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VANDREZA CARDOSO BORTOLOTTO

INVESTIGAÇÃO DA AÇÃO DA CRISINA E OS POSSÍVEIS MECANISMOS ENVOLVIDOS NA REDUÇÃO DO DÉFICIT DE MEMÓRIA INDUZIDO PELO HIPOTIREOIDISMO EM CAMUNDONGOS

TESE DE DOUTORADO

Uruguaiana, RS, Brasil

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Tese apresentada ao Programa de Pós-graduação *Stricto Sensu* em Bioquímica, da Universidade Federal do Pampa, como requisito parcial para obtenção do título de Doutora em Bioquímica.

Orientadora: Drª. Marina Prigol

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Uruguaiana, RS, Brasil

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Vandreza Cardoso Bortolotto

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RESUMO

A redução da secreção dos hormônios 3,5,3'-triiodotironina (T3) e 3,5,3',5'-tetraiodotironina ou tiroxina (T4) pela glândula tireoide acarreta o desenvolvimento de hipotireoidismo, uma disfunção endócrina que está associada a desordens neuropsiquiátricas e declínio cognitivo. O flavonoide 5,7-dihidroxi-2-fenil-4H-cromeno-4-ona ou 5,7-dihidroxiflavona, popularmente conhecido como crisina, apresenta ações terapêuticas já elucidadas, tais como ação neuroprotetora em diferentes estudos de desordens neurológicas, incluindo a melhora de memória em roedores. Neste sentido, o objetivo da presente tese foi investigar se a crisina é capaz de reduzir o déficit cognitivo ocasionado pelo hipotireoidismo e verificar o envolvimento dos sistemas glutamatérgico, colinérgico e neurotrofinérgico. Primeiramente os camundongos C57BL/6 fêmeas foram divididos em dois grandes grupos: controle e hipotireoidismo. O hipotireoidismo foi induzido pela exposição contínua ao fármaco antitireoideo metimazol (MTZ) 0,1% + 0,475% de sucralose, durante 31 dias na água de beber. No 31º dia foi retirado sangue da veia caudal e determinado os níveis de T3 e T4. Após os animais foram separados em quatro grupos: controle, hipotireoidismo, crisina, hipotireoidismo + crisina. A crisina (20 mg/kg) foi administrada a partir do dia 33 de tratamento, diariamente por 28 dias, via oral. Ao final do tratamento, os animais passaram por testes comportamentais de locomoção e de memória. Após os testes comportamentais, o sangue foi coletado para dosagem dos níveis plasmáticos de T3 e T4. Estruturais cerebrais (córtex pré-frontal e hipocampo) também foram coletadas para análises bioquímicas. Analisamos o efeito da crisina em atenuar esses déficits de memória por análises *in vivo, ex vivo* e análises *in sílico*. Desta forma, esta tese envolveu dois estudos principais separados em dois artigos publicados. No primeiro estudo, avaliamos a memória de longa duração através do labirinto aquático de Morris e esquiva passiva. Em ambos os testes, a crisina foi capaz de reverter o déficit de memória causado pelo hipotireoidismo. Assim, analisamos o sistema glutamatérgico, através dos níveis de glutamato e atividade da enzima Na^+,K^+-ATPase, e o sistema colinérgico através da atividade da enzima acetilcolinesterase (AChE) nas estruturas cerebrais. Os resultados demonstraram que a crisina atua aumentando os níveis de glutamato no córtex pré-frontal e aumenta de maneira parcial estes níveis no hipocampo, que estavam diminuídos nos animais com hipotireoidismo, e aumentou a atividade da Na^+,K^+-ATPase em ambas as estruturas cerebrais que estavam diminuidas nos animais com hipotireoidismo. A atividade da AChE não foi afetada com o hipotireoidismo. Com isso, destacamos a ação protetora da crisina em alguns alvos moleculares

do sistema glutamatérgico que foram alterados pelo hipotireoidismo, atenuando o prejuízo cognitivo nos animais hipotireoideos. No segundo estudo, analisamos o sistema neurotrofinérgico, através da avaliação da memória de longa duração pelo labirinto aquático de Morris; estes resultados corroboraram com os resultados do primeiro artigo, em que os animais com hipotireoidismo desmonstraram um déficit de memória, e o tratamento com crisina foi capaz de reverter este prejuízo cognitivo. Nas análises *ex vivo*, verificamos os níveis do fator neurotrófico derivado do cérebro (BDNF) e fator de crescimento do nervo (NGF) em que o hipotireoidismo diminuiu o nível dessas neurotrofinas, e a crisina foi capaz de normalizar os níveis do BDNF hipocampal e do NGF no hipocampo e córtex pré-frontal. Por fim, realizamos as análises *in sílico* através do *docking molecular*, e verificamos a alta afinidade da crisina com os receptores de neurotrofinas TrkA, TrkB e p75NTR. Nossos resultados mostram que a crisina foi efetiva em melhorar o déficit de memória ocasionado pelo hipotireoidismo, e sugerimos que seu efeito terapêutico envolve a modulação de parâmetros associados aos sistemas glutamatérgico e neurotrofinérgico.

Palavras-chave: Déficit de memória; Flavonoides; Neuroproteção; Sistema glutamatérgico; Sistema neurotrofinérgico; Hipotireoidismo.

ABSTRACT

The reduction in the secretion of the hormones 3,5,3'-triiodothyronine (T3) and 3,5,3',5'-tetraiodothyronine or thyroxine (T4) by the thyroid gland leads to the development of hypothyroidism, an endocrine dysfunction that is associated with neuropsychiatric disorders and cognitive decline. The flavonoid 5,7-dihydroxy-2-phenyl-4H-chromene-4-one or 5,7-dihydroxyflavone, popularly known as chrysin, presents therapeutic actions already elucidated, such as neuroprotective action in different studies of neurological disorders, including the memory improvement in rodents. In this sense, the aim of this thesis was to investigate whether chrysin is able to reduce the cognitive deficit caused by hypothyroidism and to verify the involvement of the glutamatergic, cholinergic and neurotrophinergic systems. Firstly, C57BL/6 female mice were divided into two large groups: control and hypothyroidism. Hypothyroidism was induced by continuous exposure to the antithyroid drug methimazole (MTZ) 0.1% + 0.475% sucralose for 31 days in drinking water. On day 31, blood was drawn from the tail vein and T3 and T4 levels were determined. Afterwards, the animals were separated into four groups: control, hypothyroidism, chrysin, hypothyroidism + chrysin. Chrysin (20 mg/kg) was administered from day 33 of treatment, daily for 28 days, orally. At the end of the treatment, the animals underwent behavioral tests for locomotion and memory. After behavioral tests, blood was collected to measure plasma levels of T3 and T4. Brain structurals (prefrontal cortex and hippocampus) were also collected for biochemical analyses. We analyzed the effect of chrysin in alleviating these memory deficits by *in vivo*, *ex vivo* and *in silico* analysis. Thus, this thesis involved two main studies separated into two published articles. In the first study, we assessed long-term memory through the Morris water maze and passive avoidance. In both tests, chrysin was able to reverse the memory deficit caused by hypothyroidism. Thus, we analyzed the glutamatergic system, through the levels of glutamate and activity of the enzyme Na+,K+-ATPase, and the cholinergic system through the activity of the enzyme acetylcholinesterase (AChE) in brain structures. The results showed that chrysin acts by increasing glutamate levels in the prefrontal cortex and partially increasing these levels in the hippocampus, which were decreased in animals with hypothyroidism, and increased the activity of Na+,K+-ATPase in both brain structures which were decreased in animals with hypothyroidism. AChE activity was not affected by hypothyroidism. Thus, we highlight the protective action of chrysin on some molecular targets of the glutamatergic system that were altered by hypothyroidism, attenuating cognitive impairment in hypothyroid animals. In the

second study, we analyzed the neurotrophinergic system, through the assessment of long-term memory using the Morris water maze; these results corroborate the results of the first article, in which animals with hypothyroidism showed a memory deficit, and the treatment with chrysin was able to reverse this cognitive impairment. In the ex vivo analyses, we verified the levels of brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) in which hypothyroidism decreased the level of these neurotrophins, and chrysin was able to normalize the levels of hippocampal BDNF and of the NGF in the hippocampus and prefrontal cortex. Finally, we performed the *in silico* analysis through molecular docking, and verified the high affinity of chrysin with the neurotrophin receptors TrkA, TrkB and p75NTR. Our results show that chrysin was effective in improving the memory deficit caused by hypothyroidism, and we suggest that its therapeutic effect involves the modulation of parameters associated with the glutamatergic and neurotrophinergic systems.

Keywords: Memory deficit; Flavonoids; Neuroprotection; Glutamatergic system; Neurotrophinergic system; Hypothyroidism.

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LISTA DE ABREVIATURAS

ACh - Acetilcolina

AChE - Acetilcolinesterase

AMPA - α-amino-3-hidroxi-5-metil-4-isoxazolpropionico

AMPc - Monofosfato cíclico de adenosina, do inglês c*yclic adenosine monophosphate*

BDNF - Fator neurotrófico derivado do cérebro, do inglês *brain-derived neurotrophic factor*

CaMKII - Proteína quinase dependente de Ca^{2+}/calmodulina, do inglês *Ca^{2+}/calmodulin-dependent protein kinase*

CNTF - Fator neurotrófico ciliar, do inglês *ciliary neurotrophic factor*

CREB - Proteína de ligação responsiva ao AMPc, do inglês *cAMP responsive-element binding protein*

DA - Doença de Alzheimer

GDNF - Fator neurotrófico derivado da linha celular glial, do inglês *glial cell line-derived neurotrophic factor*

HT - Hormônios tireoidianos

iGluR - Receptores ionotrópicos

KA - Ácido caínico, do inglês *kainic acid*

LTD - Depressão de longo prazo, do inglês *Long-Term Depression*

LTM - Memórias de longa duração, do inglês *Long-Term Memory*

LTP - Potenciação à longo prazo, do inglês *Long-Term Potentiation*

mGLUR – Receptores metabotrópicos

mRNA – Ácido ribonucleico mensageiro

MTZ – 1-metil-2-mercaptoimidazol, metimazol

NGF - Fator de crescimento do nervo, do inglês *nerve growth factor*

NMDA - N-metil D-aspartato

NT3 - Neurotrofina 3

NT4 - Neurotrofina 4

PKA - Proteína quinase A, do inglês *protein kinase A*

PKC - Proteína quinase C, do inglês *protein kinase C*

PPF - Facilitação de pulso emparelhado, do inglês *Paired-Pulse Facilitation*

PTU - 6-propil-2tiouracil, propiltiouracil

RMSD - Desvio médio da raiz quadrática, do inglês *Root Mean Square deviation*

SNC - Sistema nervoso central

STM - Memórias de curta duração, do inglês *Short-Term Memory*

T3 - 3,5,3'-triiodotironina

T4 - 3,5,3',5'-tetraiodotironina, tiroxina

TGF - fator de transformação de crescimento, do inglês *transforming growth factor*

TR – Receptores tireoidianos, do inglês *thyroid receptors*

TRH - Hormônio liberador de tireotrofina, do inglês *Thyrotropin releasing hormone*

TSH - Hormônio estimulador da tireoide, do inglês *Thyroid stimulating hormone*

SUMÁRIO

APRESENTAÇÃO

Na primeira parte da presente tese, encontram-se as seções INTRODUÇÃO e REVISÃO BIBLIOGRÁFICA, estas apresentam informações sobre o tema abordado, seguidas pela JUSTIFICATIVA dos estudos e OBJETIVOS.

A metodologia realizada e os resultados obtidos que compõem esta tese estão apresentados sob a forma de artigos científicos já publicados, os quais se encontram no item RESULTADOS.

Após os artigos, há uma breve DISCUSSÃO, bem como as CONCLUSÕES da tese em que apresentamos interpretações gerais sobre os estudos e as PERSPECTIVAS FUTURAS.

Por fim, as REFERÊNCIAS BIBLIOGRÁFICAS referem-se às citações que aparecem nesta tese, com exceção ao item RESULTADOS.

1. INTRODUÇÃO

Os hormônios tireoidianos (HT), 3,5,3'-triiodotironina (T3) e 3,5,3',5'-tetraiodotironina ou tiroxina (T4) são fundamentais para o processo de desenvolvimento e de homeostase do organismo, e o desequilíbrio nos níveis destes hormônios, pode ocorrer patologias como hipertireoidismo (aumento exacerbado nos níveis dos HT) e hipotireoidismo (diminuição na síntese dos HT). O hipotireoidismo é uma disfunção hormonal complexa (ONCU *et al.*, 2004), manifestado em grande parte por uma lenta desaceleração de todas as funções corporais (GREENSPAN & DONG, 2004). Além do distúrbio metabólico geral, a anormalidade da produção dos HT pode ocasionar déficits intelectuais e comportamentais que podem afetar a homeostase dos indivíduos que apresentam esta patologia e resultar em doenças neurológicas como o estresse, depressão, ansiedade e déficit de memória (HAYAT *et al.*, 2010).

Deve-se levar em consideração que esse déficit de memória induzido pelo hipotireoidismo, com o passar dos anos pode ser um fator de risco para a Doença de Alzheimer (DA), uma vez que 36% das pessoas que possuem DA demonstram uma irregularidade nos níveis dos HT (ACCORRONI *et al.*, 2017). O déficit de memória ocasionada pelo hipotireoidismo ainda não possui uma fisiopatologia definida, sendo necessário novos estudos que tentem elucidar estas alterações.

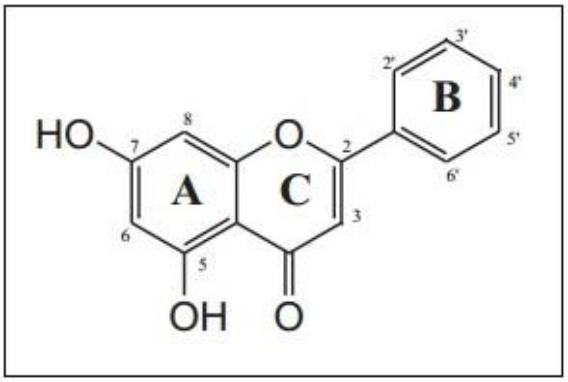
Para o estabelecimento da memória, é necessário que o cérebro passe por três fases principais de processamento, sendo elas: aquisição/aprendizagem; formação/consolidação; e evocação da informação (IZQUIERDO, 2002). Desta forma, a consolidação da memória acaba ativando cascatas bioquímicas que atuam em diversas moléculas de sinalização até a indução da síntese de novas proteínas (KANDEL *et al.*, 2014), como por exemplo: a ligação entre o neurotransmissor glutamato e seus receptores, que irá estimular o influxo intracelular de íons, ativando vias intracelulares que resultam na modulação da transcrição e síntese proteica (este processo será melhor elucidado na sessão 2.7).

Assim sendo, alterações de algumas vias cerebrais estão envolvidas no déficit de memória ocasionado pelo hipotireoidismo, provocando alterações morfológicas neuronais (SALA-ROCA *et al.*, 2008). Desta forma, tomamos como exemplo, os sistemas glutamatérgicos, colinérgico e neurotrofinérgico que são estudados nesta tese, estes podem estar envolvidos, uma vez que estes podem ser ativados ou inativados em algum momento das fases do processamento da memória.

Sabendo que, o tratamento do hipotireoidismo é a ingestão de comprimidos contendo T4, esta pode não ser totalmente eficaz na melhoria dos sintomas patológicos, uma vez que, nesta desordem endócrina, pode ocorrer também a diminuição dos receptores tireoidianos (TR, do inglês *thyroid receptors*). Desta forma, novas abordagens terapêuticas, como a associação de T4 com outros compostos pode tornar-se uma opção de tratamento.

Há diversos compostos naturais que estão sendo estudados a vários anos para fins terapêuticos, dentre eles estão os flavonoides que possuem atividades biológicas através de vias de sinalização em muitas patologias (NABAVI, 2015). Neste grande grupo se encontra a 5,7-Dihidroxiflavona (crisina) (Fig. 1).

Figura 1 - Estrutura química da crisina.



Fonte: Arquivo próprio (2021).

A crisina foi extraída pela primeira vez do maracujá do mato (*Passiflora caerulea*) e utilizada no estudo de Pearce *et al.*, (1984), sendo identificada nesse estudo sua atividade antialérgica. Em contrapartida, ficou conhecida principalmente por sua atividade antioxidante (PUSHPAVALLI *et al.*, 2010; CIFTCI *et al.*, 2012); pela inibição da enzima aromatase (KAO *et al.*, 1998) a qual atua no processo de conversão da testosterona, aumentando a massa muscular em praticantes de exercício físico - atualmente comercializada como suplemento alimentar, e pela atividade anti-inflamatória (AHAD *et al.*, 2014; FENG *et al.*, 2014; XIAO *et al.*, 2014; LI *et al.*, 2019). Também foi identificado o seu efeito neuroprotetor no déficit de memória em diferentes protocolos: Souza *et al.*, (2015) em animais idosos, possuindo ainda um efeito de agente anti-idade; Goes *et al.*, (2018) e Krishnamoorthy *et al.*, (2019) na doença de Parkinson; Sarkaki *et al.*, (2019) na isquemia cerebral; e Shooshtari *et al.* (2020) na hipoperfusão e reperfusão cerebral. Ainda, a crisina mostrou-se eficiente em atenuar o estado

tipo-depressivo induzido pelo hipotireoidismo, através da modulação das vias serotoninérgica e dopaminérgica, sem modificar os níveis de HTs, atuando, portanto, no sistema nervoso central (SNC) (BORTOLOTTO *et al.*, 2018).

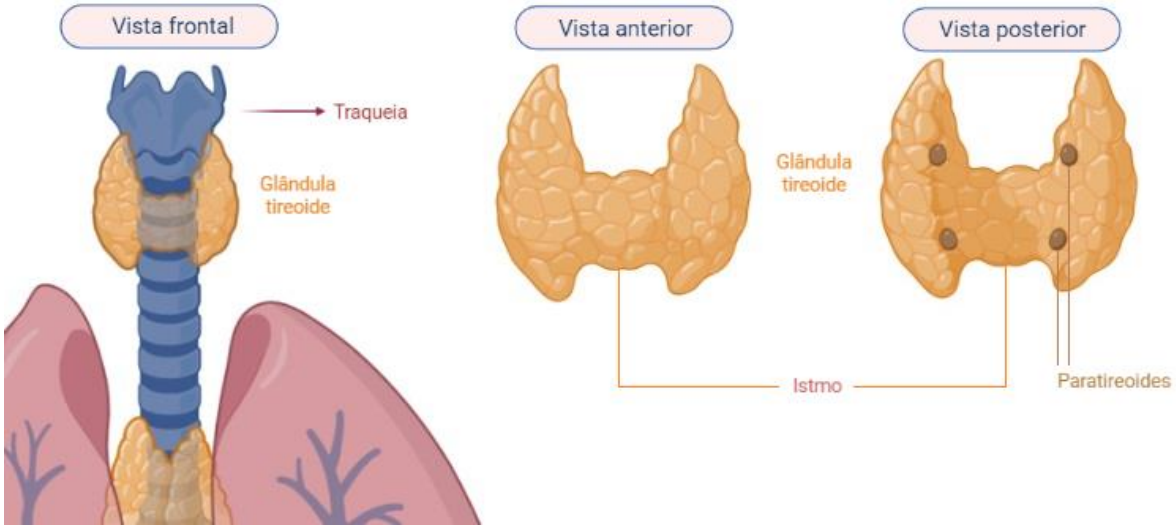
Com base no exposto, esta tese tem como objetivo investigar o possível efeito protetor do flavonoide crisina sobre o déficit de memória ocasionado pelo hipotireoidismo, e verificar o envolvimento dos sistemas glutamatérgico, colinérgico e neurotrofinérgico que podem estar modificados nos animais hipotireoideos.

2. REFERENCIAL TEÓRICO

2.1 Glândula tireoide

A glândula tireoide é responsável por numerosos processos em nosso organismo, como manutenção da homeastose, inclusive é uma das maiores glândulas presente no corpo. Considerando sua anatomia, podemos dizer que a tireoide possui o formato de "borboleta", situa-se aderida na parte anterior e lateral da laringe e da traqueia (Fig. 2), e contém dois lobos, sendo o esquerdo e o direito unidos por um istmo de parênquima glandular.

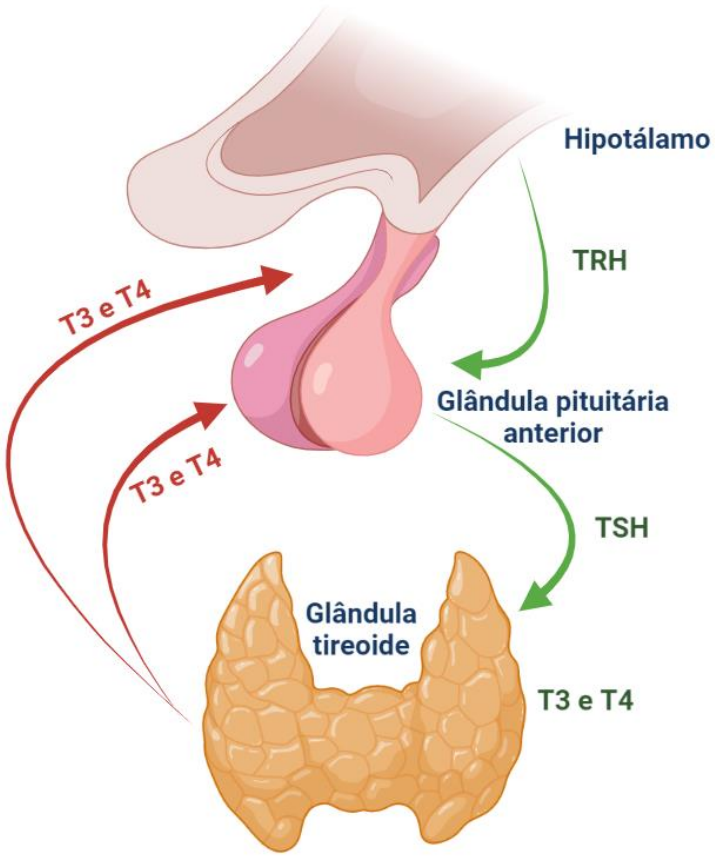
Figura 2 - Ilustração esquemática da anatomia da glândula tireoide.



