45], so this is one explanation for our data being more expressive in the hippocampus. Our data suggest a connection between memory deficit and the impair of glutamate neuronal levels and hippocampal Na^+, K^+-ATPase in hypothyroidism, considering a reduction of these levels and activities, respectively. Taking into consideration the findings of this study, we can imply that chrysin is a pharmacological option, with possible fewer side effects, both as adjuvant for the treatment for hypothyroidism.

5. Conclusion

TH dysregulation has been implicated in severe cognitive and behavioral abnormalities related to dysfunctions of neural systems. In this work, our findings demonstrate the memory deficits caused by hypothyroidism in female mice, and the beneficial effect of chrysin treatment. Furthermore, this study suggests that glutamate levels and Na^+, K^+-ATPase activity contributes to the chrysin attenuating the deficits memory caused by hypothyroidism.

Author contributions

MP access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

VCB and MP conceived and designed the project.

VCB, SMA, FCP, MRP, MTP, LBM, FPA, and EASM performed the behavioral experiments.

VCB, SMA, FCP and MRP performed the biochemical measurements and acquisition of data.

VCB, GPG and MP interpreted of data and statistical analysis.

VCB drafting of the manuscript.

Critical revision of the manuscript for important intellectual content: GPG and MP.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest in the present work.

Acknowledgments

The authors gratefully acknowledge the financial support received from CNPq (307099/2017-2), FAPERGS and UNIPAMPA. This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brazil (CAPES) - Finance Code 001, and for the support of research grants provided by CAPES.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.physbeh.2020.112892.

References

[1] A. Accorroni, G. Chiellini, N. Origlia, Effects of thyroid hormones and their metabolites on learning and memory in normal and pathological conditions, Curr. Drug Metab. 18 (2017) 225–236, https://doi.org/10.2174/1389200218666170116112407.

[2] S. Sampaolo, A. Campos-Barros, G. Mazziotti, S. Carlomagno, V. Sannino, G. Amato, C. Carella, G. Di Iorio, Increased cerebrospinal fluid levels of 3,3′,5′- triiodothyronine in patients with Alzheimer's disease, J. Clin. Endocrinol. Metab. 90 (2005) 198–202, https://doi.org/10.1210/jc.2004-1083.

[3] J. Mandal, A. Chakraborty, A.K. Chandra, Altered acetylcholinesterase and Na + -K + ATPase activities in different areas of brain in relation to thyroid gland function and morphology under the influence of excess iodine, 8 (2016) 1564–1573.

[4] S.E. Fanibunda, L.A. Desouza, R. Kapoor, R.A. Vaidya, V.A. Vaidya, Thyroid Hormone Regulation of Adult Neurogenesis, 1st ed., Elsevier Inc., 2018, https://doi.org/10.1016/bs.vh.2017.04.006.

[5] S.Y. Cheng, J.L. Leonard, P.J. Davis, Molecular aspects of thyroid hormone actions, Endocr. Rev. 31 (2010) 139–170, https://doi.org/10.1210/er.2009-0007.

[6] A.W. Grant, Thyroid hormones and the brain, Front. Neuroendocrinol. 22 (2001) 1–17, https://doi.org/10.1006/frne.2000.0208.

[7] I. Astapova, A.N. Hollenberg, The in vivo role of nuclear receptor corepressors in thyroid hormone action, Biochim. Biophys. Acta - Gen. Subj. 1830 (2013) 3876–3881, https://doi.org/10.1016/j.bbagen.2012.07.001.

[8] Q. Zhang, Z. Bai, Y. Gong, X. Liu, X. Dai, S. Wang, F. Liu, Monitoring glutamate levels in the posterior cingulate cortex of thyroid dysfunction patients with TE-averaged PRESS at 3T, Magn. Reson. Imaging. 33 (2015) 774–778, https://doi.org/10.1016/j.mri.2015.03.004.

[9] S.E. Fanibunda, L.A. Desouza, R. Kapoor, R.A. Vaidya, V.A. Vaidya, Thyroid hormone regulation of adult neurogenesis, Vitam. Horm. 106 (2018) 211–251, https://doi.org/10.1016/bs.vh.2017.04.006.

[10] C.G. Vasilopoulou, C. Constantinou, D. Giannakopoulou, P. Giompres, M. Margarity, Effect of adult onset hypothyroidism on behavioral parameters and acetylcholinesterase isoforms activity in specific brain regions of male mice, Physiol. Behav. 164 (2016) 284–291, https://doi.org/10.1016/j.physbeh.2016.06.016.

[11] M.E. Appleyard, Acetylcholinesterase induces long-term potentiation in CA1 pyramidal cells by a mechanism dependent on metabotropic glutamate receptors, Neurosci. Lett. 190 (1995) 25–28, https://doi.org/10.1016/0304-3940(95)11491-E.

