2017

Quantification of Brain-Region Specific Alterations in Insulin-Like Growth Factor-1 Expression During Developmental Hypothyroidism

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By

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A Thesis Submitted in Partial Fulfillment of the

Requirements for the Degree of

Master of Science

in

Biological Sciences

Minnesota State University, Mankato

Mankato, Minnesota

December 2017

October 13th, 2017

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This thesis has been examined and approved by the following members of the student's committee.

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Abstract

Insufficient thyroid hormone (TH) during development results in permanent neurological deficits. These deficits are the result of perturbed TH-mediated brain development. Interestingly, insufficient insulin-like growth factor 1 (Igf-1) during development results in neurological deficits that are similar to those reported for developmental hypothyroidism. This observation suggests that deficits associated with low TH during development may be the result of altered Igf-1 expression in the developing brain. To test this, timed-pregnant mice were treated with thyroid gland inhibitors from gestational day 16 (GD16) until postnatal day 21 (P21) to induce a hypothyroid state. A parallel set of untreated timed-pregnant mice were used as controls. Brains from exposed and control pups were collected at P7, P14, P21, and P42 and processed for detecting Igf-1 mRNA by RNA isolation and reverse-transcriptase quantitative real-time PCR. Trunk blood was collected to measure serum thyroxin (T4) by ELISA. Body weights and total wet brain weights were also quantified. Developmentally hypothyroid mice weighed significantly less at all ages. Wet brain weights were significantly smaller in hypothyroid mice at P7, P21, and P42. Igf-1 mRNA levels did not differ significantly in the cortex compared to euthyroid controls. However, Igf-1 mRNA levels were significantly decreased in the hippocampus at P7, and in the cerebellum at P14. Interestingly, Igf-1 mRNA levels were increased in hypothyroid mice at P42 in the hippocampus. Because treatment of goitrogens ceased at P21, any differences seen at P42 are the result of permanent TH deficit during early development. Since this result was not reflected in decreased Igf-1 expression at P42 in any region, it can be assumed that TH has more complex

mechanistic actions than just regulating Igf-1 gene expression. It is also safe propose brain Igf-1 mRNA expression is affected by low serum TH; yet the way Igf-1 gene expression is perturbed is regionally and temporally specific. These findings identify a novel, previously unconsidered, mechanism by which thyroid hormone insufficiency during development results in neurological deficits.

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Introduction

Congenital hypothyroidism (CH) is a condition in which an individual is born with a partial or complete lack of thyroid gland function (Brent, 1999). This deficit of thyroid hormone (TH) occurs in approximately 1 in 3500 births, and if untreated, can lead to permanent neurological deficits such as low IQ (McMorris et al., 1986). Multiple lines of evidence indicate that these permanent neurological deficits are the result of decreased amounts of TH during fetal and early post-natal brain development (Zoeller and Rovet, 2004). Interestingly, decreased production of local insulin-like growth factor 1 (Igf-1) in the brain during development is reported to produce neurological deficits that are similar to that of CH (Beck et al., 1995; Dyer et al., 2016). It is therefore reasonable to suggest that perturbed Igf-1 signaling, as a result of low thyroid hormone during development, may be responsible for some of the permanent cognitive deficits associated in CH.

The goal of this study is to analyze Igf-1 mRNA expression using quantitative real-time reverse-transcription PCR in three well known targets of TH action in the developing brain - cortex, hippocampus, and cerebellum - of hypothyroid and euthyroid mice. The working hypothesis is that: low TH during perinatal development reduces the temporal expression of Igf-1 mRNA in the cortex, hippocampus, and cerebellum in hypothyroid mice when compared to wildtype mice. The corollary hypothesis is that hypothyroidism-induced reductions in brain Igf-1 mRNA results in the permanent neurological deficits observed in CH.

Literature Review

Hypothalamic-Pituitary-Thyroid Gland Axis

1. Overview

Thyroid hormone production is controlled by the hypothalamic-pituitary-thyroid gland (HPT) axis (Zoeller et al., 2007). The HPT axis is conserved throughout vertebrates and is well-defined in human and murine models. The periventricular nucleus of the hypothalamus releases thyrotropin-releasing hormone (TRH) into the hypophyseal portal system. Thyrotropes of the anterior pituitary gland respond to TRH by releasing thyroid-stimulating hormone (TSH) into the blood stream. TSH acts on the thyroid gland stimulating the production and release of TH. Circulating levels of TH levels in serum are tightly controlled by TH acting through negative feedback at the level of anterior pituitary gland and hypothalamus.

The thyroid gland secretes mainly two forms of TH: active receptor ligand triiodothyronine (T3) and an inactive prohormone thyroxine (T4) (Zoeller et al., 2007). The difference between the active (T3) and inactive (T4) form is the number of iodide (I^-) anions conjugated to a tyrosine molecule, with four I^- on T4 and three I^- on T3. All forms of TH are hydrophobic, but require a protein transporter to travel across cell membranes. TH enters target cells where deiodinase enzymes can metabolize TH to the active T3 or inactive metabolites.

Thyroid hormone receptors (TRs) are ligand regulated transcription factors that control gene expression. Two TRs exist in the mammalian genome: thyroid hormone receptor-alpha (Thra, TRα) and thyroid hormone receptor-beta (Thrb, TRβ). TRs bind

to areas of DNA called thyroid response elements (TREs). Also bound to the TRE is another receptor, creating either a heterodimer or a homodimer. A homodimerization would be two TRs of the same kind. A heterodimerization would be either two different TRs, or one TR and another complementary receptor like a retinoic X receptor (RXR) or a vitamin D3 receptor (VD3R). Furthermore, complexed receptor cofactors depend on the TRs liganded state and can either activate (Co-A) or repress (Co-R) gene transcription in the presence or absence of TH, respectively (Zoeller et al., 2007).

2. Deiodinase Regulation

The availability of TH to regulate gene expression is controlled locally in cells by different deiodinase enzymes. Deiodinase 2 (DIO2) converts inactive T4 into active T3 by removing an iodide (I^-) anion through outer ring 5'-deiodination (Zoeller et al., 2007; Ng et al., 2013). In the brain, DIO2 is predominately expressed in astrocytes and tanycytes (Guadano-Ferraz et al., 1997). Deiodinase 3 (DIO3) functions to inactivate TH by removing the correct I^- anion from T4, then converting T4 to reverse-T3 (rT3) and T3 to T2 (3,5-diiodo-L-thyronine). DIO3 is primarily expressed in neurons (Tu et al., 1999). By directly regulating the amount of T3, deiodinases act in a paracrine fashion to indirectly regulate TH responsive genes, which has been shown in multiple animal models (Freitas et al., 2010).

4. Receptor Regulation

Encoded by genes *Thra* and *Thrb*, TRs are cellular homologues of the viral oncogene v-erb-A protein product (Weinberger et al., 1986). *Thra* transcribes for receptors TRα1 and TRα2, and *Thrb* transcribes TRβ1 and TRβ2. TRα1, TRβ1, and

TRβ2 are ligand binding transcription factors that act as direct regulators of TH expression. TRα2 is non-ligand binding, meaning it cannot bind the necessary ligand subunits to regulate TH expression. TRs are found throughout the CNS in areas such as the cortex, hypothalamus, hippocampus, striatum, and cerebellum (Bradley et al., 1992). Specifically TRα1 is expressed in developing and adult neurons as well as developing Purkinje cells and tanycytes in the cerebellum (Wallis et al., 2010). TRα1 covers all T3 binding during fetal brain development while TRβ1 and TRβ2 show minimal expression in fetal brain development (Bradley et al., 1992; Yen, 2001). TRβ increases forty-fold from birth to two weeks after birth, and remains high throughout life (Yen, 2001). TRβ1 and –β2 can be found in neurons which feedback on the HPT axis to regulate systemic TH release (Flamant et al., 2006).