Fonte: Arquivo próprio (2021).

A principal função da tireoide é sintetizar os hormônios T3 e T4, através da sinalização do hormônio liberador de tireotrofina (TRH – do inglês *Thyrotropin releasing hormone*) pelo hipotálamo para a glândula pituitária anterior que irá sintetizar a produção do hormônio estimulador da tireoide (TSH – do inglês *Thyroid stimulating hormone*). Este por sua vez, vai sinalizar à glândula tireoide para então produzir T3 e T4 (Fig. 3). Esta síntese pode igualmente acontecer por retroalimentação negativa dos hormônios tireoideos (BIANCO, 2002; CAMPBEL, 2008; STATHATOS, 2012).

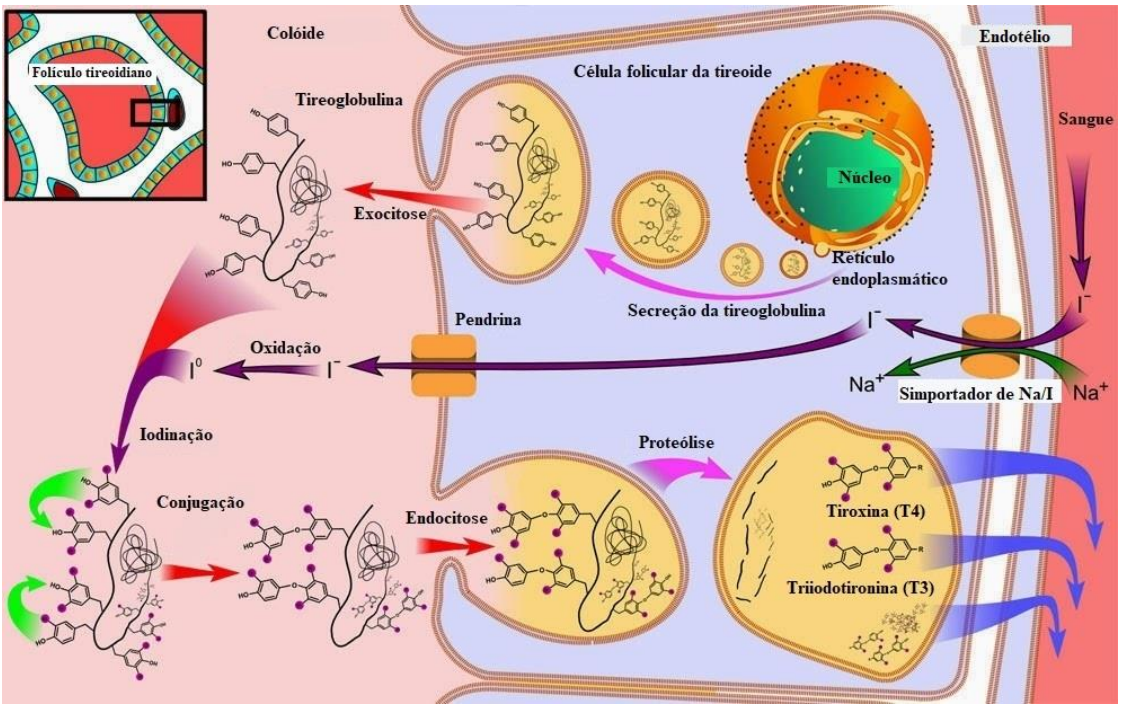
Figura 3 - Regulação da secreção dos hormônios da tireoide.



Fonte: Arquivo próprio (2021).

Os HT são sintetizados a partir do iodeto proveniente da dieta, e o iodo (I^-) que se encontra na tireoide, passa a ser oxidado com resíduos de tireoglobulina (moléculas de tirosina), que são sintetizados pelo retículo endoplasmático. As tireoglobulinas passam para o colóide a partir de exocitose, o I^- passa para o colóide pela proteína pendrina, sofrendo oxidação e tranformando-se em I^0. A junção destes (tireoglobulina e I^0) é chamada de iodinação, podendo esta, ser com um ou dois resíduos de I^0. Após se sucede a conjugação ou condensação, que é a ligação de duas moléculas tireoglobulina, podendo, ao final, ter três ou quatro I^0, para formar T3 e T4, respectivamente. Após esta etapa, acontece a endocitose a célula folicular, para posteriormente acontecer a proteólise que produz o T3 e T4 livres, que serão liberados na circulação sanguínea (Fig. 4).

Figura 4 - Biossíntese dos hormônios tireoidianos.



Fonte: Arquivo próprio (2017) adaptado de Boron (2003).

2.2 Disfunção da glândula tireoide

Os HTs são denominados como hormônios metabólicos do organismo, sendo o T4 mais abundante e conhecido por ser o precursor ou pró-hormônio do T3 que é a forma hormonal funcionalmente mais ativa (STOICA *et al.*, 2007). Esses hormônios são importantes no crescimento, desenvolvimento e homeostase do organismo (WHITE, 2010), ainda possuem efeitos sistêmicos e vias metabólicas, como regulação da temperatura, desenvolvimento, diferenciação e funcionamento de tecidos como o do SNC (NUNES, 2003). Quando existe uma descompensação nestes hormônios, seja por elevação ou diminuição nos níveis, o indivíduo pode ser acometido por hipertireoidismo (elevação dos níveis de T3 e T4), ou hipotireoidismo (diminuição nos níveis de T3 e T4).

Em síntese, no hipertireoidismo podemos citar a doença de Graves como a causa mais comum, que ocorre quando há uma doença autoimune, em que o sistema de defesa do corpo reage contra as próprias células, atacando a glândula tireoide, situação em sua maioria de ordem

genética. A glândula tireoide fica hiperexcitada, gera HT em excesso, e em consequênca, há uma aceleração do metabolismo corporal, entrando assim em um estado metabólico aumentado e com muitos sistemas corporais desenvolvendo função anormal. Em casos mais graves, envolve vários órgãos, como por exemplo o coração, que acarreta em batimentos cardíacos acelerados e irregulares, ocasionando em insuficiência cardíaca congestiva; e também há o envolvimento do sistema ósseo, podendo causar osteoporose (ATA, 2013).

O hipotireoidismo é uma doença amplamente identificada por seus efeitos sobre os diferentes sistemas orgânicos, levando a manifestações clínicas sob vários aspectos como: bradicardia, intolerancia ao frio, efeitos neurológicos, como depressão e déficit cognitivo, que leva ao hipometabolismo do organismo (ZHU *et al.*, 2006; FARWELL & BRAVERMAN, 2006).

2.3 Hipotireoidismo

O hipotireoidismo é a alteração mais frequente da tireoide, sua prevalência em mulheres ocorre em torno de 10%, tendendo a aumentar no período da menopausa (ficando em torno de 12% a 15% nesta fase), já em homens a prevalência é menos frequente, é em torno de 3%. Em 2008, a incidência variava de 0,3% a 4,6% da população geral, sendo de cinco a oito vezes mais prevalente em mulheres que em homens (SOARES & CASTRO, 2008). Com o aparecimento do hipotireoidismo, o metabolismo desacelera, o que leva a uma produção reduzida de HT necessários para o organismo, causando uma variedade de sintomas (Tabela 1).

Tabela 1 - Sintomas mais frequentes do hipotireoidismo

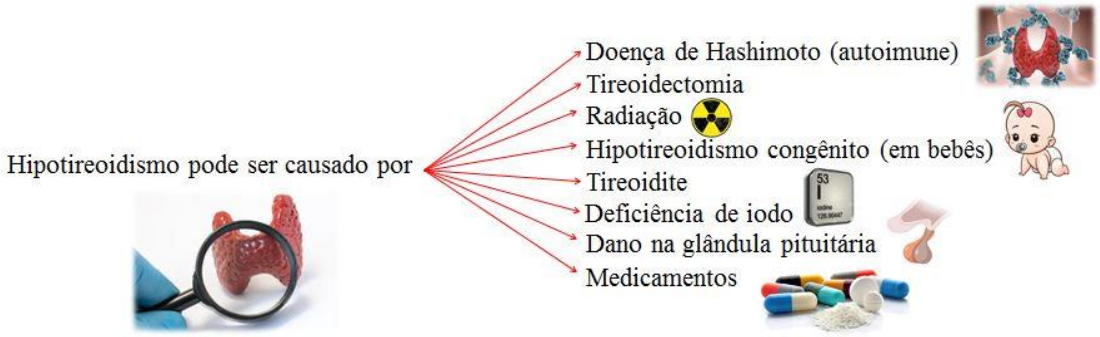
Pele seca e ressecada	Baixa energia
Cabelo seco, quebradiço, e maior queda	Diminuição do suor
Constipação	Perda de apetite
Sensibilidade ao frio	Ganho de peso leve ±5 kg
Cansaço excessivo	Dificuldade em perder peso
Síndrome do túnel do carpo	Dores musculares e nas articulações
Irritação	Parestesia
Desaceleração da frequência cardíaca	Acumulação de líquido corporal
Pressão sanguínea aumentada	Aumento dos níveis de colesterol
Esquecimento	Crescimento retardado em crianças
Depressão	Bócio
Ansiedade	Rouquidão vocal

Fonte: Arquivo próprio (2021) adaptado de ATA (2013).

O hipotireoidismo ocorre em um contexto no qual há uma insuficiencia da glândula tireoide, quando esta não produz quantidade suficiente de HT. Há diversas causas para que haja o desenvolvimento do hipotireoidismo, dentre estas, estão a doença de Hashimoto (autoimune), remoção cirúrgica da tireoide (tireoidectomia), tratamento com radiação, hipotireoidismo congênito, tireoidite, quantidade diminuida de iodo, dano na glândula pituitária e vias medicamentosas.

Na doença autoimune, conhecida como doença de Hashimoto, as células do corpo atacam os próprios órgãos, fazendo com que a glândula tireoide acabe inflamando e sofrendo danos nas próprias células, interferindo na síntese dos HT. Esta doença se desenvolve lentamente ao longo dos anos, podendo iniciar em qualquer período da vida, com a incidência tendendo a aumentar conforme a idade, e inclusive pode ocorrer na gestação, após o parto ou na menopausa (ATA, 2013) (Fig. 5).

Figura 5 – Causas do hipotireoidismo.



Fonte: Arquivo próprio (2021).

A tireoidectomia, que é a remoção cirúrgica de toda glândula tireoide ou ainda quando o tecido da tireoide remanescente não funciona mais corretamente, acaba resultando em hipotireoidismo. Este procedimento é realizado quando há nódulos ou câncer na tireoide, e na doença de Graves (ATA, 2013) (Fig. 5).

Há doenças que são tratadas com radiação, como a doença de Hodgkin, linfoma ou câncer de cabeça ou pescoço e, outra opção para doenças que são tratadas com radiação, existe o iodo radioativo, que pode ser usado no tratamento na doença de Graves, bócio nodular e câncer na tireoide. Estes tipos de radiação destroem permanentemente a glandula tireoide, ocasionando o seu não funcionamento, diminuindo a síntese dos HT e consequentemente causando o hipotireoidismo (ATA, 2013) (Fig. 5).

O hipotireoidismo congênito acontece quando os bebês nascem sem ou com a tireoide parcialmente formada, devido a tireoide ectópica, ou por medicamentos que a mãe ingere durante a gestação, a prevalência é de 1 em 4000 bebês (ATA, 2013) (Fig. 5).

Ainda, entre os tipos de hipotireoidismo, a tireoidite é a inflamação na glândula tireoide, ela acontece geralmente por uma infecção viral ou um ataque autoimune (como doença de Hashimoto, tireoidite pós-parto ou tireoidite silenciosa). Se viral, o funcionamento da tireoide pode retornar ao normal, entretanto se autoimune, as estimativas apontam que uma entre quatro pessoas continuam com o hipotireoidismo de forma permanente. O hipotireoidismo nestes casos ocorre devido a tireoidite liberar todos os hormônios armazenados ao mesmo tempo,

ocorrendo um hipertireoidismo passageiro, e após este período a tireoide se torna subativa (ATA, 2013) (Fig. 5).

O hipotireoidismo causado pela deficiência de iodo, ocorre, pois, para a síntese dos hormônios tireoidianos é necessário ter iodo disponível no sangue, e este é provindo de alimentos, como frutos do mar, produtos lácteos, etc. A deficiência deste mineral é a causa mais comum do hipotireoidismo, porém o excesso, geralmente por suplementos dietéticos de algas marinhas, pode piorar ou causar o hipotireoidismo, assim com o excesso de iodo a glândula diminui a produção de HT, em consequência aumenta seu tamanho, formando bócio (ATA, 2013) (Fig. 5).

Ainda, considerando que a glândula pituitária possui um papel essencial para síntese dos hormônios tireoidianos, pois é ela que sinaliza à tireoide a produção de T3 e T4 (Fig. 3 e 4), uma lesão, tumor, radiação ou cirurgia, pode acarretar em hipotireoidismo no organismo (ATA, 2013) (Fig. 5).

Por fim, alguns medicamentos podem interferir na capacidade da glândula tireoide de sintetizar os hormônios, levando ao hipotireoidismo, como as tionamidas, e os inibidores iônicos que interferem na concentração de iodo pela tireoide (Fig. 5).

2.4 Drogas que interferem na função da glândula tireoide

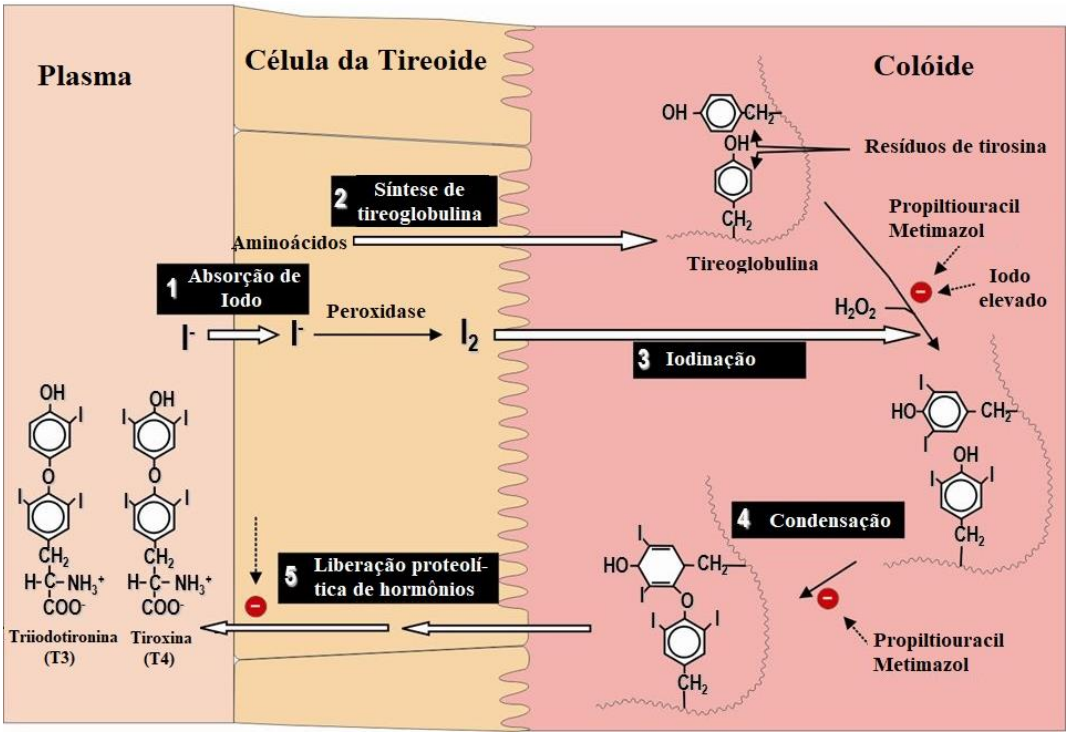
Existem drogas que interferem no funcionamento da glândula tireoide e em sua capacidade de produzir T3 e T4. Estas são ânions hidratados, monovalentes e com tamanho similar ao iodo, por exemplo o fluoborato (BF_4) e perclorato (ClO_4^-), que são classificados como inibidores iônicos, sendo este último bastante empregado como “descarga” para iodo inorgânico no teste diagnóstico de organificação (LAZARUS, 1998; KIBIRIGE *et al.*, 2013). Outros íons, como o lítio, inibem a liberação dos hormônios tireoideos (KIBIRIGE *et al.*, 2013).

Clinicamente, as drogas antitireoidianas mais usadas são as tioureilenos no tratamento do hipertireoidismo: 6-propil-2tiouracil (propiltiouracil - PTU) e 1-metil-2-mercaptoimidazol (metimazol - MTZ). Elas são utilizadas principalmente na doença de Graves, uma vez que ambas interferem na incorporação de iodo nos resíduos tirosil e também impedem a formação

das iodotironinas, ou seja, inibem a síntese de hormônios tireoidianos por bloquearem a atividade da tireoperoxidase (TAUROG, 2000).

Há evidências de que estes fármacos inibem a iodetação dos resíduos de tirosil na tireoglobulina, sendo assim, eles cessam as reações de oxidação catalisadas pela tireoperoxidase por atuarem como substratos para o suposto complexo iodo-peroxidase, inibindo competitivamente a interação com a tirosina (RANG *et al.*, 2012), ou seja, para que a reação de acoplamento seja mais sensível a drogas tireoideas do que a reação de iodação (TAUROG, 2000) (Fig. 6).

Figura 6 – Atuação de tioureilenos (MTZ e PTU) na glândula tireoide.



Fonte: Arquivo próprio (2017) adaptado de Harvey *et al.* (2006).

Em relação as ações dos tioureilenos, eles podem agir em dois locais na glândula tireoide, ambos na síntese dos hormônios. Podem atuar na condensação dos resíduos de tireoglobulina ligados aos I^0, como demonstrado no item 4 (Fig. 6), ou inibem a enzima tireoperoxidase como demonstrado no item 3 (Fig. 6), inibindo assim a ligação de iodo aos resíduos de tirosina, sendo esta a maior ação dos tioureilenos, principalmente do MTZ.

2.5 Hipotireoidismo e distúrbios neuropsiquiátricos

Como a glândula tireoide é essencial para o funcionamento normal de todo organismo, já tem sido evidenciado uma associação entre o hipotireoidismo e distúrbios neuropsiquiátricos, principalmente depressão, ansiedade, déficit da memória e dificuldade em aprendizagem (BORTOLOTTO *et al.*,2018; VAN BOXTEL *et al.*, 2004; GUIMARÃES *et al.*, 2009).

Esta associação também é demonstrada em tratamentos com animais de experimentação, por indução do hipotireoidismo, por exemplo: com drogas antitireoideas; tireoidectomia, indução de hipotireoidismo congênito, entre outras (WILCOXON *et al.*, 2007). Há mecanismos que tentam explicar esta associação, tais como: alterações morfológicas nos neurônios do hipocampo nas regiões CA1 e CA3 (SALA-ROCA *et al.*, 2008) e modulação de rotas apoptóticas (SINHA *et al.*, 2009; ZHANG *et al.*, 2009). Levando em consideração estas alterações mencionadas, é essencial o estudo por compostos a fim de diminuí-las, e conseguir identificar as vias afetadas e os possíveis mecanismos de ação responsáveis pelo déficit de memória.

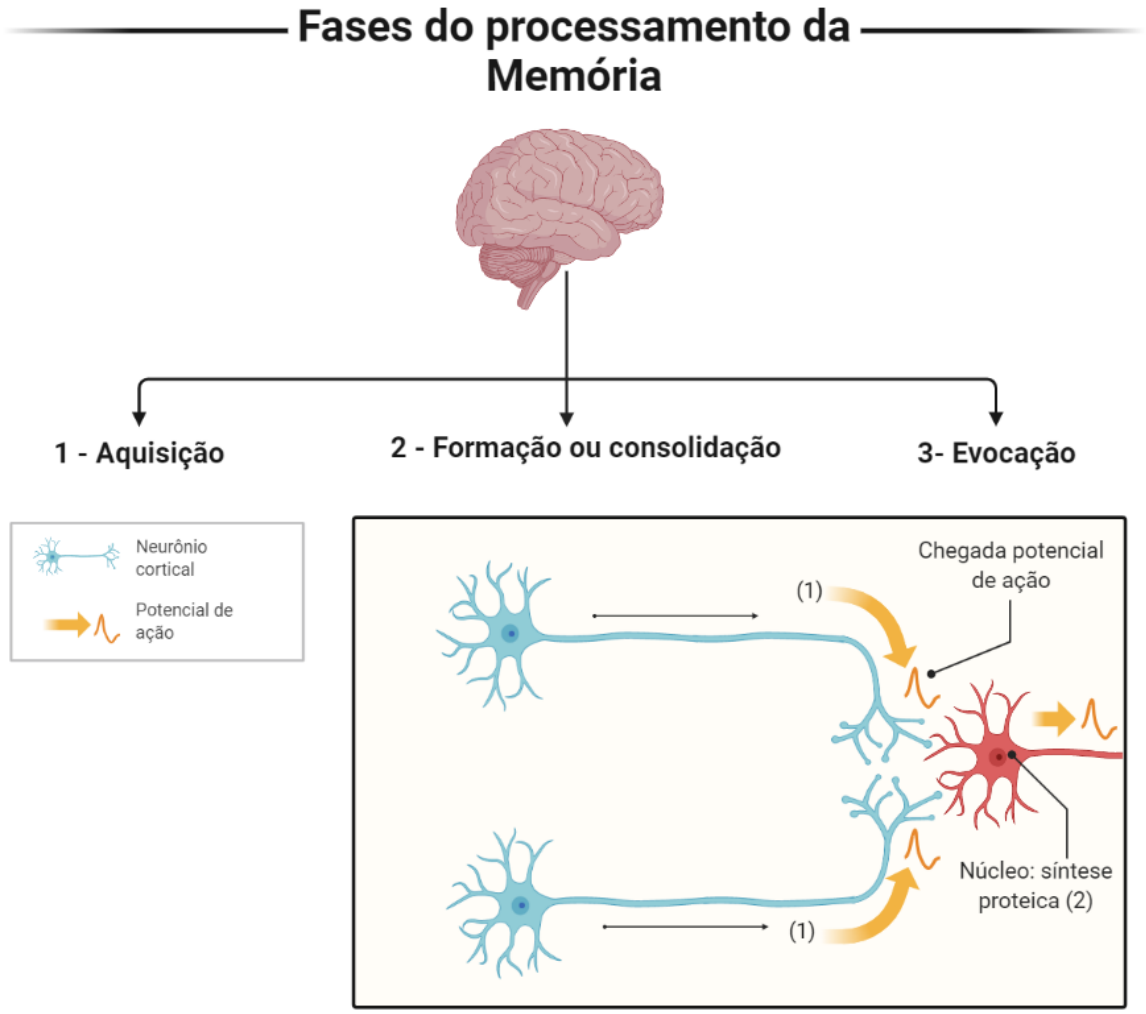
2.6 Memória

O aprendizado deriva do armazenamento da informação como efeito da prática, da experiência, da introspecção, resultando em uma alteração relativamente permanente do comportamento real ou potencial. A informação originada pelo aprendizado é chamada de memória (CAMMAROTA *et al.*, 2007; IZQUIERDO & MCGAUGH, 2000; IZQUIERDO & MEDINA, 1997; KANDEL & SQUIRE, 2000). Alguns estudiosos da neurociência vêem a consolidação celular como um processo que dura poucas horas no qual as memórias são transformadas de um estado mais lábil para um estado mais estável (BLISS & COLLINGRIDGE, 1993; MALENKA & NICOLL, 1999; KANDEL E SQUIRE, 2000; IZQUIERDO & MCGAUCH, 2000; IZQUIERDO & MEDINA, 1997; IZQUIERDO *et al.*, 2006).