[12] G. Pushpavalli, P. Kalaiarasi, C. Veeramani, K.V. Pugalendi, Effect of chrysin on hepatoprotective and antioxidant status in D -galactosamine-induced hepatitis in rats, Eur. J. Pharmacol. 631 (2010) 36–41, https://doi.org/10.1016/j.ejphar.2009.12.031.

[13] Y. Kao, C. Zhou, M. Sherman, C.A. Laughton, S. Chen, Molecular basis of the inhibition of human aromatase (Estrogen synthetase) by flavone and isoflavone phytoestrogens: a site-directed mutagenesis study, Environ. Health Perspect. 106 (1998) 85–92.

[14] C. Wolfman, H. Viola, A. Paladini, F. Dajas, J.H. Medina, Possible anxiolytic effects of chrysin, a central benzodiazepine receptor ligand isolated from Passiflora Coerulea, Pharmacol. Biochem. Behav. 47 (1994) 1–4.

[15] J.F. Rodríguez-landa, F. Hernández-lópez, J. Cueto-escobedo, E.V. Herrera-huerta, E. Rivadeneyra-domínguez, B. Bernal-morales, E. Romero-avendaño, Chrysin (5,7-dihydroxyflavone) exerts anxiolytic-like e ff ects through GABAA receptors in a surgical menopause model in rats, Biomed. Pharmacother. 109 (2019) 2387–2395, https://doi.org/10.1016/j.biopha.2018.11.111.

[16] C.B. Filho, C.R. Jesse, F. Donato, R. Giacomeli, M.G.D.E. Gomes, A.T.R. Goes, S.P. Boeira, Chronic unpredictable mild stress decreases BDNF and NGF levels and Na^+, K^+-ATPase activity in the hippocampus and prefrontal cortex of mice: antidepressant effect of chrysin, Neuroscience 289 (2015) 367–380, https://doi.org/10.1016/j.neuroscience.2014.12.048.

[17] V.C. Bortolotto, F.C. Pinheiro, S.M. Araujo, M.R. Poetini, B.S. Bertolazi, M.T. de Paula, L.B. Meichtry, F.P. de Almeida, S. de Freitas Couto, C.R. Jesse, M. Prigol, Chrysin reverses the depressive-like behavior induced by hypothyroidism in female mice by regulating hippocampal serotonin and dopamine, Eur. J. Pharmacol. 822 (2018), https://doi.org/10.1016/j.ejphar.2018.01.017.

[18] C.B. Filho, C.R. Jesse, F. Donato, L. Del Fabbro, M.G. de Gomes, A.T.R. Goes, L.C. Souza, R. Giacomeli, M. Antunes, C. Luchese, S.S. Roman, S.P. Boeira, Neurochemical factors associated with the antidepressant-like effect of flavonoid chrysin in chronically stressed mice, Eur. J. Pharmacol. 791 (2016) 284–296, https://doi.org/10.1016/j.ejphar.2016.09.005.

[19] C.B. Filho, C.R. Jesse, F. Donato, L. Del Fabbro, M. Gomes de Gomes, A.T. Rossito Goes, L.C. Souza, S.P. Boeira, Chrysin promotes attenuation of depressive-like behavior and hippocampal dysfunction resulting from olfactory bulbectomy in mice, Chem. Biol. Interact. 260 (2016) 154–162, https://doi.org/10.1016/j.cbi.2016.11.005.

[20] T. Farkhondeh, S. Jalali, S. Samarghandian, F. Samini, Effects of chrysin on serum corticosterone levels and brain oxidative damages induced by immobilization in rat, Cardiovasc. Haematol. Disord. Targets. 19 (2019) 1–6, https://doi.org/10.2174/1871529X19666190618144440.

[21] L.C. Souza, M.S. Antunes, C.B. Filho, L. Del Fabbro, M.G. De Gomes, A.T.R. Goes, F. Donato, M. Prigol, S.P. Boeira, C.R. Jesse, Flavonoid chrysin prevents age-related cognitive decline via attenuation of oxidative stress and modulation of BDNF levels in aged mouse brain, Pharmacol. Biochem. Behav. 134 (2015) 22–30, https://doi.org/10.1016/j.pbb.2015.04.010.

[22] A.T.R. Goes, C.R. Jesse, M.S. Antunes, F.V. Lobo Ladd, A.A.B. Lobo Ladd, C. Luchese, N. Paroul, S.P. Boeira, Protective role of chrysin on 6-hydroxydopamine-induced neurodegeneration a mouse model of Parkinson's disease: involvement of neuroinflammation and neurotrophins, Chem. Biol. Interact. 279 (2018) 111–120, https://doi.org/10.1016/j.cbi.2017.10.019.