GHRH-GH-IGF Axis

1. Overview

The hypothalamus releases growth hormone releasing hormone (GHRH) into the hypophyseal portal system, which binds GHRH receptors on somatotropes of the anterior pituitary gland. In response, the anterior pituitary gland then releases growth hormone (GH), which acts on the liver to produce insulin-like growth factor 1 (Igf-1). IGF proteins are small peptides which share homology to insulin and have two isoforms: Igf-1 and Igf-2. Insulin-like growth factors circulate in the plasma attached to Igf binding proteins (IGFBPs) and must be released from IGFBP binding to signal. Free IGFs bind IGF receptors located on target cells to elicit a response. Serum Igf-1 is controlled, in-part, through negative feedback at the level of the hypothalamus,

inhibiting GHRH release. Igf-1 and Igf-2 are both needed for normal *in utero* growth, but only Igf-1 is needed for postnatal development (D'Ercole et al., 2002).

2. Igf-1 Receptors

Igf-1 receptors are tyrosine kinase receptors similar in structure to insulin receptors. There are two types of Igf receptors: Igf-1R and Igf-2R. Igf-1R binds both Igf-1 and Igf-2 but has higher affinity for Igf-1, while Igf-2R only binds Igf-2. Igf-1R induces cell signaling through the PI3K-AKT pathway and the Wnt pathways to induce cell proliferation (Wang et al., 2010). Igf-1R reaches peak expression during late embryonic stages and decreases to basal levels soon after birth (Feldman et al., 1997). Igf-1R expression is similar to that of Igf-1 with expression described in the cortex, cerebellum, hippocampus, choroid plexus, and olfactory bulbs (Feldman et al., 1997).

3. Igf Binding Proteins

Igf-1 travels throughout the body bound to carrier or binding proteins (IGFBPs). These binding proteins provide a plasma reservoir for IGF proteins and slow the degradation of IGFs, which in turn increases Igf-1 half-life. There are six BPs that have a high binding affinity for Igf-1: IGFBP-1, IGFBP-2, IGFBP-3, IGFBP-4, IGFBP-5, and IGFBP-6. Of these IGFBP proteins, IGFBP-2 and IGFBP-4 are the most abundant in central nervous system (Dyer et al., 2016). IGFBP-2 and IGFBP-4 expression peaks during brain development (Dyer et al., 2016), indicating the importance of tight control of Igf-1 action during that time.

Sites of Thyroid Hormone and Igf-1 Action

1. Oligodendrocytes and Myelination

Mice lacking Igf-1 show deficient myelination throughout the brain. The corpus callosum displays decreased myelination and a reduction in thickness disproportionate to brain size by 70%. The anterior commissure was also disproportionately decreased to total brain size, and white matter tracts in the spinal cord were decreased (Beck et al., 1995). These effects were hypothesized to be caused by a decreased total number of oligodendrocytes throughout the brain; however, myelination was not completely abolished, suggesting other regulators of myelination. Cerebrum cell cultures treated with Igf-1 also show increased numbers of oligodendrocytes, increasing in number as the dosage increased (Beck et al., 1995).

Similarly, myelination is reduced in developmentally hypothyroid animals, as TH plays an important role in the differentiation, proliferation, and maturation of oligodendrocytes throughout development (Rodriguez-Pena, 1999). Furthermore, myelin-basic-protein (MBP), a major component of myelin, is regulated directly by TH with a TRE -186 to -169 base pairs from the MBP promoter (Farsetti et al., 1991). Oligodendrocytes, which express MBP, are decreased in number under CH conditions throughout development, with TH replacement treatment shown to be unsuccessful at rescuing cell count at any age (Schoonover et al., 2004). Collectively, these reports indicate that both TH and Igf-1 play an important role in regulating myelination during neurodevelopment.

2. Parvalbumin Neurons

In Igf-1 KO mice, parvalbumin neurons are decreased disproportionately to total brain size by 50%. Specifically, in the dentate gyrus of the hippocampus, parvalbumin neurons were reduced by approximately 35%. However, the granule cells

of the dentate gyrus were reduced in total volume by 59%; which can be partially attributed to the loss of parvalbumin neurons. This suggests that Igf-1 is involved in the generation of dentate granule neurons (Beck et al., 1995).

In the developmentally hypothyroid rat brain, a reduction of parvalbumin-positive interneurons is observed in the neocortex and hippocampus, but no overall change in GABAergic neurons was detected (Gilbert et al., 2007). These observations suggest that it is not the numbers of interneurons affected by developmental TH insufficiency, rather the phenotypic expression of parvalbumin within these cells. A decrease in parvalbumin-positive interneurons was observed in these regions throughout the perinatal period and into adulthood even after restoring a euthyroid state. Furthermore, T4 injections during the early postnatal period rescue parvalbumin expression (Gilbert et al., 2007). Considering these results, Igf-1 and TH are both crucial for the proper development of parvalbumin interneurons during brain development.

3. Neurogenesis

Igf-1 expression is typically found in areas where adult neurogenesis occurs, like the hippocampus and cerebellum; however, expression can be detected as early as embryonic day 18 (E18) with gradual increase until about postnatal day 20 (P20) (D'Ercole et al., 2002). Sharp increased expression of Igf-1 from P0 to P14 then coincides with neuronal proliferation. After P14 to about P30, a decrease in Igf-1 to adult levels corresponds to decreased neuronal proliferation (Bartlett et al., 1991).

TH is also crucial for neurogenesis during fetal and postnatal development. Decreased TH during early embryogenesis leads to disrupted neuronal migration in the cortex and hippocampus, as well as decreased glial cell proliferation in the cerebellum (Ausó et al., 2004; Martinez et al., 2011). More recently, models of iodine deficiency during fetal development show enhanced neuronal differentiation in the subventricular zone and hippocampus (Kapoor et al., 2012). During postnatal and adult neurogenesis, decreased levels of TH lead to decreased myelination, synaptogenesis, and neuronal migration and differentiation (Préau et al., 2015). The effects of decreased TH on neurogenesis are similar to that of decreased Igf-1 levels in the same regions of the developing brain.

TH and Igf-1 Cross Talk

1. Phenotypic Similarities between Congenital Hypothyroidism and Igf-1 Deficiency in Animal Models

A disproportionate decrease in body to brain weight has been seen in Igf-1 deficient mice, along with an increased number of stillbirths in Igf-1 -/- mice (Beck et al., 1995). These characteristics are similar to the etiology of congenital hypothyroidism, which includes low birth weight (Brent, 1999), limited bone growth, and Cretinism, a disease characterized by deafness and irreversible mental retardation. (Flamant et al., 2006).

2. Regulation of Systemic Igf-1 by Thyroid Hormone

It has been shown that thyroid hormone directly effects systemic Igf-1 levels and extra-hepatic Igf-1 production, which has been reported in animal models.

Decreased levels of TH lead to inhibited levels of circulating GH and thereby reduces peripheral Igf-1. There is also decreased expression of growth hormone receptors (GHr) mRNA in hypothyroid rat livers (Chang et al., 2014), potentially leading to decreased release of Igf-1. Interestingly, Igf-1 levels are only restored if T3 or T4 is given back (Chang et al., 2014) but not GH alone (Burstein et al., 1979). These results suggest low thyroid hormone disrupts the GH-Igf axis on a systemic level.

3. Thyroid Hormone Regulation of Locally Produced Igf-1

Recently it has been hypothesized that thyroid hormone directly regulates Igf-1 produced in bone, indicating that growth retardation in hypothyroid animals may be a result, in part, from reduced Igf-1. A study focusing on bone acquisition in mice supports the notion that prepubertal levels of TH are more important than GH levels for local Igf-1 production (Xing et al., 2012). This study shows a strong positive correlation between increasing administration of T3 treatments and rising levels of Igf-1 in the bone. It also shows that a natural increase in the amount of circulating T3 occurs just prior to an Igf-1 increase in bone during early development. Furthermore, Igf-1 transcription was also shown to be directly regulated by a TRE in the IGF-1 gene which can be activated by TRα in osteoblasts (Xing et al., 2012).