A memória é resultante de três tipos/fases de processamentos (Fig. 7), no qual um deve ser executado com êxito para o outro funcionar, a aquisição, a formação, e a evocação de

informações. A aquisição é o processo de aprendizado – *só se "grava" aquilo que foi aprendido* - ou seja, processo no qual as novas informações são tratadas e processadas pela primeira vez. A formação, também chamada de consolidação, é o processo de armazenamento e fixação deste evento aprendido recentemente – *assim o que foi aprendido é "gravado"*. Já a evocação da memória, significa recordar, lembrar do que foi aprendido anteriormente – *só se "lembra" o que foi "gravado" e "aprendido"* (IZQUIERDO, 2002). É preciso considerar que, essas três fases de processamento dependem da ativação de redes complexas de neurônios, no qual um sinal elétrico chega ao seu destino final através de conexões neuronais, ocorrendo uma série de processos bioquímicos, como a síntese proteica, formando assim a memória.

Figura 7 – Fases do processamento da memória.



Fonte: Arquivo próprio (2021).

Para ocorrer o processamento de informações é necessário um circuito neuronal complexo (CAMATS PERNA & ENGELMANN, 2017), que inclui a ativação das estruturas cerebrais, como por exemplo, do córtex pré-frontal e do hipocampo (TANIMIZU *et al.*, 2017). As estruturas cerebrais são excelentes "discos rígidos", que conseguem adquirir e armazenar as memórias declarativas, mas o hipocampo é a estrutura central da formação das memórias, sendo este o centro da plasticidade sináptica, e a sua atividade é amplamente auxiliada por outras áreas como amígdala e o córtex entorrinal (IZQUIERDO *et al.*, 2013).

As memórias podem ser divididas e classificadas de acordo com o seu conteúdo, duração ou natureza do arquivo.

Referente ao conteúdo da memória, elas podem ser classificadas como declarativas e/ou explícitas ou não declarativas e/ou implícitas.

- Declarativas ou explícitas: descritas por meio de palavras em humanos, sendo estas subdivididas em episódica, abrangindo memória temporal (ex.: autobiografia), e em memória declarativa semântica, envolvendo conceitos atemporais (ex.: conhecimentos, cultura) (SQUIRE,1992; BONINI, 2006).
- Não declarativas ou implícitas: memórias que não podem ser descritas, sendo subdividida em: declarativa de representação perceptual, envolvida com representações conhecidas (ex.: imagens e sons) (IZQUIERDO, 2002); memória de procedimento ou hábitos, habilidade e regras (ex.: andar de bicicleta) (IZQUIERDO *et al.*, 2013); e memória não declarativa associativa que lida com dois ou mais estímulos, ou de um estímulo a uma única resposta (ex.: condicionamento pavloviano, condicionamento operante) (IZQUIERDO & MCGAUGH, 2000; BONINI, 2006; SQUIRE, 1992).

Referente à duração, as memórias podem ser classificadas como de curta ou longa duração.

- Memórias de curta duração *(Short-Term Memory (STM)*): esta memória é adquirida em poucos segundos ou minutos e dura por minutos ou horas, enquanto ocorre a consolidação da memória de longa duração (IZQUIERDO *et al.*, 1998a; IZQUIERDO *et al.*, 1998b; IZQUIERDO *et al.*, 1998c; MCGAUGH, 1966; WOLFMAN *et al.*, 1994; BONINI, 2006).

- Memórias de longa duração *(Long-Term Memory (LTM))*: esta memória é usada para designar lembranças que duram ao menos 24 horas, sendo durável por horas, dias ou anos (IZQUIERDO *et al.*, 1998a; IZQUIERDO & MEDINA, 1995; MCGAUGH, 1966; IZQUIERDO *et al.*, 1998d; WOLFMAN *et al.*, 1994; IZQUIERDO *et al.*, 1999). Esta duração pode ser devido ao seu mecanismo de consolidação, como por exemplo expressão gênica e síntese proteica, levando a neuroplasticidade (GULYAEVA, 2017). As LTM ainda podem ser subdivididas em associativas e não associativas, dependendo dos mecanismos utilizados para sua aquisição. As associativas dependem de uma ligação entre um evento específico e um estímulo para sua formação, já as não associativas são formadas quando há exposições contínuas ou repetidas a novos estímulos (VIANNA *et al.*, 2000; IZQUIERDO & MEDINA, 1997; ZHU *et al.*, 1997; THIEL *et al.*, 1998; EICHENBAUM, 1999; MCGAUGH, 2000).

Referente à natureza de arquivo, as memórias podem ser: STM, LTM e transitórias.

- STM e LTM, já descritas anteriormente.
- Transitórias: memória imediata ou de trabalho (do inglês *working memory)*. Esta memória imediata ou operacional dura de segundos a alguns minutos (GOLD & MCGAUGH, 1975; MARKOWITSCH, 1997; IZQUIERDO *et al.*, 1999). Serve para gerenciar a realidade, correspondendo ao processamento contínuo das informações adquiridas recentemente, permitindo o raciocínio e o planejamento do comportamento, dependente do córtex pré-frontal. Porém, esta memória não armazena arquivos, ou seja, não forma a memória e não há como evocá-la futuramente (BONINI, 2006; IZQUIERDO, 2002).

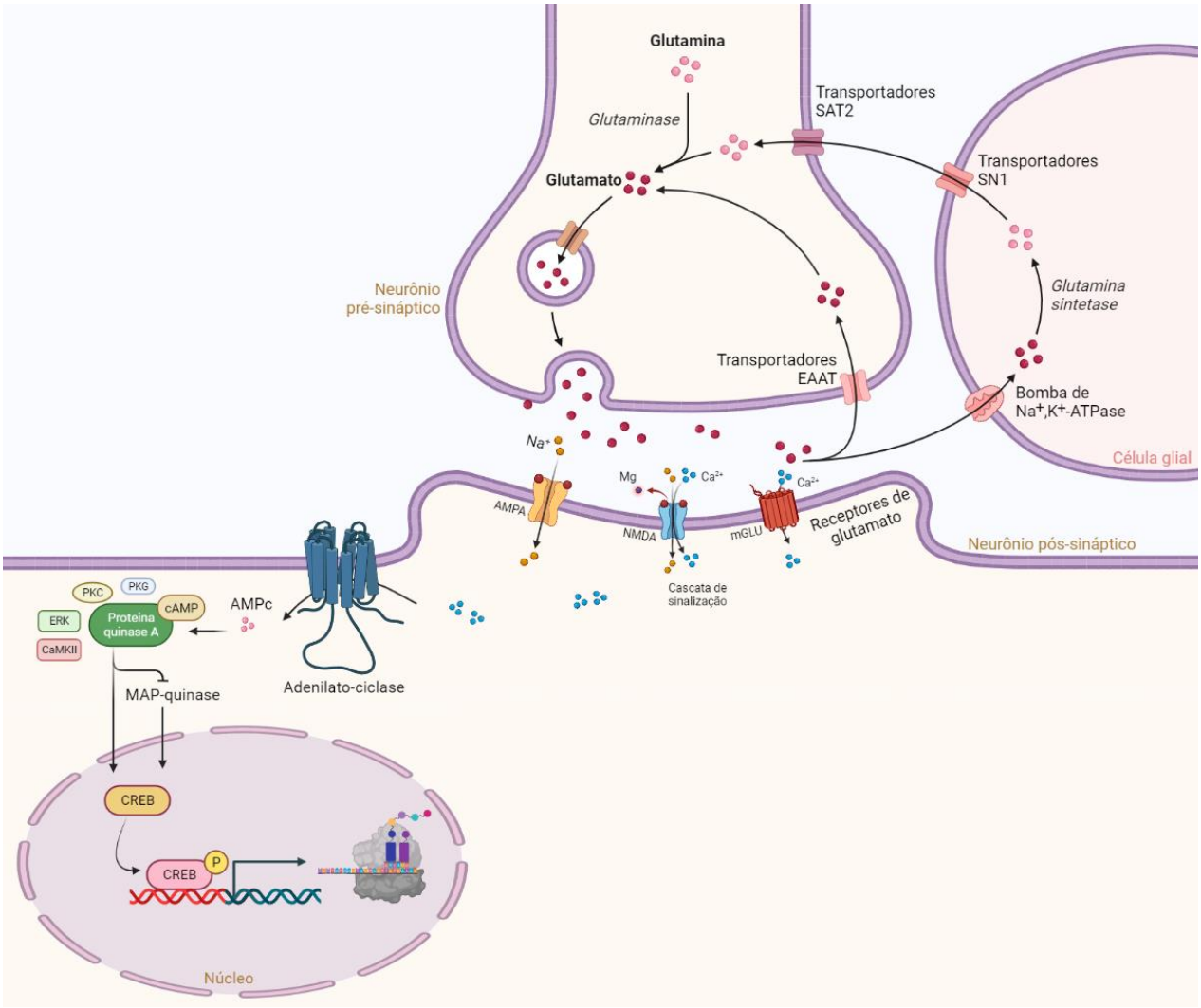
2.7 Vias relacionadas à memória

Sabe-se que a aquisição, formação e evocação da memória não são processos isolados, assim, nesta presente tese, demonstraremos algumas partes centrais de vias relacionadas com a memória, dentre elas estão as vias glutamatérgica, colinérgica e neurotrofinérgica.

O sistema glutamatérgico produz o principal neurotransmissor excitatório do SNC, o glutamato, o qual, está diretamente envolvido nos processos de plasticidade sináptica e em funções neuronais como a aprendizagem e memória (WILLARD & KOOCHEKPOUR, 2013; DANBOLT, 2001). Porém, o conteúdo elevado de glutamato, com aumento dos níveis de Ca^{2+}, é tóxico para a rede neural, ou seja, a ativação exacerbada dos receptores glutamatérgicos pode ocasionar a morte neuronal (BLEICH *et al.*, 2003; PURVES *et al.*, 2001). O glutamato possui dois tipos de receptores, os ionotrópicos (iGluR): N-metil-D-aspartato (NMDA), α-amino-3-hidroxi-5-metil-4-isoxazolpropionico (AMPA) e ácido caínico (KA); e os metabotrópicos (mGLUR): receptores acoplados às proteínas ligantes de nucleotídeos da guanina (proteínas G). A ativação dos receptores iGluR produz respostas pós-sinápticas excitatórias, principalmente pelo AMPA, ainda medeiam a neurotransmissão excitatória rápida e são canais com grande permeabilidade de Na^{+} e K^{+}, e com baixa permeabilidade de Ca^{2+}; os receptores NMDA possuem um papel essencial no desenvolvimento da plasticidade sináptica (Fig. 8) (BLEICH *et al.*, 2003; PURVES *et al.*, 2001).

Logo, o sistema glutamatérgico possui uma importância vital para a plasticidade sináptica, já que a potenciação à longo prazo (LTP, do inglês *Long-Term Potentiation*) – em suma, é o fortalecimento sináptico de longa duração (COBAR *et al.*, 2017) - depende da interação do neurotransmissor glutamato com o receptor NMDA para que se estabeleça um fortalecimento sináptico duradouro (LOMO, 2003). As teorias do aprendizado que melhor explicam a representação e armazenamento da memória no cérebro são os mecanismos celulares e moleculares envolvidos na LTP (BLISS & COLLINGRIDGE, 1993).

Figura 8 – Mecanismos moleculares para consolidação da memória.



Fonte: Arquivo próprio (2021) adaptado de Kandel *et al.* (2014).

A figura 8, demonstra alguns mecanismos envolvidos com a consolidação da memória, iniciando pela formação e liberação do glutamato na fenda sináptica, em que este se ligará a algum de seus receptores. Quando houver a ligação entre glutamato e AMPA, haverá concomitantemente a entrada de Na^+ no citoplasma do neurônio pós sináptico. Se a ligação for com o receptor NMDA, permitirá a saída de Mg e entrada de Ca^{2+}. A ligação com mGLU se dará pela entrada de Ca^{2+} na célula neuronal, aumentando a concentração deste íon intracelularmente. Este influxo de Ca^{2+}, ativa a adenilato-ciclase, que gera monofosfato cíclico de adenosina (AMPc, do inglês c*yclic adenosine monophosphate*), estimulando proteínas quinases, como a proteína quinase A (PKA, do inglês *protein kinase A*) que irá ativar a MAP-quinase. Esta por sua vez será translocada ao núcleo para fosforilar a proteína de ligação

responsiva ao AMPc (CREB, do inglês *cAMP responsive-element binding protein*) e pós-fosforilação o CREB irá ativar fatores de transcrição, induzindo a síntese proteica.

Vale ressaltar que, além de sintetizar novas proteínas, este mecanismo molecular pode levar ao crescimento de novas conexões sinápticas, podendo ainda, ativar RNA mensageiro (mRNA) nos dendritos, ocorrendo outras cascatas de sinalização em que sucederá ao aumento do número de receptores AMPA de longa duração na membrana pós sináptica e também ao aumento da liberação de glutamato (KANDEL *et al.*, 2014).

Adicionalmente, a concentração de proteínas como a quinase dependente de Ca^{2+}/calmodulina (CaMKII, do inglês *Ca^{2+}/calmodulin-dependent protein kinase*) e a quinase C (PKC, do inglês *protein kinase C*) são alteradas pelo hipotireoidismo (WANG *et al.*, 2014a), e estas estão envolvidas com a consolidação da memória (Fig. 8).

O sistema colinérgico, especificadamente o neurotransmissor acetilcolina (ACh), possui a função de neuromodulador no SNC, possuindo a capacidade de alterar a resposta de neurônios que atuam a mudanças das condições do meio ambiente, assim, promove o rearranjo das redes neuronais, contribuindo para a plasticidade sináptica (PICCIOTTO *et al.*, 2012; ITO & SCHUMAN, 2008). A ACh tem ação sobre dois tipos de receptores: muscarínicos que são metabotrópicos (ativados pela muscarina) e os nicotínicos que são ionotrópicos (ativados pela nicotina), funcionando como canal iônico de Na^+ e K^+ (HARVEY & SHAHID, 2012; JONES *et al.*, 2012; SADAVA *et al.*, 2011).

Desta forma, a enzima acetilcolinesterase (AChE), regula os níveis de ACh na fenda sináptica. A AChE atua na hidrólise a ACh em colina e acetato/ácido acético (MANDAL *et al.*, 2016), e tem sido estudada como estratégia terapêutica para o tratamento de distúrbios cognitivos, como a DA, por participar das fases de processamento da memória (ROBINSON *et al.*, 2011). Neste sentido, um grupo de pesquisa tem estudado os efeitos dos tratamentos de T4 em associação com a donepezila (um tratamento para DA, inibidor da AChE – aumentando a concentração de ACh na fenda sináptica) em animais com hipotireoidismo (WANG *et al.*, 2014a; 2014b; YANG *et al.*, 2015; WANG *et al.*, 2017). Seus resultados demonstram a melhora de marcadores neuronais do sistema colinérgico, nas expressões gênica e proteica e, visualização neuronal através da atuação da associação dos tratamentos de T4 e donepezila nas estruturas encefálicas de hipocampo (WANG *et al.*, 2014a; 214b; YANG *et al.*, 2015) e córtex pré-frontal (WANG *et al.*, 2017).

Contudo, segundo o bulário da Agência Nacional de Vigilância Sanitária (ANVISA) em 2021, a donepezila possui diversos efeitos adversos, desde os mais comuns (69% dos pacientes testados) até os efeitos colaterais no SNC (20% dos pacientes), sendo essencial a busca por novos compostos com menos efeitos adversos, que possuam efeitos na modulação da consolidação da memória.

Como nos sistemas glutamatérgicos e colinérgicos, a plasticidade neuronal possui função essencial na aquisição da memória, uma vez que este está relacionado com a flexibilidade do SNC, já que o cérebro sofre mudanças como resultados de experiências (DUFFAU, 2006). Assim, a plasticidade sináptica é um processo contínuo que permite o remodelamento do mapa sináptico a curto, médio e longo prazo, para aperfeiçoar o funcionamento das redes cerebrais e elaboração de novos circuitos (DUFFAU, 2006).

Na neuroplasticidade, possuímos uma família de neurotrofinas, que são cruciais para a formação e manutenção à longo prazo de conexões neurais no cérebro (MCPHEE *et al.*, 2020). São elas: fator neurotrófico derivado do cérebro (BDNF, do inglês *brain-derived neurotrophic factor*); fator de crescimento do nervo (NGF, do inglês *nerve growth factor*); fator neurotrófico ciliar (CNTF, do inglês *ciliary neurotrophic factor*); fator de transformação de crescimento (TGF, do inglês *transforming growth factor*); fator neurotrófico derivado da linha celular glial (GDNF, do inglês *glial cell line-derived neurotrophic factor*); neurotrofina 3 (NT3) e neurotrofina 4 (NT4) (MCPHEE *et al.*, 2020).

A principal função das neurotrofinas, é atuar na sinaptogênese, corroborando na propagação e fortalecimento das sinapses (HUANG & REICHARDT, 2001; POO, 2001), sendo cruciais para aprendizagem e LTP (MCPHEE *et al.*, 2020). Para isso, é necessário que ocorra a modulação do sistema neurotrofinérgico, este acontece a partir da comunicação neuro-glial, em que a plasticidade tanto comportamental quanto neural - aspectos importantes cerebrais -, atuam para promoção do aprendizado, formação e consolidação da memória e LTP hipocampal (YIRMIYA & GOSHEN, 2011).

Atualmente, há um predomínio de estudos com o BDNF, sabe-se que este está envolvido nos mecanismos responsáveis pela indução e manutenção da LTP e o proBDNF facilita a depressão de longo prazo (LTD, do inglês *Long-Term Depression*) hipocampal (NUMAKAWA *et al.*, 2010), além disso está implicado na formação de sinapses e no crescimento da espinha dendrítica no hipocampo (BAMJI *et al.*, 2016). Em conjunto com as demais neurotrofinas,

possuem enorme importância para estudos em modelos de déficit de memória, levando em consideração que a neuroplasticidade sináptica atua na consolidação da memória.

2.8 Hipotireoidismo e o déficit de memória

Está bem descrito na literatura que animais com hipotireoidismo apresentam déficits de aprendizagem e memória, e sabe-se que os hormônios da tireoide são essenciais para o desenvolvimento normal do hipocampo, que é crítico para a memória e particularmente para aprender e recordar associações entre estímulos visuais e verbais (REID *et al.*, 2007; TONG *et al.*, 2007; WILCOXON *et al.*, 2007; REIS-LUNARDELLI, 2007; DIAS *et al.*, 2012; WHEELER *et al.*, 2015).

Ainda é importante salientar que 36% das pessoas que possuem a DA demonstram ter anormalidade nos níveis dos HT (ACCORRONI *et al.*, 2017), e também uma diminuição dos receptores dos HT (SAMPAOLO *et al.*, 2005), levando em consideração que os pacientes com hipotireoidismo apresentam comportamentos similares aos pacientes com DA, podemos observar além da diminuição de aprendizagem e comprometimento de guardar memórias, o desenvolvimento de psicose, alucinações e confusão mental (ACCORRONI *et al.*, 2017).

Considerando que o hipotireoidismo acarreta esses déficits de aprendizagem, podendo ser um sintoma precoce que demonstra predisposição do paciente à DA, é necessário a busca por estudos que expliquem como essa deficiência ocorre. Sabe-se que estes déficits estão envolvidos com a perda/diminuição da função dos HT ou TR no SNC, podendo acarretar em redução significativa da LTP e facilitação de pulso emparelhado (PPF, do inglês *Paired-Pulse Facilitation*), ambos são mecanismos ligados a formação da memória e aprendizado (SUI *et al.*, 2006; ZHU *et al.*, 2006), atua também na diminuição da plasticidade neuronal (SALAZAR *et al.*, 2017; BAGHCHEGHI *et al.*, 2018; CHAALAL *et al.*, 2018), e diminuição da neurogênese (YIRMIYA & GOSHEN, 2011), em alterações nos níveis de neurotransmissores colinérgicos (HUSAIN *et al.*, 2018), na ação da $Na,^{+}K^{+}$-ATPase (CARAGEORGIOU *et al.*, 2007), desempenhando o funcionamento nos fatores relacionados a inflamação (ALZOUBI & ALKADHI, 2014) e aumentando o estresse oxidativo (PAN *et al.*, 2013), levando a prejuízos

na aprendizagem, formação e consolidação da memória e neuroplasticidade (YIRMIYA & GOSHEN, 2011).

