Triiodothyronine Treatment Reverses Depression-Like Behavior in a Triple-Transgenic Animal Model of Alzheimer's Disease.

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Abstract

Introduction: Alzheimer disease's (AD) is a neurodegenerative disorder and it is known that the central nervous system (CNS) is an important target of thyroid hormones (TH). Moreover, an inverse association between serum triiodothyronine (T3) levels and the risk of AD was reported.

Aim: To evaluate the effects of T3 treatment on the depression-like behavior observed in male transgenic 3xTg-AD mice. **Main methods:** Animals were divided into 2 groups, one group received daily intraperitoneal injections of L-T3 (T3 group), and the other received an equivalent volume of 0.9% saline (Control group). The experimental protocol lasted 21 days, and behavioral tests were conducted on days 15-19. At the conclusion of the experiment, the TH profile and hippocampal gene expression were evaluated.

Key findings: It was observed that the T3-treated group presented significantly increased serum T3 and decreased thyroxine (T4) levels. When compared to control hippocampal samples, the T3 group exhibited attenuated glycogen synthase kinase-3 (GSK3), metalloproteinase 10 (ADAM10), amyloid-beta precursor-protein (APP), serotonin transporter (SERT), 5HT1a receptor, monocarboxylate transporter 8 (MCT8) and bone morphogenetic protein 7 (BMP-7) gene expression levels and augmented superoxide dismutase 2 (SOD2) and Hairless gene expression levels. Additionally, T3-treated animals displayed reduced immobility time in both the tail suspension and forced swim tests, and in the latter presented a higher latency time when compared to the control group.

Significance: Taken together, our results demonstrate that in an AD mouse model, T3 supplementation promotes improvements in depression-like behavior, likely through the modulation of the serotonergic pathway, and also attenuated disease progression.

1. Introduction

Alzheimer's disease (AD) is the most common cause of dementia in the aging population. It has been reported that among AD cases, less than 1% of the patients are under 60 years of age and more than 40% are over the age of 85 (Reitz et al. 2011). It is an irreversible and severe neurological disorder that can slowly progress for years (Stelzmann et al. 1995). Pathologically, AD is characterized by extensive neuronal loss accompanied by accumulated neurofibrillary tangles and amyloid plaques in the brain (Karch and Goate 2015). In addition to the well-documented cognitive decline, natural AD progression includes functional losses and behavioral/psychological changes, which are present in up to 90% of these patients (Masters et al. 2015). While patients commonly present depression, AD-related behavioral impairment is highly heterogeneous (Burns 1990; Teri et al. 1992; Cummings 2000). These cognitive symptoms are likely due to the fact that the serotonergic system is involved in mood and memory and that serotonergic projections to the hippocampus regulate synaptic plasticity processes (Fernandez et al. 2017).

The onset and progression of AD are due to a combination and an accumulation of genetic and environmental factors (Van Rensburg et al. 2006; Letra et al. 2014). For example, mutations in the genes encoding for *beta-amyloid* precursor protein (APP), *apolipoprotein E* (apoE), *presenilin 1* (PSEN1) and *presenilin 2* (PSEN2) have been consistently associated with AD onset (Lambert et al. 2002; Xia et al. 2015). Since those genes are located on different chromosomes, the disease onset likely occurs through a common neuropathogenic pathway. Nevertheless, genetic alterations cannot account for and do not explain all AD cases (Iacono et al. 2014; Xia et al. 2015). Recently, evidence has underpinned the concept that AD is a metabolic and degenerative disease (Procaccini et al. 2016). Also, studies have shown that, at some level, insulin, growth hormone (GH), insulin-like growth factor (IGF-1), cholesterol, adipokine and thyroid hormone (TH) are involved in AD pathogenesis (Fu et al. 2010; Manuel Gomez Saez 2012; Zheng and Tong 2017; Bavarsad et al. 2019).

It is well known that the central nervous system (CNS) is an important target for TH action (Oliveira et al. 2015; Zendedel et al. 2016). During brain maturation, THs play roles in development, differentiation, myelination, cycle stabilization, and also participate in neural and glial signaling (Manzano et al. 2007; Ahmed et al. 2008; Mendes-de-Aguiar et al. 2008). Previous studies investigating hypothyroidism in adult rats showed reductions in features such as, differentiation, survival, and hippocampal progenitor cell neurogenesis, which probably contribute to the observed cognitive and behavioral deficits since these symptoms were reversed following hormone replacement (Desouza et al., 2005; Moog et al., 2017; Zhang et al., 2006). Furthermore, in clinical practice, TH therapy has shown to significantly improve cognition and emotions in patients with AD (Bauer et al. 2008).

In vivo studies have demonstrated that APP gene and protein expression, as well as APP secretase cleavage products, are influenced by thyroid status (O'Barr et al. 2006). In addition, other studies have shown that a negative correlation exists between rT3 or rT3/T3 levels in the cerebral spinal fluid (CSF) and cognitive performance in AD patients (Accorroni et al. 2017). It has been also demonstrated that in euthyroid patients, attenuated T4 concentrations and augmented serum TSH levels are associated with AD-related encephalic changes (Choi et al. 2017).

