

Sustained Pituitary T3 Production Explains the T4-mediated TSH Feedback Mechanism

Alice Batistuzzo,¹  Federico Salas-Lucia,¹  Balázs Gereben,² Miriam O. Ribeiro,³ and Antonio C. Bianco¹ 

¹Section of Adult and Pediatric Endocrinology, Diabetes and Metabolism, University of Chicago, Chicago, IL 60637, USA

²Laboratory of Molecular Cell Metabolism, Institute of Experimental Medicine, Budapest, H-1083, Hungary

³Developmental Disorders Program, Center for Biological Sciences and Health, Mackenzie Presbyterian University, Sao Paulo, SP, 01302-907, Brazil

Correspondence: Antonio C. Bianco, MD, PhD, Section of Adult and Pediatric Endocrinology, Diabetes and Metabolism, University of Chicago, 5841 South Maryland Ave, Chicago, IL 60637, USA. Email: abianco1@uchicago.edu.

Abstract

The regulation of thyroid activity and thyroid hormone (TH) secretion is based on feedback mechanisms that involve the anterior pituitary TSH and medial basal hypothalamus TSH-releasing hormone. Plasma T3 levels can be “sensed” directly by the anterior pituitary and medial basal hypothalamus; plasma T4 levels require local conversion of T4 to T3, which is mediated by the type 2 deiodinase (D2). To study D2-mediated T4 to T3 conversion and T3 production in the anterior pituitary gland, we used mouse pituitary explants incubated with ¹²⁵I-T4 for 48 hours to measure T3 production at different concentrations of free T4. The results were compared with cultures of D1- or D2-expressing cells, as well as freshly isolated mouse tissue. These studies revealed a unique regulation of the D2 pathway in the anterior pituitary gland, distinct from that observed in nonpituitary tissues. In the anterior pituitary, increasing T4 levels reduced D2 activity slightly but caused a direct increase in T3 production. However, the same changes in T4 levels decreased T3 production in human HSkM cells and murine C2C12 cells (both skeletal muscle) and mouse bone marrow tissue, which reached zero at 50 pM free T4. In contrast, the increase in T4 levels caused the pig kidney LLC-PK1 cells and kidney fragments to proportionally increase T3 production. These findings have important implications for both physiology and clinical practice because they clarify the mechanism by which fluctuations in plasma T4 levels are transduced in the anterior pituitary gland to mediate the TSH feedback mechanism.

Key Words: thyroid, deiodinase, pituitary, TSH, feedback

Abbreviations: Cyclo B, Cyclophilin B; D1, type 1 deiodinase; D2, type 2 deiodinase; FBS, fetal bovine serum; HS, horse serum; P-S, penicillin-streptomycin; PTU, propylthiouracil; UbD2, ubiquitinated D2.

Dio2 encodes the type 2 deiodinase (D2), which has a relatively high affinity for T4 and converts T4 to T3 in the brain, brown adipose tissue, endothelial cells, testes, placenta, and bone, but only minimally in skeletal muscle. It is believed that this pathway produces a substantial fraction of the circulating T3, namely approximately 70% in humans and 40% in rats, with the residual either coming directly from the thyroid gland (15% in humans and 40% in rats) or produced via the type 1 deiodinase (D1) (1, 2).

D2 exhibits a unique characteristic, which is an exquisite sensitivity to its natural substrate, T4 (3). On interaction with T4, D2 is ubiquitinated and inactivated because of a conformational change and is eventually degraded in the proteasome system (4). Thus, D2 exhibits a variable half-life, which is several hours in the absence of T4 (hypothyroidism) or just a few minutes in the presence of excess T4 (hyperthyroidism) (5, 6). This is interpreted as an autoregulatory mechanism that adjusts the rate at which T4 is converted to T3, maintaining the T3 homeostasis (the biologically active thyroid hormone). For example, during iodine deficiency, thyroidal secretion of T4 decreases and the fractional conversion of

T4 to T3 is accelerated (because there is more D2 available), preserving plasma T3 and systemic thyroid hormone signaling (7–10).

D2 is also expressed in the pituitary gland (highly coexpressed in the TSH-secreting cells), where it plays a role in the T4-mediated TSH feedback mechanism (11–15). The pituitary gland expresses the thyroid hormone receptor beta2, which binds to T3 incoming from plasma and mediates the inhibition of the TSH β gene. At the same time, incoming T4 from plasma must be converted to T3 via D2 (inside the pituitary thyrotrophs) to trigger the feedback mechanism. D2-generated T3 binds to thyroid hormone receptor beta2 and inhibits the expression of TSH β gene (16, 17). Because D2 is an endoplasmic reticulum resident protein (as opposed to D1, which is located in the plasma membrane), D2-generated T3 stays inside the cells much longer (it equilibrates much slower with the plasma) before exiting to the plasma and ends up contributing substantially to the thyroid hormone receptors (TR) occupancy (18–20).

Nonetheless, although the discovery of the D2 pathway elegantly explains how plasma T4 inhibits TSH secretion, it does

not account for its homeostatic regulation of the fractional conversion of T4 to T3. In other words, if we consider the homeostatic regulation of D2 activity provided by the T4-induced D2 ubiquitination and degradation in the proteasomes, TSH secretion would hardly change in the face of falling or surging plasma T4 levels. We have previously reported that D2 activity in rat TSH tumor cells and mouse TSH cell lines exhibited an atypical regulation by T4 (21). But, Dio2 expression in these cells is extremely high by virtue of the tumoral nature of these cells. Thus, we could not ascertain to what extent T4-induced D2 downregulation was being disrupted by the extremely high rates of D2 synthesis. In the present investigation, we set up an *in vitro* system in which explants of mouse anterior pituitary glands were incubated for 48 hours (viable up to 96 hours) in the presence of known concentrations of free ^{125}I -T4. The results revealed that the substrate-mediated regulation of pituitary T4 to T3 conversion (via D2) is unique in that it is poorly responsive to fluctuations in plasma T4 (in contrast to skeletal muscle cells and bone marrow tissue). The identification of this unique D2 behavior resolves the paradox created with the discovery of the homeostatic regulation of D2 by T4, fully explaining its role in the TSH feedback mechanism.

Materials and Methods

Animals

The experiments were planned following the American Thyroid Association guide to investigating thyroid hormone economy and action in rodent and cell models (22) and were approved by the University of Chicago Institutional Animal Care and Use Committee protocol # 72577. Females and males 4- to 7-week-old mice (B6 $\times$ FVB/Ant) were used throughout the experiments. All mice were kept at room temperature (22 °C), with a 12-hour dark/light cycle, and were fed a chow diet. For the experiments, mice were euthanized by asphyxiation in a CO₂ chamber, and blood was collected immediately after from the heart. The pituitary gland, bone marrow, and kidney were harvested for studies *in vitro*.

Anterior Pituitary Explants

Whole pituitary glands were dissected and humidified with 1 μL of Hanks' Balanced Salt solution (HBSS, Gibco) and the posterior pituitary was dissected out under a stereomicroscope (Nikon SMZ-745 T) (Fig. 1A-C). For the experiments using sliced anterior pituitary glands, each anterior pituitary was placed on top of a Petri dish, humidified with 1 μL Hanks' Balanced Salt solution, and thinly chopped. The sliced glands were then transferred to an Eppendorf containing cold high-glucose DMEM (Gibco), kept in groups of 3 throughout the procedure. Immediately afterward, the medium was removed, the pituitary glands were rinsed twice with PBS (Gibco) and preincubated for 1 hour at 37 °C in a humidified 5% CO₂ atmosphere. Medium consisted of phenol red-free high-glucose DMEM/Nutrient Mixture F-12 (DMEM/F12, Gibco), supplemented with 1% of fetal bovine serum (FBS, Bio-Techne) or .1% BSA (GoldBio), 1% of penicillin-streptomycin solution (P-S, Gibco), 1% of GlutaMAX (Gibco), and .029 to 100 nM T4 (Sigma-Aldrich). As in the plasma, the fraction of free T4 in the medium depends on the concentration of thyroid hormone-binding proteins. These figures have been determined experimentally for

.1-4% BSA, 10% FBS, 10% thyroid hormone-depleted FBS (22). Therefore, the free fraction of T4 in .1% BSA is 3.5% of the total T4 concentration (22).