In the brain, Kline and colleagues reported a decrease in Igf-1 positive cells in the motor cortex of developmentally hypothyroid mice when compared to euthyroid mice (Kline, 2016). Moreover, this study reported that T4 and serum Igf-1 levels were positively correlated (Kline, 2016).

Together, this evidence suggests that circulating thyroid hormone controls the expression of both local brain-derived and hepatic (systemic) Igf-1. These findings bring me to my working hypothesis that: low TH during perinatal development reduces local brain-derived Igf-1 mRNA in the cortex, hippocampus, and cerebellum in hypothyroid mice when compared to wildtype mice. The corollary hypothesis is that hypothyroidism-induced reductions in brain Igf-1 mRNA result in the permanent neurological deficits observed in congenital hypothyroidism.

Materials and Methods

Animal Care

This study used C57BL/6J mice. This highly inbred strain was supplied by Jackson Laboratories. All experimental procedures were approved by the Minnesota State University, Mankato Institutional Animal Care and Use Committee (IACUC).

Animal Treatment

Timed pregnant dams were split into two treatment groups: euthyroid (control) and hypothyroid. Hypothyroidism in dams was induced by providing drinking water containing 0.01% bovine serum albumin (BSA), 0.02% methimizole (MMI), and 1% potassium perchlorate ($KClO_4$). Control (euthyroid) animals received drinking water with 0.01% BSA only. The treatments were initiated on embryonic day 14.5 (E14.5) and continued until postnatal day 21 (P21). Euthyroid pups were weaned from their mother at P21 while hypothyroid pups were weaned at P35 to allow for further development. The sample size for each treatment group at each developmental time

point assessed was n = 5. Two samples were lost during processing, leaving an n = 4 for the cortex and hippocampus at P7.

Sacrifice and Dissection

Euthyroid and hypothyroid pups were sacrificed at ages P7, P14, P21, and P42. As per protocol, pups were euthanized using CO_2 gas followed by decapitation using sharp scissors. Immediately following decapitation trunk blood was collected and allowed to coagulate on ice. The brain was then carefully dissected from the skull and total wet brain weight was measured on an analytical scale. Next, the cerebellum was removed from the cerebrum, placed in a 2mL tube, frozen on dry ice, and stored in a -80^{o}C freezer. The remaining cerebrum was concomitantly frozen on dry ice and stored at -80^{o}C until microdissection could occur.

Serum Analysis

Trunk blood was collected at all experimental ages investigated. Whole blood was allowed to coagulate on wet ice for no less than 20 minutes and no longer than one hour. Serum was isolated by centrifuging trunk blood for 10 minutes at eight relative centrifugal force (rcf) and stored at -20^{o}C until an enzyme-linked immunosorbent assay (ELISA) could be performed to measure circulating hormone levels. A Human T4 ELISA Test Kit was used to analyze T4 levels in the serum, following manufacturer protocol. Resulting concentrations were calculated based on assay standards.

Microdissection for Cortex and Hippocampal Regions

Frozen cerebral tissue samples were mounted in Optimal Cutting Temperature (OCT) Compound and sectioned in the coronal plane at 20 microns until the corpus

callosum could be identified by methyl blue staining. Once reached, the brain was removed from the OCT compound, placed in a coronal brain matrix containing 1mm intervals, and a slice extending 1mm posterior from the corpus callosum was collected at Bregma 1.09mm and Interaural 4.88mm. Using the Allen-Brain atlas, microdissection of the motor cortex included removing tissue inferior to the corpus callosum, and tissue lateral to the motor cortex (Figure 1A). Following cortex collection, the brain was then remounted in OCT and the microdissection steps were repeated to collect the hippocampus at Bregma -1.43 mm and Interaural 2.36mm (Figure 1B). Cerebral tissue samples were collected for five pups at each age interval. Sections were placed in a collection tube and immediately homogenized with 900µl of QIAzol Lysis Reagent using a rotor stator homogenizer for 30 to 60 seconds. The processed homogenate was then stored at -80°C until RNA isolation could occur.

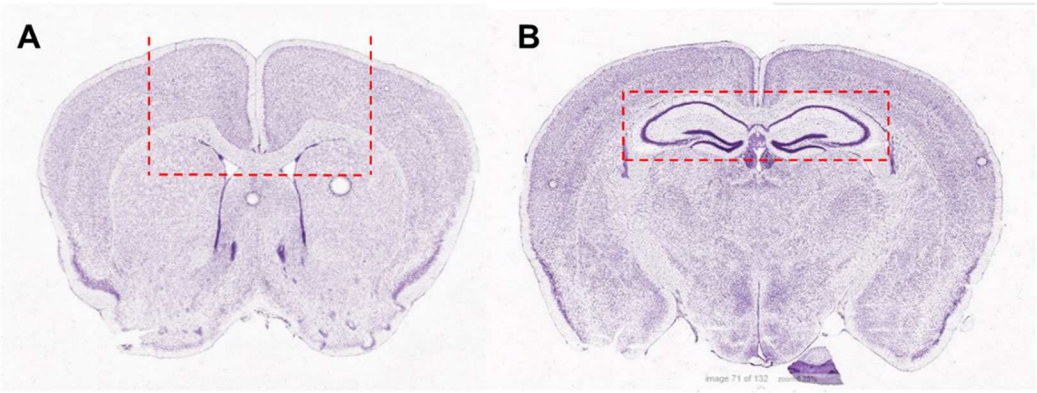

Figure 1. Coronal sections of the murine brain. Regions outlined in red represent the microdissected regions of the motor cortex and corpus callosum (left) and also the hippocampus (right). Regions were identified using the Allen-Brain Atlas.

RNA Isolation

The RNA isolation procedure followed the protocol provided by the Qiagen RNeasy Plus Universal Handbook: RNeasy Plus Universal Mini Kit with few modifications. Dissected tissues were combined with 900µl of QIAzol Lysis Reagent and homogenized using a rotor stator homogenizer for about 30 seconds until homogenous. Homogenates were then stored at -80°C for no longer than five days until RNA processing could continue. Following a seven-minute room temperature incubation after thawing, 100µl gDNA Eliminator Solution was added to the homogenate and shaken vigorously for 15 seconds. 180µl of chloroform was added and shaken again for 15 seconds. The homogenate was incubated at room temperature for two minutes followed by centrifuging at 12,000 x g for 15 minutes at 4ºC. The aqueous layer was transferred to a new 2ml tube, 600µl of 70% ethanol was added and mixed by pipetting. The sample was then transferred to an RNeasy Mini spin column and centrifuged for 15 seconds at 10,000 rpm. An additional on-column DNase treatment was completed for cerebellum only. RNA loaded columns were then wash with 700µl of prepared RWT buffer and then twice with 500µl of Buffer RPE. Samples were then centrifuged at full speed for one minute to remove any remaining buffer. The RNA was eluted from the column by adding 30µl of RNase-free water and centrifuged at 10,000rpm for one minute. For the cerebellum, an additional 30µL elution with RNase-free water was completed. For the cerebral tissues, the initial 30µL elution was reused a second time to elute any remaining RNA. RNA concentration was determined using a spectrophotometer and sample quality was determined using 1% bleached-agarose gel electrophoresis. 1% household bleach (8.25% sodium

hypochlorite) was added to the gel in order to stabilize RNA degradation against ribonucleases (Aranda et al., 2012). Cerebellar samples were then diluted three-fold with RNase-free water. RNA samples were stored at -80°C.

cDNA Synthesis using Reverse Transcription

Isolated mRNA was reverse transcribed into cDNA according to SuperScript IV VILO Master Mix protocol. Each reaction mixture included 4µL of SuperScript IV VILO Master Mix, 1µg of mRNA, and filled to a total of 20µL with RNase-free water. The samples were incubated at 25°C for 10 minutes, temperature then increased to 50°C for 10 minutes, and finally increased again to 85°C for 5 minutes. This process annealed the oligonucleotides to the mRNA, reverse transcribed the RNA, and inactivated the reverse transcriptase enzyme.

