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Barhl1 is directly regulated by thyroid hormone in the developing cerebellum of mice

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ABSTRACT

Thyroid hormones (THs) are essential for the brain development. Despite considerable effort, few genes directly regulated by THs have been identified. In this study, we investigate the effects of THs on the regulation of Barhl1, a transcription factor that regulates sensorineural development. Using DNA microarray combined with chromatin immunoprecipitation (ChIP-chip), we identified a TRβ binding site in the promoter of Barhl1. The binding was further confirmed by ChIP-PCR. The site is located approximately 755 bp upstream of the transcription start site. Reporter vectors containing the binding site or mutated fragments were transfected into GH3 cells. T3 treatment decreased the transcriptional activity of the wild fragment but not the mutant. Two 28 bp oligonucleotides containing sequences that resemble known TH response elements (TREs) were derived from this binding site and DNA–protein interaction was performed using electrophoretic mobility shift assays (EMSA). Binding analysis in a nuclear extract containing TRβ revealed that one of these fragments bound TRβ. This complex was shifted with the addition of anti-TRβ antibody. We investigated Barhl1 expression in animal models and TH-treated cultured cells. Both long term treatment with 6-propyl-2-thiouracil and short-term treatment with 0.05% methimazole/1% sodium perchlorate (both treatments render mice hypothyroid) resulted in up-regulation of Barhl1. TH supplementation of hypothyroid mice caused a decrease in the expression of Barhl1 compared to control animals. Similarly, the expression of Barhl1 in cultured GH3 decreased with the addition of T3. Given the important role of Barhl1 in brain development, we propose that perturbations of TH-mediated transcriptional control of Barhl1 may play a role in the impaired neurodevelopment induced by hypothyroidism.

1. Introduction

Thyroid hormones (THs) have long been recognized as critically important for normal brain development [1]. Disruption of TH production during fetal and early neonatal development impairs sensorimotor function, and leads to intellectual deficits in experimental animals and humans [2]. THs exert their effect by enhancing or blocking the regulation of genes through various mechanisms. The classical mechanism involves the interaction of TH and TH receptors (TRs) that bind to TH response elements (TREs) as homodimers, or as heterodimers with the retinoid X receptor. As a ligand-activated transcription factor, TR recruit coactivators or corepressors depending on the presence or the absence of TH and thereby regulates gene expression [3]. Recent studies suggest that microRNAs play an important role in TH-regulated control of target gene expression [4]. In addition, TH action may also operate through nongenomic mechanisms that appear to be relevant for the proliferation and motility of certain cells [5]. Determining the identity and function of the genes regulated by TH in the brain is critical to understanding the basis of TH-mediated processes in brain development and differentiation.

We [6,7] and others [8–12] have attempted to identify TH-responsive genes in the developing brain using various approaches. We previously used chromatin immunoprecipitation combined with DNA microarray analysis (ChIP-chip) on juvenile mouse cerebellum to identify genes that were directly bound by TRβ in their promoter regions [7]. Several transcription factors were included in this list of 91 genes. Since transcription factors play a fundamental role in the regulation of many genes and pathways, we predict that these transcription factors will be highly relevant for mediating TH action.

Barhl1 is a transcription factor that was identified as bound by TRβ in our earlier ChIP-chip study. Barhl1, a mammalian homolog of the Drosophila BarH genes, is involved in sensorineural development and neurogenesis [13]. Barhl1 null mice exhibit deficiencies

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in radial migration and increased cell death of cerebellar granular cells, which is consistent with phenotypes that arise under conditions of developmental hypothyroidism [14].

In the present study, we identify and characterize direct binding sites of TRβ in the promoter region of Barhl1. We also examine the transcription of Barhl1 in TH aberrant animal models and cells treated with T3. The results lead us to hypothesize that perturbation of TH-mediated transcriptional control of Barhl1 may play a role in impaired neurodevelopment resulting from TH insufficiency.

2. Materials and methods

2.1. Animals and tissue collection

All animal handling procedures adhered to the Canadian Council on Animal Care guidelines and were approved by the Health Canada Animal Care Committee prior to the initiation of the study.