To evaluate the T4 to T3 conversion and the T3 production, the medium was then removed, the anterior pituitaries rinsed twice with PBS, and 3 glands were placed on a cell culture insert with a pore size of .4 μm (Millicell) in a 24-well plate (Fig. 1D) and incubated at 37 °C in a humidified 5% CO₂ atmosphere. Incubations lasted 24 to 96 hours in the presence of approximately 800 K cpm/mL ^{125}I -T4 (PerkinElmer Life and Analytical Sciences, Inc; specific activity: 1080-1320 μCi [NEX111H100UC] or 5700 μCi [NEX111X100UC]). At the indicated times, the medium was collected and processed for quantification of ^{125}I production. The glands were rinsed with PBS, centrifuged, and resuspended in RIPA buffer (Thermo Scientific), sonicated with 3 pulses (set at 40% amplitude), and protein quantified using the Pierce BCA protein assay kit (Thermo Scientific). In some experiments, as indicated, the D2 assay was performed in the presence of 1 mM propylthiouracil (PTU) to inhibit possible contamination with D1.

Dio3 is not expressed in the anterior pituitary gland (23), except in its neural portion—the posterior pituitary (24). We wished to confirm the absence of D3 activity in our settings because D3 inactivates thyroid hormone and is known to cause localized reduction in T3 signaling (25). Here, we assessed D3 activity in anterior pituitaries by measuring T3 to T2 conversion in the presence of ^{125}I -T3 for up to 44 hours. Whereas substantial amounts of ^{125}I -T3 were distributed to the pituitary gland, no 3,5- ^{125}I -T2 was detected (Supplementary Fig. 1A-C (26)).

Primary Cultures

Bone marrow from 2 femoral bones/mouse was collected by centrifugation (27) with modifications. Femoral bones were dissected, the proximal and distal ends removed, and the bone cut in half. Each half was placed in a microfuge tube supported by a microfilter cartridge (without the filter) and centrifuged for 1 minute at 2000g, 4 °C. Pellet was resuspended with 100 mL of ice-cold DMEM, transferred to another tube, and placed on ice until the end of dissections. Each resuspended bone marrow was combined in the same microfuge tube and, at the end of dissections, centrifuged for 1 minute at 2000g, 4 °C. Supernatant was removed, the pellet was resuspended in 7 mL ice-cold PBS, and 500- μL aliquots were transferred to new microfuge tubes and centrifuged for 1 minute at 2000g, 4 °C. The pellets were resuspended in 1 mL of DMEM/F12, supplemented with .1% of BSA, 1% of P-S, 1% of GlutaMAX, and .029 to 10 nM T4, at 37 °C, transferred to a cell culture insert in a 24-well plate (same as the anterior pituitary gland). Kidneys were dissected and kept on ice-cold DMEM. At the end of the dissections, kidneys were transferred to an ice-cold plate and thinly fragmented with a blade. Tissue fragments (about 5 mg) were transferred to 2-mL Eppendorf tubes and resuspended with medium at 37 °C, containing DMEM/F12, supplemented with .1% of BSA, 1% of P-S, 1% of GlutaMAX, 44 mM of sodium bicarbonate, 20 mM of HEPES (Gibco), and .029 to 10 μM T4. Each tube cap was perforated with a 16-gauge disposable needle to allow ventilation during incubation. Bone marrow and kidney fragments were incubated at 37 °C in a humidified 5% CO₂ atmosphere for 24 to 48 hours in the presence of

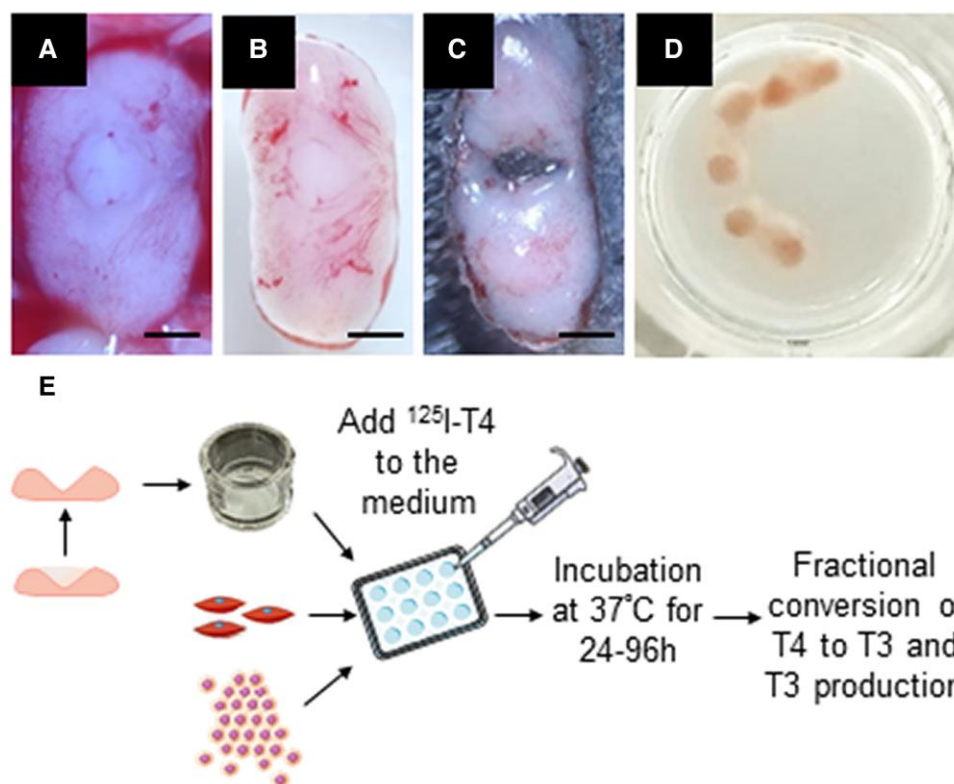


Figure 1. Isolation of the mouse anterior pituitary. Photographs of (A) intact pituitary gland in its natural anatomic location in the mouse, (B) on a Petri dish, (C) on a Petri dish after the posterior pituitary was removed, and (D) a cell culture insert containing 3 anterior pituitary glands. (E) Experimental design. (A-C) Scale bar = .55 mm.

800 K cpm/mL ^{125}I -T4. At the indicated time, the medium was collected and processed for quantification of ^{125}I production. Protein was quantified using the Pierce BCA protein assay kit (Thermo Scientific).

