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# Thyroid hormone suppression of β-amyloid precursor protein gene expression in the brain involves multiple epigenetic regulatory events

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ABSTRACT

Thyroid hormone (T3) suppresses cerebral gene expression of the β-amyloid precursor protein (APP), an integral membrane protein that plays a key role in the onset and progression of Alzheimer's disease. However, the mechanisms by which T3 signaling pathways inhibit *APP* gene transcription in the brain remain unclear. By carrying out chromatin immunoprecipitation with neuroblastoma cells and primary rat brain tissue, we show for the first time that thyroid hormone receptors (TRs) directly bind at the *APP* gene *in vivo* at a promoter region containing a negative T3-response element. We further show that T3 treatment decreases both histone H3 acetylation and histone H3 lysine 4 methylation at the *APP* promoter and that chemical inhibitors of histone deacetylases and histone lysine demethylase abrogate T3-dependent *APP* silencing. Our findings thus suggest that TRs actively facilitate T3-dependent silencing of *APP* gene expression via the recruitment of distinct histone modifying enzymes associated with transcriptional repression.



## 1. Introduction

Alzheimer's disease (AD) is a degenerative disorder of the brain causing significant problems with memory, thinking and behavior. A major feature of AD is the pathological deposition of β-amyloid protein aggregates in the brain (termed 'amyloid plaques') that are proteolytically derived from the β-amyloid precursor protein (APP) (Hardy and Higgins, 1992). The amyloid cascade hypothesis postulates that the aberrant accumulation of β-amyloid protein triggers neurodegeneration in AD brains (Hardy and Selkoe, 2002; Hardy and Higgins, 1992; Wilquet and De Strooper, 2004) and is supported by the fact that to date, all inherited AD-related mutations increase the build up of β-amyloid plaque (reviewed in Hardy, 2006). Since β-amyloid protein accumulation is dependent on large amounts of APP substrate, it follows that deregulated *APP* gene expression contributes to AD pathogenesis. Indeed, higher levels of *APP* mRNA are found in AD brains (reviewed in Theuns and Van Broeckhoven, 2000), and mutations in the *APP* promoter that increase *APP* transcription are correlated with AD (reviewed in Theuns et al., 2006). Therefore, efforts to identify the transcription factors and mechanisms regulating *APP* gene expression may have important implications for the onset and progression of AD and could lead to new ways to treat AD patients.

Thyroid hormones (thyroxine or T4, and its physiologically active metabolite 3,5,3′-triiodothyronine or T3) are essential for normal brain development and function (Bernal, 2005). Intriguingly, several recent studies show that T3 treatment suppresses *APP* gene expression in both neurological cell lines (Belandia et al., 1998; Latasa et al., 1998; Villa et al., 2004) and in the brains of mice (O'Barr et al., 2006). The findings thus suggest that low levels of T3 in the brain might contribute to the development of AD by increasing *APP* expression, and consequently, by increasing circulating levels of β-amyloid protein. Notably, some clinical studies show an association between hypothyroidism and increased risk of AD (Breteler et al., 1991; Ganguli et al., 1996; van Osch et al., 2004). Moreover, reduced levels of T3 and elevated levels of biologically inactive reverse T3 (rT3) have been reported in the cerebral spinal fluid of euthyroid patients with AD (Sampaolo et al., 2005). Thus, localized "brain hypothyroidism" likely exists in some AD patients with normal serum levels of thyroid hormone, possibly due to the aberrant activity of brain-specific deiodinases (i.e., the enzymes that convert T4 into T3 or inactive rT3) (Dumitrescu and Refetoff, 2007; Sampaolo et al., 2005) or to defective transport of T3/T4 across the blood-brain barrier (Ceballos et al., 2009; Morte et al., 2010; Refetoff and Dumitrescu, 2007).

*Abbreviations:* APP, β-amyloid precursor protein; CDS, charcoal/dextran-stripped; ChIP, chromatin immunoprecipitation; FBS, fetal bovine serum; H3-Ac, acetylated histone H3; HDAC, histone deacetylase; HKDM, histone lysine demethylase; H3K4, histone H3 lysine 4; H3K4-2me, dimethylated histone H3K4; LSD1, lysine specific demethylase 1; NCoR, nuclear receptor corepressor; nTRE, negative TRE; SMRT, silencing mediator of retinoid and thyroid receptor; TR, thyroid hormone receptor; TRE, T3-response element; TSA, trichostatin A.