More recently, an inverse association between serum free T3 and the risk of AD was identified (Quinlan et al. 2019). Although this work is very relevant, the mechanisms by which the decreased serum free T3 levels increase the risk of developing this neurodegenerative disease have not been elucidated. Moreover, peripheral and central administration of T3 improved memory deficits in AD animal models (Farbood et al. 2017). However, the clinical significance of those findings remains unclear. Notably, behavioral alterations, including depression, are commonly observed at different stages of AD, as well as in other neurodegenerative diseases, reaching 80–90% of prevalence in some studies (Steinberg et al. 2004; Cortés et al. 2018). It should be pointed out that T3 alone or in combination with other antidepressants has been successfully used for the treatment of major depression (Cooper-Kazaz and Lerer 2008; Kelly and Lieberman 2009; Touma et al. 2017). Thus, we hypothesized that T3 treatment could reverse the depression-like behavior through molecular changes in the hippocampus of transgenic AD mice.

2. Experimental Procedure

2.1. Animals

Six-month-old male transgenic 3xTg-AD (APP^{swe}, PS1^{m146v}, tau^{P301L}) mice, weighing between 20 and 35 grams, were employed for the study. This animal model of AD expresses three dementia-related transgenes, APPSWE, PS1M146V, and tauP301L, and the progression of the pathophysiological alterations is age-dependent. A previous study showed that at six months, these animals already developed associative learning and spatial working memory deficits, as well as an impairment in contextual fear conditioning (Webster et al. 2014). The animals were supplied by The Jackson Laboratory and were maintained in the vivarium at the Universidade Presbiteriana Mackenzie. The mice were housed in groups of up to 5 animals, in plastic boxes, and maintained in a temperature-controlled room (20-24°C), with a 12-hour light-dark cycle. The mice had free access to food and water throughout the experimental protocol. All procedures were performed in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and were approved by the Animals Ethics Committee of the Universidade Federal de São Paulo (UNIFESP), under protocol # 1880171017. The 3xTg-AD and 3xTg-WT animals were divided into two groups, with one group receiving T3 (T3 group) and the other saline (Control group).

2.2. Experimental Protocol

Hypertriiodothyronemia was induced with a 20 ng/g replacement dose of triiodothyronine (T3) (L-T3, Sigma-Aldrich, St. Louis, MO, USA) administered by IP injection. This dose was considered to be five times greater than the physiological dose. Stock solutions of T3 (1 µg/µL) in 40 mM sodium hydroxide, pH=10 were prepared and frozen until the day of use (Bianco et al. 2014). The solutions were adjusted to pH=7.7 and IP injections were administered using a 1.0 ml syringe. Control animals received injections of 0.9% saline that had the same volume as the injections received by the T3 group. The treatment lasted 21 days, and on days 15-19, behavioral tests were performed.

2.3. Behavioral tests

The behavioral test battery involved open field, tail suspension and forced swimming tests, with a random double-blind distribution. All tests were performed at 48-hour intervals and the testing order was determined according to the degree of invasiveness, starting with the open field, followed by the tail suspension and lastly forced swimming test. Each experimental group contained 4 or 6 mice. All tests were carried out between 9:00 AM and 12: 00 PM, and the researcher present during each test remained outside the experimental area, only entering the room between trials. Each test was recorded with an overhead camera and behavioral parameters were later analyzed by at least two researchers.

2.3.1. Open-Field Test

The open-field test arena was constructed of acrylic, with square, rectangular or circular enclosures (25 to 250cm²), a white floor labeled with black symmetrical squares and walls to prevent animal escape. The

animals were placed in the center of the arena for 5 minutes, and the total number of squares crossed, center ratio, time in center zones and rearing were assessed. Exploratory activity is related to the total distance traveled during the test, vertical activity is associated with the number of rearing, and anxiety-like responses are linked to the center ratio.

2.3.2. Tail Suspension Test

This tail suspension test is based on the observation that when rodents are placed in an inescapable situation they initially exhibit escape-oriented movements and then assume an immobile posture. In this test, the plight involves hemodynamic stress applied by hanging the animals by the tail (Thierry et al. 1986). In our protocol, the mice were suspended in a rectangular box with a suspension bar of sufficient size, in a location along the bar that was away from the walls of the box (Can et al. 2011). The test was videotaped for five minutes, and the immobility time and latency to the first immobility episode were recorded.

2.3.3. Forced Swim Test

This forced swim test is also based on the development of an immobile posture immediately following a stressful situation (Porsolt, R.D., Le Pichon, M., Jalfre 1977) and involves individually placing each mouse in a water-filled polypropylene cylinder (radius=30 cm; depth=50 cm) at 25°C. The animals are unable to escape or touch the bottom of the vessel. Animals were submitted to the test for 5 minutes and immobility time and latency to the first immobility episode were recorded. It is known that immobility time is indicative of low resilience and highly associated with depression-like behavior (Porsolt et al. 1979).

