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A Direct Comparison of Thyroid Hormone Receptor Protein Levels in Mice Provides Unexpected Insights into Thyroid Hormone Action

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Background: Thyroid hormone (TH) action is mediated by three major thyroid hormone receptor (THR) isoforms α1, β1, and β2 (THRA1, THRB1, and THRB2). These THRs and a fourth major but non-TH binding isoform, THRA2, are encoded by two genes *Thra* and *Thrb*. Reliable antibodies against all THR isoforms are not available, and THR isoform protein levels in mammalian tissues are often inferred from messenger RNA (mRNA) levels.

Methods: We generated knock-in mouse models expressing endogenously and identically 2X hemagglutenin epitope (HA)-tagged THRs (THRA1/2, THRB1, and THRB2), which could then be detected by commercially available anti-HA antibodies. Using nuclear enrichment, immunoprecipitation, and Western blotting, we determined relative THR protein expression in 16 mouse organs.

Results: In all peripheral organs tested except the liver, the predominant THR isoform was THRA1. Surprisingly, in metabolically active organs such as fat and muscle, THRB1 protein levels were up to 10 times lower than that of THRA1, while their mRNA levels appeared similar. In contrast to peripheral organs, the central nervous system (CNS) had a unique pattern with relatively low levels of both THRB1 and THRA1, and high levels of THRA2 expression. As expected, THRB2 was highly expressed in the pituitary, but a previously unknown sex-specific difference in THRB2 expression was found (female mice having higher pituitary expression than male mice). Higher THRB2 expression appears to make the central axis more sensitive to TH as both serum thyrotropin and *Tshb* mRNA levels were lower in female mice.

Conclusions: Direct comparison of THR protein abundance in different organs using endogenously tagged HA-THR mouse lines shows that expression of THR isoforms is regulated at transcriptional and posttranscriptional levels, and in organ-specific manner. The prevalence of THRA1 and low abundance of THRB1 in majority of peripheral tissues suggest that peripheral actions of these isoforms should be revisited. A unique pattern of high THRA2 in CNS warrants further exploration of this non-TH binding isoform in brain development. Finally, THRB2, in addition to cell-specific control, is also regulated in a sex-specific manner, which may change the hypothalamus–pituitary–thyroid axis set point and perhaps metabolism in males and females.

Keywords: nuclear hormone receptor, isoform specificity, thyroid hormone receptor (TRH), endogenous gene tagging, pituitary

Introduction

Thyroid hormones (TH) are essential in human development and for metabolic function in adults. Regulation of transcription of multiple genes in different tissues is believed to underlie most of the actions of TH. TH action is mediated by three major thyroid hormone receptor (THR) isoforms: α1, β1, and β2 (THRA1, THRB1, and THRB2), encoded by two genes *Thra* and *Thrb* (Fig. 1).

Thra1 is expressed at the messenger RNA (mRNA) level in a wide variety of tissues and organs and is necessary for a number of mammalian processes, such as brain and bone development, adipogenesis, and cardiac activity (1–5). In mammals, a second isoform termed THRA2 is expressed from the *Thra* locus. THRA2 is identical to THRA1 in its N-terminus; but due to alternative splicing, THRA2 contains a truncated ligand binding domain (LBD) (6–9). Significantly, different C-terminus renders THRA2 unable to bind TH and appears to change its

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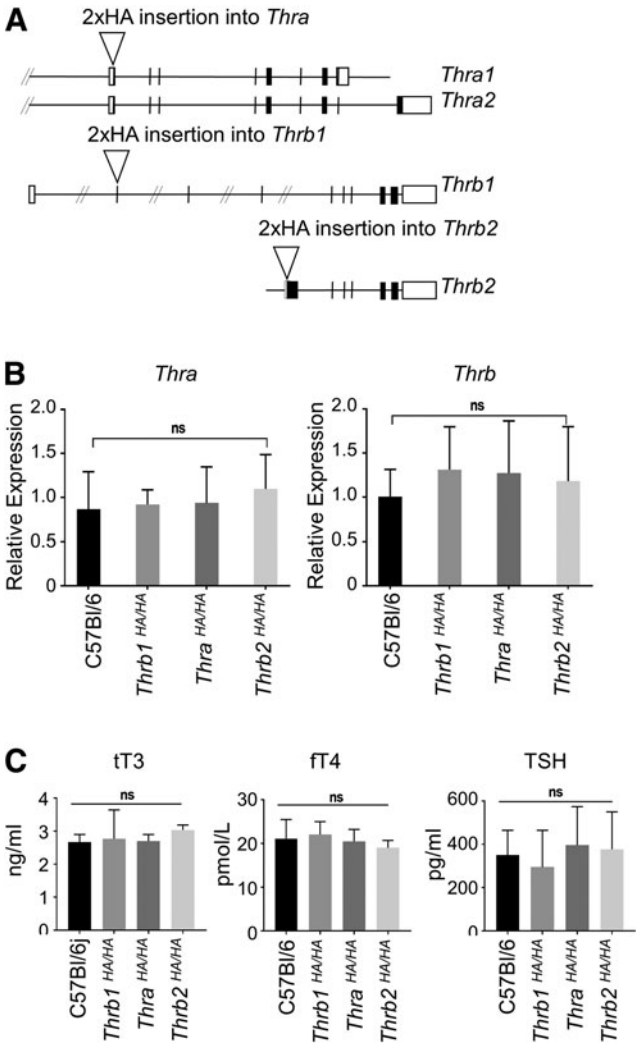

FIG. 1. CRISPR Cas9-mediated knock-in of 2XHA tag. (**A**) Schematic representation of the mouse Thra and Thrb genes with respective location of HA-coding sequences for $Thra^{HA}$, $Thrb1^{HA}$, and $Thrb2^{HA}$. Open triangles indicate position of insert, solid rectangles and vertical lines represent coding exons, and open rectangles represent untranslated portions of exons. Long introns (horizontal lines) interrupted by double slash. (**B**) Relative expression of *Thra* and *Thrb* in homozygous knock-in mice livers compared with WT C57BL/6. Gene expression levels were determined by qRT-PCR and normalized to *β-actin* ($n > 4$). (**C**) Knock-in animals showed no significant difference in serum thyroid hormone level or TSH compared with WT. Adult male mice (4–6 months old) of each genotype were used for T3 ($n = 3$), fT4 ($n > 6$), and TSH ($n > 6$) measurement. Error bars are SD. One-way ANOVA was used for statistical analysis. ANOVA, analysis of variance; fT4, free thyroxine; HA, hemagglutenin epitope; qRT-PCR, quantitative reverse transcription PCR; SD, standard deviations; tT3, total triiodothyronine; TSH, thyrotropin; WT, wild type.

ability to bind partners on DNA or co-regulators via protein–protein interactions. Neural tissues are reported to have high levels of *Thra2* expression (10–12). Like *Thra1*, *Thrb1 is* expressed at the mRNA level in a wide variety of tissues and organs, such as the liver, heart, kidney, fat, and lung (5,13–16). Exploiting the potential importance of THRB1 in regulating cholesterol metabolism, THRB-selective ligands were developed and tested in human clinical trials (17–19). The THRB2 isoform is generated by alternative transcriptional site utilization and splicing of the *Thrb* locus, resulting in a different N-terminal A/B domain in THRB2 versus THRB1. This domain is also the most divergent domain among the THR isoforms, while the central DNA-binding domain (DBD) and LBD show more than 90% homology among the three isoforms at the protein level. Unlike the more broadly expressed *Thra1* and *Thrb1*, *Thrb2* expression is restricted to the pituitary thyrotrophs, hypothalamic TRH neurons, cone cells of the retina, and hair cells in the cochlea (20–25).