[23] X.L. He, Y.H. Wang, M.G. Bi, G.H. Du, Chrysin improves cognitive deficits and brain damage induced by chronic cerebral hypoperfusion in rats, Eur. J. Pharmacol. 680

(2012) 41–48, https://doi.org/10.1016/j.ejphar.2012.01.025.
[24] A. Krishnamoorthy, M. Sevanan, S. Mani, M. Balu, S. Balaji, P. Ramajayan, Chrysin restores MPTP induced neuroinflammation, oxidative stress and neurotrophic factors in an acute Parkinson's disease mouse model, Neurosci. Lett. (2019) 134382, , https://doi.org/10.1016/j.neulet.2019.134382.
[25] L. Del Fabbro, A.R. Goes, C.R. Jesse, M.G. Gomes, L.C. Souza, F.V.L. Ladd, A.A.B.L. Ladd, R.V.N. Arantes, A.R. Simionato, M.S. Oliveira, A.F. Furian, S.P. Boeira, Chrysin protects against behavioral, cognitive and neurochemical alterations in a 6-hydroxydopamine model of Parkinson's disease, Neurosci. Lett. 706 (2019) 158–163, https://doi.org/10.1016/j.neulet.2019.05.036.
[26] A. Sarkaki, Y. Farbood, S. Mohammad, T. Mansouri, M. Badavi, L. Khorsandi, M.G. Dehcheshmeh, M.K. Shooshtari, Chrysin prevents cognitive and hippocampal long-term potentiation deficits and inflammation in rat with cerebral hypoperfusion and reperfusion injury, Life Sci. (2019), https://doi.org/10.1016/j.lfs.2019.04.027.
[27] T. Li, J. Ma, X. Han, Y. Jia, H. Yuan, S. Shui, Chrysin ameliorates cerebral ischemia/reperfusion (I/R) injury in rats by regulating the PI3K/Akt/mTOR pathway, Neurochem. Int. 129 (2019) 104496–104504, https://doi.org/10.1016/j.neuint.2019.104496.
[28] A. Accorroni, F.S. Giorgi, R. Donzelli, L. Lorenzini, C. Prontera, A. Saba, A. Vergallo, G. Tognoni, G. Siciliano, F. Baldacci, U. Bonuccelli, A. Clerico, R. Zucchi, Thyroid hormone levels in the cerebrospinal fluid correlate with disease severity in euthyroid patients with Alzheimer's disease, Endocrine 55 (2017) 981–984, https://doi.org/10.1007/s12020-016-0897-6.
[29] J. Li, J.M. Paulson, F.D. Ye, M. Sung, A.N. Hollenberg, S.B. Rutkove, Reducing systemic hypermetabolism by inducing hypothyroidism does not prolong survival in the SOD1-G93A mouse, Amyotroph. Lateral Scler. 13 (2012) 372–377, https://doi.org/10.3109/17482968.2012.662988.
[30] R.N. Walsh, R.A. Cummins, The open-field test: a critical review, Psychol. Bull. 83 (1976) 482–504, https://doi.org/10.1037/0033-2909.83.3.482.
[31] R. Morris, Developments of a water-maze procedure for studyng spatial learning in the rat, J. Neurosci. Methods. 11 (1984) 47–60.
[33] E.R.A.Y. Detrait, É. Hanon, B. Dardenne, Y. Lamberty, The inhibitory avoidance test optimized for discovery of cognitive enhancers, Behav. Res. Methods. 41 (2009) 805–811, https://doi.org/10.3758/BRM.41.3.805.
[36] L. Han, L. Zhang, L. Li, Y. Sun, Y. Wang, L. Chen, Y. Guo, Y. Zhang, Q. Zhang, J. Liu, Neuropharmacology activation of serotonin 2C receptors in the lateral habenular nucleus increases the expression of depression-related behaviors in the hemiparkinsonian rat, Neuropharmacology 93 (2015) 68–79, https://doi.org/10.1016/j.neuropharm.2015.01.024.
[37] C.H. Fiske, Y. Subbarow, The colorimetric determination of phosphorus, J. Biol. Chem. 66 (1925) 375–400.
[38] G.L. Ellman, K.D. Courtney, V. Andres, R.M. Featherstone, A new and rapid colorimetric determination of acetylcholinesterase activity, Biochem. Pharmacol. 7 (1961) 88–95 http://www.ncbi.nlm.nih.gov/pubmed/13726518.
[39] M.M. Bradford, A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254, https://doi.org/10.1016/0003-2697(76)90527-3.
[40] Y. Baghcheghi, F. Beheshti, M.N. Shafei, H. Salmani, H.R. Sadeghnia, The effects of vitamin E on brain derived neurotrophic factor, tissues oxidative damage and learning and memory of juvenile hypothyroid rats, Metab. Brain Dis. 33 (2017) 713–724.