2.4. Total RNA Isolation and Real-Time PCR

Two days after the last behavior test, animals were anesthetized with ketamine (100 mg/ml) and xylazine (20 mg/ml) administered by IP injection in a dose of 0.2 ml of ketamine and 0.1 mL of xylazine per 100g of animal weight. Blood samples were collected from the left ventricle and stored in a freezer at -20°C. These samples were later used for determining serum T3 and total T4 levels. Hippocampal samples were collected, immediately frozen in liquid nitrogen and stored at -80°C. Total RNA from brain tissue corresponding to the murine hippocampus was isolated using the TRIzol® reagent (Invitrogen, Carlsbad, CA, USA), according to the RNA extraction protocol provided by Invitrogen, and RNA concentrations were determined spectrophotometrically by recording the absorbance readings at 260 and 280 nm (NanoDrop® ND-1000 UV-Vis, Delaware, USA). Approximately 2 µg of total RNA from each sample was used to perform the reverse transcriptase reactions. First-strand cDNAs were synthesized using the MML-V reverse transcriptase (Invitrogen, Carlsbad, CA, USA). From the obtained cDNA, cycle curves were performed with each primer set. Real-Time PCR (RT qPCR) was performed using the EVA Green RT-PCR assay (Solis Biodyne, European Union) and run on an ABI PRISM 7700 Sequence Detector (ABI Applied Biosystems). The primers were manufactured by Integrated DNA Technologies (IDT, Sao Paulo, SP, Brazil) and are listed in Table 1.

Table 1
List of primers used for RT qPCR.

Gen Bank	Coded Protein	Function	Primers
GAD1 (NM_008077.5)	Glutamate decarboxylase 1 (GAD67)	GABA synthesis	F: 5'-CTCAGGCTGTATGTCAGATGTTTC-3' R: 5'-AAGCGAGTCACAGAGATTGGTC-3'
GAD2 (NM_008078.2)	Glutamate Decarboxylase 2 (GAD65)	GABA synthesis	F: 5'-TCAACTAAGTCCCACCCTAAG-3' R: 5'-CCCTGTAGAGTCAATACCTGC-3'
TPH2 (NM_173391.3)	Tryptophan hydroxylase (TPH2)	Serotonin synthesis	F: 5'-AGTCTACATCCATCCCAACTGCTG-3' R: 5'-CATTCCTCGCACAATTCCAGTCG-3'
HT1RA (NM_008308.4)	5HT1a receptor	Serotonin Gi protein-coupled receptor	F: 5'-GTGAGAGGAAGACAGTGAAGC-3' R: 5'-CCGTGAGAGGAAGACAGTGAAGAC-3'
SLC6A4 (NM_010484.2)	Serotonin transporter (SERT)	Serotonin Reuptake	F: 5'-CTCACCAGCAGG ACAGAAAG -3' R: 5'-CTCATCTTCACCATTATCTACTTCAG-3'
Bdnf (NM_001048139.1)	Brain-derived neurotrophic factor (BDNF)	Neuroplasticity	F: 5'-AGCAGAGTCCATTGAGCACC-3' R: 5'-TGGCTTGACAGCGAGGAAAA-3'
NTF3 (NM_001164035.1)	Neurotrophin 3 (NTF3)	Maintenance of the nervous system	F: 5'-GGAGTTTGCCGGAAGACTCTC-3' R: 5'-GGGTGCTCTGGTAATTTTCCTTA-3'
NGF (NM_013609.3)	Nerve Growth Factor (NGF)	Nerve growth stimulating	F: 5'-CCAGTGAAATTAGGCTCCCTG-3' R: 5'-CCTTGGCCAAAACCTTTATTGGG-3'
APP (NM_001198823.1)	Amyloid Beta Precursor Protein (APP)	Formation of amyloid plaques	F: 5'- GCGGCAACAGGAACAACCTTT- 3' R: 5'- AACTTTGGGTTGACACGCTG- 3'
GSK3 β (NM_001031667.1)	Glycogen synthase kinase-3 β (GSK3 β)	Regulates beta- amyloid peptides	F: 5'-TCCTTGGACAAAGGTCTTCCG-3' R: 5'-GCGGTAGGTGTGTACTCCAG-3'
ADAM10 (NM_007399.3)	A disintegrin and metalloproteinase 10 (ADAM10)	Cleavage of TNF- alpha and E-cadherin	F: 5'-GAAGATGGTGTGTCGACAG-3' R: 5'-TTTCCATACTGACCTCCCAGC-3'
BACE1 (NM_011792.5)	β -Site APP cleavage enzyme 1 (BACE1)	Formation of amyloid beta peptide	F: 5'-CCCAGAGATCCCAGGGGAAAAG-3' R: 5'-GGAGGCGGAAAGGCTTGT-3'