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Physiological T3 action is mediated predominantly through thyroid hormone receptors (TRs), members of the nuclear receptor superfamily that regulate transcription from target genes bearing T3-response elements (TREs) (Yen, 2001; Zhang and Lazar, 2000). Two TR subtypes, TRα and TRβ, are expressed in the brain (Bernal, 2005). In the presence of T3, TRs predominantly bind at positive TREs as heterodimers with retinoid X receptors (RXRs) and recruit potent histone lysine acetyltransferases and histone arginine methyltransferases that in turn, modify chromatin structure and facilitate transcriptional activation (reviewed in Glass and Rosenfeld, 2000; Tsai and Fondell, 2004). In the absence of T3, RXR/TR heterodimers remain bound at positive TREs and are able to repress transcription by recruiting nuclear receptor corepressor (NCoR) or silencing mediator of retinoic and thyroid hormone receptors (SMRT) (Hu and Lazar, 2000). These negative cofactors interact with histone deacetylase complexes that deacetylate lysine residues of $NH_2$-terminal histone tails thus rendering a more 'compact' chromatin structure that is resistant to transcription. Paradoxically, TRs can also facilitate T3-dependent gene repression, but only from distinct gene promoters containing negative TREs (nTREs), poorly defined DNA elements generally containing one or more TR-binding half-sites and usually located close to, and sometimes downstream from, the transcription start site (Hollenberg et al., 1995; Williams and Brent, 1995; Wood et al., 1989). The mechanisms by which TRs silence gene expression in a T3-dependent manner remain poorly understood despite the apparently large number of negatively regulated TR-target genes (Bernal, 2005; Feng et al., 2000; Williams and Brent, 1995; Yen, 2001).

The observation that T3 suppresses *APP* gene expression suggests that TRs bind at the *APP* gene and negatively regulate transcription. Indeed, *in vitro* binding studies show that recombinant TRs bind at a putative nTRE in the human *APP* promoter (positions +80 to +96) (Belandia et al., 1998) and further suggest a passive mechanism of repression by TR involving competition with Sp1 for a common binding site (Villa et al., 2004). Nevertheless, it remains unclear whether TRs actually bind at the *APP* gene *in vivo* and whether or not the repression mechanism additionally involves alterations in histone acetylation/methylation patterns and chromatin structure at the *APP* promoter. In this study, we reasoned that chromatin immunoprecipitation (ChIP), a powerful technique that can identify regulatory transcription factors and covalent histone modifications at native gene promoters *in situ*, would be an ideal experimental tool for investigating the mechanisms of T3-regulated *APP* expression *in vivo* in neuronal tissues. Using such an approach with human brain-derived neuroblastoma cells and primary brain tissue derived from rats, we show for the first time that endogenously expressed TRs directly bind at the *APP* gene *in vivo* at the promoter region containing the nTRE. Moreover, we show that addition of T3 triggers a marked decrease in histone H3 acetylation and H3 lysine 4 (H3K4) methylation, both alterations strongly correlated with transcriptional repression and heterochromatin (Berger, 2007; Strahl and Allis, 2000). We also found that when neuroblastoma cells are treated with chemical inhibitors of histone deacetylases (HDACs) or histone lysine demethylases (HKDMs), T3-dependent *APP* silencing is abrogated. Our findings thus suggest a novel T3-dependent repression mechanism that is actively facilitated by TR via the recruitment of negative coregulatory factors and histone modifying enzymes to the *APP* promoter.

## 2. Materials and methods

### 2.1. Antibodies, hormones and inhibitors

Antibodies against TR (sc-772) and α-Tubulin (sc-8035) were from Santa Cruz Biotechnology (Santa Cruz, CA); antibodies against acetylated-H3 (06-599) and dimethylated H3K4 (07-030) were from Millipore (Billerca, MA); monoclonal antibodies recognizing all three isoforms of APP (2450) were from Cell Signaling (Danvers, MA). Trichostatin A was from Millipore and Tranylcypromine was from Biomol (Plymouth Meeting, PA). T3 (3,3′,5-triiodo-L-thyronine) was from Sigma (St. Louis, MO).

### 2.2. Cell culture

SH-SY5Y cells were cultured in 10 cm culture dishes under a 5% CO2 atmosphere in DMEM: F12 (1:1) (Fischer Scientific, Pittsburgh, PA) supplemented with 10% fetal bovine serum (FBS; Gemini Bio-Products, West Sacramento, CA), 2 mM Glutamine, 100 units/ml penicillin/streptomycin (Invitrogen, Life Technologies, Inc., Carlsbad, CA). The medium was changed approximately every 24 h. For assays involving hormone/inhibitor treatment, SY-SY5Y cells were grown in medium containing 5% Charcoal/Dextran-stripped (CDS)–FBS (Gemini) for 72 h and then treated with T3 (1 μM), and in some cases TSA (50 nM) or Tranylcypromine (5 μM) for 24 h. The cultured cells were harvested for RNA, protein and chromatin extraction as outlined below.