2.2. Chronic hypothyroid developmental mouse model

Pregnant C57BL/6 mice were treated with 0.1% 6-propyl-2-thiouracil (PTU) (Sigma–Aldrich, Oakville, ON, Canada) from gestation day (GD) 13 to post-natal day (PND) 15. Animals were sacrificed under isoflurane by cervical dislocation. Cerebellum tissues were taken from male pups on PND 15 and stored at −80 °C. For details see [6].

2.3. Acute hypo/hyperthyroid developmental mouse models

Pregnant C57BL/6 mice were allowed to deliver and litter naturally. Mice were rendered hypothyroid by providing sucrose drinking water ontaining 0.05% 1-mehtyl-2-mercapto-imidazol (MMI, Sigma–Aldrich), 1% sodium perchlorate from PND 12 to PND 15, alongside control mice (euthyroid; sucrose water only). Four hours before sacrifice on PND 15, a group of hypothyroid pups received i.p. injections of T4/T3 at 25 μg/2.5 μg/100 g body weight, while other pups were injected with vehicle (PBS in 1.1 mM NaOH). The cerebellum was frozen in liquid nitrogen and stored at −80 °C. For details see [7].

2.4. Chromatin immunoprecipitation (ChIP) and DNA microarrays (chip)

ChIP was performed with anti-TRβ antibody (PAI-213, Affinity Bioreagents, Golden, CO) or rabbit IgG (EZ ChIP, Millipore Corporation, Danver, MA) in cerebellum of euthyroid pups on PND 15. Genomic regions enriched by ChIP were identified with Agilent custom microarrays. For further details see [7].

2.5. ChIP-PCR

Primers targeting TRβ enriched region were designed using BeaconDesigner 2.0 software (Premier Biosoft, PaloAlto, CA). PCRs were performed using AmpliTaq (Perkin Elmer Life Sciences, Woodbridge, ON, Canada) with TRβ immunoprecipitated DNA (TRβ-IP), IgG immunoprecipitated DNA (IgG-IP) or total input DNA (TI) pooled from 3 independent samples as templates.

2.6. Reporter plasmid construction

The promoter region of Barhl1 from −755 to -402 bp from the transcription start site (TSS) was PCR amplified using primers containing *XhoI* and *HindIII* sites and mouse genomic DNA as template. The amplified fragment was cloned into the reporter vector pLuc-MCS (Stratagene, La Jolla, CA) to create pLuc-bar. A QuickChange Multi site-directed mutagenesis kit (Agilent Technologies, Mississauga, ON, Canada) was used to make mutant pLuc-bar-mu which has six mutated oligonucleotides in the potential half-TRE region. All construct sequences were confirmed by sequencing.

2.7. Cell culture, transfection, and reporter assay

Gene expression: GH3 cells (1.2×10^5) were seeded in each well of 6-well plates with F12 medium containing 10% dextran-coated charcoal-treated foetal bovine serum (FBS) for 24 h. T3 (1 nM) was added for 24 h and RNA was extracted. Reporter assays: 24 h before transfection, GH3 cells (5×10^4) were seeded in each well of 12-well plates with F12 medium containing 10% dextran-coated charcoal-treated FBS. Each luciferase reporter plasmid construct (200 ng) was co-transfected with 100 ng of pRL-TK (Promega, Madison, WI) and 100 ng TRβ expression vector (Origene, Rockville, MD) into GH3 cells using 1.2 μl of FUGENE 6 (Agilent). Twenty four hours after transfection, cells were treated with vehicle (PBS containing 6.6 μM NaOH) or 1 nM T3. Cells were harvested 24 h after treatment, and then firefly and renilla luciferase activities were determined in the cell lysates using a Veritas luminometer with the Dual-luciferase reporter assay system (Promega). Firefly luciferase activity was normalized with renilla luciferase activity to correct transfection efficiency. Each condition was performed in triplicate and experiments were repeated three times.