Cell Cultures

The nontumoral spontaneously immortalized cell lines C2C12 (28) (ATCC Cat# CRL-1772, RRID:CVCL_0188) and LLC-PK1 (29) (ATCC Cat# CL-101, RRID:CVCL_0391) were obtained from ATCC, and the primary human myoblasts (HskM) were obtained from Gibco. Cells were grown at 37 °C in a humidified 5% CO_2 atmosphere. C2C12 cells were cultured in high-glucose DMEM with 10% FBS until they reached a confluence of 90%. Cells were subcultured in 12-well tissue culture plates (10^4 cells/well) in the presence of DMEM supplemented with 20% FBS plus 1% P-S until they reached a confluence of 50%. To induce differentiation, the cells were rinsed twice with PBS and finally cultured for 5 days in differentiation media containing DMEM, 2% horse serum (HS, Gibco), 1% of P-S, and 1% insulin–transferrin–selenium (Gibco) (30). LLC-PK1 cells were grown in high-glucose DMEM supplemented with 5% FBS plus 15 mM HEPES and subcultured in 12-well tissue culture plates (10^4 cells/well) (31). HskM cells were cultivated for 48 hours with low-glucose DMEM supplemented with 2% HS to induce differentiation to myotubes. To evaluate the T3 production, the cells were rinsed with PBS and incubated with DMEM containing .1% BSA and .029 to 100 nM of T4 in the presence of 500 to 800 K cpm/mL ^{125}I -T4 for 24 hours. After 24 hours, the medium

was collected and processed for quantification of T3 production. Cells were rinsed with PBS, harvested with RIPA buffer, sonicated with 3 pulses (set at 40% amplitude), and protein quantified using the Pierce BCA protein assay kit.

Quantification of T3 Production

A total of 50 μL of medium was collected in duplicate in 1.7-mL Eppendorf tubes at the indicated times and combined with 33.3 μL HS and 16.6 μL of trichloroacetic acid 50%. The tubes were vortexed for 3 minutes and centrifuged for 3 minutes at 21 300g. The supernatant was collected and the amount of radioactive iodine (from ^{125}I -T4 deiodination) was measured in a 2470 Automatic Counter Wizard2 (Perkin-Elmer) as previously described (32). Negative (background) controls consisted of the same medium incubated in the absence of pituitary glands or cells. In the experiments using bone marrow and kidney fragments, the background was established by incubating the tissues with 10 nM T4 or 10 μM T4, respectively, for D2 and D1. The fractional conversion of T4 to T3 was calculated as a function of the total ^{125}I -T4 added to the medium after the ^{125}I activity was multiplied by 2 to correct for the random deiodination of the outer ring. D2-mediated T3 production was calculated as a function of the total T4 (cold) added to the medium and expressed per milligram of protein and hour as indicated.

Measurement of TSH and Tshb mRNA

To assess TSH secretion from anterior pituitary explants, we incubated each gland individually in medium containing 1% charcoal-stripped serum (Gibco) and the indicated amounts

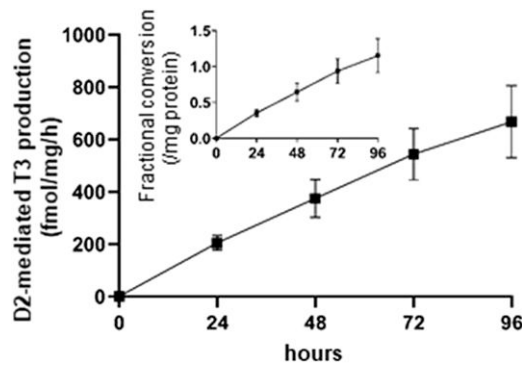


Figure 2. Anterior pituitary explant. D2-mediated T3 production over time in female mouse glands incubated with 20 pM free ^{125}I -T4 (800 K cpm/mL). The inset shows the fractional conversion of T4 to T3 for each time-point. Each well contained $.17 \pm .006$ mg of protein. Values are expressed as mean $\pm$ SEM ($n = 3$).

of T4 or T3 (Sigma-Aldrich). Medium was collected after 12 hours, 24 hours, and 72 hours of incubation, stored at -80°C , processed for TSH analysis using a MILLIPLEX rat TH panel kit (Millipore Corp.), and read on a BioPlex (Bio-Rad). In some experiments as indicated, incubations were performed in the presence of $1\ \mu\text{M}$ of TRH (Sigma-Aldrich). After 72 hours of incubation, the anterior pituitary glands were rinsed twice with PBS and stored at -80°C for further analyses of Tshb mRNA expression.

RNA Isolation and Real-time Quantitative PCR

Total RNA was isolated from anterior pituitary glands using the RNAqueous-Micro Kit (Invitrogen) including DNase treatment. A total of $1.0\ \mu\text{g}$ total RNA was used to produce cDNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche). Gene expression was measured by RT-quantitative PCR (StepOnePlus real-time PCR system; Applied Biosystems) using PowerUp SYBR Green Master Mix (Applied Biosystem). Cyclophilin B (Cyclo B) was used as a housekeeping internal control gene. Results were expressed as the ratio of Tshb mRNA to Cyclo B mRNA levels. The pair of primers were mTshb: sense $5'$ -TCTGTCGGTATTGTATGAC- $3'$ and antisense $5'$ -GCGGCTTGGTGCAGTAGTTG- $3'$; mCyclo B: sense $5'$ -GGAGATGGCAGGAGGAA- $3'$ and antisense $5'$ -GCCCGTAGTGCTTCAGCTT- $3'$.

Statistical Analysis

All data were analyzed using PRISM software (GraphPad Software 9.5.1, Inc) and expressed as mean $\pm$ SEM. A Student t -test was used to compare 2 groups. Pearson correlation was used for the analyses of Tshb mRNA levels. Significance was set at $P < .05$ to reject the null hypothesis.

Results

To standardize the in vitro measurements of T3 production in anterior pituitary gland explants, whole female mouse pituitary glands were dissected and the posterior pituitary was removed under a stereomicroscope (Fig. 1A-E). The anterior pituitary was incubated with ^{125}I -T4, in a medium containing 1% FBS, and at the indicated times medium was collected and processed for ^{125}I quantification. The fractional conversion of T4 to T3 increased over time, reaching between 40% and

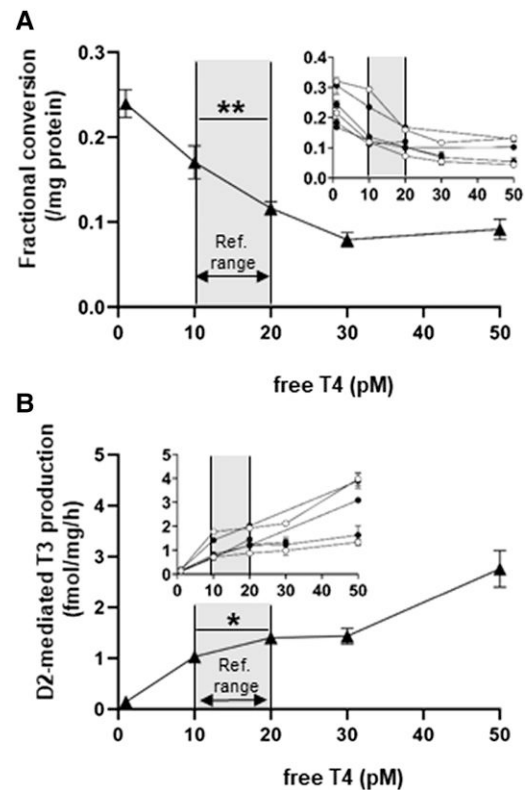


Figure 3. Anterior pituitary explant. Effects of increasing T4 concentration on the conversion of T4 to T3 in glands of female and male mice. (A) Pituitary explants incubated in media containing 800 K cpm/mL ^{125}I -T4 for 48 hours. Shown is the fractional conversion of 7 experiments measuring T4 to T3 conversion in the presence of 1, 10, 20, 30, or 50 pM fT4. $**P = .01$. The inset shows each of the seven experiments individually; open symbols represent experiments with females, closed symbols males. (B) Calculated D2-mediated T3 production for each fT4 level-point shown in (A). $*P = .005$. The inset shows as in (A). The number of entries for each fT4 level-point varied between 11 and 18. Each well contained $.36 \pm .008$ mg of protein. Values are expressed as mean $\pm$ SEM.