### 2.3. Animals and treatment

Male Sprague–Dawley rats (Charles River Laboratories, Inc., Wilmington, MA) weighing 239 ± 14.7 g were housed in a room with controlled light, temperature, and humidity. Two groups of rats were used for this study and each group had 5 animals. Group 1: vehicle (10% m-pyrol, 5% ethanol, 5% cremaphor, and 80% water) ($n = 5$) and Group 2: T3 (924 nmol/kg/day) in the same volume of vehicle ($n = 5$). Each animal was dosed daily via oral gavage for 7 days. Body weights were taken on days 1, 4 and 7. On day 7, heart rate was measured by lead II ECG approximately 1 h post dose under isofluorane anesthesia. Animals were killed by $CO_2$ exposure. Approximately 3 ml of whole blood was collected by terminal cardiac puncture and processed for serum T3 levels (see below). Brain tissue was excised and split into tubes either containing TRIZOL for mRNA analysis or snap frozen in liquid nitrogen and stored at −80 °C for subsequent chromatin preparation. All animal procedures followed the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the Animal Use and Care Committee of Eurofins Product Safety Labs.

### 2.4. RNA extraction, cDNA synthesis, and q-PCR

Total RNA was extracted from SH-SY5Y cells and primary rat brain tissue using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. RNA concentration was measured by spectrophotometry at 260 nM. Reverse transcription was performed using 1 μg of total RNA, 1 unit MMLV-Reverse Transcriptase (Invitrogen), 10 pM oligo-dT and 500 μM deoxynucleoside triphosphates (dNTPs). Semi-quantitative PCR was performed using Taq polymerase (Denville Scientific, NJ) with specific primers (below) for 30 cycles. Real time PCR was performed using the Opticon Continuous Fluorescence Detection System (MJ Research, Waltham, MA) using a power SYBR® Green PCR Mix (Applied Biosystems, UK). The following primers were used for both semi-quantitative and real time PCR, *APP*: For-5′ATT CAG ATC CAT CAG GGA CC3′ and Rev-5′AAA CAT CCA TCC TCT CCT GG3′; *hTRα1*: For-5′AAC AGA AGC CAA GCA AGG3′ and Rev-5′TTT CAG GGA ACA TTG GCC3′; *hTRβ1*: For-5′ACA GTA TGA CAG AAA ATG GC-3′ and Rev-5′AAT GGC TCT TCC TAT GTA GG3′. qPCR primers specific for the *Dio1* gene expression were described previously (Belakavadi and Fondell, 2010).

### 2.5. Metabolic labeling

SH-SY5Y cells were grown to 80% confluency in MEM (Sigma) containing 5% CDS–FBS for 72 h with or without T3 (1 μM). Cells were washed once with PBS, labeled with [$^{35}$S] methionine (100 μCi/ml) in methionine-free DMEM (Sigma) for 30 min in 5% CDS–FBS and then cultured in MEM containing 5% CDS–FBS for 6 h. The conditioned medium was collected, incubated overnight with anti-APP antibodies at 4 °C, and then incubated with 10 μl pre-equilibrated protein G-agarose for an additional 2 h at 4 °C. The immunoprecipitate was collected by centrifugation, washed 4× in PBS, resuspended in 20 μl SDS-loading buffer, boiled for 5 min at 95 °C and then resolved on 6% SDS-PAGE. The gels were then dried and subjected to autoradiography.

### 2.6. Western blotting

SH-SY5Y cells ($1 \times 10^6$) were seeded in 6-well culture dishes in a medium containing 5% CDS–FBS. After 72 h, cells were treated with or without T3 (1 μM) for 72 h in 5% CDS–FBS and then harvested in cell lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 5 mM EDTA 0.5% NP-40). Equal amounts of protein were resolved on 6% SDS-PAGE and immunoblotting was performed as described earlier (Sharma and Fondell, 2002) using anti-APP antibodies.

### 2.7. Chromatin immunoprecipitation

Chromatin immunoprecipitation was carried out essentially as described earlier (Sharma and Fondell, 2002). In brief, SH-SY5Y cells were cultured for 72 h in