Primer Testing

Igf-1 mRNA primers were used at concentrations of 0.3µM and 0.5µM on euthyroid and hypothyroid cDNA samples which were either diluted at a 1:1 ratio with water or kept neat. Primers were amplified with cDNA samples using a standard PCR amplification of 40 repetitions of 95°C for 15 minutes, 94°C for 15 seconds, 60°C for 15 seconds, and 72°C for 30 seconds. Table 1 shows each tested primers forward and reverse sequence, accession number, and band size.

	Sequence	Accession #	Size
Primer A (Harvard Bank)	F: CTGGACCAGAGACCCTTTGC R: GGACGGGGACTTCTGAGTCTT	NM_010512.5	268 bp
Primer B (Ge et al., 2015)	F: CAACTCCCAGCTGTGCAATT R: GCCAGGTGAACACAAAACT	NM_001111276.1	165 bp
Primer C (Omazic et al., 2001)	F: TGGATGCTCTTCAGTTCGTG R: GCAACACTCATCCACAATGC	NM_010512.5	112 bp

Table 1. Igf-1 Primers. All primers matched to Igf-1 transcript variant one mRNA. Primer A was taken from Harvard Bank and has a predicted amplicon length of 268 base pairs. Primer B (Ge et al., 2015) and Primer C (Omazic et al., 2001) were found from previous research and have predicted band sizes of 165 and 112 base pairs.

Real Time quantitative-Reverse Transcriptase Polymerase Chain Reaction

qPCR was conducted using PowerUp Sybr Green PCR mastermix, supplied by Thermo Scientific. Primer efficiency curves were run using ten-fold dilutions for both primer sets: Igf-1 and Ppia. Samples will be plated by region of the brain and then further organized by age, for a total of six qPCR plates, two plates per region. The standard curve was a five-fold dilution of one sample of cDNA, which was used on each plate to normalize for potential cross-plating differences.

Statistical Analyses

1. Body Weight and Total Wet Brain Weight

A two-way ANOVA with treatment and age as factors was used to analyze pup body weights at P7-P21 (data combined with (Kline, 2016) for a sample size of n = 95). A Sidak's post-hoc t-test corrected for multiple comparisons after the two-way ANOVA. P42 samples were analyzed using an unpaired t-test. Total wet brain weights were analyzed in the same manner as body weights, with a sample size of n = 39.

2. ELISA Analyses

Total serum T4 levels were analyzed using the standard curve method. Experimental sample mean absorbance values were then interpolated from corresponding standards. A two-way ANOVA was used to determine statistically significant effects of age, treatment, and interaction for samples between P7-P21. A Sidak's post-hoc t-test was then used to determine significant differences between treatment groups at each age. Concentrations at P42 were analyzed using an unpaired t-test test to detect significant differences between treatment groups.

3. Quantification of Relative Igf-1 mRNA Levels

Igf-1 mRNA quantity was determined via the standard curve method for qRT-PCR. An efficiency value was determined using a calibrator sample of cDNA which underwent the same processes as experimental cDNA but was serially diluted five-fold a total of five times (100ng/μL, 20ng/μL, 4ng/μL, 0.8ng/μL, and 0.16ng/μL). Relative quantities of experimental mRNA could then be calibrated according to quantities produced by the standard curve. The same standard curve dilution set was run on all experimental sample plates to normalize for cross-plate analyses.

Quantities of relative Igf-1 mRNA were then analyzed using a two-way ANOVA to determine significant effects of time, treatment, and interaction between ages P7-P21. A Sidak's post-hoc t-test was used to correct for multiple comparisons to determine any significant differences between treatments. Igf-1 mRNA levels at age P42 were analyzed using an unpaired t-test to determine a significant difference between treatment groups.

Results

RNA Quality Testing using Bleach-Agarose Gel Electrophoresis

No genomic DNA was present along the wells in the cortex (Figure 2A), the hippocampus (Figure 2B), or the cerebellum (Figure 2C). Both 28S and 18S rRNA bands were also clearly visible at approximately 1,325 kDa and 750 kDa with no smearing, indicating high quality RNA (Figure 2).

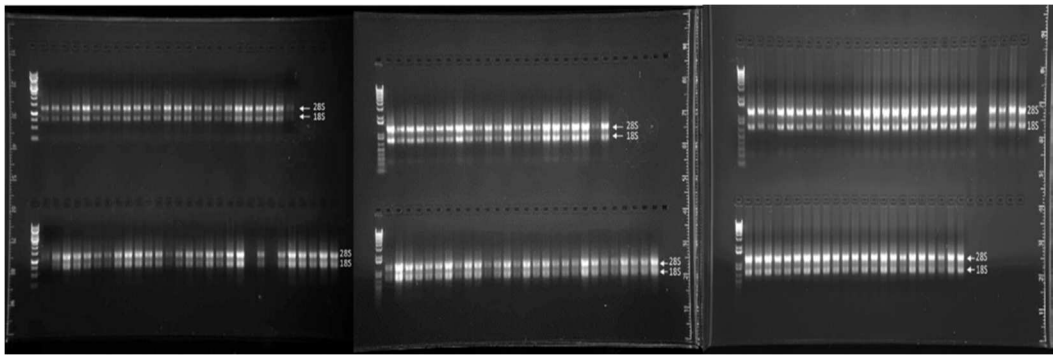

Figure 2. RNA quality tested using 1% bleach-agarose gel electrophoresis. The cortex (left), hippocampus (middle), and cerebellum (right) all have distinct 28S and 18S banding. A 1 Kb plus DNA ladder was used as reference.

Primer Efficiencies

All three predicted band sizes of the Igf-1 mRNA primers matched gel results, with no difference in amplification between primer concentrations or cDNA dilutions (Figure 3). Based on these observations, primers were used at 0.3μM with undiluted cDNA to ensure robust amplification with the least potential for primer associated anomalies.

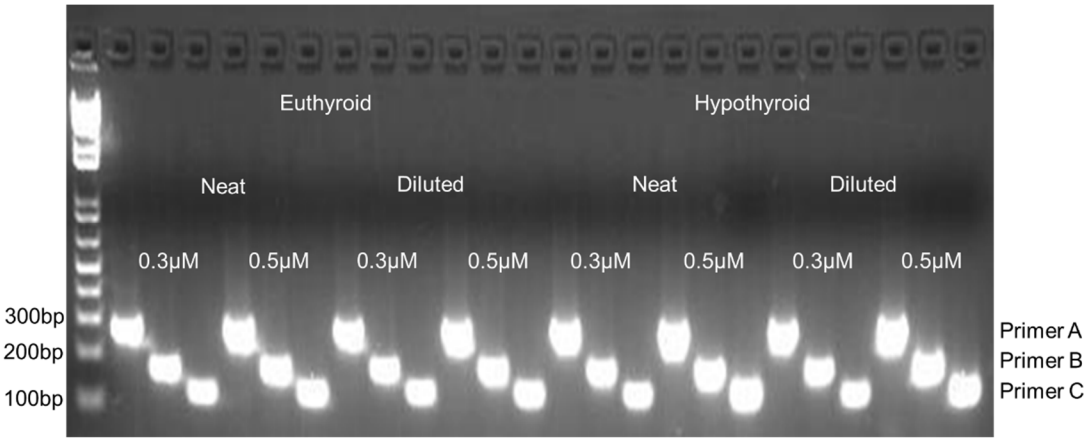

Figure 3. Igf-1 mRNA transcript variant 1 primer concentration testing. Standard PCR amplification was used with diluted and undiluted, euthyroid and hypothyroid cDNA samples to test the most efficient primer-to-cDNA concentration ratio.

