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Thyroid Hormone Levels in the Prefrontal Cortex of Post-Mortem Brains of Alzheimer's Disease Patients

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Abstract

Converging evidence suggests a possible link between thyroid state and Alzheimer's disease (AD), including a higher probability of dementia in individuals with higher TSH levels and a two-fold risk of AD in patients with hypothyroidism. Thyroid hormones modulate factors associated with AD, including amyloid precursor protein expression in the brain, suggesting a possible role for thyroid hormone in AD pathology. The present study is the first to directly evaluate brain thyroid hormone levels in AD. Triiodothyronine (T₃) and thyroxine (T₄) levels were measured with radioimmunoassay (RIA) in post-mortem samples of prefrontal cortex of patients with pathologically confirmed AD, including Braak stage I–II (*n*=8), Braak stage V–VI (*n*=8), and controls without any primary neurological disease (*n*=8). T₄ levels did not differ between groups. T₃ levels were significantly lower in Braak stage V–VI brains relative to controls, but there was no statistically significant difference between T₃ levels in Braak stage I–II versus controls. Results suggest that the conversion of T₄ to T₃ may be affected in advanced AD, perhaps due to alterations in deiodinase activity. Reduced conversion of T₄ to T₃ in AD may be associated with both AD pathology and the clinical presentation of dementia.

Keywords

Thyroid hormones; thyroxine; Alzheimer's disease; dementia

INTRODUCTION

It is well established that hypothyroidism is associated with mental status changes, including secondary or reversible dementia [1], though the relationship between thyroid disease and risk for Alzheimer's disease (AD) is unclear [2–5]. Some studies have demonstrated a relationship between subclinical thyroid disease (i.e., elevated or reduced thyroid stimulating hormone [TSH] levels with normal T₃ and T₄) and cognitive impairment and Alzheimer's disease (AD) [6]. There is a three- to four-fold higher probability of dementia among individuals with

elevated serum thyroid stimulating hormone (TSH), but without overt hypothyroidism [7], a high prevalence (41%) of autoimmune thyroid disease among familial AD kindreds [8], and a significantly higher rate of antibodies to thyroid peroxidase (TPO) in AD patients compared to controls [9]. There is also a relationship between mood state and thyroid status in euthyroid patients with AD [10].

Despite the link between HPT abnormalities and dementia, the exact nature of the relationship is unclear. Clinical studies of patients diagnosed with AD have identified HPT abnormalities in this population, including increased risk for AD associated with both lower and higher serum TSH levels. Individuals with reduced TSH levels outside of the normal range showed a greater than threefold increased risk of dementia at a two-year follow-up compared to euthyroid individuals [11]. Similarly, individuals with lower TSH values within the reference range have an increased risk for AD after controlling for the effects of potentially confounding medical comorbidities, such as diabetes mellitus and blood pressure [12]. In addition, patients with AD show significantly lower T₃ levels and a blunted TSH response to thyrotropin releasing hormone (TRH) [13]. TRH, a neuropeptide that regulates anterior pituitary release of TSH, is depleted in the hippocampus of post-mortem AD brains compared to controls [14]. Finally, there may be an important association between thyroid state and risk for future cognitive decline and dementia. Volpato and colleagues (2002) found that thyroxine (T₄) concentrations within the normal range were significantly associated with an increased risk for future cognitive decline in a large, community-based sample of older, physically impaired, non-demented women [15]. Compared with women in the highest tertile of T₄ levels, those in the lowest tertile had a twofold risk of future cognitive decline. The interrelationship between levels of thyroid hormone and AD is not without controversy, however, as higher serum total and free T₄ levels were associated with AD pathology at autopsy [16] and higher serum concentrations of T₄ were associated with worse cognition in AD [17]. Similarly, higher serum T₄ levels were associated with a better treatment response to donepezil in individuals with AD [18].