Functional and structural differences between THR isoforms have been acknowledged by many researchers (26–29). Isoform-specific action and organ-specific expression patterns contribute to the differences found in *Thra* and *Thrb* KO mice and in humans with mutations in the same genes who have TH resistance syndromes (30–33).

It has been shown that both *Thra1* and *Thrb1* mRNA are widely expressed. The relative abundance of the transcripts was assessed by northern blotting, quantitative reverse transcription PCR (qRT-PCR), microarrays, and more recently using transcriptomics approaches across large panels of tissues (34,35). While providing valuable information, mRNA levels do not address translational or posttranslational events that may affect THR protein levels. THR protein levels theoretically could have been determined by a proteomics approach (36,37), but generally low levels of endogenous nuclear receptors make this approach impractical. Furthermore, the antibodies against THR are unreliable, not widely available or in most cases no longer manufactured, prohibiting direct comparison of THR isoform relative abundance. The question then of whether THRA1 and THRB1 isoforms are widely and comparably expressed remains unanswered.

Here, we report three new mouse knock-in lines that carry identical 2XHA insertions endogenously tagging *Thra1/2*, *Thrb1*, and *Thrb2* isoforms. We decided to use a small neutrally charged N-terminal tag based on our previous research demonstrating that N-terminally hemagglutenin epitope (HA)-tagged THRs are functional and readily detectible by Western blotting (27). Importantly, CRISPR/Cas9-mediated insertions of HA-sequences did not cause detectable changes in levels of corresponding transcripts nor did they cause obvious phenotypic changes, suggesting that the HA-tagged form of each THR does not alter its function or expression.

As expected, THR levels in many tissues were low, and detection of endogenous THRs required additional efforts such as nuclear enrichment and immunoprecipitation (IP); but we were able to detect one or more THR isoforms in large array of organs. Surprisingly in all organs, except the liver, THRA1 was the predominately or exclusively (based on detection limits) expressed protein isoform. Only in the liver was THRB1 found to be expressed at higher levels than THRA1. As expected, THRB2 has limited expression and was only detected in the pituitary extracts (retina and cochlea were not evaluated). What was interesting is that THRB2 expression in female mice was higher than that in male mice; and expression of the THRB2 target gene, *Tshb*, was lower in female mice. Importantly, these HA-THR mouse lines can be used to study cell-specific expression, localization, and function of THRs; characterize isoform protein partners; study posttranslational THR modifications; and compare relative chromatin occupancy.

Materials and Methods

Generation of transgenic mice

All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Rutgers, The State University of New Jersey. All mice had C57BL/6 genetic background and were maintained on a standard chow diet (#5008; LabDiet, St. Louis, MO) with water *ad libitum* in a temperature-controlled facility with a 12-hour light/12-hour dark schedule. Unless otherwise indicated, five- to six-month-old adult animals were used for tissue collection.

Knock-in mice were generated by Genome Editing Shared Resource (GESR), Rutgers University, using pronuclear microinjection of one-cell embryos with CRISPR-Cas9 ribonuclear protein complexes and single-strand oligonucleotide donor. Thirty nanograms per microliter of Cas9 protein (Sigma–Aldrich, St. Louis, MO) was complexed with equimolar amount of crRNA and tracrRNA (see gRNA sequences below) and 50 ng/μL of donor oligo resuspended in microinjection buffer (10 mM Tris, pH 7.5, 0.2 mM EDTA).

Thrb2 2XHA tag: gRNA sequence (C68) AGCAGTTGCT GCTGGCAGCTGGG

Donor oligo sequence, IDT Ultramer, polyacrylamide gel electrophoresis (PAGE) purified (Integrated DNA Technologies, Inc., Coralville, IA): ATGCATGCGTAGAGCGTGT GTATATGTAAAGTAGAACCTGAACCTGGATAGCAA AATGTACCCATACGACGTCCCAGACTACGCTTATC CATATGATGTTCCAGATTATGCTGAATTCAACTAC TGTATGCCAGAGGTACACGAAGTGTGCCCAGCTG CCAGCAGCAACTGCTACATGCAGGTCACTGACTAC (2XHA underlined).

Thrb1 2XHA tag: gRNA (C65) GTCATACTGTTAGGA GTCATAGG

Donor: oligo sequence (IDT Ultramer, PAGE purified): GCTATTTTGTCTCTTTTAGGGAATGCCAGTACAGA AGATGACCCAGCATGACTACTAACCTATGTACCCA TACGACGTCCCAGACTACGCTTATCCATATGATGT TCCAGATTATGCTGAATTCACTCCTAACAGTATGA CAGGTATCTTTGTGTCCTTTCCTGATTTACACTGTG TGTCTTCATCTGTGTAC (2XHA underlined).

Thra 2XHA tag: gRNA (C60) CTGGATGGAATTGAAG TGAATGG

Donor: oligo sequence (IDT Ultramer, PAGE purified): CAGGCCCATTCGTTACCTGTTCTCCTCTGGGTCTGA CCCACACTCCACCTTGCTTGGCTTCTGTTCGAATTC AGCATAATCTGGAACATCATATGGATAAGCGTAGT CTGGGACGTCGTATGGGTACATTCACTTCAATTCC ATCCAGGATGCTCTCCAGCACGCCAAGAGACTGGG GTGGGCA

Two to four C57B/l6 founders were obtained for each knock-in.

Genotyping of transgenic mice

Transgenic mice were genotyped based on the presence of 2XHA insertion (60 bp).

Toes biopsies were digested in 100 μL of 25 mM NaOH and 0.2 mM EDTA (pH 12) for 20 minutes at 95°C. Toes were briefly centrifuged followed by addition of 100 μL of 40-mM Tris-HCl (pH 7.0). One microliter of this reaction was used for subsequent PCRs with the primers below, followed by gel electrophoresis:

Primers: *Thrb2* forward, 5′-TGTATGCTCTCCGAGTAT ATGCAC-3′, and reverse, CCATGTCCAAGTCAGAGTCC TTG; *Thrb1* forward, 5′-TGCCAGTGCCACATAGTAAGA C-3′, and reverse, 5′-TTGCTGCTAAGATTCTCAGGTTG G-3′; *Thra* forward, 5′-TTACTATGGGTGCTGTGCCCT AG-3′, and reverse, 5′-CCAGAGAGCTATCCATATCTG TTG-3′. PCR-generated fragments were cloned into pCR2 .1Topo (Thermo Fisher Scientific, Waltham, MA) and sequenced by the Sanger method.