Gen Bank	Coded Protein	Function	Primers
SOD2 (NM_013671.3)	Superoxide Dismutase 2 (SOD2)	Positively regulated	F: 5'-ACGTGAACAATCTCAACGCC-3' R: 5'-GTCACGCTTGATAGCCTCCA-3'
MCT8 (NM_009197.2)	Monocarboxylate transporter 8 (MCT8)	Negatively regulated	F: 5'-CCCTGGACTTAAGAAGATATACTTGCA-3' R: 5'-CCCGAAGTCCCGGCATA-3'
SLC01C1 (NM_021471.2)	Solute Carrier Organic Anion Transporter Family Member 1C1 (SLC01C1)	Positively regulated	F: 5'-GGATCCCCAGTGGGTCTGGGG-3' R: 5'-ACCAGAAAGGCACGGCTGCA-3'
BMP-7 (NM_007557.3)	Bone Morphogenetic Protein 7 (BMP-7)	Negatively regulated	F: 5'-AGAATTCTTCCACCCTCGATACC-3' R: 5'-TCCTTATAGATCCTGAATTGGGCT-3'
HR (NM_021877.3)	Hairless (HR)	Positively regulated	F: 5'- CGGAGGCAGAGCAACAAGAG-3' R: 5'- GGAGCCTAATCCTGCCATCTC-3'
RPL19 (NM_009078.2)	Ribosomal Protein L19 (RPL19)	Housekeeping (Internal Control)	F: 5'- CTGATCAAGGATGGGCTGAT-3' R: 5'- ACCCTTCCTCTTCCCTATGC-3'

2.5. Serum T3 and T4 Measurements

Serum T3 and total T4 levels were measured using the Elecsys® Kit (Roche Diagnostics, Germany), following the instructions of the manufacturer. Negative controls with Cal set were used before making the measurements and the obtained results were used for calibration. The values were measured automatically and based on a standard curve. For T3, the lower limit of detection for the kit is 1.25 nmol/l and the upper limit is 8.50 nmol/l. For T4, the lower limit of detection is 5.40 nmol/l and the upper limit is 320.0 nmol/l. None of the samples used in the data analyses had values that were outside of these ranges.

2.6. Data Analysis

The RT-PCR data were analyzed using Microsoft Excel and the samples were normalized based on the respective controls. The difference between samples was normalized according to the variation in the mean of CT (Δ CT), which was subsequently used to calculate the relative expression levels of genes of interest. The results from these experiments are expressed in arbitrary units (Livak and Schmittgen 2001).

All results are presented as the mean $\pm$ SEM. The assumption of normal data distribution was determined using the Shapiro-Wilk test. Parametric comparisons were performed with data that passed the normality test. In these cases, between-group comparisons were analyzed with the Student's unpaired t-test. On the other hand, data that did not have a normal distribution were compared using the Mann-Whitney test. The

Grubbs' test was employed for detecting outliers. Cohen's d analysis was used to evaluate the effect sizes between the groups, which is represented by the difference between means divided by the standard deviation. Effect sizes were interpreted as: small ($0.2 < d < 0.5$), moderate ($0.5 < d < 0.8$) and large ($d > 0.8$). Relationships between variables were identified with Pearson correlation analyses. Results with p values of less than or equal to 0.05 were considered statistically significant. All statistical analyses were performed with GraphPad Prism 6 statistical software (La Jolla, CA, USA).

3. Results

3.1. Serum Hormone Measurements

The serum hormone levels of controls and T3-treated mice are summarized in Table 2. We observed that mice administered T3 exhibited a significant increase in serum T3 (5.31 ± 0.53 ng/dl vs. 2.68 ± 0.25 ng/dl, $p=0.00$), and a significant decrease in total T4 (14.9 ± 1.07 µg/dl vs. 80.9 ± 3.02 µg/dl, $p<0.00$) levels when compared to control animals.

Table 2
Serum thyroid hormone levels in control and T3-treated AD mice.
Values are expressed as the mean $\pm$ SEM. n = 6 animals/group.
Different letters in the same column indicate $p<0.05$ as determined by the Student's t-test.

	Total Serum T4 (µg/dl)	Total Serum T3 (ng/dl)
Control	80.9 ± 3.02^a	2.68 ± 0.25^a
T3-Treated	14.9 ± 1.07^b	5.31 ± 0.53^b

3.2. Behavioral tests

3.2.1. Open-Field Test

The open-field test showed no statistically significant differences in the total number of squares crossed (7.40 ± 3.47 vs. 19.20 ± 7.26 , $p=0.18$), center ratio (0.48 ± 0.07 vs. 0.31 ± 0.021 , $p=0.07$), time in center zones (48.00 ± 19.79 vs. 26.20 ± 13.22 , $p=0.37$) or rearing (4.50 ± 1.04 vs. 8.40 ± 1.69 , $p=0.11$) when comparing the control and T3-treated groups (Figure 1a). However, the Cohen's d analysis showed that T3 had a strong effect on the total number of squares crossed, center ratio and rearing (Table 3).

Table 3
Effect size between groups and behavioral parameters.