The association between the HPT axis and AD is plausible given that laboratory studies implicate a strong relationship between thyroid state and factors associated with the pathogenesis of AD, including β -amyloid (A β) deposition and neuronal apoptosis. For example, T₃ plays a regulatory role of A β [19], a major component of senile plaques found in AD and central to the pathogenesis of AD [20]. Thyroid response elements (TREs) have been found on the amyloid precursor protein (APP) gene, a precursor of A β , and T₃ negatively regulates APP gene expression by directly repressing the APP promoter [21]. Cell treatment with T₃ also alters the splicing of APP and secretion of its various isoforms [19], leading to complex alterations in the expression of A β . There also appears to be a complex relationship between transthyretin (a serum transport protein), thyroid hormones, and AD pathogenesis. Transthyretin prevents the deposition of A β protein fibrils by creating soluble A β complexes [22]. In addition, transthyretin is a transport protein for T₄ and is the only carrier protein for T₄ in cerebrospinal fluid (CSF). Transthyretin is reduced in the CSF of AD patients compared to age-matched controls [23], suggesting not only an etiologic role of transthyretin in the deposition of A β in AD, but also a possible reduction in T₄ transport into the brain in AD patients. Furthermore, recent findings indicate a relationship between apolipoprotein (ApoE), transthyretin, and actin, which suggests a possible metabolic role of ApoE genetics and transthyretin in cytoskeleton biochemistry that may be associated with AD pathogenesis [24].

Despite the evidence linking the HPT axis and AD, there has been little work directly examining TH concentrations in the brains of AD patients. Circulating thyroid hormones are produced in the thyroid gland, primarily in the form of T₄, and are transported into the brain by transthyretin [25]. T₄ is then converted to its bioactive form, T₃, by 5'-iodothyronine deiodinases. T₄ conversion to T₃ is regulated in the CNS by type II deiodinase, and type III deiodinase is responsible for the degradation of T₄ to reverse T₃ (rT₃; the inactive isomer of T₃). The majority

of T₃ within the brain is produced locally by intracerebral conversion of T₄ to T₃ (75–90%), allowing the brain to maintain T₃ concentrations within very narrow limits [26].

Given the interaction of thyroid hormones with several of the key components of AD pathology and their role in cognition and mood, it is possible that an association exists between reduced thyroid hormone concentrations on a tissue level and some of the clinical features of AD. Although a causal relationship between changes in the HPT axis is unlikely, evidence from *in vivo* and *in vitro* studies support a complex relationship between thyroid hormones and AD that may have some relevance for adjunctive treatment and management of AD. The aim of the present study was to measure both T₄ and T₃ concentrations in the prefrontal cortex of post-mortem brains of patients diagnosed with Braak I–II or Braak V–VI AD compared to age-matched controls with no pathological evidence of disease. If deiodinase activity is disrupted in AD, T₃ should be lower in brains of AD patients, along with somewhat higher levels of T₄. In contrast, a disturbance in T₄ transport activity should result in both decreased T₃ and T₄.

METHODS

Human Tissues

All brains were obtained at autopsy with a post-mortem interval of 2 to 24 hours and stored in the Brain Collection of the Division of Neuropathology at Rhode Island Hospital. All participants provided informed consent for brain donation prior to their death. AD brains were pathologically confirmed and classified according to the Braak neuropathological staging of Alzheimer-related changes, where stages I through VI represent increasing levels neuropathological changes [27]. Control brains were obtained from age-matched hospital patients with no history or evidence of neurological and neurodegenerative disease. A clinical synopsis of each case is provided in Table 1. There were no significant group differences in age or gender. The prefrontal cortex (Brodmann area 10) was sampled for three groups - Braak stage I–II (*n*=8), Braak stage V–VI (*n*=8), and control brains (*n*=8). The prefrontal cortex was selected for two primary reasons. This region contains one of the highest concentrations of thyroid hormone receptors in the CNS [28], and neuropsychological and neuroimaging studies suggest primarily frontal-subcortical system dysfunction in patients with thyroid disease [1, 29].

Tissue Preparation and Hormone Measurement

The original protocol for extraction of TH from brain tissue was adopted from Pinna *et al.* (1999) as follows [30]. Frozen tissue samples were weighed and cut into approximately 50 mg sections. These were kept on ice for the remainder of the procedure. Tissue samples were homogenized in 2 ml of 100% methanol. An additional sample of control tissue was simultaneously homogenized in 1.5 ml of 100% methanol and 0.5 ml of buffer containing radiolabeled T₃. This homogenate was labeled the “recovery sample” and it underwent the same procedures as all the other samples. The homogenates were collected and each tube used for homogenization was washed with another 2 ml of 100% methanol, which was then added to the homogenates. These mixtures were centrifuged at 3000 r.p.m. for 15 min at 4°C. The supernatants were collected, the pellets washed with another 3 ml of 100% methanol and then centrifuged at 3000 r.p.m. for 15 min at 4°C. The supernatants were again collected and added with the previously collected supernatants to poly-prep ion-exchange chromatographic columns prefilled with AG 1-X8 resin (BioRad, Hercules, California) kept at room temperature. The hormones were purified by washing the columns with the following solutions in the following order: 2 ml acetate buffer pH 7, 2 ml 100% ethyl alcohol, 2 ml acetate buffer pH 7, 2 ml acetate buffer pH 7, 2 ml 100% ethyl alcohol, 2 ml acetate buffer pH 7, 2 ml acetate buffer pH 4, 2 ml acetate buffer pH 3, 2 ml 1% acetic acid, 2 ml 35% acetic acid, 0.5 ml 70%