Protein extraction and Western blot analysis

Isolated tissues were snap-frozen in liquid nitrogen. Liver, cardiac, adipose, muscle, and kidney tissues were grinded in liquid nitrogen with a mortar and pestle. Smaller tissues were homogenized in appropriate buffer using Bullet Blender homogenizer (Next Advance, Laboratory Supply Network, Atkinson, NH) and 0.2 mm Zirconium Oxide Beads.

Protein lysates were generated by homogenizing tissue on ice in radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 1% Igepal CA-630, 50 mM Tris-Cl pH 8.3, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS]) together with additional protease inhibitors (cOmplete™; Roche, Basel, Switzerland). BCA Protein Assay Kit (Pierce, Thermo Fisher Scientific) was used to determine protein concentrations. Lysates were separated using gradient (4–20%) Mini-Protean TGX precast gels (Bio-Rad Laboratories, Inc., Hercules, CA).

Samples were transferred onto Nitrocellulose 0.2 μm membrane (Bio-Rad Laboratories, Inc.) stained with Ponceau S (Fisher Scientific, Hampton, NH), washed in phosphate-buffered saline with 0.2% Tween 20 (PBST), and blocked for one hour at room temperature in 2% nonfat dry milk in PBST. Then, the samples were incubated with anti-HA-peroxidase-conjugated antibody (1:1000, 11867423001; Millipore Sigma, Burlington, MA) overnight at 4°C. Membranes were washed three times for 15 minutes each in PBST. Blots were imaged at ChemiDoc Touch Imaging System (Bio-Rad Laboratories, Inc.). ImageLab 6 (Bio-Rad Laboratories, Inc.) was used to process images and quantify band intensity.

Nuclear enrichment

Frozen powdered or minced tissue sample ($\sim$200 mg) was mixed to 400 μL of hypotonic buffer (10 mM KCl, 10 mM HEPES 0.1 M EDTA, 1 mM DTT, pH 8.0) with one protease inhibitor tablet (Roche, Fisher Scientific) and 1 mM DTT and homogenized using Bullet Blender homogenizer and 0.2–0.5 mm Zirconium Oxide Beads. Homogenate was separated from beads and left to swell on ice for 30 minutes; 25 μL of 20% Igepal CA-630 (Millipore Sigma) was added for every 400 μL of sample and vortexed for 20 seconds; nuclei were collected by centrifugation at 13,000 *g* for 20 seconds. Nuclear-enriched pellet was resuspended in 30 μL of SDS lysis buffer (10 mM Tris-HCl pH 6.8, 10% SDS, 20% glycerol) and heated at 95°C for 10 minutes. Protein concentration was calculated using absorbance of diluted sample at 280 nm; 2 μg of protein was used for Western blotting. Histones were used as loading control.

IP of HA-tagged proteins

RIPA protein extracts were sonicated in ice to disrupt the membranes and nucleic acids. The protein concentration of

starting total protein extract was measured by BCA colorimetric assay and SDS-PAGE stained with Coomassie blue. Several immunopurifications were performed for each genotype. Four hundred milliliters of RIPA buffer containing protease inhibitors and 3 mg of total protein were mixed with 15 μL of anti-HA magnetic beads (Pierce, Thermo Fisher Scientific). The samples were incubated at 4°C with rotation overnight and washed three times for 10 minutes with RIPA. Thirty microliters of SDS lysis buffer was used to elute proteins for 10 minutes at 95°C; 5 μL of 6×Laemmli sample buffer with beta-mercaptoethanol was added to each sample.

RNA extraction and qRT-PCR

RNA was isolated from powdered aliquot of the same tissues that were used for protein extraction, using TRIzol (Invitrogen; Carlsbad, CA) according to the manufacturer's instructions. Complementary DNA was synthesized using iScript Reverse Transcription Supermix (Bio-Rad Laboratories, Inc). quantitative PCR (qPCR) was performed using the following primers:

Thrb1 forward, 5′-CCTGGATCCTGACGATGTGAA-3′, and reverse, 5′-ACAGGTGATGCAGCGATAGT-3′; *Thrb* forward, 5′-AACCAGTGCCAGGAATGTCG-3′, and reverse, 5′-CTCTTCTCACGGTTCTCCTC-3′; *Thra* forward, 5′-GGCTGTGCTGCTAATGTCAA-3′, and reverse, 5′-CTTTAGACTTCCTGATCCTCAAAG-3′; *Thra1* forward, 5′-GTCTCTGACGCCATCTTTGAAC-3′, and reverse, 5′-ACTCGACTTTCATGTGGAGGA-3′; *Tshb* forward, 5′-GTGCTGGGTATTGTATGACACG-3′, and reverse 5′-CTGGTATTTCCACCGTTCTGTAG-3′.

Thyrotropin and TH measurement

Serum thyrotropin (TSH) was measured by Luminex® MAGPIX®, using a Milliplex® Map Mouse Pituitary Magnetic Bead Panel (EMD Millipore, Billerica, MA). Bio-Plex Manager™ was used for interpolation of the concentration of the sample from the standard curve. Serum total triiodothyronine (tT3) and free thyroxine (fT4) were measured using Mouse Free Thyroxine and Mouse Tri-iodothyronine ELISA Kits (Aviva Systems Biology, San Diego, CA).

Results

Generation and testing of 2XHA-tagged THR knock-in mice

Knock-in mice carrying 2XHA tag inserted after the first ATG of *Thrb1*, *Thrb2*, and *Thra* (Fig. 1A) were created in GESR, Rutgers University, via CRISPR/Cas9-mediated approach. Two to four 2XHA-positive C57Bl/6 founders were obtained for each insertion. Progeny of each founder was confirmed by PCR and Sanger sequencing. The area of insertion, plus 1 kb upstream and 1 kb downstream of the insertion, was sequenced to ensure in-frame insertion of the tag, and lack of genome alteration and point mutations in surrounding area. Positive F1 heterozygous mice were bred with wild-type (WT) C57Bl/6. Back-breeding into a C57Bl/6 background was repeated seven times to eliminate background mutations potentially generated by the CRISPR/Cas9 technique. F7 heterozygous mice were crossed with their siblings to generate 2XHA-tagged homozygous animals.

Homozygous $Thrb1^{HA/HA}$, $Thrb2^{HA/HA}$, $Thra^{HA/HA}$ mice developed and grew normally, had a normal life span, and were fertile. No significant differences in body weight or hormone levels, such as tT3, fT4, or TSH, were observed between knock-in and WT mice (Fig. 1C). We also did not notice phenotypic or HA-protein abundance variations among individual founder lines (data not shown). Finally, HA tag insertions did not alter mRNA levels of corresponding genes in the liver or pituitary (Fig. 1B and section ''Expression of THR isoforms in the pituitary of adult knock-in mice'').