The selected Igf-1 Primer C (Omazic et al., 2001) and the control primer Ppia (cyclophilin-A) were then tested by qRT-PCR for amplification efficiency on a euthyroid sample diluted ten-fold five times (100ng/μL, 10ng/μL, 1ng/μL, 0.1ng/μL, and 0.01ng/μL). As shown in Figure 4, the critical threshold of Igf-1 Primer C amplification was $C_t = 18$, while $C_t = 12$ for Ppia primers. The amplification of all five serial dilutions at the same cycle indicates only the target gene was amplified, meaning the primers were efficient and specific.

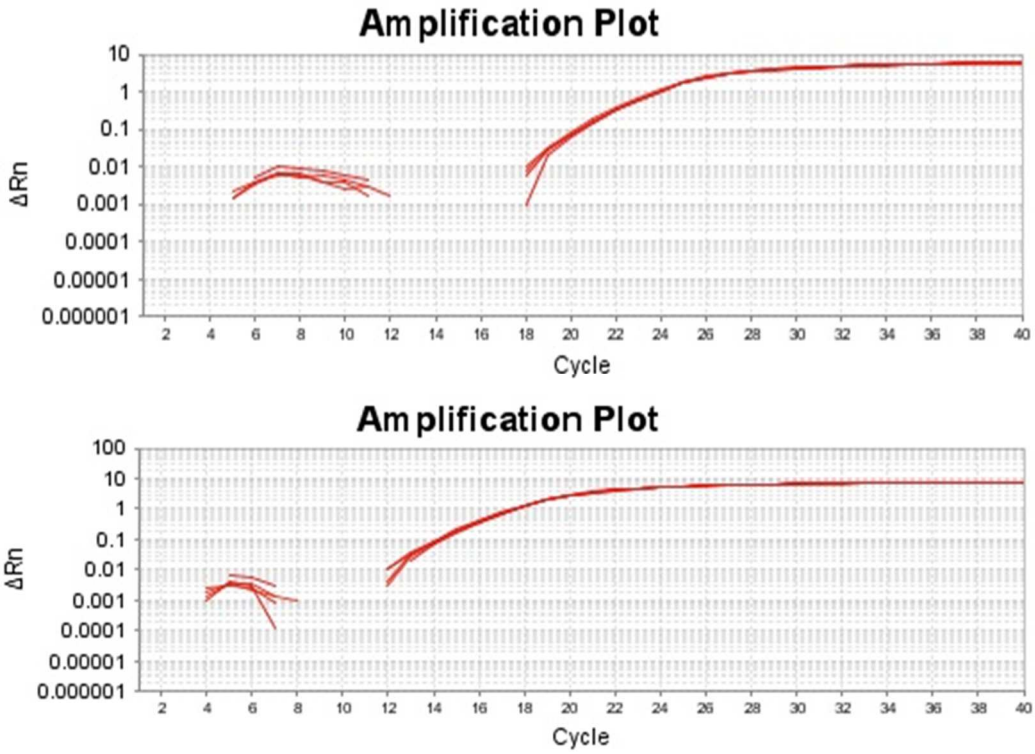

Figure 4. Igf-1 mRNA and Ppia primer amplification plots. Primers specifically amplified the selected Igf-1 mRNA sequence (top) of all tissue samples. Experimental control gene cyclophilin-A, Ppia (bottom), also showed specific amplification.

Body and Brain Weights

A significant effect of age ($F_{(2,74)} = 121.2$, $p < 0.0001$) and treatment ($F_{(1,74)} = 80.91$, $p < 0.0001$) was found for body weights. An interaction was also significant ($F_{(3,94)} = 18.88$, $p < 0.0001$). A Sidak's post-hoc t-test deteremined a significant decrease in hypothyroid pup body weights at P7 ($t = 2.457$, $p = 0.0482$), P14 ($t = 4.325$, $p = 0.0001$), and P21 ($t = 8.70$, $p < 0.001$). An unpaired t-test determined a significant decrease in hypothyroid pup body weights compared to euthyroid pups at P42 ($t = 8.298$ $p < 0.0001$ (Figure 5A)).

A significant effect of age ($F_{(2,27)} = 62.63$, $p < 0.0001$) and treatment ($F_{(1,27)} = 10.58$, $p = 0.0031$) was observed for wet brain weights (Figure 5B). Hypothyroid pups

had significantly lower brain weights when compared to controls at all developmental time points except for P14. These results indicate that hypothyroid pups were significantly developmentally stunted in growth compared to euthyroid controls. More importantly, retarded growth size persisted in hypothyroid pups after they were weaned from goitrogens.

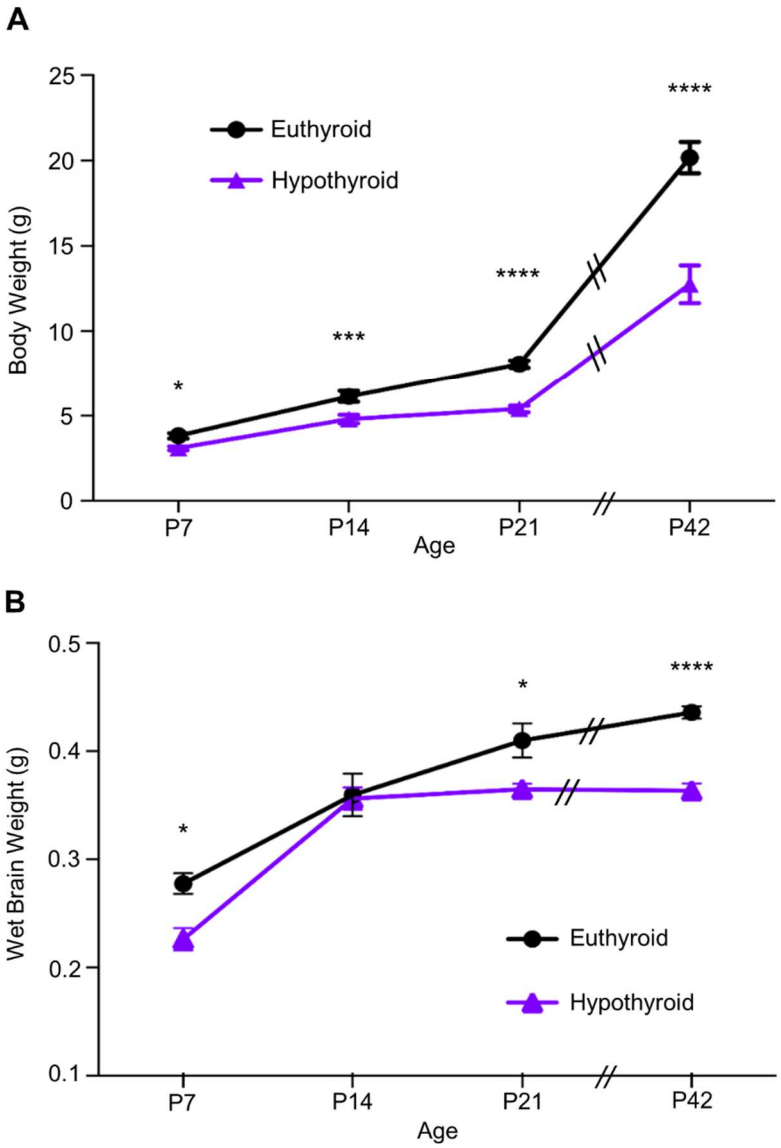

Figure 5. Body weight and total wet brain weight differences between developmentally euthyroid and hypothyroid mice. Average body weight (top) at sacrifice and average wet brain weight (bottom) were analyzed using a two-way ANOVA with a Sidak post-hoc test. Body weights were significantly reduced at all ages in hypothyroid mice compared to euthyroid. Wet brain weights were also significantly decreased in hypothyroid mice at ages P7, P21, and P42. *p<0.05, ***p<0.001, ****p<0.0001

Circulating T4 Levels

A significant effect was found for age ($F_{(2,30)} = 38.64$, $p < 0.0001$), treatment ($F_{(1,30)} = 461.80$, $p < 0.0001$), and an interaction ($F_{(2,30)} = 38.11$, $p < 0.0001$). There was a significant reduction of circulating T4 in developmentally hypothyroid pups at ages P7, P14, and P21. At P42, following withdrawal of goitrogens at P21, no significant difference between treated and control animals was observed; indicating treated pups returned to a euthyroid state (Figure 6).