2.9 Flavonoides

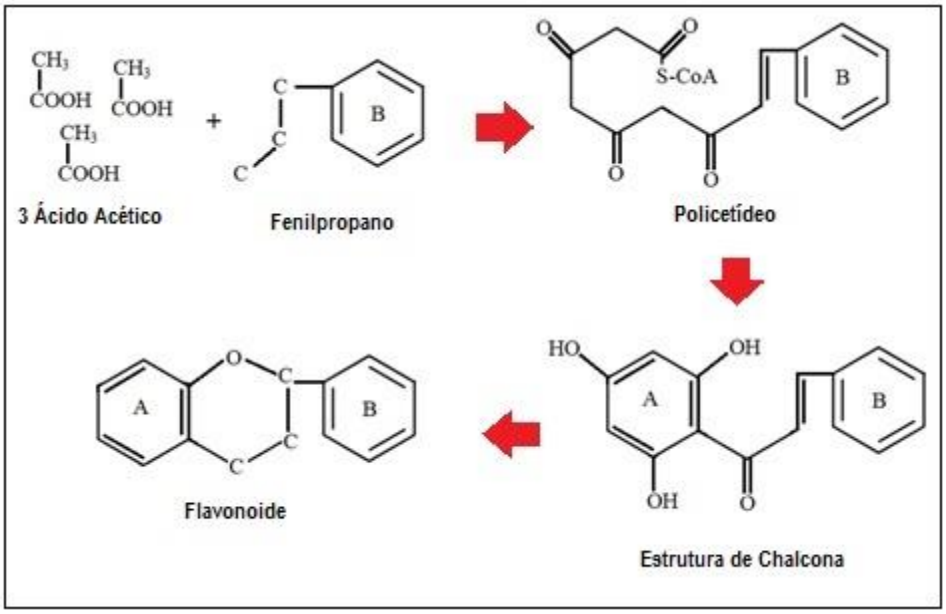
Os flavonoides (ou bioflavonoides) são metabólitos secundários das plantas, encontrados em diversos alimentos, como frutas e hortaliças e também em algumas bebidas. Possuem atividades biológicas para a saúde humana, e várias vias de sinalização envolvidos em muitas doenças (NABAVI, 2015).

Os flavonoides podem ser encontrados em duas configurações: ligados a carboidratos (forma glicosilada) e de formas livres (forma agliconada – como a maioria dos flavonoides). Estes possuem propriedades químicas e podem ser considerados como substâncias anfipáticas. A absorção da forma glicosilada se sucede após a hidrólise da molécula no trato digestivo, transformando-se assim na forma agliconada (livre), esta por sua vez inclui reações de glucuronidação e sulfatação, que podem ser seguidas de efluxo, culminando na eliminação via sistema excretor e dióxido de carbono (mediada por bactérias do trato intestinal), ou ainda absorção e distribuição para os tecidos via sistema circulatório (YAO *et al.*, 2004).

Os flavonoides são capazes de modular a atividade de enzimas e afetam o comportamento de muitos sistemas celulares, possuindo atividade antihepatotóxica, antialérgica, anti-inflamatória e até mesmo antitumoral (DI CARLO *et al.*, 1999).

A biossíntese dos flavonoides (Fig. 9) inicia-se através de duas vias: ácido chiquímico (sintetiza fenilpropano, derivado de ácido cinâmico) e acil polimeanato. O fenilpropano atua como ponto de partida para que, com a adição de 3 moléculas de ácido acético, forma-se policetídeo. Através de hidroxilações e reduções, há o fechamento do anel aromático, para que, após formar Chalcona, tenha-se a estrutura do flavonoide.

Figura 9 - Biossíntese dos flavonoides.

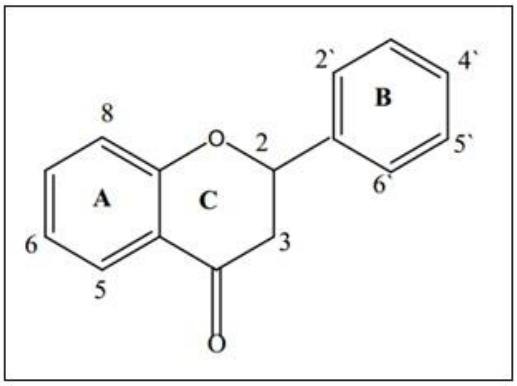


Fonte: Arquivo próprio (2021) adaptado de Di Carlo *et al.* (1999).

Os flavonoides são subdivididos em classes, sendo as principais: flavonas, flavonóis, chalconas, auronas, flavanonas, flavanas, antocianidinas, leucoantocianidinas, proantocianidinas, isoflavonas e neoflavonoides (BRAVO, 1998).

Sua estrutura básica consiste em um núcleo fundamental, constituído de quinze átomos de carbono arranjados em três anéis (C6-C3-C6), sendo dois anéis fenólicos substituídos (A e B) e um pirano (cadeia heterocíclica C) acoplado ao anel A (Fig. 10) (DI CARLO *et al.*, 1999).

Figura 10 - Estrutura básica dos flavonoides.



Fonte: Arquivo próprio (2021) adaptado de Dornas *et al.* (2007).

2.9.1 Crisina

Dentre os flavonoides encontrados na natureza, temos a 5,7-dihidroxi-2-fenil-4H-cromeno-4-ona ou 5,7-dihidroxiflavona, popularmente conhecida como crisina, que é um derivado da flavona hidroxilada, e um flavonoide agliconado, encontrada principalmente no maracujá do mato (*Passiflora caerulea* L.), própolis, mel de abelha, e nas espécies de plantas como a *Passiflora incarnata* L. (ESCUREDO *et al.*, 2012; WILLIAMS *et al.*, 1997).

Estruturalmente, a crisina contém dois anéis de benzeno (A e B) e um anel heterocíclico contendo oxigênio, possui a presença de ligação dupla C2–C3 com um grupo carbonil ligado ao 4º carbono do anel C. Com base nessa classificação estrutural, a crisina se enquadra na classe de flavonas, possuindo também contém grupos hidroxil (-OH) nos carbonos 5 e 7 do anel A (Fig. 1). Ao contrário de vários flavonoides, a crisina não possui oxigenação no anel B, principalmente, a diferença na oxigenação do anel A é responsável por vários derivados da crisina como wogonin, baicalein e oroxilina A (BALTA *et al.*, 2015).

As atividades biológicas da crisina estão associadas à ausência de oxigenação nos anéis B e C, acompanhadas de efeitos anti-inflamatórios. Também é descrito que a atividade antioxidante de diferentes flavonas se baseia na diversidade estrutural desses compostos químicos, nos quais a presença do grupo carbonila no carbono 4 e a ligação dupla entre os carbonos 2 e 3 estão essencialmente ligadas à atividade antioxidante da crisina (NAZ *et al.*, 2019).

Por essas especificações e efeitos benéficos constatados, a crisina parece ser um composto promissor, e a partir disto, há um grande número de trabalhos na literatura que apontam suas propriedades farmacológicas, por exemplo:

- Atividade antialérgica (PEARCE *et al.*, 1984);
- Atividade anticonvulsiva (MEDINA *et al.*, 1990);
- Atividade ansiolítica (WOLFMAN *et al.*, 1994; RODRÍGUEZ-LANDA *et al.*, 2019);
- Atividade antiviral (CRITCHFIELD *et al.*, 1996);
- Inibição da enzima aromatase (KAO *et al.*, 1998; GAMBELUNGHE *et al.*, 2003);
- Atividade antiestrogênica (MACHALA *et al.*, 2001);
- Efeito anti-hipertensivo (VILLAR *et al.*, 2002; VEERAPPAN & MALARVILI, 2019);
- Inibição da angiogênese tumoral (FU *et al.*, 2007);
- Efeito inibitório na formação de fibrina sobre a proteína beta amiloide (AKAISHI *et al.*, 2008);

- Efeito anti apoptótico (IZUTA *et al.*, 2008; KANG *et al.*, 2004; GEORGE *et al.*, 2017; XU *et al.*, 2019);
- Atividade antioxidante (PUSHPAVALLI *et al.*, 2010; CIFTCI *et al.*, 2012);
- Atividade anti-inflamatória por diferentes mecanismos investigados (BAE *et al.*, 2011; AHAD *et al.*, 2014; FENG *et al.*, 2014; XIAO *et al.*, 2014, LI *et al.*, 2019);
- Atividade antiasmática (WADIBHASME *et al.*, 2011);
- Atividade anticancerígena (LI *et al.*, 2011);
- Efeito antineoplásico (PICHICHERO *et al.*, 2011; DELDAR *et al.*, 2018; YUFEI *et al.*, 2020; CHEN *et al.*, 2020);
- Inibição das histonas deacetilases (SUN *et al.*, 2012);
- Inibição das DNA topoisomerases (RUSSO *et al.*, 2012);
- Ação positiva no sistema reprodutor masculino em camundongos (CIFTCI, *et al.*, 2012; DEL FABBRO *et al.*, 2019c);
- Prevenção da progressão metastática em células de câncer de mama (LIRDPRAPAMONGKOL *et al.*, 2013);
- Inibição de fator de necrose tumoral- α (TNF-α) e interleucina-1β (IL-1β) (BAI *et al.*, 2013);
- Atividade anti-hipercolesterolêmica (ANANDHI *et al.*, 2013);
- Atividade cardioprotetora (TESTAI *et al.*, 2013; YANG *et al.*, 2018; RANI & ARYA, 2020);
- Prevenção da osteoporose (ZENG *et al.*, 2013);
- Efeito supressivo no fator de crescimento endotelial vascular (VEGF) induzindo angiogênese (TIAN *et al.*, 2014);
- Efeito anti-hiperlipidêmico (ZARZECKI *et al.*, 2014);
- Efeitos protetores em lesões na medula espinhal (KANDHARE *et al.*, 2014);
- Efeito antiaterogênico em modelo experimental de aterosclerose (ANANDHI *et al.*, 2014);
- Atividade renoprotetiva (KANG *et al.*, 2015);
- Efeito neuroprotetor atuando como um agente antienvelhecimento (SOUZA *et al.*, 2015);
- Efeito neuroprotetor na doença de Parkinson (GOES *et al.*, 2018; AHMED *et al.*, 2018; KRISHNAMOORTHY *et al.*, 2019; DEL FABBRO *et al.*, 2019b);

- Efeitos epigenéticos (KANWAL *et al.*, 2016);
- Supressão dos efeitos de dermatite atópica (CHOI *et al.*, 2017);
- Efeito do tipo antidepressivo em modelos com diferentes indutores (FILHO *et al.*, 2015; 2016a; 2016b; BORTOLOTTO *et al.*, 2018; FARKHONDEH *et al.*, 2020);
- Melhora a deficiência visual causado pelo diabetes (KANG *et al.*, 2018);
- Efeito neuroprotetor em encefalopatia hepática (EL-MARASY *et al.*, 2018);
- Melhoria da endometriose, induzindo apoptose (RYU *et al.*, 2019);
- Efeito anti-isquêmico (EL KHASHAB *et al.*, 2019);
- Prevenção de deficiências cognitivas e de potenciação à longo prazo devido à isquemia cerebral (SARKAKI *et al.*, 2019);
- Melhoria do dano causado por lesão cerebral traumática (RASHNO *et al.*, 2019);
- Melhoria de anormalidades glicolipídicas relacionadas à idade (FARKHONDEH *et al.*, 2019);
- Ação de respostas imunes (DEL FABBRO *et al.*, 2019a);
- Melhoria da esteatose não alcoólica (PAI *et al.*, 2019b);
- Interferência nos efeitos da frutose em nível intestinal, diminuindo a possibilidade de síndrome metabólica (ANDRADE *et al.*, 2019);
- Melhoria pós isquemia cerebral (LI *et al.*, 2019);
- Efeito antiobesidade (PAI *et al.*, 2019a);
- Ação anti hepatotóxica (MOHAMMADI *et al.*, 2019);
- Efeito antinociceptivo (HONG *et al.*, 2020);
- Melhoria dos sintomas da doença de Huntington (HAIDER *et al.*, 2020);
- Efeito regenerante ósseo e, reparo de defeitos ósseos em conjunto de células-tronco da polpa dentária (HUO *et al.*, 2021);
- Entre outros efeitos benéficos evidenciados na literatura.

A crisina está no mercado farmacêutico como suplemento alimentar, principalmente para fins de hipertrofia muscular, pela inibição da enzima aromatase, aumentando os níveis de testosterona (KAO *et al.*, 1998). Com a sua popularidade e demais benefícios já descritos, especialmente pela sua atividade antioxidante (PUSHPAVALLI *et al.*, 2010; CIFTCI *et al.*, 2012) e ansiolítica (WOLFMAN *et al.*, 1994), atualmente existem diversas marcas no mercado de crisina encapsulada nos mais variados valores (500 a 800 mg/cápsula).

2.10 Crisina e o déficit de memória

Os produtos naturais, originados de plantas, tem sido alvo de uma fonte importante de agentes terapêuticos, muitos dos quais constituem modelos para a síntese de um grande número de fármacos (CALIXTO, 2005). A partir disto, as plantas medicinais tornaram-se importantes objetos das indústrias farmacêuticas e de centros de pesquisas na busca de novos fármacos com maior atividade farmacêutica/terapêutica e menores efeitos colaterais (EVANS, 1996). Com a crisina não é diferente, após algum tempo de estudo, esta se tornou conhecida por seus benefícios já estabelecidos - alguns citados na seção 2.9.1. Em relação à memória, alguns trabalhos que demonstraram os efeitos promissores da crisina em roedores estão descritos a seguir.

No estudo de Souza *et al.* (2015) a crisina demonstrou diminuir o déficit memória em animais idosos, o que foi verificado a partir de testes comportamentais e marcadores bioquímicos como do estresse oxidativo, incluindo medidas dos níveis de espécies reativas (RS), atividades da superóxido dismutase (SOD), da catalase (CAT), da glutationa peroxidase (GPx) e, ainda da Na^+,K^+-ATPase e medida dos níveis de BDNF nas estruturas cerebrais de córtex pré-frontal e hipocampo, atuando como um agente anti-idade.

No estudo de Goes *et al.* (2018) os autores apontam a crisina com potente ação neuroprotetora na doença de Parkinson, pois observaram melhora da neuroinflamação a partir da mensuração dos níveis de citocinas e fator nuclear kappa B (NF-kB), potencial antioxidante reativo total (TRAP) e reatividade antioxidante total (TAR), de neurotrofinas (BDNF, GDNF, NGF), e dos níveis de dopamina, ácido 3,4-di-hidroxifenilacético (DOPAC) e ácido homovanílico (HVA), no estriado cerebral.

Krishnamoorthy *et al.* (2019) demonstraram a ação neuroprotetora da crisina em doença de Parkinson, avaliando marcadores de estresse oxidativo como glutationa redutase (GSH), SOD, peroxidase lipídica (LPO), expressão gênica por PCR-RT das neurotrofinas (BDNF e GDNF) e da transportador de dopamina (DAT) e α-sinucleína (SYN), expressão proteica de NFκB, NRF2, IL1β e BDNF, imunohistoquímica para tirosina hidroxilase e fator de necrose tumoral- α (TNF-α), dosagens de dopamina, DOPAC e HVA, na substância negra compacta e estriado.

Ainda, no estudo realizado por Sarkaki *et al.* (2019) foi verificado que a crisina é capaz de melhorar os danos causados por isquemia cerebral, incluindo deficiências cognitivas e de LTP, analisado com o auxílio da eletrofisiologia, teste de memória espacial e, por dosagens de TNF-α e IL-1β no hipocampo.

Neste sentido, o estudo de Shooshtari *et al.* (2020) reafirmam a ação neuroprotetora da crisina, desta vez em modelo de hipoperfusão e reperfusão cerebral em ratos. Foi avaliado a memória de esquiva passiva, avaliação de GPx, malondialdeído (MDA), prostaglandina E2 (PGE2) e óxido nitrico NO, avaliação histológica de Coloração de Nissl e contagem de densidade neuronal de sobrevivência. Os resultados cerebrais do hipocampo total e regiões, indicaram que a crisina possui atividade neuroprotetora pela diminuição da hiperemia reativa, redução do estresse oxidativo e modulação de biomarcadores endógenos.

A partir destes estudos mencionados, sabemos que a crisina possui efeitos que diminuem o déficit de memória, por diferentes vias. Desta forma, a reversão do déficit de memória induzido pelo hipotireoidismo, se torna uma opção de estudo, levando em consideração a ausência do efeito da crisina neste modelo de disfunção endócrina.

Assim, a crisina pode atuar melhorando os déficits de memória induzido pelo hipotireoidismo, no SNC, uma vez que em um estudo anterior, a crisina demonstrou atenuar a sintomatologia da depressão em animais com estado tipo-depressivo induzido pelo hipotireoidismo, sem alterar os níveis dos HT (BORTOLOTTO *et al.*, 2018). Desta forma, pode-se avaliar a ação neuroprotetora da crisina nas vias glutamatérgica, colinérgica e neurotrofinérgica, sem que haja atuação/secreção na glândula tireoide, em animais com hipotireoidismo.

3. Justificativa

O hipotireoidismo é a alteração mais frequente da glândula tireoide, possuindo uma prevalência em torno de 10% nas mulheres, e considerando o fato de que a doença majora no período da menopausa (ficando em torno de 12 a 15%), as alterações que ocorrem devido a esta disfunção endócrina, principalmente no SNC, precisam de maior atenção.

Sabendo que esta alteração tireoidiana está associada a desordens neuropsiquiátricas, como o déficit de memória, é imprescíndivel estudos que mostrem as vias neuronais que possam estar alterando a consolidação da memória em animais com hipotireoidismo, da mesma forma, buscar compostos naturais com potencial terapêutico nestas vias, modulando possíveis mecanismos moleculares envolvidos no déficit de memória, como por exemplo, nos sistemas glutamatérgico, colinérgico e neurotrofinérgico.

A partir disto, os compostos naturais possuem uma gama de propriedades farmacológicas bem estabelecidas, principalmente os flavonoides, citamos como composto de estudo o flavonoide crisina, sobretudo por atuar no SNC. É importante ressaltar que este possui efeitos colaterais diminuídos ou ausentes, o que é bastante interessante para a indústria farmacêutica e para os pacientes.

Desta forma, sabendo que a crisina já é comercializada, esta tese oferece entendimento para o reposicionamento deste suplemento, o qual possui diversos efeitos benéficos já relatados. Enfatizamos a sua ação neuroprotetora, em que poderia ser um adjuvante ao tratamento das consequências neurológicas causadas pelo hipotireoidismo.

Sendo assim, esta tese tem como finalidade promover embasamento na busca de estratégias terapêuticas/reposionamento da crisina para o tratamento de déficit de aprendizado e memória causado pelo hipotireoidismo, através da análise de marcadores moleculares dos sistemas glutamatérgico, colinérgico e neurotrofinérgico.

4. Objetivos

4.1 Objetivo geral

Investigar se a crisina é capaz de reduzir o déficit cognitivo ocasionado pelo hipotireoidismo e verificar o envolvimento dos sistemas glutamatérgico, colinérgico e neurotrofinérgico.

4.2 Objetivos específicos

- Investigar se a crisina possui ação moduladora no déficit de memória espacial e aversiva, ocasionado pelo hipotireoidismo;
- Verificar o envolvimento do sistema glutamatérgico no déficit cognitivo ocasionado pelo hipotireoidismo e na possível ação da crisina;
- Avaliar o envolvimento do sistema colinérgico no déficit cognitivo ocasionado pelo hipotireoidismo e na possível ação da crisina;
- Analisar o envolvimento do sistema neurotrofinérgico no déficit cognitivo ocasionado pelo hipotireoidismo e na possível ação da crisina;
- Analisar a afinidade da crisina com os receptores neurotrofinérgicos: TrkB, TrkA e p75NTR, por meio do *docking molecular*.

5. Resultados

Os resultados que fazem parte desta tese estão apresentados sob a forma de 2 artigos científicos.

Os itens Introdução, Materiais e Métodos, Resultados, Discussão e Referências, encontram-se nos próprios Artigos Científico.

Artigo 1:

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

Publicado na revista: Physiology and Behavior.

Artigo 2:

Intitulado: Chrysin restores memory deficit in hypothyroidism mice: behavioral, neurochemical and computational approaches involving the neurotrophinergic system.

Publicado na revista: Journal of Psychiatric Research

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5.1 Artigo científico I

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

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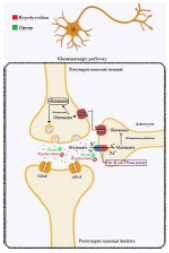
GRAPHICAL ABSTRACT



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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.

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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

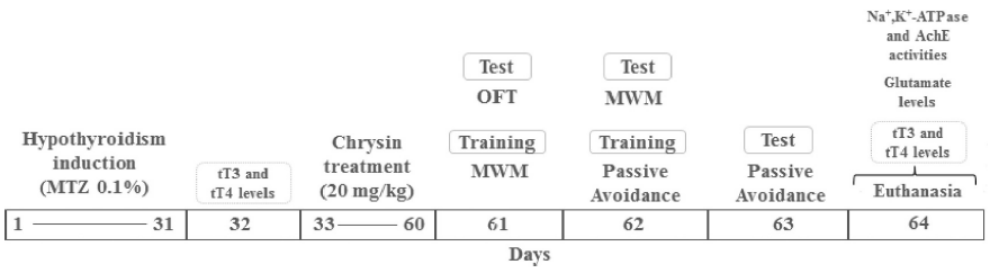

Fig. 1. Experimental protocol.

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.