Behavioral Parameter		Cohen's <i>d</i>
Open Field Test	Total squares crossed	1.01
	Center ratio	1.34
	Time in center zone	<i>0.62</i>
	Rearing	1.27
Tail Suspension Test	Latency to immobility	<i>0.61</i>
	Immobility Time	1.91
Forced Swim Test	Latency to immobility	2.08
	Immobility time	3.59
Numbers in italics represent a moderate magnitude of effect, whereas numbers in bold represent a large magnitude of effect.		

3.2.2. Tail Suspension Test

In the tail suspension test (Figure 1b), the T3 treated animals had a latency time (36.25 ± 12.76 s) that was similar to control mice (63.75 ± 29.04 s, $p=0.42$) (Fig.2A). In contrast, the T3-treated group exhibited a significant reduction in immobility time when compared to control mice (132.50 ± 18.31 s vs. 64.50 ± 17.19 s, $p=0.03$). Additionally, the Cohen's *d* analysis indicated that T3 induced a strong effect on immobility time (Table 3). Furthermore, Pearson correlation analyses showed that immobility time was strongly correlated with SERT ($r = 0.80$), HTR1a ($r = 0.93$), GSK3 β ($r = 0.95$), ADAM10 ($r = 0.95$) gene expression, as well as serum T4 levels ($r = 0.74$).

3.2.3. Forced Swim Test

As shown in Figure 1b, in the forced swim test, which is a classic test for evaluating depression-like behavior, the T3-treated animals exhibited longer latency (9.75 ± 5.60 s vs. 105.80 ± 32.16 s, $p=0.03$) and a shorter immobility time (124.50 ± 9.94 s vs. 53.00 ± 9.95 s, $p=0.01$) than control mice. Cohen's *d* analysis indicated that T3 treatment had a strong effect on latency to immobility and immobility time (Table 3). Moreover, Pearson correlation analyses detected a strong correlation between immobility time and SERT ($r=0.78$), SOD2 ($r = 0.87$) and GSK3 β ($r=0.86$) gene expression, as well as serum T4 levels ($r=0.91$).

3.3. Hippocampal Gene Expression

The Cohen's *d* analysis detected a strong effect on the gene expression levels of SERT, HTR1a, SOD2, MCT8, BMP7, Hairless, GSK3 β and ADAM10 in T3-treated mice (Table 4). Moreover, Pearson correlation analyses identified a strong correlation between serum T3 levels and the expression of serotonergic

system-related genes, including SERT ($r = 0.89$) and HTR1a ($r = 0.78$). Likewise, serum T3 levels were also strongly correlated with MCT8 ($r = 0.87$) and BMP7 ($r = 0.77$) gene expression levels, which have also been shown to be regulated by thyroid hormones. With regards to AD-related genes, there was also a positive correlation found between serum T3 levels and ADAM10 ($r = 0.71$) and GSK3 β ($r = 0.71$) expression levels, and serum T4 levels were correlated with ADAM10 ($r = 0.74$) and GSK3 β ($r = 0.84$) expression. A strong correlation between HT1r expression and ADAM10 ($r = 0.84$) and GSK3 β ($r = 0.74$) expression was also observed.

Table 4
Effect size on gene expression between the control and T3-treated groups.

Coded Protein	Cohen's <i>d</i>
GAD 65	0.33
GAD 67	0.13
TPH2	<i>0.50</i>
SERT	1.84
HTR1a	1.32
BDNF	0.45
NGF	<i>0.53</i>
NTF3	0.03
SOD2	1.79
MCT8	1.97
SLC01C1	0.76
BMP7	2.48
Hairless	1.71
BACE1	0.36
GSK3 β	1.50
ADAM10	1.67
Numbers in italics represent a moderate magnitude of effect, whereas numbers in bold represent a large magnitude of effect.	

4. Discussion

The findings of this study showed that T3 supplementation in the transgenic AD mouse model (APPswe, PS1m146v, tauP301L, 3xTg-AD) promotes improvements in depression-like behavior. These positive

effects appear to be mediated through the modulation of the serotonergic pathway. Previous studies have shown that thyroid hormones play important roles in a variety of organs, including the CNS and an association between AD and hormonal disturbances, such as in hypo- and hyperthyroidism, have been reported (O'Barr et al. 2006; Alois Gessl, Rosa Lemmens-Gruber 2012; Chaker et al. 2016). Herein, we showed that the T3 IP injections increased serum T3 levels and decreased T4 levels and that T3-treated animals presented gene expression level changes that were indicative of a hyperthyroid status in the hippocampus. These results not only demonstrate the clinical accuracy of our hypertriiodothyronemia model but are also in line with previous studies (Hamidi et al. 2010; Denereaz and Medicine 2014). Notably, T3-treated animals displayed signs of improved AD-related depression-like behavior in this model, which was accompanied by the downregulation of genes related to the hippocampal serotonergic system. However, there were no detectable alterations in expression patterns of genes related to the GABAergic system and neurotrophins.