65%/mg protein after 66 hours. Using these figures (and the concentration of T4 in the FBS = $14.6\ \mu\text{g/dL}$ [manufacturer's certificate of analysis]), we subsequently calculated the T3 produced over time, which reached between 3.7 to $6.2\ \text{pmol/mg}$ protein by 66 hours of incubation. We next sliced female and male mouse anterior pituitary glands before incubation to increase the area of contact between the cells and the medium. Both the fractional conversion of T4 to T3 and T3 production increased substantially, reaching between 25% to 100% and 2.6 to $10.5\ \text{pmol/mg}$ protein in 1 experiment. However, the inter-experiment variability increased as well, which led us to move forward with intact anterior pituitary glands.

In the next set of experiments, we wished to study the effects of different T4 concentrations in the local conversion of T4 to T3 and T3 production. In this case, intact anterior pituitary glands were incubated in a medium without FBS but containing .1% BSA, which allows us to manipulate the free T4 concentration (22). We first repeated the time-course experiments up to 96 hours, noticing that the conversion rates of T4 to T3 increased over time, from $35.3 \pm 4.9\%$ at 24 hours to $115 \pm 23\%$ at 96 hours when anterior pituitaries from female mice were incubated in the presence of 20 pM free T4 (the physiological free

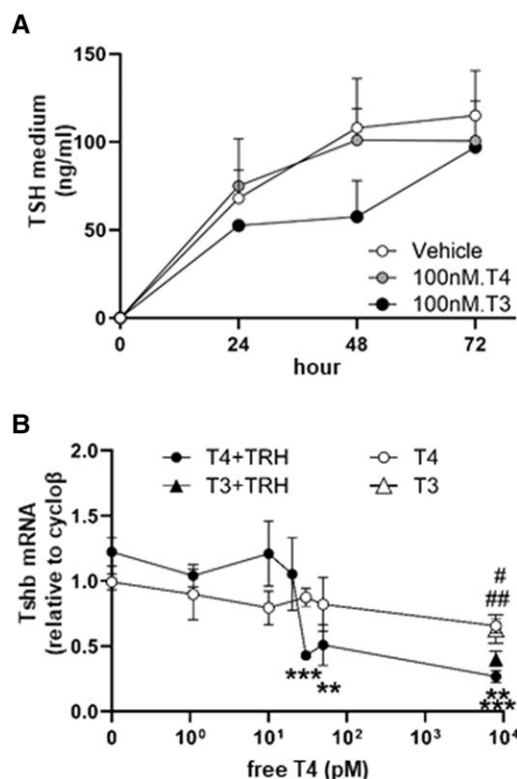


Figure 4. Anterior pituitary explant. Effects of increasing T4 concentration on TSH secretion and Tshb expression in glands of male mice. (A) TSH concentration in conditioned medium of anterior pituitary explants incubated with no T4 (vehicle) or 100 nM T4 or 100 nM T3 for 72 hours. (B) Expression of Tshb mRNA in anterior pituitary explants incubated with no T4 (vehicle), 1, 10, 20, 30, or 50 pM-ft4, 100 nM T4, or 100 nM T3 for 72 hours. Closed symbols indicate anterior pituitary glands incubated with 1 μ M TRH during the last 24 hours. ** P < .01 (50 pM ft4 and 100 nM T3 vs vehicle in the group with 1 μ M TRH); *** P < .001 (30 pM ft4 and 100 pM ft4 vs vehicle in the group with 1 μ M TRH); # P < .05 (100 nM T4 vs vehicle); ## P < .01 (100 nM T3 vs vehicle). Values are expressed as mean $\pm$ SEM.

T4 concentration in the plasma is 10–20 pM). During this time, T3 production increased from 204 ± 27 fmol/mg protein at 24 hours to 667 ± 137 fmol/mg protein at 96 hours (Fig. 2).

T3 Production in Anterior Pituitary Mouse Explants at Different Free T4 Concentrations

We next conducted 7 independent experiments in which anterior pituitary gland explants from both female and male mice were incubated with variable free T4 levels (1–50 pM) for 48 hours. At 1 pM free T4, the fractional conversion of T4 to T3 varied between 20% and 30% and, as expected, decreased with the increase in the free T4 levels (Fig. 3A). However, the calculated T3 production increased progressively with the increase in free T4 levels (Fig. 3B). This occurred because the drop in the fractional conversion of T4 to T3 was not sufficient to offset the increase in free T4. Specifically within the normal free T4 reference range, T3 production increased from $1 \pm .12$ to $1.4 \pm .09$ fmol/mg protein/h as the T4 levels increased from 10 to 20 pM (Fig. 3B). Notably, no differences in T4-regulated fractional conversion or T3 production were observed between male and female anterior pituitaries (Supplementary Fig. 2A–B (26)), suggesting that the well-known sexual dimorphism in TSH regulation (33–35) does not involve major differences in localized T3 production.

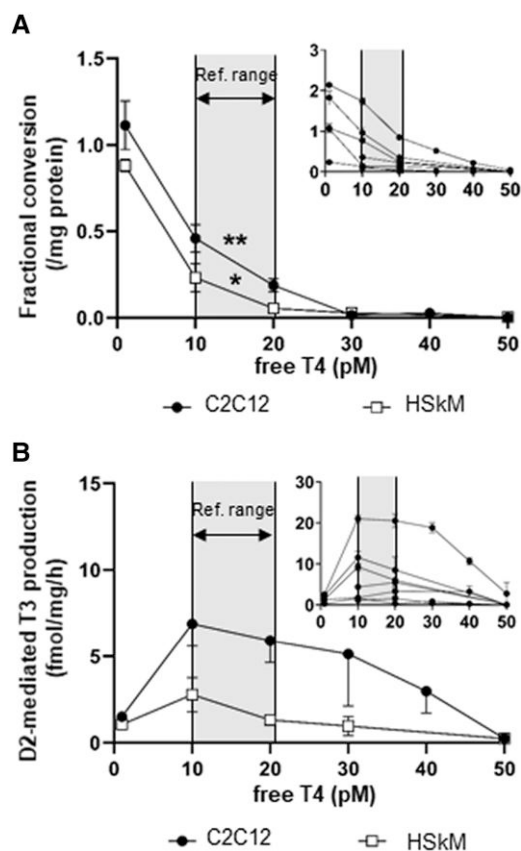


Figure 5. Skeletal muscle cells. Effects of increasing T4 concentration on the conversion of T4 to T3 in (A) C2C12 and HSkM cells incubated in medium containing 800 K cpm/mL ¹²⁵I-T4 for 24 hours. Shown is the fractional conversion of 7 experiments measuring T4 to T3 conversion in the presence of 1, 10, 20, 30, 40, or 50 pM ft4. * P = .049; ** P = .004. Open squared symbols represent experiments with HSkM cells, closed circular symbols C2C12 cells. The inset shows each of the 7 C2C12 experiments individually. (B) Calculated D2-mediated T3 production for each ft4 level-point shown in (A). The inset is as in (A). The number of entries for each ft4 level-point varied between 8 and 23. Each well contained .15 $\pm$.003 mg of protein (C2C12 cells) or .09 $\pm$.004 mg of protein (HSkM cells). Values are expressed as mean $\pm$ SEM.