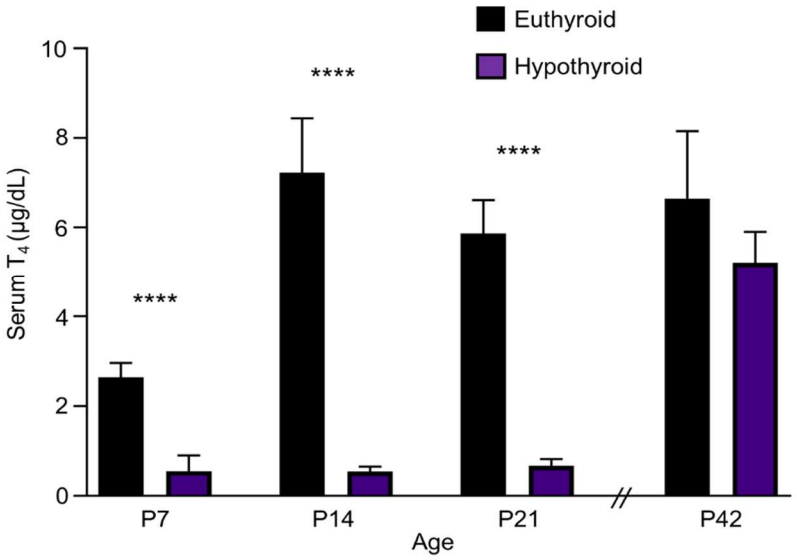

Figure 6. Serum T4 levels in developmentally euthyroid and hypothyroid mice. A two-way ANOVA and a Sidak's post hoc test determined a significant reduction in circulating T4 levels at ages P7, P14, and P21 in hypothyroid mice. No significance was found at age P42 using an unpaired t test. ****p<0.0001

Igf-1 Expression Throughout Neurodevelopment

To understand changes in Igf-1 mRNA levels during brain development, relative Igf-1 mRNA levels obtained by qRT-PCR from euthyroid mice were analyzed by a two-way ANOVA for ages P7-P42 using brain region and developmental age as

factors. There was a significant effect of age ($F_{(3,45)} = 53.74$, $p < 0.0001$), and region ($F_{(3245)} = 12.02$, $p < 0.0001$), along with an interaction between the two variables ($F_{(6,45)} = 6.968$, $p < 0.0001$) meaning the effects of treatment depended on age. A Sidak post-hoc test determined that the cortex had significantly greater relative gene expression compared to the cerebellum and hippocampus at P14.

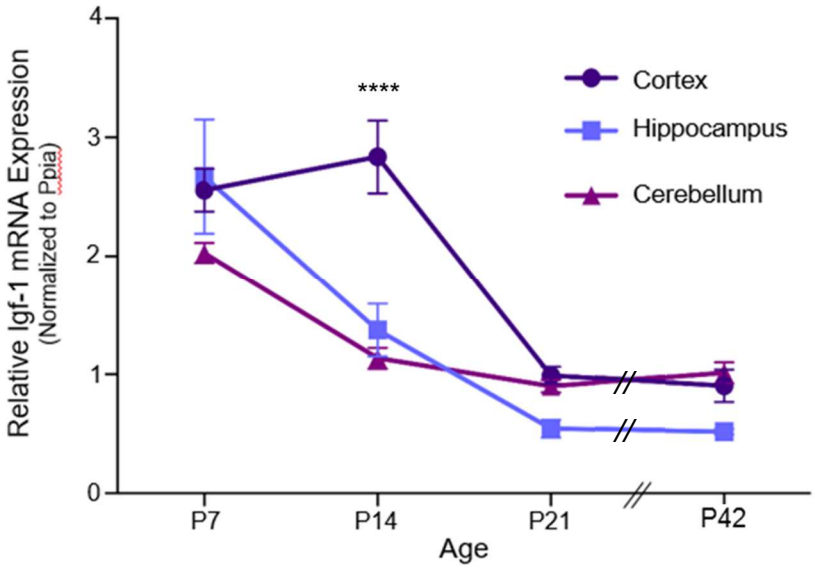

Figure 7: Relative Igf-1 mRNA expression under euthyroid conditions in the developing motor cortex, hippocampus, and cerebellum. Igf-1 gene expression was normalized to Ppia mRNA levels. 2-way ANOVA found a significant effect and interaction of age and treatment. A Sidak post-hoc test determined a significant increase in Igf-1 expression in the cortex at P14 compared to expression in the hippocampus and cerebellum. ****p<0.0001.

Relative Igf-1 mRNA Expression under Euthyroid and Hypothyroid Conditions

1. Motor Cortex

Cortical Igf-1 mRNA levels were analyzed using a two-way ANOVA for ages P7-P21 (Figure 8). A significant effect of age ($F_{(2,23)} = 11.61$, $p = 0.0003$) was seen in the cortex; yet, there was no significant effect of treatment or an interaction between the two. A Sidak post-hoc t-test indicated no differences in Igf-1 mRNA levels at any

ages investigated in the developing cortex. No significant difference was observed at age P42.

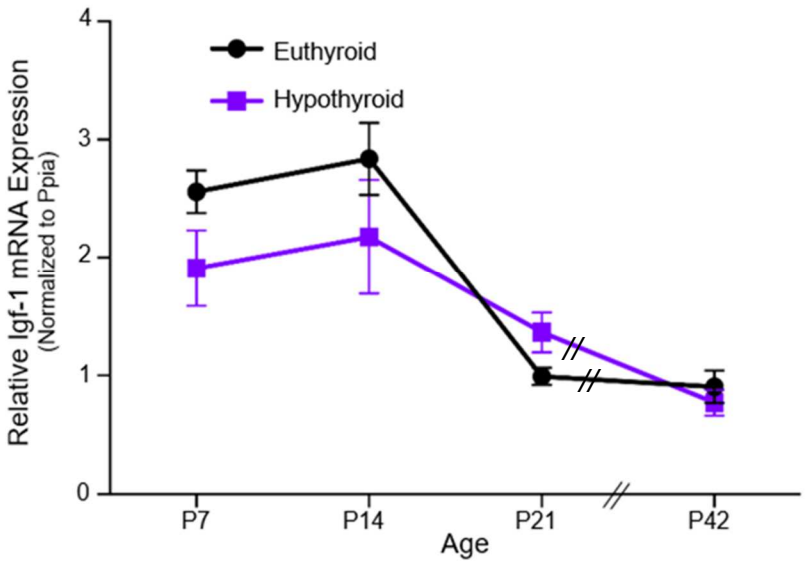

Figure 8. Relative Igf-1 expression in the developing cortex during neurodevelopment. A two-way ANOVA and a Sidak's post-hoc test found no significant differences between hypothyroid and euthyroid mice at ages P7-P21. An unpaired t test found no significant difference between groups at P42.