acetic acid. The hormones were eluted with 2 ml of 70% acetic acid. At this point the radioactivity of the recovery sample was measured using a gamma counter, and it was compared to the radioactivity of 500 μ l of buffer containing radiolabeled T_3 .

TH concentration in the eluates was measured using T_4 and T_3 RIA kits (ICN Diagnostics, Costa Mesa, CA) in accordance with manufacturer's instructions. All standards and samples were run in duplicate. For T_4 , 25 μ l of standards or samples were added to antibody-coated (Ab-coated) tubes along with 1 ml of buffer containing radiolabeled T_4 . The solution was mixed gently and the tubes incubated at room temperature for 1 hr. At the end of this time, the liquid was decanted from all tubes, which were then allowed to drain in an inverted position for at least 1 min. The radioactivity bound in the Ab-coated tubes was counted for 1 min with a gamma counter.

For T_3 measurements, 100 μ l of standards or samples were added to Ab-coated tubes along with 1 ml of buffer containing radiolabeled T_3 . This solution was mixed and the tubes incubated at 37°C for 1 hr. After this time, the liquid was decanted from all tubes and each tube washed with 2 ml of distilled water. The tubes were allowed to drain in an inverted position for at least 1 min. The radioactivity bound in the coated tubes was then counted for 1 min with a gamma counter.

Several steps were taken to further adapt the above protocols to account for current laboratory conditions, pH of the extract, measurement of TH in tissue, and low tissue concentrations of T_4 and T_3 . First, an appropriate volume of NaOH was added to the eluates to bring their pH to 7. Second, a purification step was added where the eluates were dried in a speed vacuum pump overnight and then suspended in a PBS buffer. The RIA protocol was then followed without the need to adjust the pH with NaOH. Third, in order to further concentrate the hormones and maximize the yield, 30 mg of tissue was homogenized in 100 μ l of 5% TCA, centrifuged, the supernatant mixed with Tris buffer and TH assayed without purification. Additionally, the Ab and bound hormones had to be detached from the RIA tubes and placed in test tubes suitable for measurement with the gamma counter present in the current laboratory. This was accomplished with 0.1 M citric acid containing 0.1% SDS and vigorous vortexing for 1 min. Finally, stripped human serum (free of hormone; ICN Diagnostics, Costa Mesa, CA) had to be added to the RIA mixture in order to provide the protein matrix necessary for hormone binding to the Ab.

The final determinations of TH levels in brain tissue were thus made as follows. Frozen tissue samples were weighed and cut into 100 mg pieces. They were homogenized in 200 μ l of 5% TCA and centrifuged at 14,000 r.p.m. for 10 mins. The supernatant was collected and mixed 2:1 with 4M Tris buffer. TH concentrations were measured in duplicate using RIA kits. For T_4 , the sample was mixed 1:1 with stripped human serum. 25 μ l of this new solution or 25 μ l of standards were added to Ab-coated tubes along with 1 ml of buffer containing radiolabeled T_4 , and this incubated at room temperature for 1 hr. After decanting and draining, the tubes were filled with 1 ml of 0.1 M citric acid containing 0.1% SDS. Each tube was vortexed for 1 min and the liquid then transferred to smaller test tubes, which were placed in a gamma counter and the radioactivity counted for 1 min.

The final T_3 measurements were obtained by mixing the 100 μ l of the sample, 150 μ l of stripped human serum and 1 ml of buffer containing radiolabeled T_3 in Ab-coated tubes. These were incubated for 1 hr at 37°C. After decanting, washing with distilled water, and draining, the Ab-coated tubes were filled with 1 ml of 0.1 M citric acid containing 0.1% SDS. Each tube was vortexed for 1 min and the liquid then transferred to smaller test tubes, which were placed in a gamma counter and the radioactivity counted for 1 min.