To verify that this was D2-mediated T3 production, we incubated the anterior pituitary explants with 10 nM T4, which is sufficient to saturate the D2 pathway and outcompete the ¹²⁵I-T4, but not the D1 pathway (because it has a 1000-fold higher K_m) (22). Indeed, this caused the apparent fractional conversion of T4 to T3 to drop to zero, confirming that it is saturable with relatively low free T4 concentrations (data not shown). The absence of significant contribution of the D1 pathway was confirmed in a separate experiment with a medium containing 1 mM PTU, known for inhibiting D1 (36) but not D2 activity (37). No difference was observed in the fractional conversion of T4 to T3 or in the T3 production in anterior pituitary glands incubated with PTU for 48 hours in the presence of 1 or 20 pM of free T4 (Supplementary Fig. 3A–B (26)). Altogether, these results support the idea that D1 does not contribute to the T3 production in the anterior pituitary gland.

TSH Expression and Secretion in Anterior Pituitary Explants

To assess the impact of local T3 production in the expression of TSH, we next monitored TSH levels in the medium of

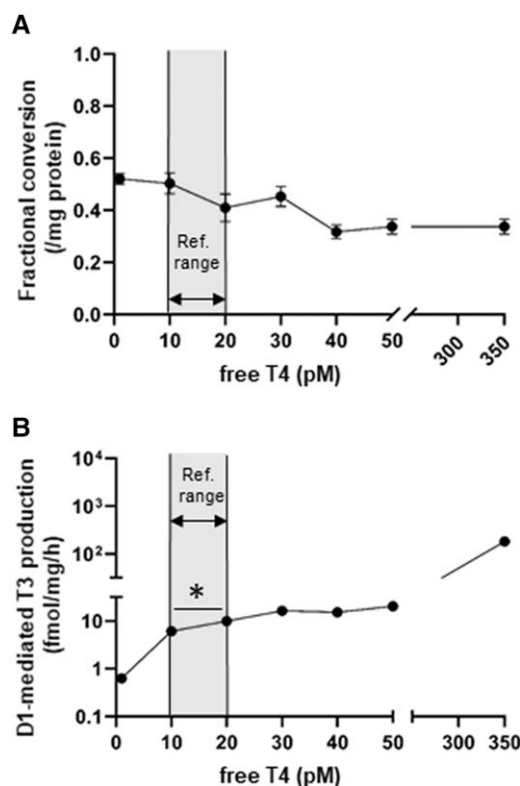


Figure 6. Kidney cells. Effects of increasing T4 concentration on the conversion of T4 to T3 in LLC-Pk1 cells. (A) LLC-Pk1 cells incubated in medium containing 700 K cpm/mL 125 I-T4 for 24 hours in the presence of 1, 10, 20, 30, 50, and 350 pM fT4. (B) Calculated D1-mediated T3 production for each fT4 level-point shown in (A). * $P = .026$. The number of entries for each fT4 level-point varied between 3. Each well contained $.14 \pm .005$ mg of protein. Values are expressed as mean $\pm$ SEM.

pituitary explants at 24-hour intervals for 72 hours. There was marked secretion in the first 24 hours, which was followed by less pronounced secretion at 48 to 72 hours. Unexpectedly, the addition of 100 nM T4 (total) to the medium did not affect TSH secretion (Fig. 4A). We next tested the effects of a TR-saturating concentration of T3 (100 nM total) and detected an inhibition in TSH secretion at the 48-hour time-point, but the difference was not significant. Given that TSH secretion from anterior pituitary explants is poorly (or not at all) responsive to T3, we next asked whether measuring mRNA for *Tshb* would serve our purpose. Indeed, incubation with increasing concentrations of T4 progressively reduced *Tshb* mRNA levels ($r = -.87$; $P < .01$) up to a maximum of $\sim 35\%$ inhibition with 10^4 pM T4 (Fig. 4B). A similar level of inhibition was achieved with 100 nM T3. We next attempted to elevate the overall level of *Tshb* mRNA expression by adding 1 μ M TRH during the last 24 hours of the incubation, but the overall inhibition caused by either T4 or T3 was not affected (Fig. 4B).

T3 Production in Skeletal Muscle and Kidney Cell Lines at Different Free T4 Concentrations

To assess T4 to T3 conversion outside the anterior pituitary gland, we used a similar setup to examine T4 to T3 conversion rate and the production of T3 in C2C12 (murine) and HSkM (human) cells. C2C12 cells are known for expressing

exclusively Dio2 on differentiation to myocytes (38, 39), and HSkM cells are primary cells obtained from a donor's quadriceps femoral (32, 40). Across 7 independent experiments, we observed that the fractional conversion of T4 to T3 was maximal at 1 pM free T4 concentration, about 1.1/fmol protein in the C2C12 cells and .9/fmol protein in the HSkM cells (Fig. 5A). With the increase in free T4 concentration to 10 pM, the fractional conversion decreased in both cell lines, but the T3 production increased to about 6.5 fmol/mg in the C2C12 cells and 2.8 fmol/mg in the HSkM cells (Fig. 5A-B). With further increases in the free T4 concentration, the T3 production reduced progressively, reaching zero at 50 pM T4 in both cell lines (Fig. 5A-B). Specifically within the normal free T4 reference range, T3 production remained stable at about 6.0 fmol/mg protein/h (C2C12 cells) and 2.5 fmol/mg protein/h (HSkM cells) as the T4 levels increased from 10 to 20 pM (Fig. 5B). Similar results were obtained with freshly isolated mouse bone marrow incubated with 125 I-T4 for 48 hours (Supplementary Fig. 4A-B (26)).

We next used the same setup to study the fractional conversion of T4 to T3 and T3 production in the pig renal epithelial cell line LLC-Pk1, known for expressing exclusively Dio1 (31). As expected, the fractional conversion remained stable throughout different concentrations of free T4 (from 1 pM fT4 [$52 \pm .02\%$] to 350 pM fT4 [$34 \pm .03\%$]) (Fig. 6A). Therefore, the D1-mediated T3 production continually increased, from $.63 \pm .02$ fmol/mg/h at 1 pM fT4 to 180 ± 19 fmol/mg/h at 350 pM fT4 (Fig. 6B). Similar results were obtained with freshly isolated mouse kidney fragments incubated with 125 I-T4 for 24 hours (Supplementary Fig. 5A-B (26)).

Discussion

In the present investigation, we developed a pituitary ex vivo system to study localized T3 production with subsequent reduction in *Tshb* mRNA levels. This led to the discovery that the D2 pathway is distinctly regulated in the anterior pituitary gland when compared with a nonpituitary D2-expressing tissue such as the skeletal muscle or bone marrow. Whereas in the skeletal muscle (differentiated in vitro) and bone marrow cells, the increase in T4 concentration markedly downregulated D2 activity and reduced the overall production of T3 (Fig. 5A-B), in the explant of the anterior pituitary gland the reduction in D2 activity was not sufficient to offset the increase in T4 concentration, resulting in a progressive increase in T3 production (Fig. 3A-B). These observations have important physiological and clinical implications. They explain how the fluctuation in plasma T4 levels is transduced in the anterior pituitary gland to control the TSH feedback mechanism. They also provide a mechanistic basis for why LT4-treated patients can maintain normal serum TSH levels in the setting of relative lower levels of plasma T3 (41).

A unique aspect of the D2 pathway is that it is downregulated by T4 in cell cultures (3) and in transplanted pituitary glands (42). T4 binds to D2 and disrupts its natural dimeric conformation, exposing 2 lysin residues that become discoverable and targetable by WSB-1 and MARCH6, 2 ubiquitin ligases previously involved in D2 ubiquitination (43-45). Ubiquitinated D2 (UbD2) is inactive and it is retrotranslocated to the cytoplasm and proteasomes via p97/Atx3 complex, where it is terminally degraded (46). In some tissues, however, UbD2 can be reactivated by deubiquitination via USP33 and USP20 and rescued

from proteasomal degradation (47). This elaborate mechanism is interpreted as a homeostatic response to fluctuations in plasma T4 levels that aims at stabilizing T3 production. Our present experiments in the skeletal muscle cells illustrate this. The T4 to T3 fractional conversion decreased significantly with the increase in free T4 from 10 to 20 pM, as a result of T4-induced loss of D2 activity (48). However, despite the increase in free T4 levels, the T3 production remained stable (Fig. 5A-B). This homeostatic mechanism has been detected in rodents and patients transitioning from hypothyroidism to thyrotoxicosis (49, 50).