HA-tagged THR isoforms in peripheral tissues

Nuclear receptors, including THRs, are proteins of low abundance and are a major challenge to detect and measure accurately (36,37). Consistent with this observation, we were unable to detect HA-THRs by Western blotting of total protein extracts from all organ tested, except the pituitary. To enrich for HA-THRs before a Western blot analysis, two approaches were employed: (i) Nuclear fraction enrichment and (ii) IP using an anti-HA antibody coupled to magnetic beads. The nuclear extract approach was attempted for all organs tested. Among these organs, only the liver and lung showed detectable HA-THRA1/2 and HA-THRB1 protein levels (representative Western blots are shown at Fig. 2A). In $Thrb1^{HA/HA}$ animals, HA-THRB1 was detected at ~55 kDa (481 aa). In $Thra^{HA/HA}$ animals, two major bands were detected at ~58 and ~50 kDa corresponding to HA-THRA2 (512 aa) and HA-THRA1 (430 aa). No HA-THRB2 (495 aa) was detected in the peripheral organs of $Thrb2^{HA/HA}$ mice; and therefore, $Thrb2^{HA/HA}$ mice serve as a negative control in these experiments. The predominant THR isoform in the liver was THRB1 (Fig. 2A), and the relative abundance of THRA1 was about 50% of THRB1. HA-THRA2 was undetectable in the liver nuclear extracts; but after IP, THRA2 was determined to be about 10% of THRA1 in the liver. In contrast, HA-THRA1 was highly expressed in the lung (Fig. 2A); and HA-THRA2 was also present in lung nuclear extracts, but at lower levels (40–60% of that of HA-THRA1). HA-THRB1 was below detection levels in the nuclei isolated from $Thrb1^{HA/HA}$ lung. No significant sex-specific differences were found in THR isoform expression in peripheral organs among the HA lines (data not shown).

IP with an anti-HA antibody allowed detection of at least one THR isoform in every organ we evaluated. The method has intrinsic variability due to differences in individual tissue composition or organ structure. To minimize variability, we used identical methods and total protein content of starting material and repeated experiments multiple times using independent mouse samples. Representative Western blots are shown in Figure 2B; the data points on the graph represent individual IPs from independent samples. Similar to results with nuclear extracts, no significant sex-specific differences were found in THR isoform expression among the HA lines from IP samples (data not shown).

HA-THRA1 was detected in the heart, muscle, kidney, spleen, intestine, and adipose depots. Relative to HA-THRA1, HA-THRB1 was detected at low levels in the heart, kidney, spleen, intestine, and adipose depots (4- to 10-fold lower density on Western blot analysis). Most peripheral organs also expressed HA-THRA2 but only at 10–30% of HA-THRA1 levels, and HA-THRA2 was below detection levels in muscle.