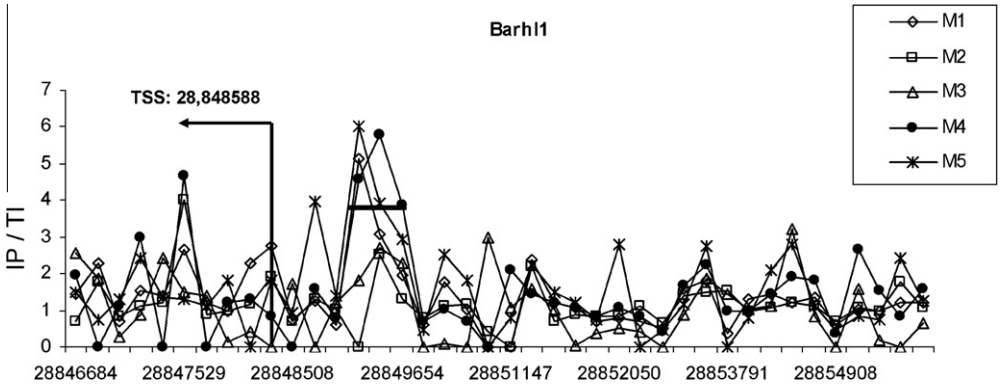

Fig. 1. TRβ binding sites identified in the promoter of Barhl1 using ChIP-chip. The *X* axis shows the probe location in the genome (mm 6, chromosome 2) and the *Y* axis shows the enrichment ratio (IP/TI). Five mouse cerebella from different litters (indicated with M1 to M5) were analyzed and showed high reproducibility. A TRβ binding site (shown as a black bar) was detected approximately 755 bp upstream of the TTS.

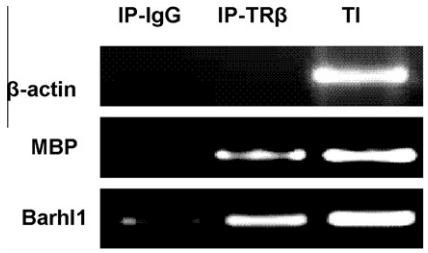

Fig. 2. Confirmation of the TRβ binding site identified in the promoter of Barhl1. Independently prepared IgG-IP, TRβ-IP and TI from three mouse cerebella were pooled and PCRs were performed with primers covering the binding site (black bar in Fig. 1, −755 to −402 bp). β-Actin was used as a negative control. MBP containing a validated TRβ binding site was used as a positive control.

2.8. Electrophoretic mobility shift assay (EMSA)

The non-radioactive LightShift Chemiluminescent EMSA Kit (Pierce Biotechnology, Rockford, IL) was used to detect DNA–protein interactions. Twenty-eight bp long oligonucleotides (Eurofins MWG Operon, Huntsville, AL) containing potential TRE sequences found in the promoter region of Barhl1 (from −608 to −581 and from −495 to −467) were labelled using a biotin 3' end labelling kit (Pierce Biotechnology). Complementary oligonucleotide sequences were annealed in 100 mM NaCl using the following program: 95 °C for 5 min; decrease 1 °C per cycle for 1 min for 30 or 40 cycles; hold at melting temperature for 30 min; decrease 1 °C per cycle for 1 min to room temperature.

Nuclear extracts (12 μg, prepared with NE-PER nuclear and cytoplasmic extraction reagents, Pierce Biotechnology) of mouse cerebellum on PND 15 were incubated with 1 nM biotin labelled probes (1× binding buffer, 5 mM $MgCl_2$, 0.05% NP40, 2.5% glycerol, and 50 ng/μl poly dI-dC) in the presence or absence of anti-TRβ antibody (Affinity Bioreagents) for 25 min, with or without preincubation with 250 or 500 nM non-labelled probes for 10 min. Reaction solutions were electrophoresed through a 5% TBE gel at 100 V for 90 min. The gels were electrophoretically transferred at 100 V for 1 h on ice to a positively charged nylon membrane (Pierce Biotechnology). The signal was detected according to manufacture's instructions with a G:Box.