Since it is known that thyroid hormones, including T3, have antidepressant effects, it is plausible that potentiation of monoaminergic neurotransmission could account for the observed T3-mediated behavioral effect, likely through direct modulation of serotonergic system gene expression. According to Bauer et al. (2002), thyroid hormones exert a modulatory effect on depressive behavior by decreasing 5HT1a receptor sensitivity in some areas of the brain (Bauer et al. 2002). More recently, higher hippocampal 5HT1a receptor densities were found in rats with hypothyroidism, possibly representing an early response to the local synaptic loss of serotonin receptors (Lee et al. 2018). Additionally, a substantial body of evidence indicates that central 5-HT metabolism is altered in response to changes in thyroid status (Newman et al. 2000; Lifschytz et al. 2006). For instance, hypothyroid rats were found to have increased levels of 5-HIAA, a 5-HT metabolite, throughout the brain and spinal cord, which was reversed with hormonal replacement. In fact, in thyroid intact rats, T3-releasing implants suppressed 5-HIAA levels within 7 days, in a dose-dependent manner (Henley and Koehnle 1997). Consistent with those findings, our study shows that thyroid hormones modulate the serotonergic pathway, consequently influencing the depression-like behavior commonly associated with AD.

Additionally, it was recently shown that 5HT1a autoreceptors and SERT play an important role in the regulation of serotonergic neurotransmission, through negative feedback and neurotransmitter reuptake mechanisms, respectively (Quentin et al. 2018). In the present study, T3 treatment attenuated 5-HT1a and SERT gene expression in this AD mouse model, suggesting that serotonergic neurotransmission is influenced by T3, which could contribute to the observed antidepressant action. This proposal is further reinforced by the strong correlation between serum T3 levels and the expression of genes involved in these processes, and the strong correlation between the serotonergic system-related genes and the antidepressant parameters associated with each test, especially the tail suspension test.

Interestingly, the beneficial aspects of serotonergic neurotransmission modulation are not restricted to the improvement of AD-affected behaviors but are also involved in delaying disease progression. According to Noristani et al. (2012), a high tryptophan diet reduces CA1 intraneuronal β -amyloid in the 3xTg-AD mouse model, suggesting that enhanced tryptophan intake and consequent increase in 5-HT

neurotransmission could effectively reduce plaque formation in AD (Noristani et al. 2012). In addition, fluoxetine stimulates type 2A protein phosphatases (PP2A), consequently activating Wnt/ β -catenin signaling and inhibiting the hippocampal GSK3 β activity. Furthermore, fluoxetine treatment also reduces APP cleavage and A β generation, thus suggesting that serotonergic targets could be exploited in both AD prevention and treatment (Huang et al. 2018).

In this context, the modulation of 5-HT receptors, especially 5-HT_{2A}, 5-HT_{2C}, and 5-HT₄ receptors, may be beneficial for AD management (Thathiah and De Strooper 2011). The 5-HT₄ receptor seems to be the most promising since it stimulates the non-amyloidogenic cleavage of APP, which is associated with neuroprotection (Cochet et al. 2013). Chronic administration of 5-HT₄ agonists also decreases β -amyloid and neuroinflammation in the brain, slowing AD progression. Interestingly, the inactivation of 5-HT₆ receptors, by different antagonists, has been shown to promote better cognitive performance in numerous behavioral tests (Upton et al. 2008; Ramírez 2013). The beneficial effects of 5-HT receptor modulation in promoting the non-amyloidogenic pathway appears to be mediated by a direct interaction with α -secretase ADAM10 or β -secretase BACE1 (Cochet et al. 2013).

A previous study with AD patients showed that lower hippocampal connectivity is accompanied by higher local neuronal metabolism (Tahmasian et al. 2015). Additionally, a recent review proposed that the role of 5HT_{1a}, in terms of synaptic plasticity, changes with aging, transitioning from a stimulated to inhibited state (Duda et al. 2018). This same review also provided evidence for serotonin-mediated inhibition of GSK3 β expression, via 5HT₁ receptors (Duda et al. 2018). Moreover, 5-HT_{1a} receptor activation was shown to increase the inhibitory serine-phosphorylation of GSK3 β (Li et al. 2004). On the other hand, the dopaminergic system can reduce GSK3 β serine-phosphorylation through D₂ receptor activation, which is due to the inactivation of Akt by protein phosphatase-2 in a β -arrestin2-driven protein complex (Beaulieu et al. 2005). In a mechanism similar to β -arrestin2 recruitment, 5-HT_{2a} receptor activation reduces serine-phosphorylation of GSK3, thereby increasing its activity (Li et al. 2004). Consistent with these observations, GSK3 β inhibitors have been shown to reduce depressive-like behavior in animal models of depression (Duda et al. 2018). Therefore, further studies are needed to understand how T3 treatment can modulate the Akt/GSK3 signaling pathway in AD animal models so that we can gain a better understanding of how T3 reduces depression and downregulates AD-related genes.