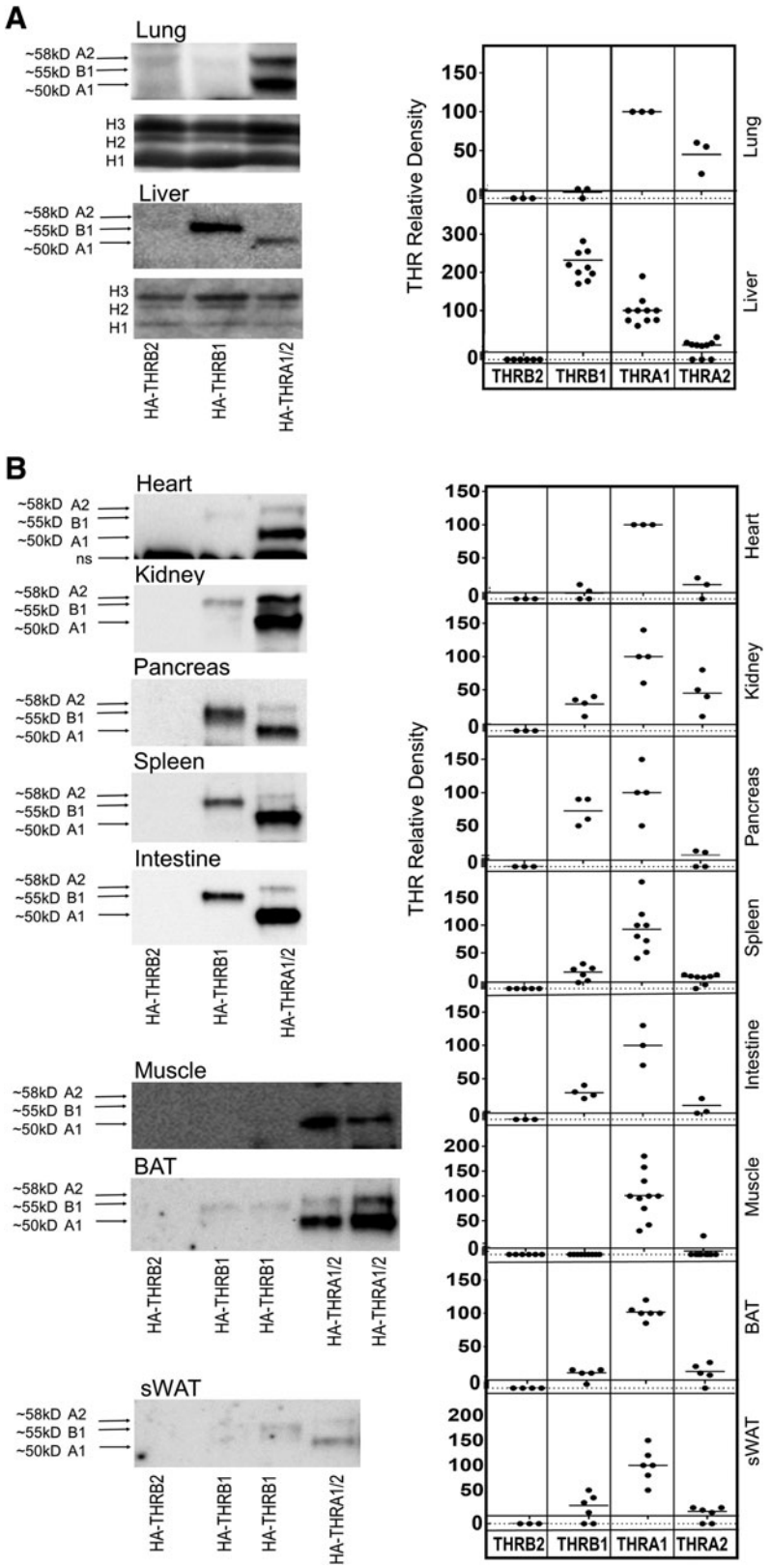

FIG. 2. THR isoform expression in peripheral organs. (**A**) Representative Western blots (4–20% acrylamide gel, anti-HA antibody) showing HA-THRA1/2 (430 and 512 aa) in the lung, and HA-THRB1 (481 aa) and HA THRA1 in the liver nuclear extracts. No HA-THRB2 (495 aa) was detected in these organs. Histones were used for a loading control. Graphs represent relative band density of THRs from individual animals. Average density of THRA1 was assumed 100%. Both sexes were used for the liver (AM = 6, AF = 4, B2M = 4, B2F = 2, B1M = 6, B1F = 3), spleen (AM = 5, AF = 3, B2M = 3, B2F = 2, B1M = 5, B1F = 3), BAT (AM = 4, AF = 2, B2M = 2, B2F = 2, B1M = 3, B1F = 2), and sWAT (AM = 4, AF = 2, B2M = 2, B2F = 1, B1M = 4, B1F = 2); male tissues were used for the lung, heart, intestine, and pancreas. (**B**) Representative Western blots (4–20% acrylamide gel, anti-HA antibody) showing HA-THR proteins immunoprecipitated from various peripheral organs. *Thrb2*$^{HA/HA}$ mice served as negative controls. Data points on the graph represent relative densities of immunoprecipitated THRs from individual animals with average THRA1 density from the same experiment at 100%. Data points below the line represent failure to detect protein. AF, THRA females; AM, THRA males; B1F, THRB1 females; B1M, THRB1 males; B2F, THRB2 females; B2M, THRB2 males; sWAT, subcutaneous white adipose tissue; THR, thyroid hormone receptor.

Both THRA1 and THRB1 isoforms are reported to be expressed in adipose depots and play important roles in metabolism (16,38). Our data, however, show that HA-THRA1 is the predominantly expressed isoform in both brown adipose tissue (BAT) and subcutaneous white adipose tissue (sWAT), although the yield of either THR isoform from sWAT was lower. Several attempts at IP of HA-THRs from inguinal WAT (iWAT) yielded undetectable amounts of any HA-THR protein. Strikingly, HA-THRA1 is up to 10-fold more abundant in BAT than

THRB1, yet THRB1 is reported to be a critical regulator of activation of BAT-like program and *Ucp1* gene expression in adipose tissues (3,38,39).

Comparison of relative THR transcript and protein abundance

Two recent studies (34,35) reported transcriptome-wide expression across multiple tissues and organs using RNA-Seq by next-generation sequencing. This approach provides transcript quantification relative to the total transcriptome, which allows for comparisons among different transcripts within many tissues and organs. Therefore, we generated an ''electronic northern'' using data from the study of Li *et al.* (34) (Fig. 3A). With exception of the liver, RNA-Seq data represented tissues from two male and two female mice (data points on Fig. 3A). While no overt sex-specific differences in THR transcripts were seen in peripheral organs, the limited number of samples did not allow statistical analysis. The ratio between *Thrb* and *Thra* (combined males and females, four to eight samples total, Fig. 3B) showed a 4- to 10-fold decrease in *Thrb* transcripts versus *Thra*, except in the liver where *Thrb* is a predominant transcript. Very high *Thra* transcript abundance in the brain ($\sim$10 times of that in other tissues) also resulted in a strikingly low *Thrb/Thra* ratio. We next compared expression of *Thrb1* and *Thra1* using qRT-PCR and primer sets directed at isoform-specific regions of each transcript (Fig. 3C). Because the expression of housekeeping genes used for normalization varies from tissue to tissue, we normalized *Thrb1* to *Thra1* expression levels (*Thrb1/Thra1*, Fig. 3C). RNA was obtained from selected organs of the HA knock-in mice. With exception of muscle and heart, the *Thrb1/Thra1* expression profiles for most tissues were similar to *Thrb/Thra* values from RNA-seq. We used the same approach to assess THR transcripts in fat depots that are not represented in published transcriptome-wide approach: BAT, sWAT, and iWAT.

In general, a 5- to 10-fold increase in *Thra* over *Thrb* transcripts (see ratio in Fig. 3B, C) results in 5- to 10-fold increase of THRA1/THRB1 density on Western blots; however, this correlation does not hold true in several organs and points out the limitation of using RNA analyses to infer THR protein levels. For example, both qRT-PCR and RNA-seq predicted high expression of *Thrb* relative to *Thra* in the liver, kidney, BAT, and muscle, and lower relative levels of *Thrb1*