2.9. Gene expression RT-PCR

Total RNA was extracted from cerebellum or cultured cells using Trizol, and reverse transcribed into cDNA using SuperScript III (Invitrogen, Burlington, ON, Canada). Quantitative PCR was performed with a CFX96 real-time PCR detection system using SYBR-Green (Bio-Rad Laboratories, Mississauga, ON, Canada).

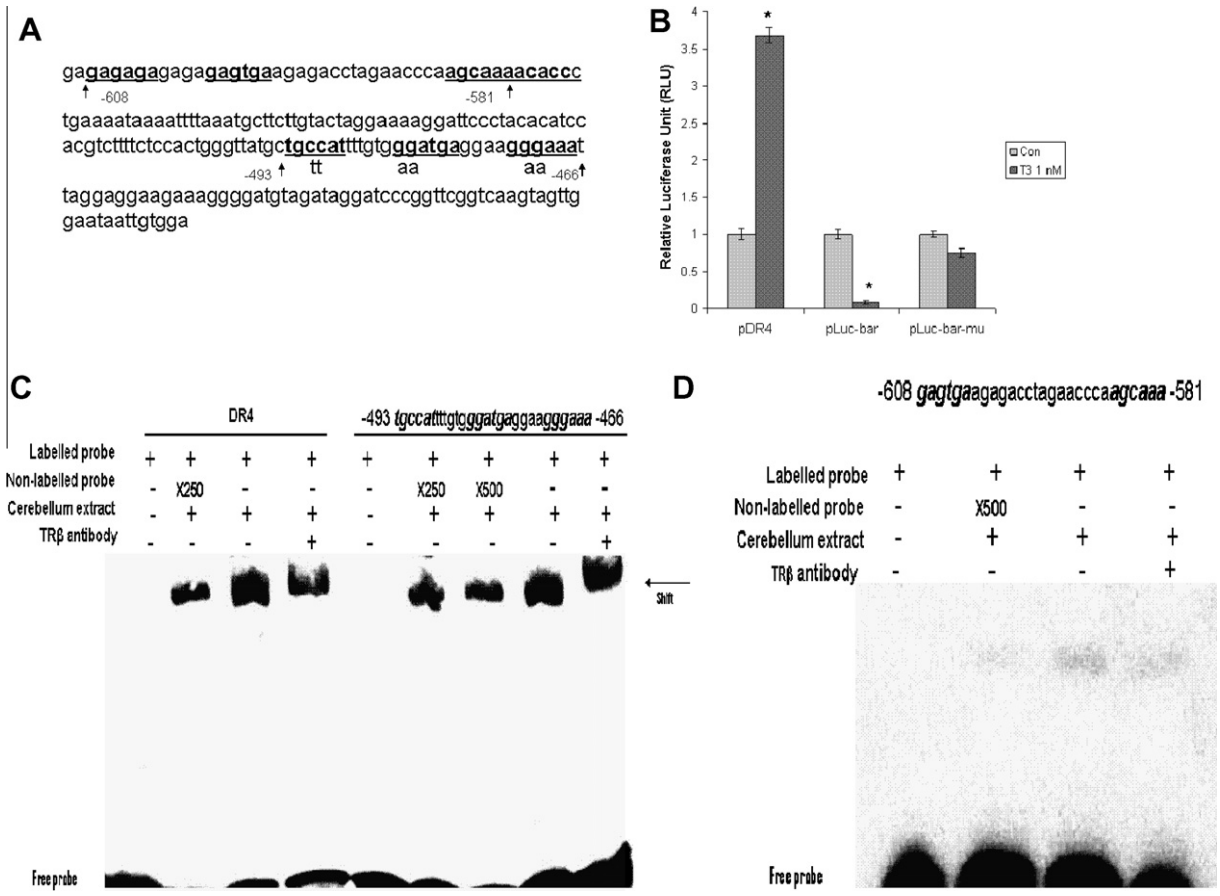

Fig. 3. Characterization of the TRβ binding site in the promoter of Barhl1. A. Seven potential TRE half sites identified in the region of −755 to −402 bp are underlined and in bold print. The reporter plasmid pLuc-bar contains an insertion of the fragment from −755 to −402. The mutant plasmid pLuc-bar-mu has six site mutations as indicated in italic below the underlined original bases. Two oligonucletides were used as probes for EMSA (from −608 to −581 bp and from −493 to −466 bp as indicted with arrows). B. Transcriptional activity of various reporter plasmids. pDR4 was used as a positive control. Various reporter plasmids were transfected into GH3 cells with cotransfection of TRβ and pRL-TK (as a transfection efficiency control). Each condition was performed in triplicate and experiments were repeated three times. Values are mean ± S.E. ($n = 3$). $^{*}p < 0.05$. C. EMSA on the fragment from −493 to −466 bp. The non-radioactive LightShift Chemiluminescent EMSA Kit was used. The fragment was labelled with biotin and incubated with nuclear extract from cerebellum in the presence or absence of anti-TRβ antibody with or without preincubation with 250 times or 500 times non labelled probes. Experiments were repeated three times and one representative example is shown here. D. EMSA on fragment from −608 to −581 bp.

Table 1
Serum T4 levels of male pups on PND 15.

	T4 ± S.E. (ug/dl)	p
Chronical Hypothyroid (n = 4)*		
Control	10.11 ± 0.89	
PTU (0.1%)	0.23 ± 0.05	<0.001
Transient models (n = 3)*		
Control	10.15 ± 0.63	
Hypo	1.97 ± 0.28	0.001 (Comparing with control)
TH replacement	18.40 ± 3.54	0.03 (Comparing with control)

* Pups were taken from different litters.

2.10. Statistical analysis

Data are expressed as mean ± standard error. Significant differences compared to controls were determined using a 2-tailed Student's *t*-test and were deemed significant if $P < 0.05$.

3. Results

3.1. Identification of a potential TRE in the promoter region of Barhl1 using ChIP-chip.