The amount of radioactivity remaining in the RIA tubes, corresponding to the amount of radiolabeled TH bound to the Ab, was detected with the gamma counter for all standards and samples. The maximum amount of binding was obtained from a hormone-free standard. The level of binding for all other standards and samples was calculated as a percentage of this maximum binding. A standard curve was then plotted for known concentrations of T₄ and T₃ and the corresponding percent of radiolabeled tracer binding (Figs. 1a and 1b). Two samples were run independently and plotted on the standard curve, and the standard curves indicate that the assay kits used in the study were appropriate and stable. The level of T₄ and T₃ in all of the samples was determined from the standard curve.

Statistical Analysis

Group differences were examined by one-way analysis of variance (ANOVA). Significant main effects and interactions were followed by Tukey post-hoc comparisons. An alpha level of $p < 0.05$ was used throughout.

RESULTS

The mean radioactivity level for each group was measured according to level of T₃/T₄ in the samples and adjusted for tissue weight. There were no significant group differences [$F(2,23) = 3.1, p > .05$] in T₄ level (ng/mg tissue). Results are presented in Fig. (2). In contrast, there was a main effect of group [$F(2,23) = 8, p < .05$] for T₃. Follow-up Tukey's *t*-tests for independent samples indicated significantly lower tissue T₃ content in the Braak V–VI group compared to controls [$t(14) = 2.82, p < .05$]; there were no differences in T₃ levels between Braak I–II and controls (see Fig. 3).

DISCUSSION

Results revealed comparable concentrations of T₄ in the prefrontal cortex of early (Braak stage I–II) and advanced (Braak stage V–VI) AD brains compared to controls. T₃ concentrations were significantly lower in Braak V–VI brains compared to controls, though there was no statistically significant difference between Braak I–II brains and controls.

The mechanism underlying reduced T₃, but not T₄, concentrations in prefrontal cortex of AD brains is unclear, but data from previous studies examining *in vivo* thyroid hormone concentrations, metabolism, and transport in AD patients suggest that disruption of at least two possible mechanisms could explain these findings, including: (1) reduced transport of thyroid hormone into the brain due to alterations in transthyretin (a transporter peptide for T₄ into the brain) activity; or (2) a disruption in deiodinase which would affect the conversion of T₄ to T₃ or the degradation of T₄. Transthyretin, a transport protein for T₄, is reduced in the CSF of AD patients compared to age-matched controls [23,31]. Alternatively, the transport of T₄ may be upregulated in advanced AD through a feedback mechanism designed to compensate for diminished bioactivity of thyroid hormone. Given that there were no group differences in tissue T₄ content, however, an overall reduction in thyroid hormone due to reduced T₄ transport is not supported.

Alternatively, lower T₃ tissue content could be explained by alterations in deiodinase activity. T₄ conversion to T₃ is regulated by type II deiodinase, and type III deiodinase is responsible for the degradation of T₄ to rT₃ (the inactive isomer of T₃). Alterations in type II deiodinase in AD brains would produce the lowered T₃ levels observed in the current study. Interestingly, the possibility of reduced conversion of T₄ to T₃ in the CNS is supported by a recent study which demonstrated that AD patients who were responders to donepezil presented with higher total T₄ and free T₄ levels before treatment and showed a significant reduction in these levels after treatment [18]. It is possible that reductions in serum T₄ in that study were due to enhanced

conversion of T_4 to T_3 by donepezil. Conversely, an upregulation of type III deiodinase, responsible for degradation of T_4 to the inactive rT_3 , would also lead to a decrease in T_3 . In fact, patients with AD have been shown to have significantly increased rT_3 levels and an increased rT_3 to T_4 ratio in the CSF, suggesting an abnormal intracerebral thyroid hormone metabolism and possibly brain hypothyroidism in AD [32]. Because rT_3 was not measured in this study, future studies are necessary to further address these competing hypotheses.