2. Hippocampus

In the developing hippocampus, there was a significant effect of age ($F_{(2,23)}$ = 19.39, $p < 0.0001$) and a significant interaction ($F_{(2,23)}$ = 8.466, $p = 0.0018$) between age and treatment. Igf-1 mRNA levels were significantly reduced ($t = 3.687$, $p = 0.0037$) at P7 in hypothyroid mice when compared to euthyroid mice. Gene expression levels between hypothyroid and euthyroid mice did not differ significantly at P14 or P21. At P42, Igf-1 mRNA levels were significantly increased ($t = 4.485$, $p = 0.0028$) in developmentally hyopthyroid animals compared to developmentally euthyroid.

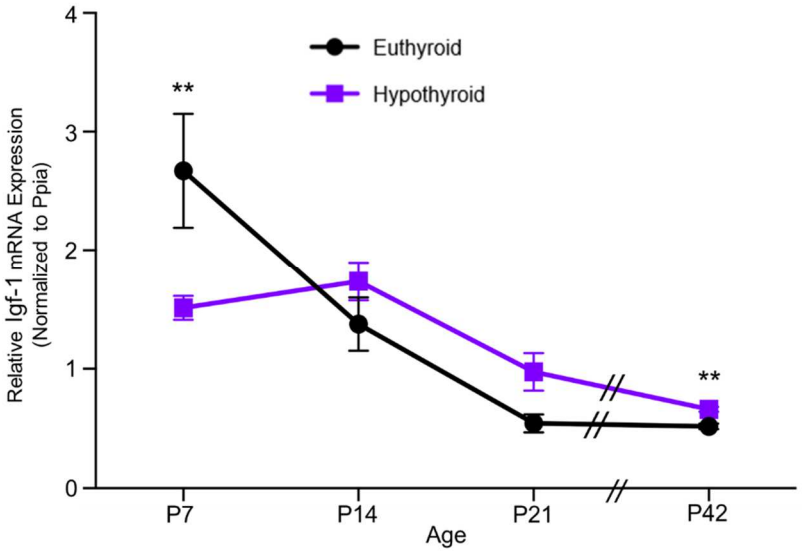

Figure 9. Relative Igf-1 mRNA expression in the developing hippocampus under hypothyroid and euthyroid conditions. Ages P7-P21 were analyzed using a two-way ANOVA and a Sidak's post-hoc test. Igf-1 mRNA levels were significantly reduced in hypothyroid mice at P7. An unpaired t test at age P42 determined significantly reduced Igf-1 gene expression in developmentally euthyroid mice compared to hypothyroid mice. **p<0.01

3. Cerebellum

In the cerebellum, Igf-1 mRNA levels indicated significant effects of age ($F_{(2,24)}$ = 137.3, $p < 0.0001$), but no effect of treatment or an interaction (Figure 10). A significant decrease in brain Igf-1 levels at P14 was found in developmentally hypothyroid mice ($t = 2.838$, $p = 0.0270$) when compared to euthyroid controls. There was no significant difference between treatment groups at any other age.

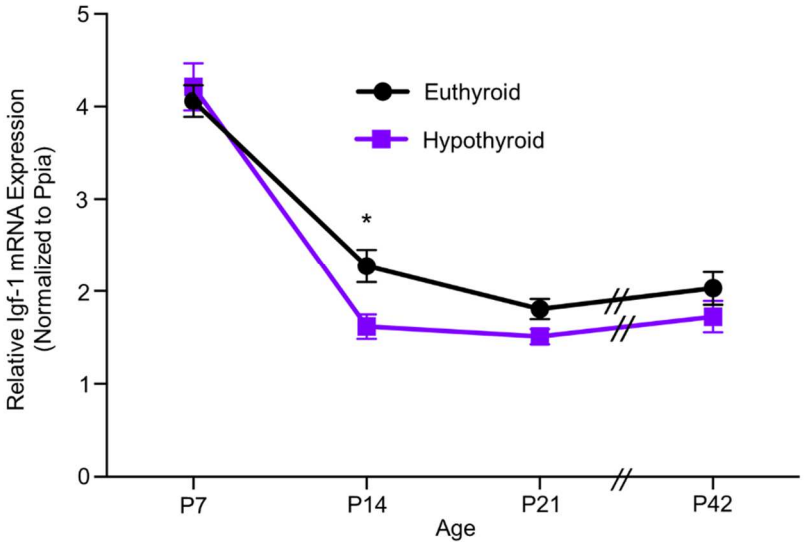

Figure 10. Relative Igf-1 mRNA expression in the developing cerebellum. A two-way ANOVA found a significant effect age, but no significant effect of treatment or an interaction for ages P7-P21. A Sidak post-hoc test determined a significant decrease of Igf-1 gene expression at P14 in hypothyroid mice when compared to euthyroid controls. An unpaired t test determined no significant difference between groups at P42. *p<0.05

Discussion

Previous studies have shown that low TH reduces the amount of serum Igf-1 (Burstein et al., 1979; Chang et al., 2014) and tissue-derived Igf-1 (Xing et al., 2012; Kline, 2016). Regulation of serum Igf-1 occurs by way of GH secreted from the pituitary gland and its ability to bind hepatic GH receptors, which low TH has been shown to decrease (Chang et al., 2014). In bone, TH, rather than GH, seems to directly regulate levels of locally produced Igf-1 (Xing et al., 2012). However, whether this is the case in other extra-hepatic tissues, such as the brain, is yet to be determined.

Hypothyroidism is defined as low levels of T4 and T3 in serum. Clinical and experimental signs of CH and developmental hypothyroidism include decreased body and brain weight. In the current experiment, goitrogen treatment resulted in significantly lower serum T4, body weight, and brain weight; indicating that mice

treated with goitrogens were indeed overtly hypothyroid. Serum T4 levels of developmentally hypothyroid mice returned to normal at P42 after goitrogens were removed at P21; therefore, it is plausible to suggest differences seen at P42 are a permanent defect resulting from low TH during development. This idea is supported by the observed decrease in brain and body weights at P42 in developmentally hypothyroid mice compared to euthyroid mice.

Although our experimental animals exhibit clear signs of overt hypothyroidism, it should be noted that tissue TH levels can be regulated locally, uncoupling TH action in tissues from circulating T4 levels. Even with a decreased serum T4, compensatory mechanisms such as the type II deiodinase enzyme can increase active T3 in tissues, which has been noted for the developing mammalian CNS (Silva and Larsen, 1982). However, once animals become overtly hypothyroid, these compensatory mechanisms fail to raise tissue T3 to normal levels, resulting in tissue hypothyroidism (Silva and Larsen, 1982). Previous work has also shown a direct correlation between circulating amounts of T4 with serum Igf-1 (Kline, 2016) indicating that the incomplete compensatory mechanisms for overt developmental hypothyroidism would also most likely be unsuccessful at compensating for the consequential decreases of Igf-1.

Deficiencies in either Igf-1 or TH lead to similar developmental defects. Because Igf-1 knockout models have a high mortality rate, deficiency models of serum Igf-1 are considered standard. Ames and Snell mice are spontaneous mutants that serve as experimental models of serum Igf-1 deficiency which also happen to be deficient in TH. These models experience decreased body weight, neuroanatomical defects, and cognitive and sensory issues as adults (Carter et al., 2002). Because the Ames and

Snell models exhibit cognitive and neurological deficits when serum Igf-1 is reduced, even though brain tissue levels remain unchanged, it is reasonable to suggest that the TH deficiency could directly or indirectly be responsible for the neurological deficits since both models are deficient in TSH. Additionally, a liver-specific Igf-1 KO model which randomly deletes gene sequences encoding serum Igf-1, reduce serum levels by 75%; however, tissue levels of local Igf-1 for this model in the heart, brain, kidney, and fat show no change in expression even though serum Igf-1 levels are reduced (Carter et al., 2002).