Using ChIP-chip, we previously found 91 genes containing TRβ binding sites [7], which included a few genes with well-established TREs (such as myelin basic protein). However, the majority of the genes identified had no previously characterized TREs associated with their promoters. Several transcriptional factors, which are considered to play critical roles in the regulation of biological pathways, were included in this list; Barhl1 (NM_019446) was one of transcription factors identified. By analyzing ChIP-chip data, we detected a TRβ binding site approximately −755 bp upstream of the TSS of Barhl1 (Fig. 1) located on chromosome 2. The enrichment ratio (TRβ-IP intensity/TI intensity) was up to 6.

3.2. Confirmation of the Barhl1 TRβ binding site using ChIP-PCR

We designed primers covering 354 bp of the TRβ binding site (from −755 to −402 bp of the TSS) in the promoter of Barhl1. PCR was used to amplify TRβ-IP or IgG-IP DNA along with TI-DNA from a pool of cerebella of 3 independent euthyroid C57BL/6 mice on PND15 (Fig. 2). The results were consistent with our ChIP-chip data and revealed strong TRβ binding within the promoter of Barhl1 in the TRβ-IP DNA.

3.3. Characterization of the TRβ binding region in the promoter of Barhl1

ChIP-chip and ChIP-PCR confirmed the binding of TRβ in the region from −755 to −402 bp from the TSS in the promoter of Barhl1. We searched for potential TREs in this region using web-based software (http://www.tre-search.com) and found that there are 7 TRE half sites (Fig. 3A, bold and underlined). A reporter construct (called pLuc-bar) was constructed by inserting this entire region into the reporter vector pLuc-MCS, transfected into GH3 cells. A luciferase reporter vector (pDR4-Luc) containing the well characterized TRE DR4, $(caggAGGTCA)_3$, was tested in parallel as a positive control. Transcriptional activity was 3.5 fold increased following 1 nm T3 treatment in pDR4-Luc, but decreased by 11 fold in pLuc-bar (Fig. 3B). When pLuc-bar-mu, which contained 6 nucleotide site mutations (indicated in Fig. 3A), was transfected into GH3 cells, T3 no longer had an effect on transcriptional activity (Fig. 3B). These data suggest that the region from −493 to −466 of the TSS in Barhl1 contains a TRE and activation of this site by liganded TR suppresses Barhl1 expression.