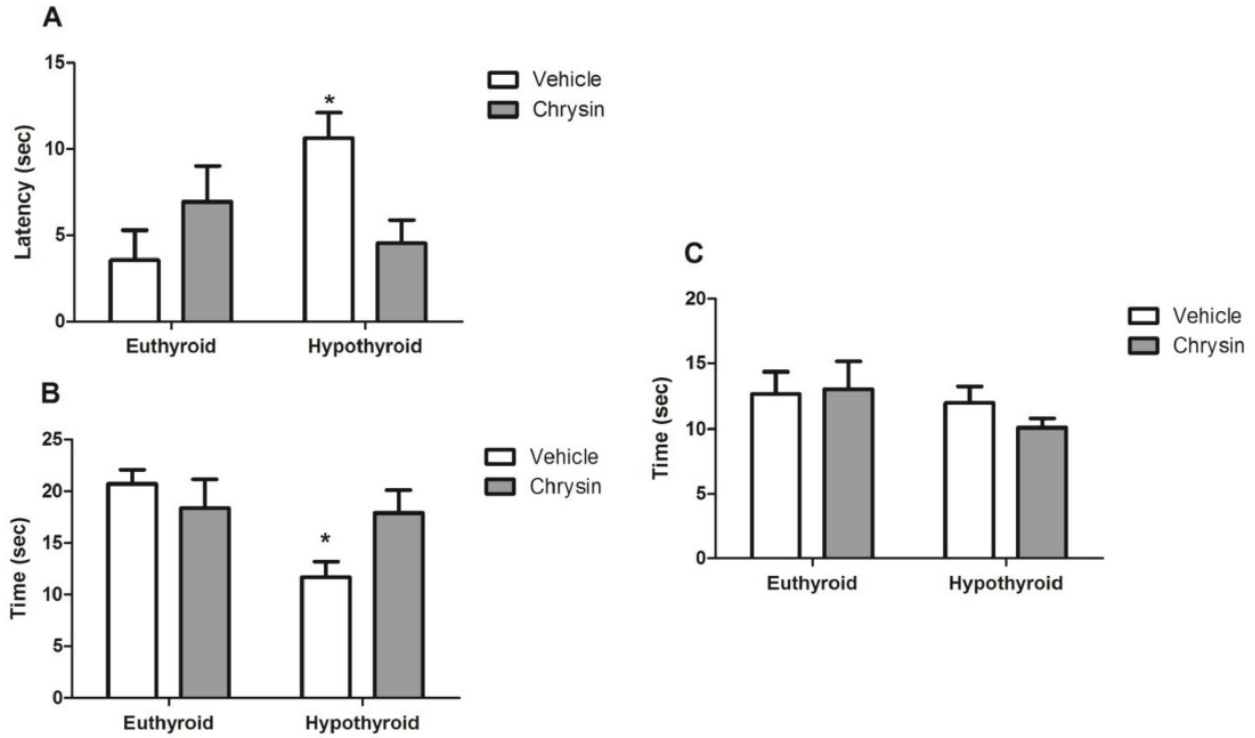

Fig. 2. Effects of hypothyroidism and chrysin on the MWM test. (A) Latency to find the quadrant of platform. (B) Time spent in the aim quadrant. (C) Time spent in the opposite quadrant. *Significant difference in relation to the euthyroid group. Data are shown as mean ± standard error of the mean (n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

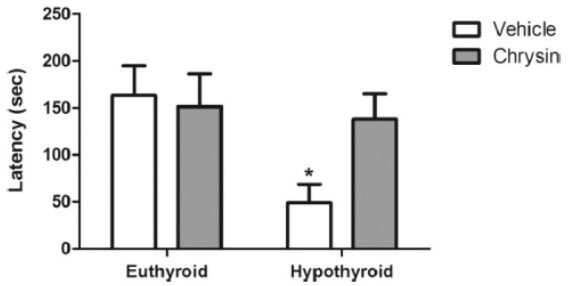

Fig. 3. Effects of hypothyroidism and chrysin on the Passive avoidance test (light and dark). The graphic shows the latency of animals to pass to the dark-side. *Significant difference in relation to the euthyroid group, euthyroid + chrysin and hypothyroid + chrysin group. Data are shown as mean ± standard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

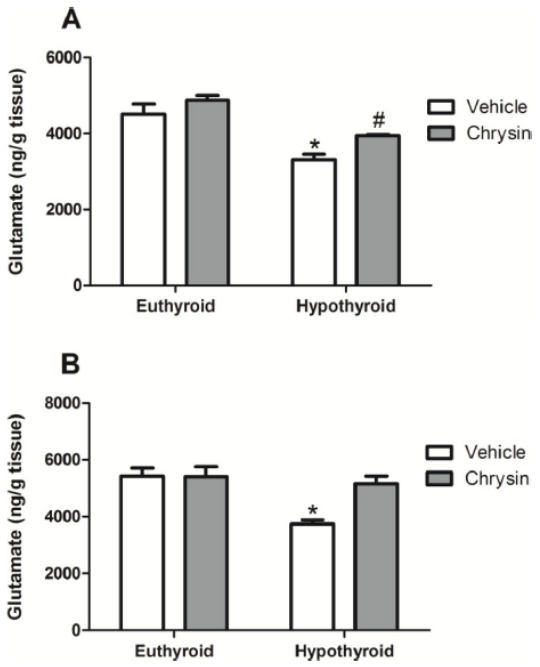

Fig. 4. Effects of hypothyroidism and chrysin on the glutamate level. (A) Hippocampus (B) Prefrontal cortex. *Significant difference in relation to the euthyroid, euthyroid + chrysin and hypothyroid + chrysin groups. #Significant difference in relation to the euthyroid and euthyroid + chrysin groups.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

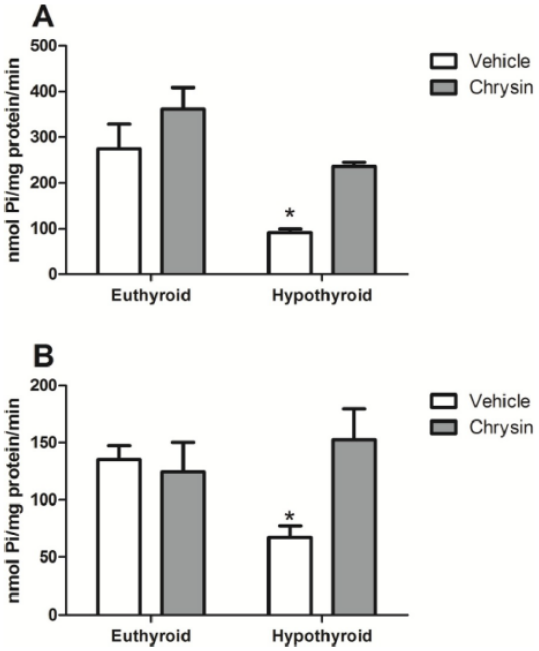

Fig. 5. Effects of hypothyroidism and chrysin on Na^+, K^+-ATPase activity. (A) Hippocampus. *Significant difference in relation to the euthyroid, euthyroid + chrysin and hypothyroid + chrysin groups. (B) Prefrontal cortex. *Significant difference in relation to the hypothyroid + chrysin group. Data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

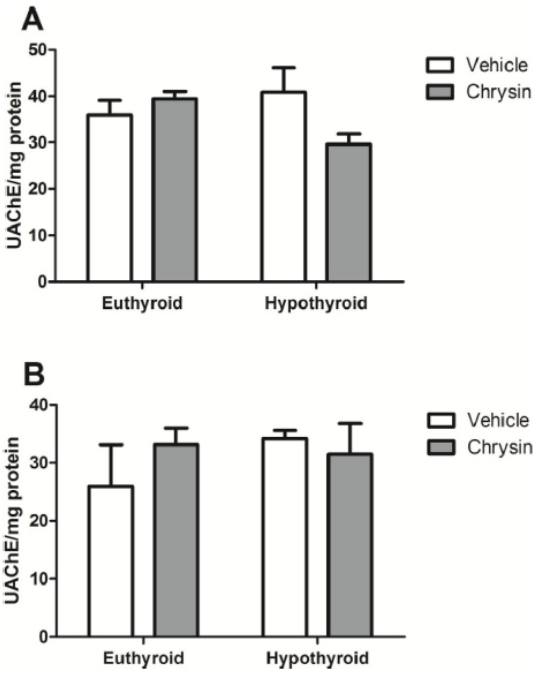

Fig. 6. Effects of hypothyroidism and chrysin on AChE activity in the hippocampus (A) and the prefrontal cortex in (B). Data are shown as mean ± standard error of the mean (n = 4–5 per group).

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.

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Supplementary materials

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

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Supplementary material

2 Materials and Methods

2.5.1 Open-field test (OFT)

The test was performed in a 45-cm^2 plastic box with 30-cm high walls connected to a computer. The test began with the animal in the center of the device and it was observed for 5 min. The apparatus was cleaned with 70% alcohol between trials to avoid possible exterior influence on locomotion [30].

2.5.2 Morris Water Maze (MWM) test

The MWM was realized according the method of Morris [31], and modification of Souza [21], the apparatus was made of fiberglass, with 97 cm in diameter and 60 cm in height. For the acquisition phase, the tank was filled with water maintained at 22±2°C. The target platform (10 cm × 10 cm) was made of acrylic and it was submerged 1 –1.5 cm beneath the surface of the water. The starting points for the animals were marked on the outside of the pool as north (N), south (S), east (E) and west (W). Four distant visual cues (55 cm × 55 cm) were placed on the walls of the water maze room, they were all positioned with the lower edge 30 cm above the upper edge of the water tank and in the standard setting, and the position of each symbol marked the midpoint of the perimeter of a quadrant. The training session consisted of 10 consecutive trials, in 1 day, during which the animals were left in the tank facing the wall and then allowed to swim freely to the submerged platform, which was located in a constant position, equidistant from the center and the wall of the pool. If the animal did not find the platform during a period of 60 s, it was gently guided to it. When it arrived on the platform the animal stayed 10 s, and withdrawn for 20 s before being placed at the next starting point in the

tank, until completing 10 times of training. The starting points (the axis of one imaginary quadrant) varied in a pseudo-randomized manner.

Twenty-four hours after the acquisition phase, a probe trial was conducted by removing the platform and placing the mice next to it and facing the S side for 60 s. Behavioral data were recorded: latency to find the platform quadrant; time in the platform quadrant (i.e. where the platform was located on the training session); time in the opposite quadrant (i.e. quadrant opposite to the one where the platform was located in the training session).

2.5.3 Passive avoidance test

The animal learns to avoid a specific place associated to an aversive event, the reduction in step-through latency was used as an indicator of learning [33]. The apparatus consisted of two compartments (light and dark), each of them measured 18 × 16 × 18.5 cm, and it had a guillotine type door (9 x 10 cm) in the middle of the compartments to divide the walls. The compartment's floor consisted of parallel stainless steel rods (0.3 cm in diameter with 1 cm intervals), and only the dark side produced an electric shock. The mice should learn that they should avoid the dark compartment where an electric shock was applied. The task was divided in acquisition phase and test session: in the training session, the mice were allowed to explore the light compartment and a foot electric shock (60 Hz, 2 s, 0.5 mA intensity) was applied whenever the mice input on the dark compartment with a maximum of three electric shocks; after 24 hours, a retention testing session was performed without the application of shock, each animal was placed again on the light side and the latency was measured considering the time the animals stayed in the light side, and considering a cut-off time of 300 s [3].

2.8 Na^+,K^+-ATPase activity

The Na^+,K^+-ATPase activity was realized with method of Fiske and Subbarow [37]. Briefly, started with the reaction mixture for the assay containing 3 mM $MgCl_2$, 125 mM NaCl, 20 mM KCl, 50 mM Tris–HCl (pH 7.5), and distilled water. An aliquot containing the sample and dimethyl sulfoxide was added to the reaction mixture, and it was preincubated at 37°C for 10 min. The reaction was initiated by the addition of adenosine triphosphate (ATP), and incubated for 30 minutes at 37°C. The control assays were carried out under the same conditions, with the addition of 0.1 mM ouabain. The reaction was stopped by adding trichloroacetic acid (TCA) solution (10%) with 10 mM $HgCl_2$. Afterward, it was caught an aliquot of solution and added distilled water, ammonium molybdate and ascorbic acid, then these solutions were left in the dark for 7 minutes, and read in 650 nm.

2.9 Acetylcholinesterase (AChE) activity

The reaction medium was prepared containing (0.25 M KPi buffer, pH 8.0 and 5,5 0-dithiobis-2-nitrobenzoic acid (DTNB 5 mM)). It was added to the supernatant (S1) solution sample and to AcSCh 7.25 mM (2.1 mg/ml), and the reading was done for 2 min at 412 nm.

2.11 Statistical analysis

The results of HT in 32 days were analyzed using a one-sample Student t-test. Pearson's correlation coefficient was used to examine the correlation between the analyzed parameters.

3 Results

3.4 Correlation analysis

In the Pearson's correlation analysis (Table 1S), glutamate levels in the hippocampus and the prefrontal cortex were significantly negatively correlated to the MWM (A) test ($r = -0.6072$, $p = 0.0126$; $r = -0.7253$, $p = 0.0015$, respectively), the same was found in the prefrontal cortex

and the MWM (C) also correlated negatively (r= -0.3161, p = 0.2329). Na^+,K^+-ATPase activity in the hippocampus and the prefrontal cortex was also significantly negatively correlated to the MWM (A) test (r = -0.5299, p = 0.0077; r = -0.6504, p = 0.0064, respectively), and the same was found in in the prefrontal cortex and the MWM (C) also correlated significantly negatively (r= -0.5214, p = 0.0383). AChE activity in the hippocampus and the MWM (A), (B) and (C) were correlated negatively (r = -0.5259, p = 0.0172; r = -0.7035, p = 0.0005; r = -0.4437, p = 0.0500, respectively) being (A) and (B) significantly, in the prefrontal cortex and the MWM (B) test also correlated significantly negatively (r = -0.7371, p = 0.0011).

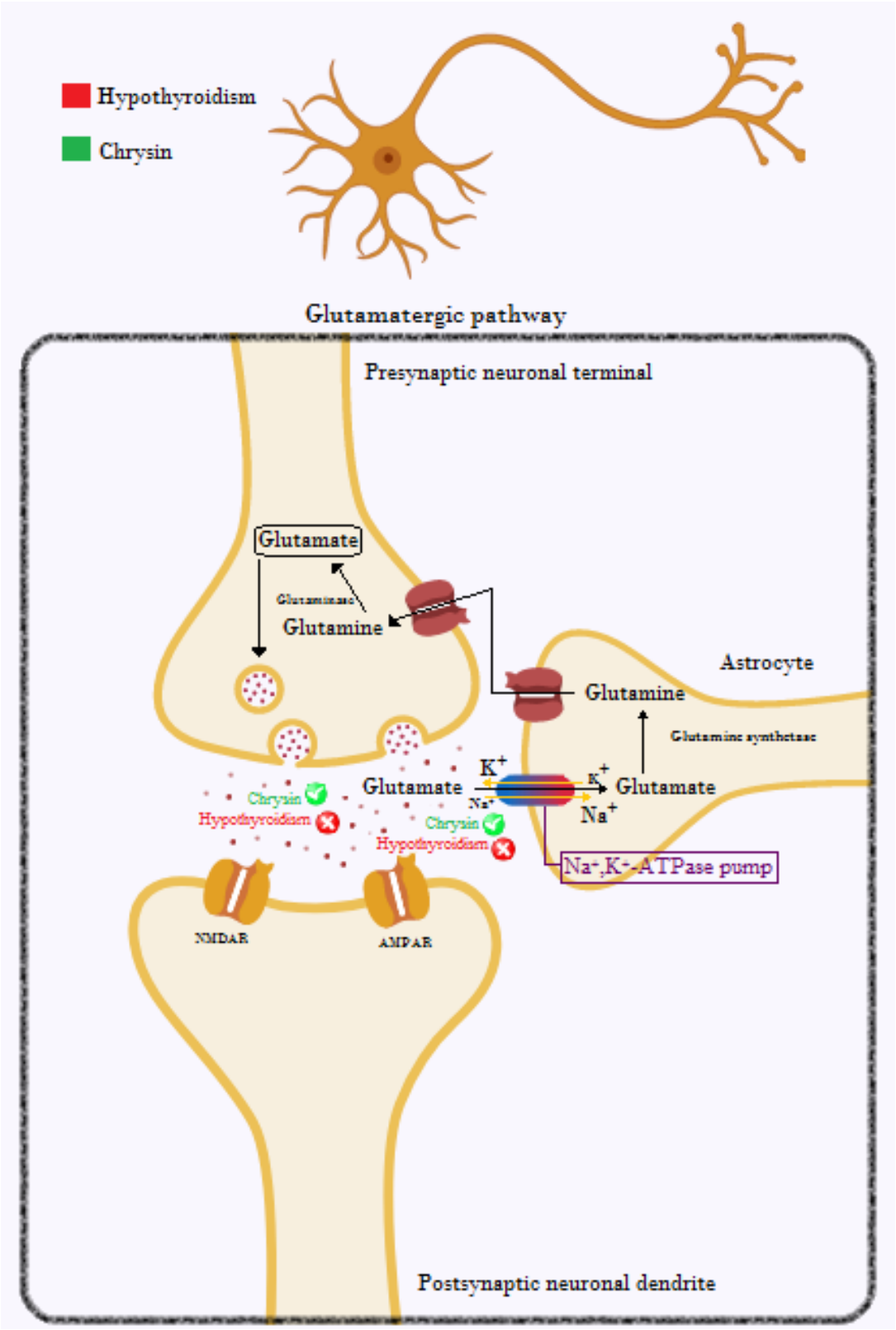
Furthermore, the correlation analysis found a significant positive correlation between the passive avoidance test and the glutamate levels in the hippocampus and the prefrontal cortex (r = 0.5820, p = 0.0180; r = 0.6389, p = 0.0077, respectively), and the hippocampus and the MWM (B) and (C) (r = 0.6916, p = 0.0030; r = 0.6601, p = 0.0054, respectively), also showed a positive significantly correlation between the prefrontal cortex and the MWM (B) (r = 0.8666, p = < 0.0001). The Na^+,K^+-ATPase activity in the hippocampus and the prefrontal cortex were also significantly positively correlated to the passive avoidance test (r = 0.3840, p = 0.0639; r = 0.2041, p = 0.4484, respectively), and the hippocampus and the MWM (B) and (C) r = 0.6176, p = 0.0037; r = 0.6355, p = 0.0026, respectively) also showed a positive significantly correlation between the prefrontal cortex and the MWM (B) (r = 0.8283, p = < 0.0001). AChE activity in the hippocampus and the prefrontal cortex was also significantly positively correlated to the passive avoidance test (r = 0.4896, p = 0.0284; r = 0.6009, p = 0.0138, respectively), and positively correlated to the prefrontal cortex and the MWM (A) and (C) (r = 0.4575, p = 0.0748; r = 0.6618, p = 0.0052, respectively) being (C) significant.

Table 1S. Effects of Chrysin on correlation between glutamate levels, Na^+,K^+-ATPase and AChE activity in hippocampus and prefrontal cortex with behavioral tests.

	r	***p***	***n***
Glutamate hippocampus x Passive avoidance	0.6340	0.0084**	16
Glutamate hippocampus x MWM (A)	-0.7077	0.0022**	16
Glutamate hippocampus x MWM (B)	0.6916	0.0030**	16
Glutamate hippocampus x MWM (C)	0.6601	0.0054**	16
Glutamate prefrontal cortex x Passive avoidance	0.8259	<0.0001***	16
Glutamate prefrontal cortex x MWM (A)	- 0.9013	<0.0001***	16
Glutamate prefrontal cortex x MWM (B)	0.8666	<0.0001****	16
Glutamate prefrontal cortex x MWM (C)	-0.3161	0.2329	16
Na^+,K^+-ATPase hippocampus x Passive avoidance	0.7294	0.0003***	20
Na^+,K^+-ATPase hippocampus x MWM (A)	-0.6113	0.0042**	20
Na^+,K^+-ATPase hippocampus x MWM (B)	0.6176	0.0037**	20
Na^+,K^+-ATPase hippocampus x MWM (C)	0.6355	0.0026**	20
Na^+,K^+-ATPase prefrontal cortex x Passive avoidance	0.8482	<0.0001***	16
Na^+,K^+-ATPase prefrontal cortex x MWM (A)	-0.8165	0.0001***	16
Na^+,K^+-ATPase prefrontal cortex x MWM (B)	0.8283	<0.0001****	16
Na^+,K^+-ATPase prefrontal cortex x MWM (C)	-0.5214	0.0383*	16
AChE hippocampus x Passive avoidance	0.4896	0.0284*	20
AChE hippocampus x MWM (A)	-0.5259	0.0172*	20
AChE hippocampus x MWM (B)	-0.7035	0.0005***	20
AChE hippocampus x MWM (C)	-0.4437	0.0500	20
AChE prefrontal cortex x Passive avoidance	0.6009	0.0138*	16
AChE prefrontal cortex x MWM (A)	0.4575	0.0748	16
AChE prefrontal cortex x MWM (B)	-0.7371	0.0011**	16
AChE prefrontal cortex x MWM (C)	0.6618	0.0052**	16

Morris Water Maze (MWM) task (A) Latency to find the quadrant of platform; (B) Time spent in the aim quadrant; (C) Time spent in the opposite quadrant; Passive avoidance test (Passive avoidance); Acetylcholinesterase (AChE) activity. *p<0.05 is considered significant.

Graphical abstract



5.2 Artigo científico II

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Chrysin restores memory deficit in hypothyroidism mice: Behavioral, neurochemical and computational approaches involving the neurotrophinergic system

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ABSTRACT

Hypothyroidism is a condition that affects multiple systems, including the central nervous system, causing, for example, cognitive deficits closely related to Alzheimer's disease. The flavonoid chrysin is a natural compound associated with neuronal improvement in several experimental models. Here, we evaluated the effect of chrysin on cognitive impairment in hypothyroid female mice by exploring neuroplasticity. Hypothyroidism was induced by continuous exposure to 0.1% methimazole (MTZ) in drinking water for 31 days. On the 32nd day, the animals showed low plasma levels of thyroid hormones (hypothyroid mice) than the control group (euthyroid mice). Subsequently, mice were intragastrically administered with vehicle or chrysin (20 mg/kg) once a day for 28 consecutive days. At the end of the treatments, behavioral tests were performed: open-field test (OFT) and morris water maze (MWM). Then, the levels of neurotrophins (BDNF and NGF) in the hippocampus and prefrontal cortex were measured and tested the affinity of chrysin with neurotrophinergic receptors through molecular docking. Hypothyroid mice showed memory deficit in the MWM and reduced neurotrophins levels in the hippocampus and prefrontal cortex, meanwhile, the chrysin treatment was able to reversed the deficit of spatial memory function and increased the levels of BDNF in hipocamppus and NGF in both structures. Additionally, molecular docking analysis showed that chrysin potentially binds to the active site of the TrkA, TrkB, and p75NTR receptors. Together, these findings suggest that chrysin reversed behavioral and neurochemical alterations associated with memory deficit induced by hypothyroidism, possibly by modulating synaptic plasticity in the neurotrophinergic system.