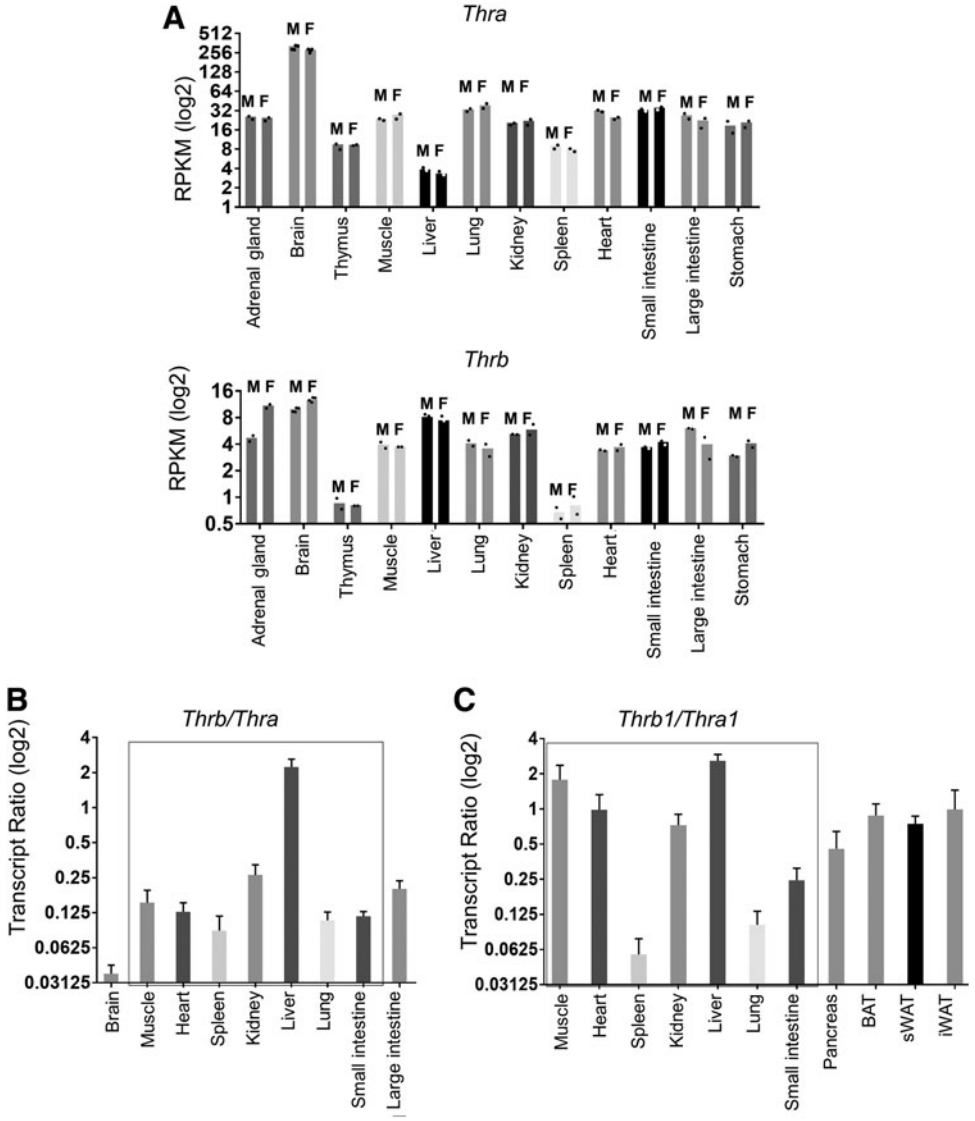

FIG. 3. THR transcripts in mouse organs. (**A**) ''Electronic northern blots'' for *Thra* and *Thrb* generated form RNA-seq data (34). Expression numbers on the Y-axis correspond to mean RPKM values from two to four males (M) and two to four females (F); individual values are shown as dots. Peripheral organs show no sex-specific difference in transcript levels; however, the low number of animals does not allow statistical analysis. (**B**) *Thrb* to *Thra* transcript ratio calculated from RNA-seq data *Thrb* RPKM/*Thra* RPKM ($n > 4$). (**C**) *Thrb1/Thra1* transcript ratio assessed by qRT-PCR. Y-axis represent $2^{-\Delta CT}$. RNA was isolated from HA knock-in animals, which are the same as used for protein analysis ($n > 6$). Rectangles enclose organs analyzed by both methods. RPKM, reads per kilobase per million.

transcripts in the lung, spleen, and small intestine. However, HA-THRB1 was low or undetectable in the heart, BAT, and muscle and present at easily detectable levels in the spleen and small intestine. Furthermore, across several organs, *Thrb* transcript is similar in the liver, kidney, heart, and BAT. Yet, THRB1 protein was easily detected in the liver nuclear extracts, which also yielded high levels of HA-THRB1 after IP, while the kidney, heart, and BAT yielded barely detectable or undetectable THRB1 by IP.

Detection of HA-tagged THR isoforms in the central nervous system

In addition to the lung and liver, THRs were detected readily in nuclear extracts from neural tissues. We tested expression of HA-THR isoforms in the hypothalamus, cerebellum, spinal cord, cortex, and olfactory bulb. We ran several experiments for each tissue (data points in the graph, Fig. 4). All neural tissues tested showed a consistent pattern of isoform expression with HA-THRA1/2 being highly expressed and HA-THRB1 being barely detectable; no sex-specific differences in expression were found (data not shown). In all tissues tested, THRA2 was 5- to 10-fold more abundant than THRA1. Publicly available microarray and sequencing data across mouse and human tissues show the highest levels of *Thra1/2* expression in various parts of the brain but do not quantify relative *Thra1 and Thra2* levels. However, it has been reported that *Thra2* mRNA levels exceed *Thra1* mRNA levels in the rodent brain (6,12,40) consistent with our THRA2 protein levels. Our data also show that THRA2 in the adult brain is in much greater relative abundance than previously appreciated and much more abundant than the previously reported ubiquitous brain expression of THRA1 using a GFP-tagged protein (4), which suggests that this non-TH binding isoform may have a unique function in the brain. Although it is likely present in many brain tissues, THRB1 protein levels are relatively low compared with THRA2 or even THRA1, and THRB1 bordered on being undetectable in many of the brain tissues tested. THRB2 is expressed in a limited number of sites (anterior pituitary, hypothalamus, retina, and inner ear) and could not be detected by Western blotting in the brain tissues we tested.

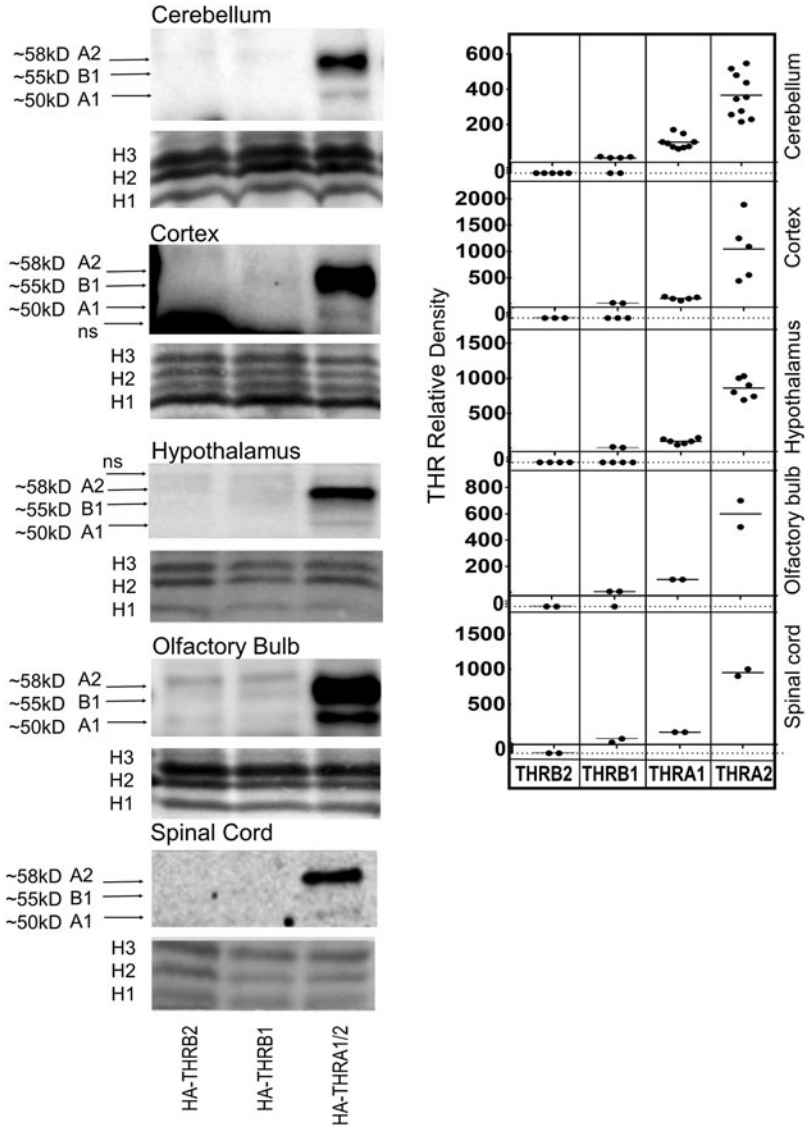

FIG. 4. THR isoform expression in the central nervous system. Representative Western blots (4–20% acrylamide gel, anti-HA antibody) showing HA-THRA1/2, and HA-THRB1 in nuclear extracts from the cerebellum, cortex, hypothalamus, olfactory bulb, and spinal cord. THRA2 is a predominant isoform in all tested tissues tested. NS marks nonspecific bands present in all samples. Histones were used for a loading control. Relative band density of THRs from several animals is shown in the graph. Average density of THRA1 was 100%. Each datum point represents an individual animal. Both sexes were used for the cerebellum (AM = 6, AF = 4, B2M = 3, B2F = 2, B1M = 3, B1F = 3), cortex (AM = 3, AF = 2, B2M = 2, B2F = 1, B1M = 3, B1F = 2), and hypothalamus (AM = 4, AF = 2, B2M = 3, B2F = 1, B1M = 4, B1F = 2); males for spinal cord and olfactory bulb. Data points below line represent failure to detect protein.