Developmental Hypothyroidism and Igf-1 mRNA Expression

TH is critical for normal brain development. Abnormal brain development in response to low TH is thought to be the result of altered gene expression. Considering the report that proposed direct regulation of Igf-1 by thyroid hormone receptors in bone and the similar neuroanatomical defects between low TH and low Igf-1 (Xing et al., 2012), we sought to characterize the effect of low TH in development on locally derived Igf-1.

1. Motor Cortex

Temporal expression patterns exhibit similar peaks and decreases under both treatment conditions. Levels increase from P7 to peak at P14, followed by a dramatic decrease until P42. Although no statistical difference in cortical Igf-1 mRNA was observed between developmentally hypothyroid and euthyroid mice, trends support a decreased Igf-1 mRNA expression under hypothyroid conditions. An increase in sample size could potentially provide the power to reach statistical significance.

Nonetheless, this observation is inconsistent with other reports that demonstrated reduced cortical Igf-1 mRNA (Elder et al., 2000) and Igf-1 positive cells (Kline, 2016).

Both microglia and oligodendrocytes are important regulators of available Igf-1 mRNA and protein levels during early postnatal development (Wilkins et al., 2001; Ueno et al., 2013). They are also both targets of TH signaling during brain development (Lima et al., 2001; Schoonover et al., 2004). Highly accumulated in white matter tracts like the corpus callosum, microglia produce Igf-1 which leads to neuronal survival during development. When microglia are transfected with Igf-1 siRNA, decreased neuronal survival followed decreased Igf-1 mRNA levels (Ueno et al., 2013).

However, in mice where microglia responses are dysregulated following fractalkine receptor *Cx3cr1*-deficiency, neuronal survival decreases following no change in Igf-1 mRNA levels. Ueno and colleagues found although Igf-1 mRNA levels hadn't changed, the IGF protein inhibitor IGFBP5 was upregulated. This upregulation of the binding protein limits the availability of free IGF-1 protein to activate its receptor leading to neuronal apoptosis in the presence of normal Igf-1 mRNA levels.

Oligodendrocytes could undergo a similar process, which are also heavily accumulated near white matter tracts, potentially explaining why there was no significant difference seen in Igf-1 mRNA levels between each treatment group. Studies focusing on Igf-1 positive cells in the motor cortex found statistically less cells in developmentally hypothyroid mice when compared to controls (Kline, 2016), suggesting a possible compensation in Igf-1 mRNA production by the remaining Igf-1 positive cells to restore overall Igf-1 expression. However, it has yet to be determined

if these cell types include microglia, and it is not known if developmental hypothyroidism leads to microglial dysregulation.

Future experiments focusing on IGF-1 protein levels should be conducted throughout the cortex to compare mRNA locations and quantities to that of protein levels to better understand Igf-1 mechanisms. Igf-1 positive cells could also be identified as a specific cell type to determine if developmental hypothyroidism effects the production of Igf-1 mRNA differently in each type.

2. Hippocampus

Temporally, we observed Igf-1 mRNA levels in the hippocampus peaked at P7, then decreased until P21 when expression reached adult levels measured at P42. At P7, Igf-1 mRNA in the hippocampus of euthyroid mice was significantly higher than in hypothyroid mice. Although Igf-1 mRNA in euthyroid animals decreased, expression under hypothyroid conditions peaked at P14 and surpassed euthyroid mRNA levels. Although the higher Igf-1 mRNA level at this age as well as P21 was not significant, Igf-1 expression was statistically higher at P42 following developmental hypothyroidism.

These results could point to a temporal shift of Igf-1 expression during development. Under normal conditions, Igf-1 mRNA levels peak early and fall until adulthood. However, under hypothyroid conditions, Igf-1 levels peak until P14 rather than P7, and then begins to fall. This delayed peak in expression could be attributed to low T3 availability. A similar shift in temporal expression in myelination under hypothyroid conditions has been previously reported (Rodriguez-Pena et al., 1993).

However, whether the delayed expression of Igf-1 is related to the altered myelination (or vice versa) remains to be tested.

More than half of all T3 availability in the brain comes from conversion of T4 by Dio2 (Bernal, 2015). Considering this, one could speculate that the ability of Dio2 activity to increase following developmental hypothyroidism, and moderate the changes in brain T3, may also be developmentally regulated. That is, increased Dio2 activity may only occur after P7, but prior to P14. Such a result would increase the amount of T3 after P7, thereby delaying peak expression of Igf-1 mRNA. This delay would cause a slowed decrease to adult Igf-1 levels, potentially leading to higher amounts of Igf-1 mRNA in hypothyroid mice into adulthood. Protein levels of Dio2 as well as mRNA levels of T3 should be examined in neural tissues, including the hippocampus, in order to elucidate these discrepancies.

3. Cerebellum

Cerebellar expression of Igf-1 at P7 was similar between euthyroid and hypothyroid animals, contradicting previous findings of decreased mRNA levels during hypothyroidism (Elder et al., 2000). This inconsistency could be due to differences in goitrogen treatment. However, more likely, the difference is due to the sensitivity of the assay utilized to quantify Igf-1 levels. Elder and colleagues used a ribonuclease protection assay, which is less sensitive than RT-qRT-PCR at determining mRNA levels.

After P7, a dramatic decrease in Igf-1 expression was seen in both euthyroid and hypothyroid conditions, plateauing from P14 to P42. Hypothyroid pups showed a

trending decrease in Igf-1 mRNA levels when compared to euthyroid pups, but was only statistically significant at P14. With an overall decreased amount of *Igf-1* gene expression under hypothyroid conditions, both euthyroid and hypothyroid cerebellums followed the same pattern of expression. Like the cortex, the cerebellum could be quick to adjust local T3 availability even in the presence of low serum T4 levels, by increasing the amount of DIO2.

Conclusions

Thyroid hormones play a crucial role in neurodevelopment, along with specific growth factors such as insulin-like growth factor 1. Deficiency in either of these two hormones lead to similar developmental abnormalities such as decreased body and brain size (Beck et al., 1995; Brent, 1999), and mental retardation (Flamant et al., 2006). Developmental hypothyroidism is known to cause neurological defects that can lead to permanent cognitive deficits if untreated early enough in development (Zoeller and Rovet, 2004). The mechanisms behind these neurological abnormalities following low circulating TH levels is yet unknown; however, TH and Igf-1 share many common sites of action throughout the developing brain. The goal of this study was to elucidate a potential relationship between circulating TH during early development and perturbed locally produced brain Igf-1 mRNA levels.

The working hypothesis of this study was that: low TH during perinatal development reduces the temporal expression of Igf-1 mRNA in the cortex, hippocampus, and cerebellum in hypothyroid mice when compared to wildtype mice. The corollary hypothesis was that hypothyroidism-induced reductions in brain Igf-1 mRNA result in the permanent neurological deficits observed in CH.

Overall, the results of this study support this working hypothesis. In the cortex and cerebellum, developmentally hypothyroid mice had decreased amounts of Igf-1 mRNA, although the difference was not always statistically significant. In the hippocampus, peak Igf-1 mRNA levels shifted to P14 in hypothyroid mice compared to P7 under euthyroid conditions. Because treatment of goitrogens ceased at P21, any differences seen at P42 are the result of permanent TH deficit during early development. This evidence is most prominent when comparing overall body weight and total wet brain weights, which are both significantly less in hypothyroid mice even when serum T4 levels are normal.

Since this result was not reflected in decreased Igf-1 expression at P42 in any region, it can be assumed that TH has more complex mechanistic actions than just regulating Igf-1 gene expression. It is also safe to propose that brain Igf-1 mRNA expression is affected by low serum TH; yet the way Igf-1 gene expression is perturbed is regionally and temporally specific. This study has identified a potential novel, previously unexplored mechanism, by which low TH during development results in neurological deficits.

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