Studies in which LT4-treated thyroidectomized patients (with normal serum TSH levels) were treated with 1 g/day PTU for 10 days led to the discovery that about 80% of the T3 production is via the D2 pathway (51). Thus, it is indeed remarkable that with relatively higher free T4 levels (ie, 50 pM, which can be reached in patients with overt hyperthyroidism), the T3 production via the D2 pathway dropped to zero in both mouse and human skeletal muscle cell lines. This indicates that it is possible to shut down D2-mediated T3 production just by exposing D2-expressing cells to too much T4. This also indicates that the D2 pathway must be greatly minimized (if at all functional) in patients with overt hyperthyroidism because the bulk of T3 production would have switched to the thyroid gland itself and the extrathyroidal T3 production via the D1 pathway. This probably explains the relatively faster drop in serum T3 levels after PTU is given to patients with hyperthyroidism (52, 53).

The homeostatic nature of D2 explains why such a drop in T3 production caused by T4 could not possibly occur in the pituitary gland, as documented in the present investigation. In the anterior pituitary, the reduction in the T4 to T3 fractional conversion that follows the increase in free T4 (from 10 to 20 pM) increased T3 production by approximately 40% (Fig. 3A-B). Although at the moment we do not have a mechanistic explanation for such a diverse regulation of D2, we speculate that this is caused by a distinct D2 ubiquitination in the anterior pituitary gland. Our previous studies indicate that D2 activity in the tumoral thyrotrophic cell lines Tt97 and TaT1 is less sensitive to inhibition with T4 (21) and much less UbD2 accumulates in an *in vitro* assay when extracts of the medial basal hypothalamus were used (14). Along with our present data, these findings suggest that the D2-ubiquitination pathway in the hypothalamus-pituitary unit has unique properties, which are distinct from the other D2-expressing tissues.

We looked at the gene expression atlas of the anterior pituitary gland and noticed that the main genes involved in D2 ubiquitination Wsb-1 (54) and March6 (55) are indeed expressed in thyrotrophs (23), which we showed coexpress Dio2. We also noticed that genes encoding the D2 deubiquitinases Usp20 and Usp33 (47) are expressed in these cells (56). Notably, in our previous studies that used *in situ* hybridization, we noticed that tanycytes are 1 of the few areas in the central nervous system that coexpress Dio2 and Usp33 (57), suggesting that deubiquitination of UbD2 in these hypophyseotrophic structures could be accelerated by default, mitigating T4-induced ubiquitination of D2. Last, we note that USP33 expression is higher in the mouse pituitary gland vs the skeletal muscle (57), which supports the differences in D2-mediated T3 production detected in the present study.

We recently observed that in patients with hypothyroidism treated with LT4, an increase in plasma free T4 levels (from 10

to 20 pM) was associated with a sharp decline in serum TSH levels (from ~3 to ~.5 mIU/L), whereas serum T3 levels remained stable (41). These observations are explained by the present findings. The persistent D2 activity in the anterior pituitary gland generates local T3 and slows down TSH secretion at the same time that the drop in D2 activity in the skeletal muscle (and supposedly other D2-expressing tissues) stabilizes T3 production (Fig. 5B) and serum T3 levels (41).

The present study is not without limitations. First, although we focused on the role played by D2 in the TSH feedback mechanism, we understand that besides thyrotrophs, Dio2 is expressed in lactotrophs, somatotrophs, and gonadotrophs, but not in the corticotrophs or endothelial cells (23). Furthermore, studies with single nuclei RNA sequencing of mouse anterior pituitary gland revealed that thyrotrophs constitute only approximately 1.5% of total pituitary cells in both sexes. The majority of cells comprise lactotrophs (~30%) in female mice and somatotrophs (~50%) in male mice (23). Therefore, rather than the thyrotrophic D2-mediated T3 production, our results reflect the total D2-mediated T3 production in the anterior pituitary gland. Second, our setup did not allow for the study of the role played by the D2 pathway in the hypothalamic median eminence, where Dio2 is highly expressed in tanycytes (58, 59). Nonetheless, our previous studies suggest that in this location as well, D2 is poorly ubiquitinated (60). Third, D1 activity has been reported in a human somatomammotrophs cell line (GX) (61) and in the rat pituitary cell line GH3 (62). In healthy human pituitaries, D1 activity was found to be very low or undetectable, whereas it was more pronounced in pituitary adenomas (lower than D2 activity (63, 64)). Dio1 is not expressed in mouse thyrotrophs, although low levels of mRNA were found in the melanotrophs and somatotrophs (23). We did not study D1-mediated T3 production in the anterior pituitary glands because the fractional conversion of T4 to T3 and the T3 production dropped to zero when pituitaries were incubated with 10 nM T4 (350pM free T4). This observation assures us that our analyses focused exclusively on the D2 pathway (22). This is illustrated by the analysis of the D1-expressing LLC-PK1 cells and in freshly obtained kidney fragments, in which the conversion of T4 to T3 was not affected by similar T4 levels (Fig. 6B).

In conclusion, here we developed an *ex vivo* setup to study D2-mediated T3 production in the murine anterior pituitary gland that remained viable for up to 96 hours. Our present findings indicate that, in contrast to the mouse and human skeletal muscle cells as well as mouse bone marrow cells, an elevation in T4 in the anterior pituitary gland is transduced intracellularly by a direct elevation in D2-mediated T3 production, with downstream effects such as reduction in TSH secretion. Although the D2 mechanism in the anterior pituitary gland was discovered in the late 1970s, it remained unclear how to reconcile the intrinsic homeostatic nature of D2 with the transduction of fluctuations in plasma T4 levels. The present results provide such an explanation through the identification of unique aspects of D2 regulation in the anterior pituitary gland.

Funding

This work was supported by the National Institute of Diabetes and Digestive and Kidney Diseases NIDDK—DK15070, DK65066, DK77148 (A.C.B.), and DK58538 (A.C.B. and B.G.).

Author Contributions

A.B.: performance of experiments, formal analysis, methodology, writing—original draft, and writing—review & editing. F.S.-L.: performance of experiments that involved UPLC separation of iodothyronines and review of the manuscript. B.G.: conceptualization, funding acquisition, and review of manuscript. M.O.R.: conceptualization and review of manuscript. A.C.B.: conceptualization, formal analysis, funding acquisition, project administration, resources supervision, methodology, validation, visualization writing—original draft, and writing—review & editing.

Disclosures

A.B. is a consultant for AbbVie, Allergan, Synthetics, Sention, and Thyron. The other authors have no relevant disclosures.

Data Availability

All data sets generated during and/or analyzed during the present study are included in the manuscript.