[41] Y. Baghcheghi, M. Hosseini, F. Beheshti, H. Salmani, A. Anaeigoudari, Thymoquinone reverses learning and memory impairments and brain tissue oxidative damage in hypothyroid juvenile rats, Arq. Neuropsiquiatr. 76 (2018) 32–40, https://doi.org/10.1590/0004-282x20170182.
[42] F. Beheshti, S. Karimi, F. Vafaee, M.N. Shafei, H.R. Sadeghnia, M.A.R. Hadjzadeh, M. Hosseini, The effects of vitamin C on hypothyroidism-associated learning and memory impairment in juvenile rats, Metab. Brain Dis. 32 (2017) 703–715, https://doi.org/10.1007/s11011-017-9954-y.
[43] T. Pan, M. Zhong, X. Zhong, Y. Zhang, D. Zhu, Levothyroxine replacement therapy with vitamin e supplementation prevents oxidative stress and cognitive deficit in experimental hypothyroidism, Endocrine 43 (2013) 434–439, https://doi.org/10.1007/s12020-012-9801-1.
[44] J.S. Wilcoxon, G.J. Nadolski, J. Samarut, O. Chassande, E.E. Redei, Behavioral inhibition and impaired spatial learning and memory in hypothyroid mice lacking thyroid hormone receptor α, Behav. Brain Res. 177 (2007) 109–116, https://doi.org/10.1016/j.bbr.2006.10.030.
[45] E. Sh, A. Allah, A.M.S. Gomaa, M.M. Sayed, The effect of omega-3 on cognition in hypothyroid adult male rats, Acta Physiol. Hung. 101 (2014) 362–376, https://doi.org/10.1556/APhysiol.101.2014.3.11.
[46] I. Amano, Y. Takatsuru, M.A. Khairinisa, M. Kokubo, A. Haijima, N. Koibuchi, Effects of mild perinatal hypothyroidism on cognitive function of adult male offspring, Endocrinology 159 (2018) 1910–1921, https://doi.org/10.1210/en.2017-03125.
[47] A. Shuaib, S. Ijaz, S. Hemmings, P. Galazka, R. Ishaqzay, L. Liu, J. Rabindran, H. Miyashita, Decreased glutamate release during hypothyroidism may contribute to protection in cerebral ischemia, Exp. Neurol. 128 (1994) 260–265, https://doi.org/10.1006/exnr.1994.1135.
[48] P.R. Lee, Ã.D. Brady, J.I.Ã. Koenig, Thyroid hormone regulation of N-Methyl- D -aspartic acid receptor subunit mRNA expression in adult brain, J. Neuroendocrinol. 15 (2003) 87–92.
[49] N.C. Danbolt, Glutamate uptake, Prog. Neurobiol. 65 (2001) 1–105, https://doi.org/10.1016/S0301-0082(00)00067-8.
[50] A. Amato, B. Barbour, M. Szatkowski, D. Attwell, Counter-transport of potassium by the glutamate uptake carrier in glial cells isolated from the tiger salamander retina, J. Physiol. 479 (1994) 371–380, https://doi.org/10.1113/jphysiol.1994.sp020302.
[51] K.B. Sánchez-Huerta, S. Montes, F. Pérez-Severiano, C. Alva-Sánchez, C. Ríos, J. Pacheco-Rosado, Hypothyroidism reduces glutamate-synaptic release by ouabain depolarization in rat CA3-hippocampal region, J. Neurosci. Res. 90 (2012) 905–912, https://doi.org/10.1002/jnr.22806.
[52] A.A. Mcdonough, T.A. Brown, B. Horowitz, R. Chiu, J. Schlotterbeck, J. Bowen, A. Schmitt, Thyroid hormone coordinately regulates Na^+-K^+-ATPase α-and β-subunit mRNA levels in kidney, Am. Physiol. Soc. 254 (1988) C323–C329.
[53] E.A. Dos Reis, L.S. De Oliveira, M.L. Lamers, C.A. Netto, A.T. de, S. Wyse, Arginine administration inhibits hippocampal Na^+, K^+-ATPase activity and impairs retention of an inhibitory avoidance task in rats, Brain Res. 951 (2002) 151–157, https://doi.org/10.1016/S0006-8993(02)03077-9.
[54] H. Carageorgiou, C. Pantos, A. Zarros, V. Stolakis, I. Mourouzis, D. Cokkinos, S. Tsakiris, Changes in acetylcholinesterase, Na^+, K^+-ATPase, and Mg^{2+}-ATPase activities in the frontal cortex and the hippocampus of hyper- and hypothyroid adult rats, Metabolism 56 (2007) 1104–1110, https://doi.org/10.1016/j.metabol.2007.04.003.