To provide direct evidence of an interaction between TRβ and the potential TREs, EMSA was performed for the fragment from −493 to −466 bp. Fig. 3C shows that non-labelled probe partly inhibits the binding of the oligonucleotide with nuclear extract, while incubation with TRβ antibody caused the band to shift up. The observation is similar to the positive control using the DR4 fragment. When a probe representing the −608 to −581 bp region was tested, a very weak association was observed that was largely displaced by excess, non-labelled probe. However, no shift was observed after preincubation with TRβ antibody (Fig. 3D), suggesting that the region from −608 to −581 bp does not likely contain active TREs. These results indicate that the 3 half TREs located in the region of −493 to −466 bp play a role in regulating the expression of Barhl1 by TRβ.

3.4. Gene expression of Barhl1 is negatively regulated by TH in animal models and cultured cells

The expression of Barhl1 was examined in cerebellum samples of mice rendered chronically hypothyroid via exposure to PTU from GD13 through to PND15, as well as in a mouse model of short-term hypothyroidism with and without TH supplementation (exposure to MMI/Perchlorate in drinking water from PND 12 to PND 15 with or without TH replacement 4 h before sacrifice) alongside euthyroid controls. The circulating thyroxine levels in

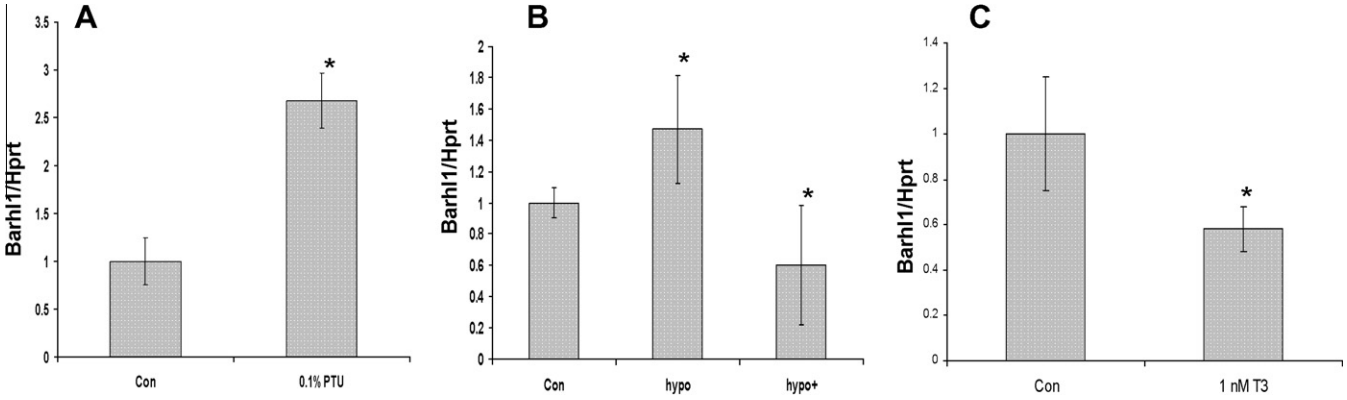

Fig. 4. Barhl1 expression in hypothyroid mouse models and in cultured cells. A. Gene expression of Barhl1 in the cerebellum of mice rendered hypothyroid by PTU treatment from GD13 to PND 15. RT-PCR was used to measure transcription in four individuals from each group. Values are mean ± S.E., $^*p < 0.05$. B. Gene expression of Barhl1 in the cerebellum of mice treated with MMI/Perchlorate (hypo) from PND 12 to PND 15. The TH supplemented group (hypo+) received an injection of TH 4 h before sacrifice on PND 15. Three individuals were analyzed per group. Values are mean ± S.E., $^*p < 0.05$. C. Gene expression of Barhl1 in GH3 cells treated with 1 nM T3. Each experiment was performed triplicate and experiments were repeated three times independently. Values are mean ± S.E., $^*p < 0.05$.