1. Introduction

Hypothyroidism results in impairments to the individual in adulthood, such as interrupted attention, depressed mood, and cognitive deficit (Baghcheghi et al., 2018). Thus, patients with hypothyroidism demonstrate certain behaviors that are related to dementia and Alzheimer's Disease (Accorroni et al., 2017). Therefore, considering that about 50 million people have some form of dementia worldwide (WHO, 2019) and that 36% of people who have some form of dementia have abnormalities in the thyroid gland (Accorroni et al., 2017), we infer that around 18 million people, with some form of dementia have thyroid gland abnormalities. This fact is significant, as the true etiology that brings these diseases together is still unknown, only the damage caused in patients with dementia is well described in the literature as: a decrease of the thyroid hormones (TH) levels (Accorroni et al., 2017), a decrease of the TH receptors (Sampaolo et al., 2005); and in rats:

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neuropathological signs, characterized by reduction of cerebral volume, abnormal tau phosphorylation and neuroinflammation associated with cognitive deficits (Chaalal et al., 2014).

Hypothyroidism is also capable of altering neuronal plasticity pathways (Chaalal et al., 2018). Learning and memory studies in animal models identified a range of gene products necessary to regulate these processes (Cunha et al., 2010). Among them are the neurotrophins, responsible for regulating neuronal plasticity by controlling neuronal cell death during brain development and play an essential role in the differentiation, survival, and neuronal growth of the peripheral and central nervous system (Barichello et al., 2013). Nerve growth factor (NGF) and the brain-derived neurotrophic factor (BDNF) play many processes in adulthood (Nagahara and Tuszynski, 2011), as the synaptic effects for plasticity, and consequent learning and memory, depending on the connection with its receptors (Cunha et al., 2010), which are the associations between the BDNF and tyrosine receptor kinase B (TrkB), NGF with tyrosine receptor kinase A (TrkA), and the p75-neurotrophin receptor (p75NTR) that acts by binding to both (BDNF and NGF) or as a subunit of Trk complexes (Chao, 1994). The effects of BDNF are extensively studied, as it acts in the formation of long-term memory (LTM), through the long-term potentiation (LTP) as a form of synaptic plasticity (Martin et al., 2000), furthermore, the hypothyroidism is capable of altering these neuronal plasticity pathways (Chaalal et al., 2018), but little is known about their effect concerning the modulation of these neurotrophins.

In our most recent and relevant study, we demonstrated that flavonoid chrysin (5,7-dihydroxyflavone) reversed the memory deficit induced by hypothyroidism through glutamatergic system modulation (Bortolotto et al., 2020). Chrysin is also well described for its neuronal effects, as an antidepressant (Bortolotto et al., 2018; Farkhondeh et al., 2019; Filho et al., 2015, 2016a, 2016b), improves memory deficit (Del Fabbro et al., 2019; Goes et al., 2018; He et al., 2012; Krishnamoorthy et al., 2019; Li et al., 2019; Sarkaki et al., 2019; Souza et al., 2015). Some of these studies, demonstrated of chrysin was associated with modulation of neurotrophins (Filho et al., 2016b; Souza et al., 2015). So, the objective of this study is to explore the action of chrysin on cognitive impairment in hypothyroid female mice and in neurotrophinergic system: levels of neurotrophins (BDNF and NGF) and affinity to chrysin with the TrkA, TrkB, and p75NTR receptors.

2. Materials and methods

2.1. Animals

The study used female C57BL/6 mice (total of 24 adults, 3–4 months) under controlled conditions, a 12:12 h light/dark cycle at 23 ± 2 °C and 50 ± 5% humidity, with food and water provided *ad libitum*. Procedures for this research were performed according to the Committee's guidelines on the Care and Use of Experimental Animal Resources. With the approval of under protocol number 024/2018, the minimum number of animals necessary for the experiment was used, ensuring the well-being and euthanasia standards. The use of females in this study is a higher prevalence of hypothyroidism in women (Morganti et al., 2005).

2.2. Drugs

Methimazole (MTZ) and chrysin were obtained from Sigma-Aldrich Chemical Co, USA - codes M8506 and C80105, respectively. Chrysin was dissolved in 80% saline and 20% polyethylene glycol (PEG).

2.3. Experimental design

2.3.1. 2.3.1 Hypothyroidism induction

For the hypothyroidism induction, we divided the animals into two groups (n = 12): control (received 0.475% sucralose in the drinking water) and MTZ (received MTZ 0.1% + 0.475% sucralose in the drinking water), for 31 days (Bortolotto et al., 2020, 2018) (Fig. 1). For confirmation of hypothyroidism induction, on day 32, after stopping exposure to MTZ and sucralose, total thyroid hormones tT3 and tT4 were measured using blood extracted from the mice tail vein (Table S2 - through a microparticle enzyme immunoassay (MEIA) method).

2.3.2. Chrysin treatment

After confirmation of hypothyroidism induction, on day 33, the groups were subdivided in 4 groups (n = 6): euthyroid; euthyroid chrysin; hypothyroid; and hypothyroid chrysin, and treatments started. Once a day, chrysin (20 mg/kg, 10 ml/kg, p.o.) was administered for 28 consecutive days; this dose utilized has already demonstrated a neuroprotective effect by studies conducted in our laboratory (Bortolotto et al., 2020, 2018; Filho et al., 2015). The groups euthyroid and hypothyroid received the vehicle (10 ml/kg, p.o.).

2.4. Behavioral assessments

2.4.1. Open field test (OFT)

On day 61, was performed evaluating the locomotor activity and validating the memory test. Each animal was placed individually at the center of the apparatus (45-cm^2 plastic box with 30-cm high walls) and observed for 5 min to record the number of rearings, distance traveled (meters) and velocity (mm/s) (Walsh and Cummins, 1976).

2.4.2. Morris water maze (MWM) test

On day 61, spatial learning and memory were assessed using the MWM test according to the method of Morris (1984), with some modifications of Souza (Souza et al., 2015). For the acquisition phase, on day 61, the mice trained for ten consecutive trials, in a fiberglass pool (with 97 cm in diameter and 60 cm in height, and 22 ± 2 °C temperature), with an acrylic platform submerged in water (1–1.5 cm), and symbols on the four walls. If the mice did not find the platform in 1 min, it was gently guided to it, remaining 10 s on top.

On day 62, after 24 h of the acquisition phase, the test was conducted by removing the platform from the pool for 1 min. The data obtained were: latency to find the platform quadrant; time spent in the platform quadrant; time spent in the opposite quadrant.

2.5. Tissue preparation

The animals were anesthetized by inhalation of isoflurane on 63 days. Blood samples were obtained using heparin as the anticoagulant, and the plasma was used to analyze tT3 tT4 levels (Table S2 - through a microparticle enzyme immunoassay (MEIA) method). The hippocampus and prefrontal cortex were removed and rapidly homogenized in 50 mM Tris-Cl, pH 7.4. The homogenate was centrifuged at 2400 g for 15 min at 4 °C, and a low-speed supernatant fraction (S1) was used for neurotropic parameters.

2.6. Neurotrophins levels

The plasticity was evaluated for BDNF and NGF's protein levels using a commercially available sandwich enzyme-linked immune sorbent assay (ELISA) kit (Chemicon International, Temecula, CA, USA). The BDNF and NGF levels were expressed as ng/g and pg/mg wet weight of tissue, respectively.

2.7. Docking molecular

The 2D structure of the compounds was drawn using Chemdraw Ultra 12.0 software, converted into 3D using the software Avogadro 0.9.4, and their geometry was optimized following MMFF94 method (Hanwell et al., 2012). The 3D X-ray crystal structures were retrieved from Protein Data Bank: TrkA (PDB code: 1WWW), TrkB (PDB code: 1HCF), and p75NTR (PDB code: 3BUK) and prepared using the software

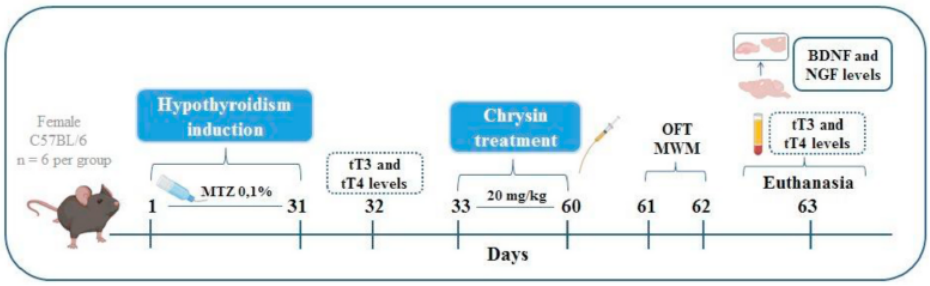

Fig. 1. Experimental protocol.

Auto Dock Tools 1.5.4 (Morris et al., 2009). The protein preparation consisted of fixing structures, deleting molecules, ions, and water, fixing hetero groups, and finally optimize the fixed structure using Gasteiger charges with 500 steps of minimization. CHIMERA 1.5.3 software was used previously to remove ligands in 3D structure and choose one chain for each receptor (Pettersen et al., 2004). We conducted the molecular docking using the software AutoDock Vina (version 1.1.1) with a grid box centered in all atom structure, allowing the program to search for additional places of probable interactions (Trott and Olson, 2010). The protein–ligand interactions were analyzed by Discovery studio visualizer 2016.

2.8. Statistical analysis

Data were checked for normality of distribution using the Shapiro-Wilk test and homogeneity using Bartlett's test. The results were compared using two-way ANOVA and Tukey *post-hoc* for multiple comparisons. Pearson's correlation coefficient was used for the estimation of the correlation between the analyzed parameters. Descriptive data were expressed as the mean ± interquartile range. Probability values less than 0.05 ($p < 0.05$) were considered statistically significant. All statistical analysis results were made by the program GraphPad Prism 9, and are shown in Table S1.

3. Results

3.1. Behavioral tests

Spontaneous locomotor activity was not affected after the treatments.

Neither MTZ nor chrysin affected spontaneous locomotor activity checked by total distance traveled, number of rearing and velocity. All these data had no significant difference (Table 1).

Administration of chrysin reversed the memory deficit after the hypothyroidism induction.

Fig. 2 shows the results obtained on the MWM test. The latency to find the aim quadrant was demonstrated in Fig. 2A. The hypothyroid group showed an increase in latency compared to all groups, and the chrysin treatment was able to reverse this deficit memory. Fig. 2B demonstrated the data of the time that animals spend in the aim quadrant; the hypothyroid group showed a decrease in the time spends in the aim quadrant compared to the euthyroid group. Neither hypothyroidism nor chrysin affected the time spent in the opposite quadrant (Fig. 2C).

Table 1
Effects of hypothyroidism and chrysin on mice subjected to the OFT.

	Distance (m)	Rearings (number)	Velocity (mm/s)
Euthyroid	19.46 ± 9.98	4.16 ± 2.04	20.89 ± 7.77
Euthyroid Chrysin	27.23 ± 11.27	2.83 ± 1.47	23.15 ± 2.85
Hypothyroid	26.31 ± 9.67	2.33 ± 1.63	24.48 ± 2.97
Hypothyroid Chrysin	18.20 ± 6.81	3.33 ± 1.50	28.66 ± 7.23

Values are expressed as means ± S.D. of 6 mice/group. $p < 0.05$ is considered significant.

3.2. Neurotrophins levels

The chrysin treatment was able to reverse the decrease in neurotrophin levels and may be responsible for the memory restoration in the hypothyroid mice.

Fig. 3 illustrates BDNF levels in the hippocampus (Fig. 3A) and prefrontal cortex (Fig. 3B), and NGF levels in the hippocampus (Fig. 3C) and prefrontal cortex (Fig. 3D).

BDNF levels were decreased in the hippocampus and prefrontal cortex of the hypothyroid group when compared to the euthyroid group. The treatment with chrysin recovered BDNF levels in hippocampus, although was unable to recover these levels in the prefrontal cortex. NGF levels were decreased in the hippocampus and prefrontal cortex of the hypothyroid group when compared to the euthyroid mice. The treatment with chrysin recovered NGF levels in both structures.

Significant correlations were found between MWM and neurotrophins levels.

After these results, knowing that chrysin can reverse the memory deficit caused by hypothyroidism and improve the decrease in neurotrophin levels in both brain structures (hippocampus and prefrontal cortex), we analyzed whether these effects were correlated through Pearson's correlation (Table 2). The results demonstrated a significant positive correlation between the BDNF hippocampus x MWM (B), BDNF prefrontal cortex x MWM (B), and NGF hippocampus x MWM (B). A significant negative correlation was found between BDNF hippocampus x MWM (A), NGF hippocampus x MWM (A), and NGF prefrontal cortex x MWM (A). Considering that the strongest correlation found in this study was between NGF prefrontal cortex x MWM (A); moderate correlations between BDNF hippocampus x MWM (A), BDNF hippocampus x MWM (B), BDNF prefrontal cortex x MWM (B), NGF hippocampus x MWM (A) and NGF hippocampus x MWM (B); and weak correlations between BDNF prefrontal cortex x MWM (A) and NGF prefrontal cortex x MWM (B).

3.3. Docking molecular

The biological effects of chrysin may be explained by the modulation of TrkA, TrkB, and p75NTR.

Significant interactions were formed between chrysin with the binding positions of TrkA, TrkB and p75NTR, shown in Figs. 4–6, respectively. The chrysin possesses a docking score of −6.2 kcal/mol on domain 5 of TrkA receptor through protein-ligand interactions as hydrogen bonds with THR352 and ASN355, with additional hydrophobic interactions such as Pi-Pi with PHE327 and Pi-Alkyl with ILE328 (Fig. 4A and B). In addition, to validate our molecular docking protocol and compare ligand interactions, we also tested as a positive control the TrkA agonist 3β 6β-dichloro-5-hydroxy-5α-Cholestane (Hannan et al., 2019). The obtained results show a molecular docking score of −6.6 kcal/mol, with protein-ligand interactions as hydrogen bond with

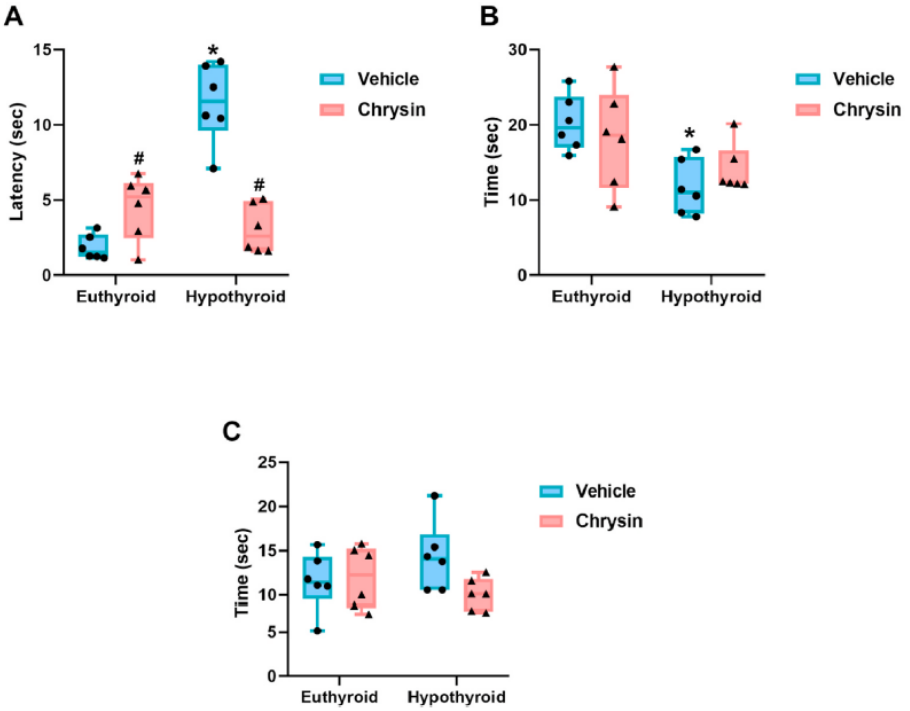

Fig. 2. Effects of hypothyroidism and chrysin on the MWM test. (A) Latency to find the quadrant of the platform. (B) Time spent in the aim quadrant. (C) Time spent in the opposite quadrant. *Significant difference compared with the euthyroid group. #Significant difference compared with the hypothyroid group. Data are shown as mean ± interquartile range (n = 6 per group).

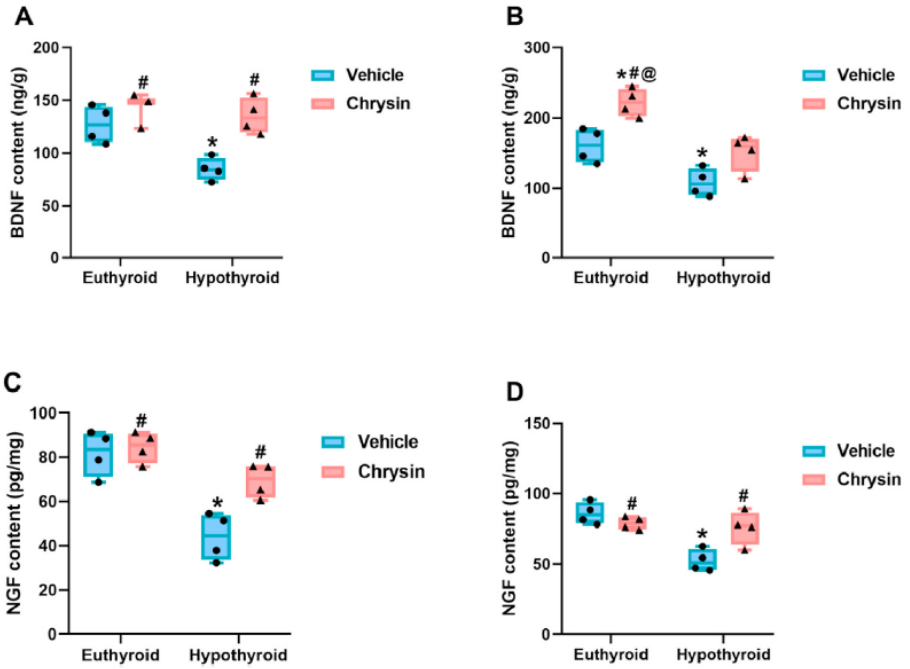

Fig. 3. Effects of hypothyroidism and chrysin on neurotrophins levels. (A) BDNF content in hippocampus. (B) BDNF content in the prefrontal cortex. (C) NGF content in hippocampus. (D) NGF content in the prefrontal cortex. *Significant difference compared with the euthyroid group. #Significant difference compared with the hypothyroid group. @Significant difference compared with the hypothyroid chrysin group. Data are shown as mean ± interquartile range (n = 3–4 per group).

Table 2
Effects of chrysin on the correlation between neurotrophins levels in the hippocampus and prefrontal cortex with MWM.

	r	P
BDNF hippocampus x MWM (A)	−0.5413	0.0304*
BDNF hippocampus x MWM (B)	0.5116	0.0428*
BDNF prefrontal cortex x MWM (A)	−0.2805	0.2927
BDNF prefrontal cortex x MWM (B)	0.5337	0.0332*
NGF hippocampus x MWM (A)	−0.6567	0.0057**
NGF hippocampus x MWM (B)	0.6243	0.0097**
NGF prefrontal cortex x MWM (A)	−0.8584	<0.0001****
NGF prefrontal cortex x MWM (B)	0.3969	0.1280

Morris Water Maze (MWM) task (A) Latency to find the platform; (B) Time in the correct quadrant. *$p < 0.05$ is considered significant.

THR325 and hydrophobic interactions as Pi-Alkyl with PHE317 and ILE328 and Alkyl with LEU322 and PHE327 (Fig. 4C and D).

The affinity and binding mode of chrysin on TrkB, revealing an energy-free binding of −6.2 kcal/mol through a hydrogen bond with THR306 and PHE306 with additional Pi-Stacked with PHE291, Pi-Alkyl with PRO304, and Pi-Sulfur with CYS345 (Fig. 5A and B). Similarly, the utilized positive control of this protocol, the potent TrkB agonist 7,8-dihydroxyflavone (Jang et al., 2010; Liu et al., 2016) presented a docking score of −6.1 kcal/mol forming hydrogen bonds with HIS335 and GLY344, Pi-Pi Stacked with PHE291 and Pi-Alkyl with CYS345 (Fig. 5C and D).

The chrysin has a docking score of −6.7 kcal/mol on p75NTR, stabilized through hydrogen bonds with TYR85, ALA82, and CYS81, Van der Waals with GLY84, and additional hydrophobic interactions such as Pi-Alkyl with VAL133 and Pi-Sulfur X with CYS97 (Fig. 6A and B). Since EVT901 is a widely accepted p75NTR antagonist, it was used as our positive control (Delbary-Gossart et al., 2016; Lee et al., 2016). EVT901 presented free binding energy of −6.7 kcal/mol with ligand-protein interactions as conventional hydrogen bond with TYR83, ARG80, and GLY84, Van der Waals with ALA82 and TYR85, Pi-Alkyl with HIS32, Pi-Stacked with TYR83, and Fluorine with CYS81 and GLN111 (Fig. 6C and D).

4. Discussion

Hypothyroidism causes some adverse effects on cognitive function (Accorroni et al., 2017; Bortolotto et al., 2020; Chaalal et al., 2014, 2018; Pan et al., 2013). Our work corroborates with these studies, showing that animals with hypothyroidism had memory loss. In addition, we showed that the neurotrophinergic system is involved in hypothyroidism, in which BDNF and NGF levels were decreased in the hippocampus and prefrontal cortex of these animals. Furthermore, our results indicate that chrysin possesses an effect in restoring the memory deficit caused by hypothyroidism, certainly by restoring the levels of BDNF in hipocamppus and NGF in both structures, and for the great affinity of chrysin for neurotrophinergic receptors.

Our data were obtained regardless of the levels of thyroid hormones that remained low in the hypothyroid groups (Table S2), as in our previous studies (Bortolotto et al., 2018, 2020). For validating the results obtained from the memory test, it was important that the OFT test was not significant, so we can consider that the results obtained in MWM.