In our study, the T3-treated group exhibited a significant reduction in GSK3 β and ADAM10 gene expression. This is particularly relevant to AD since GSK3 is a kinase that contributes to the abnormal phosphorylation during tau protein-mediated microtubule stabilization, a process that leads to neurofibrillary tangle formation (Beurel et al. 2015). Studies have shown that β -amyloid peptide formation results in abnormal APP processing and leads to the induction of tau phosphorylation and GSK3 activation (Kuruva and Reddy 2017). Based on these assumptions, GSK3 β inhibitors have great potential for treating AD. Indeed, in different AD animal models, different GSK3 β inhibitors promoted lower levels of tau phosphorylation and amyloid deposition, rescuing neuronal loss and restoring memory deficits (Serenó et al. 2009; Griebel et al. 2019). Notably, our results identified a strong correlation between

5HT1a, GSK3 β and ADAM10 gene expression, as well as with serum T3 and T4 concentrations. Thus, the T3-induced GSK3 and ADAM 10 downregulation may be indirectly mediated by the serotonergic system.

Herein, the expression of BACE1 is decreased in T3-treated animals. This aspartyl protease is responsible for the amyloidogenic cleavage of APP and it is increased in the brain of patients with AD (Sathya et al. 2012). The T3-treated animals also had reduced ADAM10 gene expression levels. Once translated, this metalloproteinase plays an important role in glutamatergic synapse modulation (Marcello et al. 2017). Indeed, several studies have shown that ADAM10 expression is reduced in patients with moderate to severe AD (Colciaghi et al. 2004; Kim et al. 2009) and it has been speculated that the attenuation ADAM10 expression would consequently increase N-cadherin levels, ultimately resulting in more AMPA receptors and larger, more stable synapses (Malinverno et al. 2010). Moreover, a recent review showed that T3 increases N-cadherin levels in stem-cells repressing EGF-EGFR and cAMP-PKA signaling (Izaguirre and Casco 2016). The Pearson correlation analyses detected a positive association between ADAM10 gene expression and the behavioral parameters of the tail suspension test, as well as with serum T3 and T4 levels. Thus, the results from the present study indicate that T3-mediated ADAM10 attenuation, increases N-cadherin levels, consequently, resulting in better glutamatergic synapses and improved depressive behavior.

Another important aspect that needs to be discussed is mitochondrial dysfunction in AD. Extensive studies have demonstrated mitochondrial degradation by autophagy and impaired oxidative phosphorylation in the brains of AD patients that is induced by reactive APP and β -amyloid (Perez Ortiz and Swerdlow 2019). These deleterious effects are also accompanied by impaired mitochondrial metabolism and increased reactive oxygen species production (Zhao and Zhao 2013). In this context, SOD nitration and inactivation of SOD was observed in the transgenic AD mice model. The decreased activity of this antioxidant enzyme can result in oxidative damage and contribute to mitochondrial dysfunction, consequently resulting in caspase activation and apoptosis (Anantharaman et al. 2006). Furthermore, mitochondrial SOD1 deficiency was shown to promote amyloid β protein oligomerization and memory loss, whereas SOD2 overexpression decreased hippocampal oxidative damage (Murakami et al. 2011). Moreover, increased SOD2 expression also prevented memory deficits in the Tg2576 AD mouse model (Massaad et al. 2009). Notably, in the present study, T3 treatment significantly increased the hippocampal SOD2 expression, which could slow AD progression.

GSK3 β also plays a key role in the regulation of mitochondrial function, modulating mitochondrial biogenesis, bioenergetics, permeability, motility and apoptosis. This protein promotes the activation of caspase-2 and caspase-8, which can induce the release of cytochrome *c*, and lead to mitochondria-dependent apoptosis (Yang et al. 2017). Therefore, the observed downregulation of GSK3 β induced by T3 treatment could represent another mechanism for reducing the impacts of mitochondrial dysfunction in AD.

It is worth mentioning that under normal conditions damaged mitochondria are removed by autophagy; however, in AD, this process is disrupted due to over-activation of the PI3K/Akt/mTOR pathway (Oddo

2012; Góes et al. 2020). As in brown adipose tissue, T3 treatment can reduce mTOR activity and stimulate mitochondrial autophagy (Yau et al. 2019). Additionally, the modulation of mTOR signaling appears to be modulated by non-genomic actions of thyroid hormones through the integrin receptor $\alpha\beta3$ (Ahmed et al. 2013). While promising, further studies will need to be conducted to assess the impact of T3 treatment on AD-related autophagy mechanisms.

A recent study revealed that T3 supplementation was capable of reversing mnemonic deficits and the progression of neuroinflammation in hypothyroid rats. In that study, T3 replacement reduced the expression of A β 42 peptide and proinflammatory markers, including TNF α (Chaalal et al. 2019). While TNF α concentrations are low in healthy adults, this cytokine is increased in the serum and CNS of AD patients (Chang et al. 2017). Interestingly, in primary cultures of astrocytes, TNF α was shown to increase APP and BACE1 expression levels (Zhao et al. 2011), and, in AD animal models, the deletion of TNF α leads to decreased levels of BACE1, ultimately reducing β -amyloid production (He et al. 2007). Furthermore, GSK β activation induces NF- κ B signaling, consequently increasing the transcription of TNF α and other pro-inflammatory cytokines (Maixner and Weng 2013). Taking together with the results from the present study, the T3-induced AD-related gene downregulation may be indirectly modulated by low levels of GSK3 β and TNF α . In figure 3, we summarize all possible T3 actions that could slow the AD progression.