Results from the current study provide preliminary evidence suggesting that T_3 , but not T_4 , is reduced in the prefrontal cortex of post-mortem brains of advanced AD patients. Results should be viewed as preliminary, however, given some methodological limitations. First, tissue samples were restricted to the prefrontal cortex, so the findings from this study may not generalize to other brain structures affected in AD. This brain region was selected because it contains one of the highest concentrations of thyroid hormone receptors in the CNS [28], and neuropsychological and neuroimaging studies suggest primarily frontal-subcortical system dysfunction in patients with thyroid disease [1,29]. However, future studies should sample from other structures affected early on in AD, such as the hippocampi. Second, it is possible that the lower T_3 levels in the advanced AD brains compared to control brains identified in this study is merely an artifact of disease severity. Serum T_3 levels are commonly reduced with an associated increase in rT_3 concentrations in patients with prolonged, nonthyroidal illness. Tissue concentrations of thyroid hormone are also reduced in most organs [33]. For example, Arem and colleagues (1993) demonstrated reduced T_3 , but not T_4 , in the cerebral cortex of patients who had died from nonthyroidal illness compared to sudden death from trauma [34]. However, inspection of the causes of death of subjects in the present study indicates no systematic bias of nonthyroidal illness versus sudden death between groups, suggesting that illness severity may not fully explain the current findings. Relatedly, serum thyroid hormone concentrations were not available, so the relationship between the brain thyroid hormone levels and serum thyroid hormone levels is unclear. Future studies should measure rT_3 concentrations and deiodinase expression and activity in the brain to more directly address this issue, and those studies are currently underway by our group. Finally, we were unable to control for any additional confounds, such as pharmacological agents, as such opiates, administered close to the time of death that could affect the HPT-axis [35]. These potential confounds should be carefully controlled for in a future, larger study.

Results from the current study provide some evidence for an association between AD and the HPT axis. These data should be viewed as preliminary given that the procedures for thyroid hormone measurement in the human brain were adapted from techniques with demonstrated validity for the rat brain and have not been widely utilized [30]. The methodology used in the current study requires replication in future studies to validate the sensitivity of thyroid hormone measurement in human brain tissue. In summary, results provide preliminary evidence to suggest that the brains of patients with advanced AD may be in a state of tissue hypothyroidism that may be associated with some of the clinical features of AD. Additional work is necessary to determine the mechanisms underlying reduced tissue T_3 content in AD brains and the clinical correlates of these changes. Clinical investigation of the possible efficacy of adjunctive T_3 treatment in AD patients may be warranted.

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Fig. (1).

a. T₄ standard curve. The two sets of data points represent results from two independently run assays, and the curve indicates that the assays are stable and reliable. Points represent percent of Ab binding of radiolabeled T₄ tracer for known concentrations of unlabeled T₄, where a sample with no T₄ exhibits 100% binding.

b. T₃ standard curve reflecting two independently run assays with acceptable reliability. Points represent the percent of Ab binding of radiolabeled T₃ tracer for known concentrations of unlabeled T₃, where a sample with no T₃ exhibits 100% binding.

Fig. (2). RIA results for T₄. Mean T₄ content that remained in the Ab-coated tubes for each group, adjusted for the weight of tissue ng/mg that was homogenized. This value corresponds to the amount of radiolabeled T₄ tracer that competed with unlabeled T₄ from the tissue extract for the Ab. Error bars represent standard deviation. There were no significant group differences ($p > .05$).

Fig. (3).

RIA results for T₃. Mean T₃ that remained in the Ab-coated tubes for each group, adjusted for the weight of tissue in ng/mg that was homogenized. This value corresponds to the amount of radiolabeled T₃ tracer that competed with unlabeled T₃ from the tissue extract for the Ab. Error bars represent standard deviation. * Compared to control samples, Braak V–VI samples had significantly lower T₃ content ($p < .05$).

Table 1
Clinical and Demographic Characteristics of Alzheimer Patients and Controls

Group	Braak Staging	Age (y)	Gender	PMI	Cause of Death
AD	I-II	91	M	8	cardiac arrest
	I-II	78	F	6	pneumonia
	I-II	69	F	20	*
	I-II	66	F	13	sudden death
	I-II	81	M	7	urinary bladder rupture
	I-II	57	F	9	pneumonia and metastatic breast cancer
	I-II	84	M	2	*
	I-II	82	M	*	dehydration
	V-VI	82	F	16	pneumonia
	V-VI	72	F	4	*
	V-VI	83	M	9	*
	V-VI	81	F	3	acute aspiration pneumonia
	V-VI	60	M	10	pneumonia
	V-VI	84	M	2	pneumonia
	V-VI	76	F	*	pneumonia and kidney infection
	V-VI	64	F	12	pneumonia
Control		67	M	23	diverticulitis
		62	F	17	pneumonia with sepsis
		61	M	21	metastatic cancer
		77	M	7	acute necrotizing pancreatitis
		63	F	19	subarachnoid hemorrhage/homicide
		52	M	*	sudden death after shunt
		86	M	13	multiple myeloma
		72	F	*	*

Note: Braak I-II=mild AD; Braak V-VI = advanced AD; PMI = postmortem interval in hours.
* unavailable.