In this sense, our results of MWM test showed that animals that had hypothyroidism delayed more time to find the aim quadrant and spent less time in that same quadrant, accordingly with others studies (Baghcheghi et al., 2017, 2018; Pan et al., 2013), while animals that were treated with chrysin delayed less time to find the aim quadrant and spent more time there, accordingly (Bortolotto et al., 2020; He et al., 2012; Souza et al., 2015). This long-term spatial deficit can be associated with several pathways; one of them may be the neurotrophinergic pathway. In summary, this system promotes neuroplasticity, which is nothing more than the brain's own ability to keep developing (Calabrese et al., 2014), promotes axonal growth, neuronal survival (Zanin et al., 2019), respond and adapt to environmental challenges, using functional

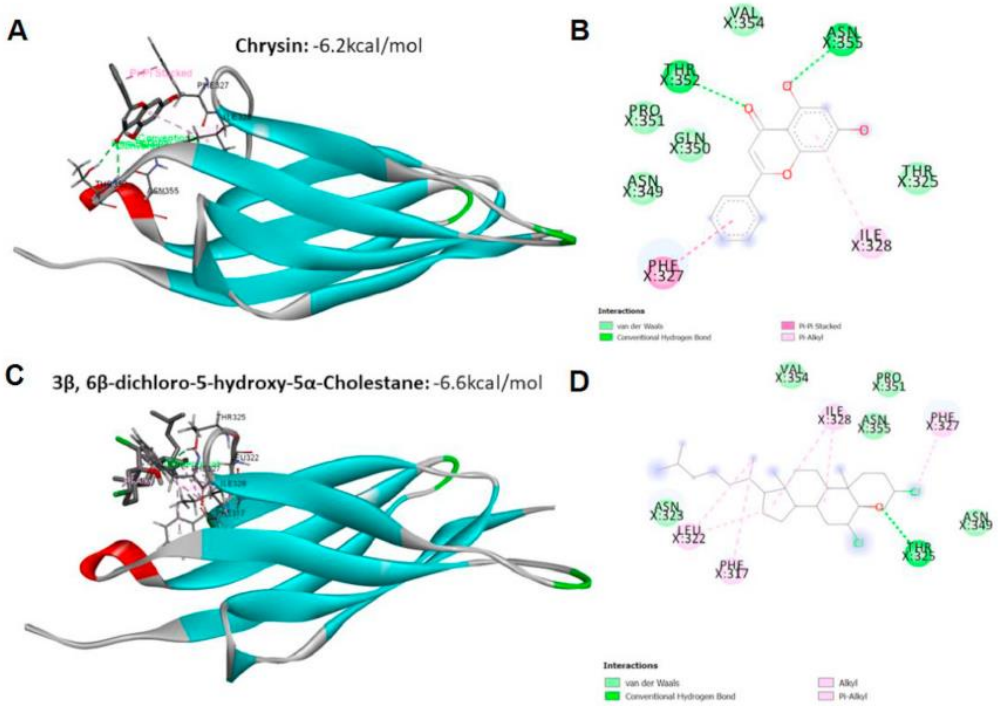

Fig. 4. Binding affinity and protein-ligand interactions of TrkA receptors with chrysin on (A) 3D representation (B) 2D representation and 3β, 6β-dichloro-5-hydroxy-5α-Cholestane on (C) 3D representation (D) 2D representation. Data were obtained using the software AutoDock Vina followed by Discovery Studio Visualizer.

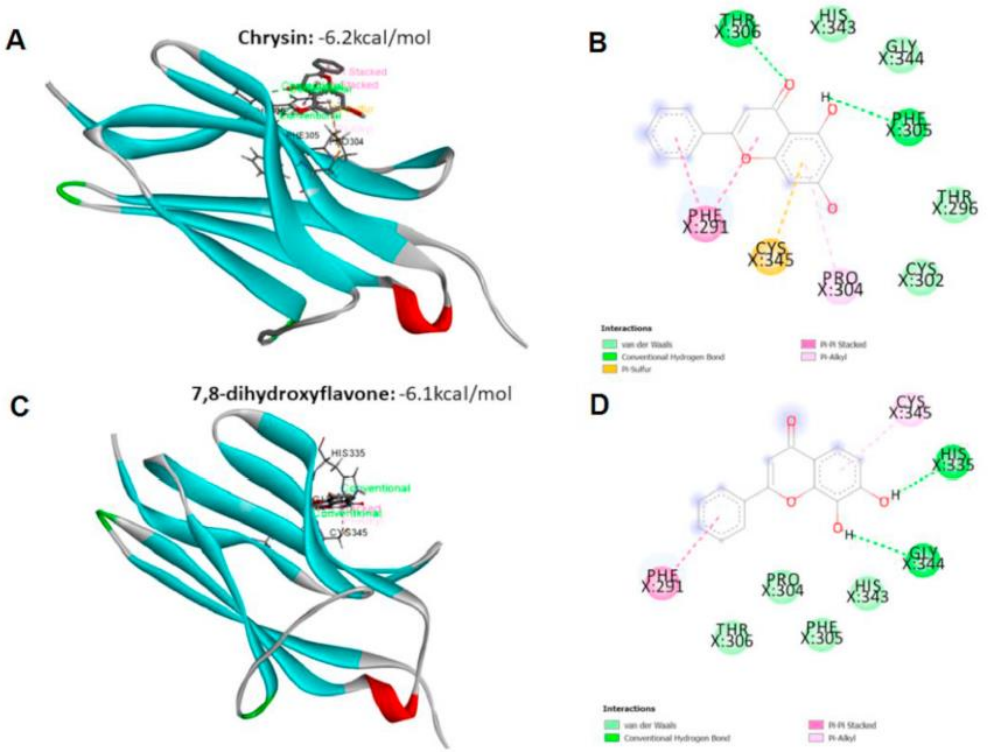

Fig. 5. Binding affinity and protein-ligand interactions of TrkB receptors with chrysin on (A) 3D representation and (B) 2D representation and 7,8-dihydroxyflavone on (C) 3D representation and (D) 2D representation. Data were obtained using the software AutoDock Vina followed by Discovery Studio Visualizer.

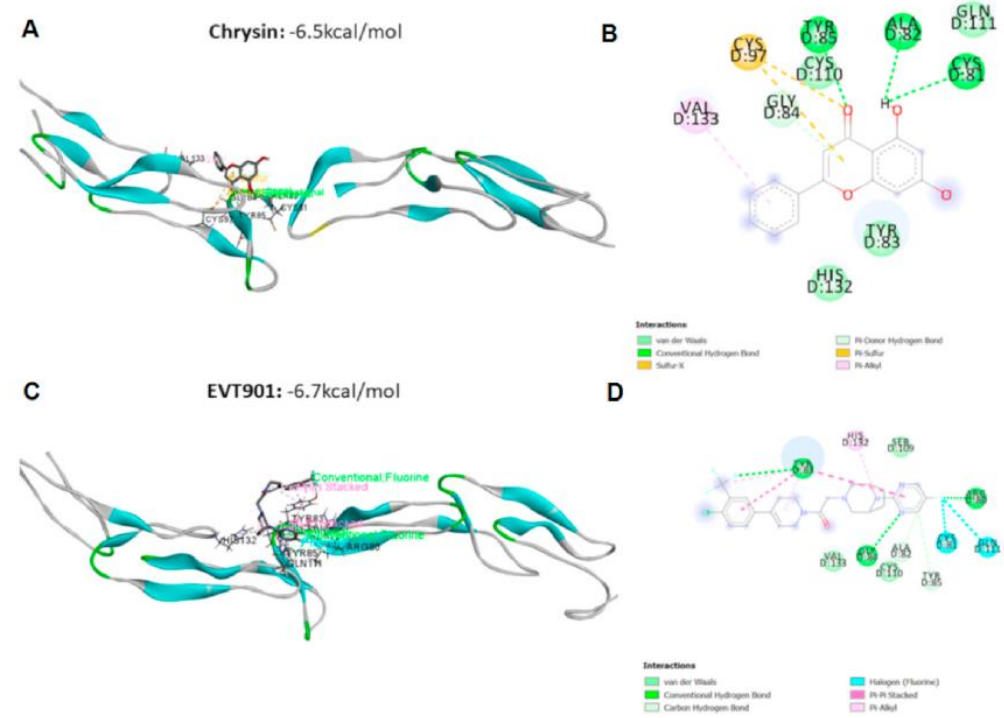

Fig. 6. Binding affinity and protein-ligand interactions of p75NTR receptors with chrysin on (A) 3D representation (B) 2D representation and EVT901 on (C) 3D representation (D) 2D representation. Data were obtained using the software AutoDock Vina followed by Discovery Studio Visualizer.

and structural mechanisms leading to the formation of novel synapses, the birth of new neurons and neuronal remodeling (Calabrese et al., 2014). Furthermore, the hippocampus is the critical region for spatial memory (Burgess et al., 2002), and for the neuroplasticity occur, the neurotrophin family of trophic factors is needed: NGF, BDNF, NT3, and NT4; on the other hand, this is only possible by signaling via Trk and p75NTR receptors (Zanin et al., 2019), also the BDNF is one of the key molecules for spatial memory (Kaptan et al., 2019).

Furthermore, taking into account that LTP is a form of synaptic plasticity (Martin et al., 2000), and one form of LTP are in the glutamatergic system, while the most prominent form is induced following activation of the N-methyl-D-aspartate (NMDA) receptor (Martin et al., 2000). In our previous study (Bortolotto et al., 2020), we showed the relationship between the glutamatergic system in animals hypothyroid and the efficiency of chrysin as the treatment. Still, certain neurotrophins have been implicated in rapid neuronal excitability control as well as in LTP (Arias et al., 2014). We can deduce that the role of learning and synaptic plasticity may be associated with the effect on the modulation of neurotrophins in hypothyroidism.

Thus, in our study, the animals with hypothyroidism have a decrease in BDNF and NGF levels in the hippocampus and prefrontal cortex, accordingly (Gilbert et al., 2016; Kim et al., 1998), showing a decline in neuronal plasticity associated with hypothyroidism. As expected, chrysin was able to reverse this decrease of BDNF in hippocampus and NGF in both structures. These results demonstrate a close relationship between the hippocampus, spatial memory and neurotrophins, mainly BDNF, for being a long treatment, probably this effect of chrysin may have been by increasing neurotrophins synthesis, may result in: facilitating the neurodevelopment, neural differentiation, cell survival, and facilitates the induction of LTP, all this plays a crucial role in synaptic plasticity and cognition (Wu et al., 2020; Yamada and Nabeshima, 2004). Our results are accordingly with previous studies (Filho et al., 2016b; Goes et al., 2018; Souza et al., 2015) still, it is essential to mention that chrysin had a per se effect BDNF on the prefrontal cortex, showing its positive action and affinity for the BDNF receptor. Thus, following our hypothesis about the relationship between memory deficit and decreased brain plasticity in hypothyroidism mice, our data are shown in Pearson's correlations. They demonstrate that when there is a more significant loss of memory, there is also a decrease in neurotrophins levels, being dependent variables.

Because of investigating the mechanism of action underlying the therapeutic effects of chrysin on memory impairment and up-regulation of BDNF and NGF levels, we evaluated the molecular affinity and binding mode of this molecule on the main receptors of the neurotrophinergic system. In this sense, Trk receptors are members of a transmembrane tyrosine kinases family composed of TrkA, TrkB, and TrkC. Interestingly, NGF primarily binds TrkA. Meanwhile, BDNF mainly interacts with TrkB, initiating receptor homodimerization and autophosphorylation of cytoplasmic tyrosine residues on the receptors (Haddad et al., 2017). The docking of these neurotrophins induces a cascade of cell signaling events culminating in the prevention of apoptotic cell death, cellular differentiation, neurogenesis, and positive feedback promoting BDNF and NGF expression (Canossa et al., 1997; Qian et al., 2006).

Our results indicate that besides the similar docking score of chrysin and 3β, 6β-dichloro-5-hydroxy-5α-Cholestane, they also stay in a similar position TrkA receptor, strongly suggesting that chrysin could also act as a TrkA receptor agonist. Interestingly, THR325 and THR352 have already been suggested as essential residues in the facilitation of NGF binding to TrkA (Scarpi et al., 2012), and ASN355 has an important rotational role for the binding receptor conformation (Benito-Gutiérrez et al., 2005; Settanni et al., 2003).

Since chrysin, formally named 5,7-dihydroxyflavone, have an extremely similar chemical structure compared to 7,8-dihydroxyflavone we had already expected a positive affinity with TrkB. Besides that, they stay in the same position on the receptor's binding site, differing from each other on the types of interactions that could indicate a weaker affinity since the interaction with HIS335 seems to be very important on TrkB agonists (Cazorla et al., 2011). Indeed, is already shown that 5, 7-dihydroxyflavone is not a potent agonist of TrkB and do not increase the levels of BDNF *in vitro* (Liu et al., 2014), although several published studies have shown the ability of chrysin upregulate the levels of BDNF in mice (Filho et al., 2015, 2016b; Souza et al., 2015). We suggest that this could be partially explained by a similar affinity with TrkB but slightly different binding modes, exerting a weaker effect on BDNF levels, being able to not exert effect per se but to counteract harmful events depending on the stimuli, or in longer treatment protocols.

Besides TrkB and TrkA, the low-affinity p75NTR is also a target for both BDNF and NGF. Growing evidence has suggested a functional role on the antagonism of p75NTR for therapeutical modulation of neuroinflammation and cell death. Considering the affinity of chrysin with TrkB and TrkA, our next step consisted on the elucidation of the binding mode of this molecule on p75ntr, to better understand if its mechanism of action involves the activation or inhibition of this receptor. Both chrysin and the positive control EVT901 have similar binding affinity and stay in very close proximity to the structure of p75NTR, which might suggest exert a similar inhibitory effect. Given these results, we suggest that the effect of chrysin might be partially attributed to the proposed inhibitory effect on p75NTR, preventing the BDNF downregulation through pro-inflammatory conditions (Calabrese et al., 2014).

This study provides a comprehensive effect of chrysin with data on the relationship between memory loss and the neurotrophic system in hypothyroidism. Our results provide a new insight that demonstrates the role of chrysin on memory improvement in hypothyroidism. This memory improvement is promoted by the modulation of BDNF and NGF, mainly in hipocamppus, probably due a long treatment with chrysin, and by its affinity through the bind with Trk or p75NTR receptors, potentiating LTP.

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, and MRP performed the behavioral experiments. MGF and LS realized the docking molecular. VCB interpreted data and statistical analysis and drafting of the manuscript. Critical revision of the manuscript: SPB and MP.

CRediT authorship contribution statement

Vandreza Cardoso Bortolotto: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. **Stífani Machado Araujo:** Data curation, Formal analysis, Investigation. **Franciane Cabral Pinheiro:** Data curation, Formal analysis, Investigation. **Márcia Rósula Poetini:** Data curation, Formal analysis, Investigation. **Luana Barreto Meichtry:** Data curation, Formal analysis, Investigation. **Mariana G. Fronza:** Data curation, Formal analysis, Investigation, Software. **Silvana Peterini Boeira:** Funding acquisition, Resources, Validation, Writing – review & editing. **Lucielli Savegnago:** Software, Validation, Visualization, Writing – review & editing. **Marina Prigol:** Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – review & editing.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jpsychires.2021.10.018.

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Supplementary material

Table S1. Statistical analysis.

Results	Interaction	
OFT (distance)	$F_{1, 20} = 4.131$, P = 0.0556	
OFT (rearings)	$F_{1, 20} = 2.899$, P = 0.1041	
OFT (velocity)	$F_{1, 20} = 0.1713$, P = 0.6834	
MWM (A)	$F_{1, 20} = 48.91$, P < 0.0001	
MWM (B)	$F_{1, 20} = 1.406$, P = 0.2496	Main effect of hypothyroidism: $F_{1, 20} = 11.51$, P = 0.0029
MWM (C)	$F_{1, 20} = 3.154$, P = 0.0909	
BDNF hippocampus	$F_{1, 11} = 4.569$, P = 0.0559	Main effect of hypothyroidism: $F_{1, 11} = 9.039$, P = 0.0119 Main effect of chrysin: $F_{1, 11} = 16.08$, P = 0.0021
BDNF prefrontal cortex	$F_{1, 12} = 0.6562$, P = 0.4337	Main effect of hypothyroidism: $F_{1, 12} = 29.63$, P = 0.0001 Main effect of chrysin: $F_{1, 12} = 21.18$, P = 0.0006
NGF hippocampus	$F_{1,12} = 6.317$, P = 0.0272	
NGF prefrontal cortex	$F_{1,12} = 12.81$, P = 0.0038	

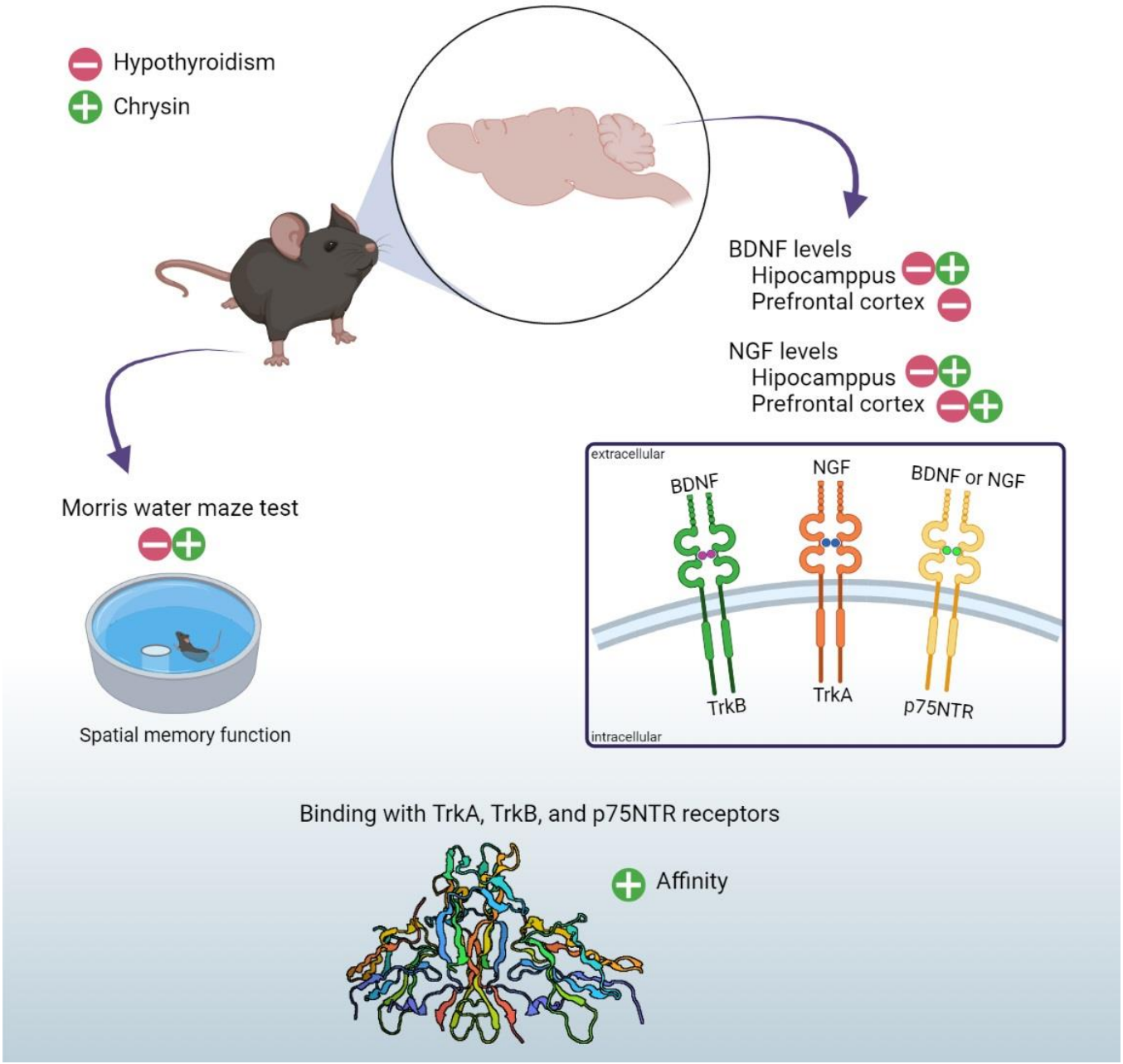
Open Field Test (OFT); Morris Water Maze (MWM) task (A) Latency to find the platform; (B) Time in the correct quadrant; (C) Time in the opposite quadrant. The results were compared using two-way ANOVA and Tukey *post-hoc* for multiple comparisons. * $p<0.05$ is considered significant.

Table S2. Plasma tT3 and tT4 total levels in mice treated with MTZ and chrysin on the 32nd and 63th day.

	tT3 (ng/dL)	tT4 (mcg/dL)
32 days		
Euthyroid	75.25 ± 5.17	1.53 ± 0.05
Hypothyroid	62.21 ± 5.21*	0.96 ± 0.20*
63 days		
Euthyroid	75.22 ± 5.12	1.52 ± 0.05
Euthyroid Chrysin	70.53 ± 0.80	1.46 ± 0.13
Hypothyroid	59.61 ± 3.68* #	0.98 ± 0.22* #
Hypothyroid Chrysin	57.30 ± 0.42* #	0.97 ± 0.21* #

Values are expressed as means ± S.D. 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.

Graphical abstract



6. Discussão

Esta tese buscou investigar o envolvimento dos sistemas glutamatérgico, colinérgico e neurotrofinérgico no déficit de memória induzido pelo hipotireoidismo, em camundongos C57BL/6 fêmeas e, possível ação terapêutica da crisina em atenuar este prejuízo cognitivo e atuar sobre estes sistemas.

Nesta tese, que está separada em dois artigos, utilizamos animais do sexo feminino, pois a prevalência do hipotireoidismo é maior em mulheres (MORGANTI *et al.*, 2005). Sendo assim, realizamos testes comportamentais padronizados para o modelo experimental utilizado, como o teste de campo aberto para avaliar a atividade locomotora, e testes de memória de longa duração como o labirinto aquático de Morris e a esquiva passiva. Não houve alteração locomotora entre os grupos experimentais. Os testes comportamentais demonstraram um déficit de memória ocasionado pelo hipotireoidismo, em ambos os estudos. Estes déficits foram atenuados pela ação da crisina, provavelmente devido a sua capacidade em atravessar a barreira hemato-encefálica, atingindo assim, alvos terapêuticos cerebrais (EL-HAWARY *et al.*, 2021).