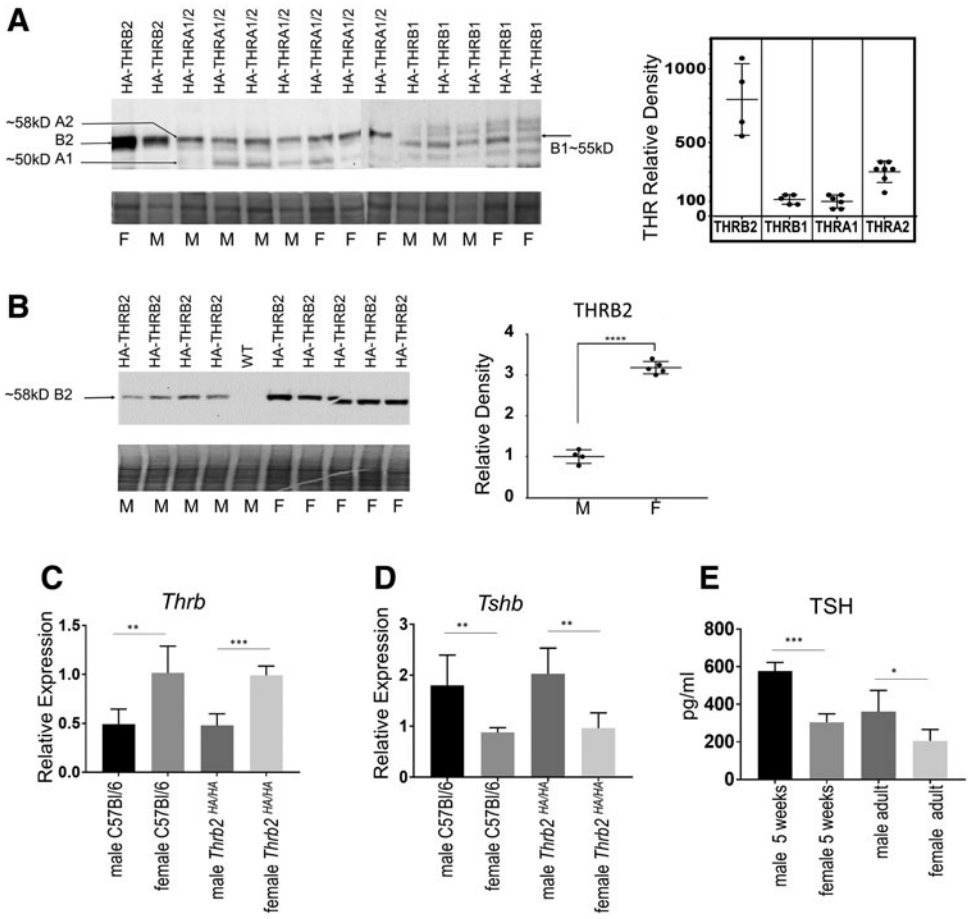

FIG. 5. THR isoform expression in mouse pituitary. (**A**) Western blots (4–20% acrylamide gel, anti-HA antibody) showing HA-THRB2 (495 aa), HA-THRA1/2 (430 and 512 aa), and HA-THRB1 (481 aa) in total protein extracts from individual pituitaries of male (M) and female (F) mice. Relative band density of THRs is shown in the graph. Average density of THRA1 was 100%. (**B**) Western blot (4–20% acrylamide gel, anti-HA antibody) showing THRB2 expression in females is significantly higher than that in males. (**C, D**) Relative expression of *Thrb* is significantly higher in female mice than in male mice of both WT and *Thrb* $^{HA/HA}$ genotypes, while *Tshb* relative expression is lower in females. Gene expression levels were determined by qRT-PCR and normalized to *β-actin*. (**E**) TSH is lower in female mice than in male mice. Younger (5 weeks old) animals show more pronounces difference than adult (5 months old) mice. Results are mean ($n > 4$), and error bars are SD. Student T-test or one-way ANOVA were used for statistical analysis. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

Expression of THR isoforms in the pituitary of adult knock-in mice

Interestingly, the pituitary was the only organ in which all THR isoforms could be readily detected in total protein extracts. *Thrb2* is known to be predominantly expressed in thyrotrophs of the anterior pituitary gland (20,41); and in *Thrb2*$^{HA/HA}$ mice, pituitary HA-THRB2 is observed as a single band of ~57 kDa (495 aa, Fig. 5A, C). As expected, this band was not present in pituitary protein samples from WT and *Thrb1*$^{HA/HA}$ mice. HA-THRB2 represented the major THR isoform in the pituitary, and its level exceeded HA-THRB1 (~55 kDa) and HA-THRA1 (~50 kDa) by approximately five- to sevenfold (Fig. 5A, B). Similar to other brain tissues, the pituitary gland has relatively high THRA2 expression (~58 kDa, two- to threefold more than THRA1 and THRB1), but THRB2 levels were about two- to threefold higher than THRA2 in Western blots. No sex-specific differences in HA-THRA1, HA-THRA2, and HA-THRB1 were found (Fig. 5A).

Unlike the other THR isoforms, HA-THRB2 showed a significant sex-specific difference, where THRB2 was more than threefold higher in female versus male pituitaries. This phenomenon was observed in multiple experiments and correlated with higher levels of *Thrb2* mRNA levels in adult WT and *Thrb2*$^{HA/HA}$ female versus male mice (Fig. 5B, C). Based on our observations, the pituitary was the only organ, and THRB2 was the only isoform in mice that displayed significant sex-specific differences in THR protein expression.

We next tested if the high levels of THRB2 altered expression of *Tshb*, a major gene target of THRB2 in the pituitary (21,42). We found lower expression of *Tshb* in both WT and *Thrb2*$^{HA/HA}$ female mice (Fig. 5D) and lower levels of serum TSH in female mice especially in younger (5 weeks) animals (Fig. 5E). A similar sex-specific difference in TSH has been observed in a number of mouse studies (43–47).

Discussion

Tagged endogenous proteins are a proven tool allowing researchers to visualize and study proteins in the absence of suitable antibodies to the endogenous target. Endogenously

tagged *Thra1-GFP* (4,48) has been used for identification of thyroid response elements in the brain. In our studies, we identically tagged individual THRB and both THRA isoforms allowing a direct comparison among endogenous THRs in multiple assays. We chose the N-terminal HA tag because our previous studies showed that this tag does not alter protein function, and there is an array of validated commercially available antibodies against HA. As with any genetic modification, HA tag insertion could affect the levels, stability, or functionality of THRs. However, we did not detect overt changes in expression of corresponding transcripts nor did we find phenotypic abnormalities in knock-in mouse lines, suggesting that HA tag insertion had no or a minimal effect on the content and activity of THRs. Importantly, using a common epitope present on four major THR isoforms allows for detection of THRs with uniform efficiency. These mouse lines can be used for assessment of relative protein abundance in organs, comparison of chromatin occupancy, accurate analysis of protein partners, assessment of subcellular localization, and other important studies.