these animals are shown in Table 1. Reduced serum T4 was associated with increased Barhl1 expression in both of these animal models (Fig. 4A and B). The increased expression was reversed by TH supplementation 4 h prior to sacrifice, demonstrating a relatively fast response to TH treatment *in vivo* (Fig. 4B). We also examined TH regulation of Barhl1 expression in different cell lines. The expression of Barhl1in the pituitary cell line GH3 was negatively regulated by TH (Fig. 4C). However TH had no effect on Barhl1 expression in mouse neuroblastoma cell Neuro-2a (data not shown). Thus, the direction of change of Barhl1 under conditions of hyper- or hypothyroidism *in vivo* and *in vitro* supports the role of TH in controlling its expression.

4. Discussion

Barhl1 is the murine homolog of the *Drosophila* gene BarH1, a homeobox-containing gene required for fate determination of external sensory organs in the fly [15]. It is exclusively expressed in the developing central nervous system. Loss of Barhl1 in mice leads to a suite of phenotypes, including attenuated cerebella foliation, defective radial migration, increased apoptotic death of granule cells [14], as well as progressive degeneration of hair cells in the organ of Corti [16]. Similarly, TH plays a role in the process of neuronal migration. Rats born to dams with moderately low TH have blurred cortical lamina and many neurons do not migrate to their normal destination [17] due to reduced transcription of Reelin and its downstream signalling [18]. Hypothyroidism appears to result in apoptosis in the cerebellum by regulating apoptotic and proliferating genes, such as cyclin D2, Bcl-2, Bax and Bag3 [6,19,20]. Impaired transactivation of TRβ leads to severe deficits in the proliferation of granular precursors and the arborisation of Purkinje cells [21]. TRβ knockout mice display abnormal cochlear development and defective auditory function [22,23]. Thus, the remarkable parallels between Barhl1 mutant phenotypes and TH deficiency suggest a potentially important role for Barhl1 in the manifestation of hypothyroid-related disease.

Barhl1 executes its function via various complex interactions. Barhl1 controls the expression of neurotrophin-3 (NT-3) [14], while Barhl1, along with two other genes (Lhx2 and Lhx9), is controlled by Math1, an essential effector for the development of cerebellar granule neurons [24]. Activation of Notch signalling inhibits the expression of Math1 through the activity of Hes5 [25]. Interestingly, many of the genes involved in these interactions appear to be responsive to TH. We previously identified Lhx2 and Hes5 as targets of TRβ using ChIP-chip [7]. Hypothyroidism leads to a 35–45% reduction in NT-3 mRNA levels, depending on postnatal stage [26,27]. These findings provide additional support to the notion that TH functions through target genes that include Barhl1 to modulate cerebellar development.

Cerebellum development requires the orchestrated regulation of spatiotemporal proliferation, differentiation and migration of granule cells. This sophisticated process is carried out by a suite of genes, some of which have been demonstrated to be regulated by TH. TH positively regulates NT-3, BDNF, reelin, Hr, Egr1, NeuroD, netrin-1 and negatively regulates N-CAM and the netrin-1 receptor Unc5h3 [6,28,29]. We now demonstrate that Barhl1 is negatively regulated by TH. An interesting paradox exists for N-CAM and Barhl1. Although N-CAM and Barhl1 null mice display abnormal neuronal migration, differentiation and synapogenesis [30], typical phenomena exhibited under conditions of developmental hypothyroidism, TH deficiency results in increased expression of both N-CAM or Barhl1 [31]. Thus, the lack of concordance between the N-CAM or Barhl1 null phenotypes and molecular response to TH is puzzling. Further work will be needed to resolve the coordination of TH signalling in determining the abundance and balance of Barhl1 expression to fine tune cerebellar development.

In conclusion, we demonstrate a TRβ binding site within the promoter of Barhl1, a gene that is critical in sensorineural development and neurogenesis. Negative regulation of Barhl1 by TH was confirmed both *in vivo* and *in vitro*. These findings provide an interesting molecular pathway to target for additional studies on the neurodevelopmental repercussions of hypothyroidism, and contribute to a better understanding of the effects of TH on brain and auditory system development.

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