Altogether, both oxidative stress and inflammatory reactions are increased in AD brain, promoting neuroinflammation, which contributes to the pathogenesis of this neurodegenerative disease (Hsieh and Yang 2013). These factors promote neuroinflammation by activating JNK and p38 MAPK signaling cascades. In transgenic mouse models of AD, the activation of these signaling cascades coincide with the age-dependent increase in amyloid deposition, tau phosphorylation (Savage et al. 2002). Moreover, neurodegenerative progression in AD are accompanied by inflammatory responses in activated glial cells, especially microglia and astrocytes, which are also responsible for initiating or exacerbating the neuropathology progression (Morales et al. 2014). Further studies will be necessary to assess the impact of T3 treatment in the regulation of these glial cells and these signaling cascades in order to reduce neuroinflammation.

In conclusion, our data suggest that T3 therapy improves the depression-like behavior observed in an AD transgenic mouse model and that these effects are mediated through the modulation of gene expression in the serotonergic pathway. The benefits of T3 treatment are not only restricted to improving AD-related depressive behavior but are also applicable to disease progression.

Declarations

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Data availability: the datasets generated and analyzed during the current study are not publicly available, but are available from the corresponding author on reasonable request.

Ethics approval: all procedures were performed in this manuscript are in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and were approved by the Animals Ethics Committee of the Universidade Federal de São Paulo (UNIFESP), under protocol # 1880171017.

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Figures

Figure 1

The results presented above show the behavioral parameters of 6-month AD male mice treated with saline (White) and T3 20ng/g (Black) (n=4-5). The evaluated parameters were: total squares crossed in the open field test (A), center ratio in the open field test (B), time in center area in the open field test (C), rearing in the open field test (D), latency to immobility and time of immobility in the forced swim test and tail suspension test (E). Data represent mean ($\pm$ SEM) analyzed by Mann-Whitney test. *p < 0.05 and **p < 0.01 when compared to control group. s: seconds.

Hippocampal Gene Expression

Figure 2

Hippocampal gene expression in 6-month old male AD mice treated with saline (White) or T3 20 ng/g (Black). n=6. Notably, there was a significant downregulation in the expression of serotonin-related genes in the T3-treated group. Data represent mean ($\pm$ SEM) analyzed by Student's unpaired t-test. The significance between groups was * $p<0.05$ and ** $p<0.01$ when compared to control group.

Figure 3

Graphic representation of possible thyroid hormone actions in the hippocampus neurons and its modulation in the expression of AD-related and behavior-related genes. In the presynaptic neuron, the interaction between T3 and thyroid receptors results in binding to TREs and culminates in the negative effect in SERT and 5-HT1A expression. The downregulation of these genes implies in low somatodendritic regulation and low 5-HT reuptake, inducing a higher serotonergic transmission. It is possible that other presynaptic 5-HT auto-receptors could be modulated by T3, such as: 5-HT1B and 5-HT1D. In the postsynaptic neuron, it is possible that the 5-HT4 receptor activation and the inactivation of 5-HT6 receptors would promote a decreased neuroinflammation and better cognitive performance, respectively. Moreover, 5-HT1a receptor activation increase the inhibitory serine-phosphorylation of GSK3 β by PI3K/akt activation and AMPc reduction. The GSK3 β inhibition has a negative effect in AD-related genes. Further studies will be needed to determine whether the genomic actions of T3 could directly reduce AD-related genes expression. In addition, the modulation of mTOR by non-genomic actions of thyroid hormones through the integrin receptor $\alpha\text{v}\beta3$ increases mitochondrial autophagy. Concomitantly, increase in SOD2 expression may decrease oxidative stress activity and apoptosis. Abbreviations: TR (Thyroid Receptor), RXR (Retinoid X Receptor), TRE (Thyroid Hormone Response Elements), T3 (Triiodothyronine), SERT (Serotonin Transporter), 5-HT1A (5-Hydroxytryptamine Receptor 1A), 5-HT1B (5-Hydroxytryptamine Receptor 1B), 5-HT1D (5-Hydroxytryptamine Receptor 1D), 5-HT4 (5-Hydroxytryptamine Receptor 4), 5-HT6 (5-Hydroxytryptamine Receptor 6), mTOR (Mammalian Target of Rapamycin), SOD 2 (Superoxide Dismutase 2), TF (Transferrin), PS2 (Inorganic Pyrophosphatase 1), PKA (Protein Kinase A), ADAM10 (A Disintegrin and Metalloproteinase 10), GSK3B (Glycogen Synthase Kinase-3 Beta), APP (Amyloid Beta Precursor Protein), TNFa (Tumor Necrosis Factor Alpha), PI3K (Phosphoinositide 3-Kinases), AKT (Protein Kinase B), Gs (Protein G), ACs (Transmembrane ACs), PS2 (Presenilin 2).