Em um primeiro momento (artigo 1), buscou-se investigar se os sistemas glutamatérgico e colinérgico estavam envolvidos na fisiopatologia do hipotireoidismo e se a crisina atuaria nestes sistemas. Neste sentido, ao final dos tratamentos, realizamos testes comportamentais relacionados a memória e aprendizagem e, após a eutanásia, realizamos dosagens do neurotransmissor glutamato, atividades da Na^+, K^+-ATPase e acetilcolinesterase, no hipocampo e córtex pré-frontal. Após estas análises, este primeiro estudo demonstrou que há uma conexão entre o déficit de memória espacial e também da memória aversiva com a diminuição dos níveis de glutamato e atividade da Na^+, K^+-ATPase e, que o tratamento com crisina foi capaz de reverter o déficit de memória e os parâmetros *ex vivo*.

Neste sentido, um grupo composto por Maragos e Greenamyre *et al.* desde 1987 (MARAGOS *et al.*, 1987; GREENAMYRE *et al.*, 1988), estudavam a relação entre o sistema glutamatérgico e a doença de Alzheimer em humanos e, este assunto continua sendo estudado experimentalmente (SONG *et al.*, 2021; TURKEZ *et al.*, 2021). Esta relação vem ao encontro com nossos resultados em um modelo de hipotireoidismo, em que este ocasiona um déficit de memória e causa prejuízos em alguns mecanismos moleculares essenciais para consolidação da memória de longa duração do sistema glutamatérgico.

Em nosso estudo, a diminuição nos níveis de glutamato, pode ser explicada pela diminuição dos mecanismos dos receptores pré-sinápticos ocasionado pelo hipotireoidismo (SHUAIB *et al.,* 1994). Ainda, a crisina mostrou-se eficaz em normalizar estes níveis, estes resultados foram demonstrados pela primeira vez até o momento em nosso estudo.

Sabe-se ainda, que quando há uma diminuição nos HT, em consequência do hipotireoidismo, há também uma diminuição na síntese de Na^+, K^+-ATPase (MANDAL *et al*., 2016), nossos resultados demonstram a atuação da crisina, somente no SNC, uma vez que os níveis dos HT se mantiveram baixos ao final dos experimentos. Esta atuação da resposta da crisina na atividade da Na^+, K^+-ATPase corrobora com estudos anteriores do nosso grupo, a partir de outros indutores de déficit de memória (FILHO *et al.*, 2015; SOUZA *et al.*, 2015). É importante ressaltar ainda que, a inibição da Na^+, K^+-ATPase, está associada a déficits cognitivos uma vez que esta interfere nos processos do SNC (MANDAL *et al*., 2016).

Na avaliação da atividade enzimática da AChE, tanto os animais eutireoideos quanto os hipotireoideos, tratados ou não com crisina, estava em equilíbrio. Uma vez que a AChE é uma enzima chave para os efeitos colinérgicos atuando nas fases de processamento da memória (ROBINSON *et al.*, 2011). Esperávamos que houvesse uma diminuição da atividade da AChE nos animais hipotireoideos, para que assim, pudéssemos analisar a ação da crisina no sistema colinérgico, pois há estudos que demonstram essa alteração no hipotireoidismo (WANG *et al*., 2014a; 2014b), contudo já foi demonstrado que a concentração cerebral de colina em humanos não estão relacionadas com os HT (ZHANG *et al.,* 2015), corroborando com nossos resultados.

Em conjunto estes dados fornecem novas evidências que demonstram a atuação da crisina como atenuante dos problemas cognitivos de longa duração causados pelo hipotireoidismo, relacionados ao sistema glutamatérgico. Sabendo que este sistema está envolvido com a formação da memória e aprendizado, por modular a plasticidade sináptica e também a LTP (MARMIROLI & CAVALETTI, 2012), seguimos ao nosso próximo estudo para analisar a plasticidade neuronal.

Em um segundo estudo (artigo 2), investigamos se havia atuação do sistema neurotrofinérgico no déficit de memória ocasionado pelo hipotireoidismo e possível atuação da crisina. Neste sentido, realizamos análises comportamentais de memória espacial, e após a eutanásia seguimos com dosagens de neurotrofinas. Observamos no hipotireoidismo que o sistema neurotrofinérgico possui os níveis de BDNF e NGF diminuídos nas estruturas cerebrais

de hipocampo e córtex pré-frontal e, que a crisina está envolvida na restauração do déficit de memória, principalmente no hipocampo. Adicionalmente, realizamos o *docking molecular* para identificar se a crisina possuía afinidade aos receptores do BDNF e NGF, assim confirmamos nossos dados, pelas interações em que a crisina é capaz de fazer, possuindo os desvio médio da raiz quadrática (RMSD, do inglês *Root Mean Square deviation*), muito similares aos controles positivos dos respectivos receptores.

Neste segundo estudo, ratificamos o papel do sistema neurotrofinérgico em camundongos fêmeas adultas com hipotireoidismo. Este sistema possui a capacidade de manter-se em desenvolvimento (CALABRESE *et al.*, 2014), promove o crescimento axonal e sobrevivência neuronal (ZANIN *et al.*, 2019), utiliza mecanismos que levam a formação de novas sinapses e de novos neurônios, ocasionando a remodelação neuronal (CALABRESE *et al.*, 2014), em resumo, este sistema é capaz de responder e se adaptar a novos desafios/tarefas.

Em um primeiro momento, observamos um déficit de memória através do teste comportamental, e também a reversão deste déficit ocasionada pela crisina. Nas análises *ex vivo*, houve uma diminuição dos níveis das neurotrofinas nas duas regiões encefálicas estudadas, estes dados estão de acordo com o estudo de Gilbert *et al.* (2016). Complementarmente, a ação da crisina em reverter a diminuição dos níveis de neurotrofinas estão de acordo com estudos anteriores do nosso grupo (FILHO *et al.*, 2016b; GOES *et al.*, 2018; SOUZA *et al.*, 2015) e, a análise de *docking molecular* confirmou a afinidade da crisina com os receptores do BDNF e NGF, assim, inferimos que estas ligações podem ter sido a causa da melhoria nos níveis destas neurotrofinas. Deste modo, estes dados em conjunto demonstraram a capacidade da crisina em modular os níveis de neurotrofinas, em consequência reduzindo o déficit de memória e ocasionando um certo desenvolvimento/plasticidade neuronal e LTP.

Sabe-se que a LTP é uma forma de plasticidade sináptica, e um tipo desta é realizado através da ativação do receptor N-metil D-aspartato (NMDAR) do sistema glutamatérgico (MARTIN *et al.*, 2000). Tomados em conjunto, os resultados dos dois estudos desta tese complementam-se, uma vez que para que ocorra a reversão do déficit de memória, é interessante que o cérebro possua plasticidade sináptica, este, por sua vez, terá LTP que será ativado por NMDAR de glutamato, sendo esta um tipo de cascata de sinalização neuronal.

Não podemos excluir que há diversas moléculas que podem ter sido ativadas e inibidas nestes sistemas, contudo, com os resultados obtidos conseguimos demonstrar que o hipotireoidismo atua ocasionando danos em marcadores celulares destas vias e, consequentemente, o déficit de memória e, que a crisina atua melhorando os danos causados pelo hipotireoidismo em alguns marcadores estudados destes sistemas, observado tanto em análises *in vivo, ex vivo* e *in sílico.*

Em suma, nossos estudos possuem um papel importante, fornecendo novas evidências que podem ser utilizadas para melhor elucidar essa disfunção endócrina, ainda, traz a crisina como uma alternativa no tratamento do déficit de memória ocasionado pelo hipotireoidismo no SNC.

7. Conclusão

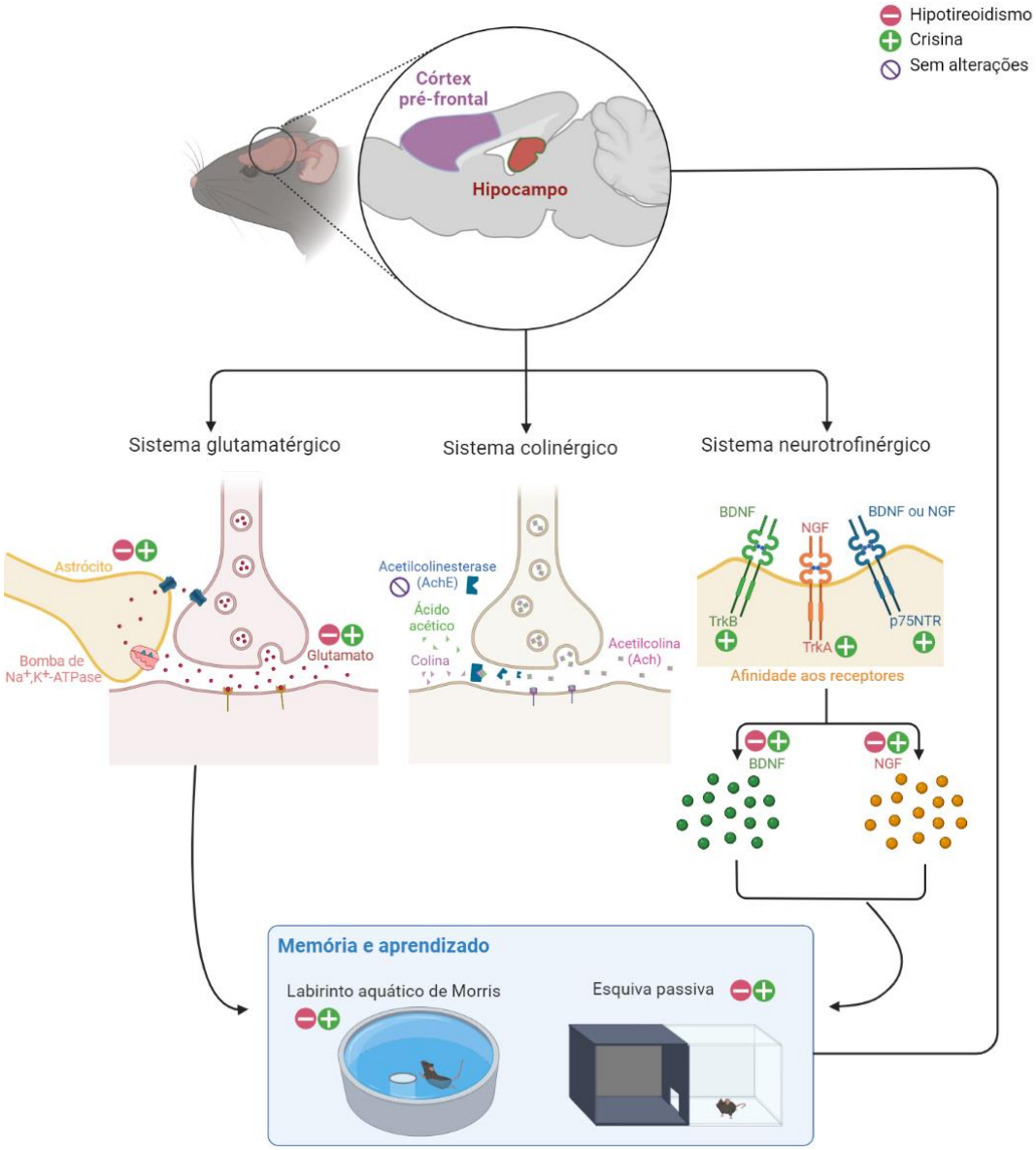
Baseado nos resultados realizados nesta tese pode-se concluir que:

1. Os animais com hipotireoidismo possuem um déficit cognitivo, tanto nas memórias espacial quanto aversiva à longo prazo, e a crisina foi capaz de reverter esses déficits;
2. Dois dos sistemas estudados estão envolvidos no déficit de memória ocasionado pelo hipotireodismo:
    a. Em relação ao sistema glutamatérgico, houve uma diminuição tanto nos níveis de glutamato quando na atividade enzimática da Na^+,K^+-ATPase nas estruturas cerebrais hipocampo e córtex pré-frontal dos animais hipotireoideos;
    b. No sistema neurotrofinérgico houve uma diminuição dos níveis das neurotrofinas em ambas estruturas cerebrais estudadas dos animais com hipotireoidismo;
3. A crisina melhorou o déficit de memória ocasionado pelo hipotireoidismo e seu efeito envolveu a modulação de parâmetros associados aos sistemas glutamatérgico e neurotrofinérgico.
4. Ainda, através de estudos *in sílico*, verificamos que a crisina possui alta afinidade com os receptores de BDNF e NGF;
5. O déficit de memória de longa duração observado no presente estudo não envolveu a atividade enzimática da AChE.

Por fim, inferimos que a crisina pode ser reposionada, sendo utilizada como estratégia suplementar, e adjuvante para o tratamento das consequências neurológicas causadas pelo hipotireoidismo, por melhorar a memória de longa duração através de mecanismos moleculares aqui demonstrados. A Fig. 11 demonstra um resumo das principais conclusões da presente tese.

Entretanto, é inegável que o hipotireoidismo pode estar atuando em outras vias neuronais, assim como a crisina também pode amenizar o prejuízo causado pelo hipotireoidismo nestas vias. Sendo assim, mais estudos são necessários para obter mais informações acerca dessa patologia.

Figura 11 – Fluxograma esquemático mostrando as principais conclusões desta tese.



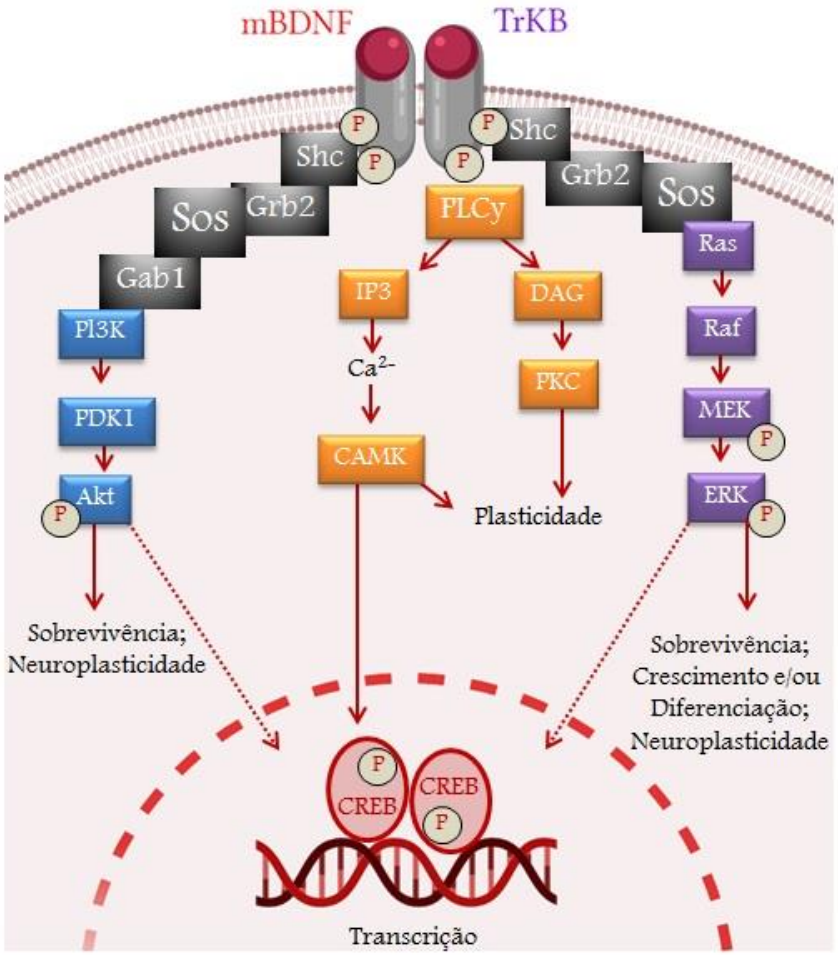
Fonte: Arquivo próprio (2021)

8. Perspectivas futuras

A fim de obter um melhor entendimento a respeito dos mecanismos de ação da crisina sobre as alterações comportamentais induzidas pelo hipotireoidismo, abaixo encontram-se algumas perspectivas futuras:

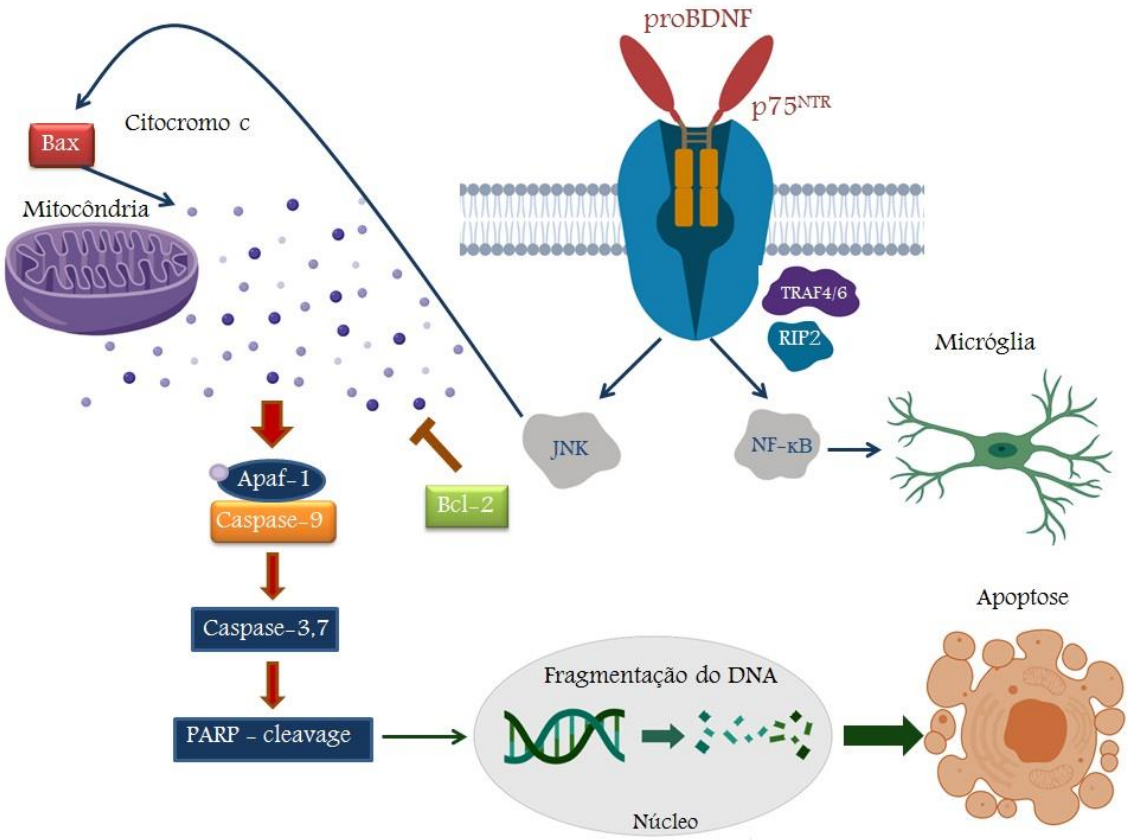
- Avaliar os efeitos da crisina sobre parâmetros comportamentais de esquiva inibitória ativa, e memória de trabalho (labirinto em Y);
- Quantificar através de *western blot* os níveis dos receptores neurotrofinérgicos e alguns marcadores da via de sinalização ativadas por mBDNF/TrkB (Fig. 12) e via de sinalização ativada pelo receptor do proBDNF (Fig. 13).

Figura 12 - Via de sinalização ativadas por mBDNF/TrkB



Fonte: Arquivo próprio (2021)

Figura 13 - Via de sinalização ativada pelo receptor do proBDNF



Fonte: Arquivo próprio (2021)

Ambas as vias são importantes para realização e esclarecimento sobre os locais em que o hipotireoidismo pode estar associado, assim como a possibilidade de restauração com a crisina. É evidente que estas vias podem estar envolvidas na atuação das neurotrofinas, principalmente no hipocampo que é a principal estrutura relacionada com a memória, assim, conseguiremos corroborar os dados obtidos no artigo II desta tese.

- Outro ponto importante é que a partir destas vias saberemos também uma porção da neuroinflamação, levando a próxima perspectiva: explorar a cascata de neuroinflamação. Sabendo que há trabalhos na literatura que demonstram a crisina como agente neuroprotetor por ser anti-inflamatório, utilizando as dosagens de citocinas, pode ser que no hipotireoidismo a via neuroinflamatória também esteja alterada.
- Ainda, realizar-se-á a imunohistoquímica das amostras cerebrais dos animais com hipotireoidismo e tratados com a crisina, quantificando as astrócitos, micróglias e morte

neuronal, uma vez que essa relação das alterações celulares dos animais que passaram por estes tratamentos ainda não foi realizada.

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Anexo

Comitê de Ética no Uso de Animais (CEUA)

MINISTÉRIO DA EDUCAÇÃO
FUNDAÇÃO UNIVERSIDADE FEDERAL DO PAMPA
(Lei nº 11.640, de 11 de janeiro de 2008)

Pró-Reitoria de Pesquisa, Pós-Graduação e Inovação (PROPII)

CEUA
Comissão de Ética em Uso de Animais

COMISSÃO DE ÉTICA NO USO DE ANIMAIS - CEUA

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CERTIFICADO DE APROVAÇÃO DE PROTOCOLO PARA USO DE ANIMAIS EM PESQUISA

Número de protocolo da CEUA: 024/2018

Titulo: Investigação da ação protetora da crisina e possíveis mecanismos envolvidos no déficit de memória induzido pelo hipotireoidismo em camundongos.

Data da aprovação: 11/07/2018

Período de vigência do projeto: 11/07/2020

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CEUA

Finalidade	**( ) Ensino (X) Pesquisa**
Espécie/Linhagem/Raça	Camundongos C57BL/6
Nº de animais	80
Peso/Idade	20 - 22 g/ 3 a 4 meses
Sexo	Fêmeas
Origem	Biotério Central da UFSM

Comissão de Ética em Uso de Animais

Cátia A. Veiverberg

Profª. Drª. Cátia Aline Veiverberg
Coordenadora Substituta CEUA/UNIPAMPA