References

- Bianco AC, Dumitrescu A, Gereben B, *et al.* Paradigms of dynamic control of thyroid hormone signaling. *Endocr Rev.* 2019;40(4):1000-1047.
- Bianco AC, Salvatore D, Gereben B, Berry MJ, Larsen PR. Biochemistry, cellular and molecular biology, and physiological roles of the iodothyronine selenodeiodinases. *Endocr Rev.* 2002;23(1):38-89.
- St. Germain DL. Hormonal control of a low Km (type II) iodothyronine 5'-deiodinase in cultured NB41A3 mouse neuroblastoma cells. *Endocrinology.* 1986;119(2):840-846.
- Gereben B, Goncalves C, Harney JW, Larsen PR, Bianco AC. Selective proteolysis of human type 2 deiodinase: a novel ubiquitin-proteasomal mediated mechanism for regulation of hormone activation. *Mol Endocrinol.* 2000;14(11):1697-1708.
- Leonard JL, Siegrist-Kaiser CA, Zuckerman CJ. Regulation of type II iodothyronine 5'-deiodinase by thyroid hormone. Inhibition of actin polymerization blocks enzyme inactivation in cAMP- stimulated glial cells. *J Biol Chem.* 1990;265(2):940-946.
- Silva JE, Leonard JL. Regulation of rat cerebrocortical and adeno-hypophyseal type II 5'- deiodinase by thyroxine, triiodothyronine, and reverse triiodothyronine. *Endocrinology.* 1985;116(4):1627-1635.
- Fukuda H, Yasuda N, Greer MA, Kutas M, Greer SE. Changes in plasma thyroxine, triiodothyronine, and TSH during adaptation to iodine deficiency in the rat. *Endocrinology.* 1975;97(2):307-314.
- Silva E. Disposal rates of thyroxine and triiodothyronine in iodine-deficient rats. *Endocrinology.* 1972;91(6):1430-1435.
- Abrams GM, Larsen PR. Triiodothyronine and thyroxine in the serum and thyroid glands of iodine-deficient rats. *J Clin Invest.* 1973;52(10):2522-2531.
- Chopra IJ, Hershman JM, Hornabrook RW. Serum thyroid hormone and thyrotropin levels in subjects from endemic goiter regions of New Guinea. *J Clin Endocrinol Metab.* 1975;40(2):326-333.
- Silva JE, Kaplan MM, Cheron RG, Dick TE, Larsen PR. Thyroxine to 3,5,3'-triiodothyronine conversion by rat anterior pituitary and liver. *Metabolism.* 1978;27(11):1601-1607.
- Abend SL, Fang SL, Alex S, Braverman LE, Leonard JL. Rapid alteration in circulating free thyroxine modulates pituitary type II 5'-deiodinase and basal thyrotropin secretion in the rat. *J Clin Invest.* 1991;88(3):898-903.
- Maeda M, Ingbar SH. Effect of alterations in thyroid status on the metabolism of thyroxine and triiodothyronine by rat pituitary gland in vitro. *J Clin Invest.* 1982;69(4):799-808.
- Fonseca TL, Correa-Medina M, Campos MP, *et al.* Coordination of hypothalamic and pituitary T3 production regulates TSH expression. *J Clin Invest.* 2013;123(4):1492-1500.
- Curry FH, Lisboa PC, Ortega-Carvalho TM, Pazos-Moura CC. The somatostatin analogue octreotide modulates iodothyronine deiodinase activity and pituitary neuromedin B. *Thyroid.* 2000;10(8):647-652.
- Shupnik MA, Chin WW, Habener JF, Ridgway EC. Transcriptional regulation of the thyrotropin subunit genes by thyroid hormone. *J Biol Chem.* 1985;260(5):2900-2903.
- Larsen PR. Thyroid-pituitary interaction: feedback regulation of thyrotropin secretion by thyroid hormones. *New Engl J Med.* 1982;306(1):23-32.
- Baqui MM, Gereben B, Harney JW, Larsen PR, Bianco AC. Distinct subcellular localization of transiently expressed types 1 and 2 iodothyronine deiodinases as determined by immunofluorescence confocal microscopy. Research support, U.S. Gov't, P.H.S. *Endocrinology.* 2000;141(11):4309-4312.
- Curcio C, Baqui MM, Salvatore D, *et al.* The human type 2 iodothyronine deiodinase is a selenoprotein highly expressed in a mesothelioma cell line. Research support, U.S. Gov't, P.H.S. *J Biol Chem.* 2001;276(32):30183-30187.
- Jo S, Fonseca TL, Bocca B, *et al.* Type 2 deiodinase polymorphism causes ER stress and hypothyroidism in the brain. *J Clin Invest.* 2019;129(1):230-245.
- Christoffolete MA, Ribeiro R, Singru P, *et al.* Atypical expression of type 2 iodothyronine deiodinase in thyrotrophs explains the thyroxine-mediated pituitary thyrotropin feedback mechanism. *Endocrinology.* 2006;147(4):1735-1743.
- Bianco AC, Anderson G, Forrest D, *et al.* American Thyroid Association guide to investigating thyroid hormone economy and action in rodent and cell models: report of the American Thyroid Association task force on approaches and strategies to investigate thyroid hormone economy and action. *Thyroid.* 2014;24(1):88-168.
- Ruf-Zamojski F, Zhang Z, Zamojski M, *et al.* Single nucleus multi-omics regulatory landscape of the murine pituitary. *Nat Commun.* 2021;12(1):2677.
- Tanaka K, Shimatsu A, Imura H. Iodothyronine 5-deiodinase in rat posterior pituitary. *Biochem Biophys Res Commun.* 1992;188(1):272-277.
- Medina MC, Molina J, Gadea Y, *et al.* The thyroid hormone-inactivating type III deiodinase is expressed in mouse and human beta-cells and its targeted inactivation impairs insulin secretion. *Endocrinology.* 2011;152(10):3717-3727.
- Batistuzzo A, Salas-Lucia F, Gereben B, Ribeiro MO, Bianco AC. Sustained pituitary T3 production explains the T4-mediated TSH feedback mechanism. *Endocrinology.* 2023. <https://doi.org/10.5281/zenodo.8237270>
- Gouveia CH, Christoffolete MA, Zaitune CR, *et al.* Type 2 iodothyronine selenodeiodinase is expressed throughout the mouse skeleton and in the MC3T3-E1 mouse osteoblastic cell line during differentiation. *Endocrinology.* 2005;146(1):195-200.
- Yaffe D, Saxel O. Serial passaging and differentiation of myogenic cells isolated from dystrophic mouse muscle. *Nature.* 1977;270(5639):725-727.
- Hull RN, Cherry WR, Weaver GW. The origin and characteristics of a pig kidney cell strain, LLC-PK. *In Vitro.* 1976;12(10):670-677.
- An X, Ogawa-Wong A, Carmody C, *et al.* A type 2 deiodinase-dependent increase in Vegfa mediates myoblast-endothelial cell crosstalk during skeletal muscle regeneration. *Thyroid.* 2021;31(1):115-127.
- Leonard JL, Ekenbarger DM, Frank SJ, Farwell AP, Koehle J. Localization of type I iodothyronine 5'-deiodinase to the basolateral plasma membrane in renal cortical epithelial cells. *J Biol Chem.* 1991;266(17):11262-11269.