Our analysis of THR organ-specific expression confirms that at least one THR isoform is present in all organs, although at low levels which is typical for nuclear hormone receptors. With the exception of the liver, the predominant THR isoform in all peripheral organs we tested is THRA1. The liver was also the only organ where THRB1 expression exceed that of THRA1. It has been reported that *Thra* and possibly *Thrb1* have zonal expression in the liver (49,50). Differential zonal expression of THRA1 versus THRB1 in the liver could result in previously uncharacterized isoform-specific functions. Alternatively, isoform-specific functions of THRA1 and THRB1 may be due to differential target gene binding. We believe that these knock-in mouse models will help to answer these questions.

Overall, the protein expression in majority of organs correlated with mRNA levels, both measured by RNA-seq or estimated by qRT-PCR. However, there are some notable exceptions, which highlight the limitations of RNA analyses for predicting THR protein levels. For example, our qRT-PCR data and previous reports (16) suggested similar *Thra* and *Thrb* transcript levels in all adipose depots. We demonstrate, however, that THRA1 abundance is greater than THRB1 in adipose depots, especially in BAT. This seemed somewhat puzzling given multiple studies purporting a direct effect of THRB1- and THRB-selective agonists on inducing a BAT-like program in adipose tissues (3,38,39,51,52). Paradoxically, however, THs and THRB-specific agonists appear to have ''whitening'' effects on BAT itself (52,53). Assuming a direct effect of THRB1 on adipose depots, our findings suggest that the abundant expression of THRA1 cannot substitute for THRB1 in adipose depots to activate thermogenic genes, which implies an isoform-specific set of target genes. Alternatively, the low relative levels of THRB1 expression in adipose depots, particularly in BAT, could suggest an indirect effect of TH perhaps mediated by THRB1 activation of the autonomic nervous system or hepatic secretion of fibroblast growth factor 21 (FGF21) (39,43,53–55).

We acknowledge that detection of THR protein may vary from organ to organ. This could explain our failure to detect THRB1 in the heart, muscle, iWAT, and some neural tissues, despite *Thrb* transcript levels that were similar or higher than that in the lung and kidney. However, we favor the hypothesis that THR mRNA translation may vary by organ given that at least one THR could be detected in every organ tested, and there is no reason to believe that one tagged THR protein is more readily detected than another.

We were surprised to find a clear difference between THR expression patterns in the brain and peripheral organs. High levels of *Thra2* transcript in mammalian neural tissues have previously been reported, which exceed mRNA levels of functional THRs, especially in perinatal period (10–12). We demonstrate that THRA2 in the brain is 4–10 times more abundant than that of THRA1, while in peripheral organs, THRA1 is a predominant isoform. As THRA2 may bind DNA *in vivo* and modulate transcription in a non-TH dependent manner, it has been proposed that THRA2 may act as a dominant-negative transcriptional factor (7–9,56–58). In this context, the adult mouse brain would then be uniquely insensitive to TH, which seems contrary to TH functional studies. A study by Salto *et al.* (59) shows that ablation of THRA2 causes a concomitant overexpression of THRA1 and suggests a role of THRA2 in fine-tuning of growth and homeostasis. However, THRA1 overexpression is coupled to THRA2 loss such that an independent role for THRA2 in controlling neural development and signaling could not be assessed. We suggest that the high abundance of THRA2 in the brain relative to other THRs, in a pattern not found in peripheral tissues, is suggestive of an TH-independent and previously underappreciated function of this isoform in the brain.

While we did not attempt to provide a comprehensive and statistical analysis of sex-specific expression of THRs in all organs, we did measure THR expression in male and female mice in most organs. There were no apparent sex-specific differences in THR protein expression in peripheral organs we studied (data not shown), but other sex-specific expression differences might be found. For example, a sexual dimorphism has been reported for *Thrb1* in adrenal gland (60) and also detected in *Thrb1/2* transcripts from the adrenal gland and brain by an ''electronic northern'' (Fig. 3A). Other approaches, such as immunostaining of tissue sections, may provide more detailed information about cell-specific THR distribution and sexual dimorphism but are beyond the scope of the current study.

Interestingly, the level of expression of all THR isoforms, and THRB2 in particular, was high enough in the pituitary to allow for Western blot detection of THRs in whole-cell protein extracts. Similar to other tissues, THRA1/2 and THRB1 did not show sex-specific differences in protein levels; however, THRB2 protein levels were clearly higher in females (Fig. 5A, B). THRB2 is reported to be the prevailing isoform in the pituitary, and this agrees well with THR transcript patterns, suggesting three to five times higher levels of *Thrb2* mRNA compared with that of *Thra* (15,61–63). We found that elevated THRB2 protein correlated with higher *Thrb* mRNA levels in females, suggesting distinct and sex-specific transcriptional regulation. The transcription start of *Thrb2* is distant from that of *Thrb1* (Fig. 1), supporting this independent regulation.

One of the essential functions of THRB2 is hormone-dependent repression of *Tshb*, which has a major role in regulating the hypothalamus–pituitary–thyroid (HPT) axis (21,42). Importantly, in the female pituitary where THRB2 expression is high, *Tshb* mRNA expression and plasma TSH

levels are correspondingly lower (Fig. 5E). Similar observations were reported by other investigators (43–46), and more comprehensively studied by McLachlan *et al.* (47) who used three mouse strains. In all reports, sex-specific difference in TSH is more prominent in younger animals and is similar to what we observed (Fig. 5E). Importantly, however, the difference in THRB2 expression in male and female mice persists in adult animals even with a lesser impact on TSH hormone levels, suggesting additional compensatory mechanisms controlling the euthyroid state in male and female mice. Recent study by Rakov *et al.* (44) also suggests that sex-specific differences are more pronounced under hyperthyroid conditions and may be influenced by multiple factors including deiodinase activity (64) and thyroxine-binding globulin concentrations.

In humans, differences in *Thrb2* and *Tshb* expression in men and women are difficult to assess, and the reference level of normal TSH is similar between sexes. However, two large cohort studies with more than 150,000 individuals in each (65,66) show that median TSH levels in thyroid disease-free adults are slightly but significantly higher in males than in females. Moreover, within the normal fT4 reference range, the median TSH levels were significantly higher at every T4 value in men versus women (65). We hypothesize that mice and human employ similar mechanisms contributing to a sexual dimorphism in their HPT axis. Based on our data, differences in THRB2 levels should be taken into account as a factor affecting thyroid status in male and female mice and possibly playing a role in human disease.

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Author Disclosure Statement

No competing financial interests exist.

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