32. Grozovsky R, Ribich S, Rosene ML, *et al.* Type 2 deiodinase expression is induced by peroxisomal proliferator-activated receptor- γ agonists in skeletal myocytes. *Endocrinology*. 2009;150(4):1976-1983.
33. Parra-Montes de Oca MA, Sotelo-Rivera I, Gutierrez-Mata A, Charli JL, Joseph-Bravo P. Sex dimorphic responses of the hypothalamus-pituitary-thyroid axis to energy demands and stress. *Front Endocrinol (Lausanne)*. 2021;12:746924.
34. Marassi MP, Fortunato RS, da Silva AC, *et al.* Sexual dimorphism in thyroid function and type 1 iodothyronine deiodinase activity in pre-pubertal and adult rats. *J Endocrinol*. 2007;192(1):121-130.
35. Schomburg L, Bauer K. Regulation of the adenohypophyseal thyrotropin-releasing hormone-degrading ectoenzyme by estradiol. *Endocrinology*. 1997;138(9):3587-3593.
36. Oppenheimer JH, Schwartz HL, Surks MI. Propylthiouracil inhibits the conversion of L-thyroxine to L-triiodothyronine. An explanation of the antithyroxine effect of propylthiouracil and evidence supporting the concept that triiodothyronine is the active thyroid hormone. *J Clin Invest*. 1972;51(9):2493-2497.
37. Larsen PR, Silva JE, Kaplan MM. Relationships between circulating and intracellular thyroid hormones: physiological and clinical implications. *Endocr Rev*. 1981;2(1):87-102.
38. Dentice M, Marsili A, Ambrosio R, *et al.* The FoxO3/type 2 deiodinase pathway is required for normal mouse myogenesis and muscle regeneration. *J Clin Invest*. 2010;120(11):4021-4030.
39. Marsili A, Tang D, Harney JW, *et al.* Type II iodothyronine deiodinase provides intracellular 3,5,3'-triiodothyronine to normal and regenerating mouse skeletal muscle. *Am J Physiol Endocrinol Metab*. 2011;301(5):E818-E824.
40. Hosoi Y, Murakami M, Mizuma H, Ogiwara T, Imamura M, Mori M. Expression and regulation of type II iodothyronine deiodinase in cultured human skeletal muscle cells. *J Clin Endocrinol Metab*. 1999;84(9):3293-3300.
41. Ettleson MD, Prieto WH, Russo PST, *et al.* Serum thyrotropin and triiodothyronine levels in levothyroxine-treated patients. *J Clin Endocrinol Metab*. 2022;108(6):e258-e266.
42. St Germain DL, Adler RA, Galton VA. Thyroxine 5'-deiodinase activity in anterior pituitary glands transplanted under the renal capsule in the rat. *Endocrinology*. 1985;117(1):55-63.
43. Sagar GD, Gereben B, Callebaut I, *et al.* Ubiquitination-induced conformational change within the deiodinase dimer is a switch regulating enzyme activity. *Mol Cell Biol*. 2007;27(13):4774-4783.
44. Zavacki AM, Arrojo EDR, Freitas BC, *et al.* The E3 ubiquitin ligase TEB4 mediates degradation of type 2 iodothyronine deiodinase. *Mol Cell Biol*. 2009;29(19):5339-5347.
45. Watanabe M, Houten SM, Matak C, *et al.* Bile acids induce energy expenditure by promoting intracellular thyroid hormone activation. *Nature*. 2006;439(7075):484-489.
46. Arrojo EDR, Egri P, Jo S, Gereben B, Bianco AC. The type II deiodinase is retrotranslocated to the cytoplasm and proteasomes via p97/Atx3 complex. *Mol Endocrinol*. 2013;27(12):2105-2115.
47. Curcio-Morelli C, Zavacki AM, Christofollete M, *et al.* Deubiquitination of type 2 iodothyronine deiodinase by von Hippel-Lindau protein-interacting deubiquitinating enzymes regulates thyroid hormone activation. *J Clin Invest*. 2003;112(2):189-196.
48. Steinsapir J, Bianco AC, Buettner C, Harney J, Larsen PR. Substrate-induced down-regulation of human type 2 deiodinase (hD2) is mediated through proteasomal degradation and requires interaction with the enzyme's Active center. *Endocrinology*. 2000;141(3):1127-1135.
49. Nicoloff JT, Lum SM, Spencer CA, Morris R. Peripheral autoregulation of thyroxine to triiodothyronine conversion in man. *Horm Metab Res Suppl*. 1984;14:74-79.
50. Silva JE, Gordon MB, Crantz FR, Leonard JL, Larsen PR. Qualitative and quantitative differences in the pathways of extrathyroidal triiodothyronine generation between euthyroid and hypothyroid rats. *J Clin Invest*. 1984;73(4):898-907.
51. Geffner DL, Azukizawa M, Hershman JM. Propylthiouracil blocks extrathyroidal conversion of thyroxine to triiodothyronine and augments thyrotropin secretion in man. *J Clin Invest*. 1975;55(2):224-229.
52. Abuid J, Larsen PR. Triiodothyronine and thyroxine in hyperthyroidism. Comparison of the acute changes during therapy with antithyroid agents. *J Clin Invest*. 1974;54(1):201-208.
53. Cooper DS, Saxe VC, Meskell M, Maloof F, Ridgway EC. Acute effects of propylthiouracil (PTU) on thyroidal iodide organification and peripheral iodothyronine deiodination: correlation with serum PTU levels measured by radioimmunoassay. *J Clin Endocrinol Metab*. 1982;54(1):101-107.
54. Dentice M, Bandyopadhyay A, Gereben B, *et al.* The Hedgehog-inducible ubiquitin ligase subunit WSB-1 modulates thyroid hormone activation and PTHrP secretion in the developing growth plate. Research support, N.I.H., extramural research support, U.S. Gov't, P.H.S. *Nat Cell Biol*. 2005;7(7):698-705.
55. Zavacki AM, Harney JW, Li J, Gereben B, Fekete C, Bianco AC. TEB4 Is an endoplasmic Reticulum ubiquitin ligase that mediates type 2 iodothyronine deiodinase (D2) degradation. presented at: 89th Annual Meeting of the Endocrine Society; 2007; Toronto, Canada.
56. Zhang X, Jiang Y, Han W, *et al.* Effect of prolonged iodine overdose on type 2 iodothyronine deiodinase ubiquitination-related enzymes in the rat pituitary. *Biol Trace Elem Res*. 2016;174(2):377-386.
57. Fekete C, Freitas BC, Zeold A, *et al.* Expression patterns of WSB-1 and USP-33 underlie cell-specific posttranslational control of type 2 deiodinase in the rat brain. *Endocrinology*. 2007;148(10):4865-4874.
58. Tu HM, Kim SW, Salvatore D, *et al.* Regional distribution of type 2 thyroxine deiodinase messenger ribonucleic acid in rat hypothalamus and pituitary and its regulation by thyroid hormone. *Endocrinology*. 1997;138(8):3359-3368.
59. Guadano-Ferraz A, Obregon MJ, St Germain DL, Bernal J. The type 2 iodothyronine deiodinase is expressed primarily in glial cells in the neonatal rat brain. *Proc Natl Acad Sci U S A*. 1997;94(19):10391-10396.
60. Werneck de Castro JP, Fonseca TL, Ueta CB, *et al.* Differences in hypothalamic type 2 deiodinase ubiquitination explain localized sensitivity to thyroxine. *J Clin Invest*. 2015;125(2):769-781.
61. Baur A, Kohrle J. Type 1 deiodinase is stimulated by iodothyronines and involved in thyroid hormone metabolism in human somatomammotroph GX cells. *Eur J Endocrinol*. 1999;140(4):367-370.
62. Baur A, Bauer K, Jarry H, Kohrle J. 3,5-diiodo-L-thyronine stimulates type 1 5'-deiodinase activity in rat anterior pituitaries in vivo and in reaggregate cultures and GH3 cells in vitro. *Endocrinology*. 1997;138(8):3242-3248.
63. Baur A, Buchfelder M, Kohrle J. Expression of 5'-deiodinase enzymes in normal pituitaries and in various human pituitary adenomas. *Eur J Endocrinol*. 2002;147(2):263-268.
64. Tannahill LA, Visser TJ, McCabe CJ, *et al.* Dysregulation of iodothyronine deiodinase enzyme expression and function in human pituitary tumours. *Clin Endocrinol (Oxf)*. 2002;56(6):735-743.