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The Role of Insulin-Like Growth Factor I in the Development of the Cerebellum in Euthyroid and Hypothyroid Mice

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By

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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

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Thyroid hormone deficiency during development is known to cause anatomical defects in the cerebellum that lead to neurological deficits. It has been observed that insufficient thyroid hormone levels also decrease the levels of brain derived Insulin-like growth factor 1 (Igf-1). Given that the deficits caused by low Igf-1 levels are similar to those caused by low thyroid hormone, we designed this study to investigate if the deficits caused in the development of the cerebellum by low thyroid hormone levels may be due to the lack of sufficient Igf-1. We also sought to determine if ectopic over expression of Igf-1 in the brain can ameliorate the defects caused by low thyroid hormone. To do this, we used a double transgenic mouse model that ectopically over expresses Igf-1 in the brain astrocytes, specifically the Bergman glia. To obtain double transgenic mice, we crossed mice that contain the tTA (tetracycline trans activator) transgene driven by the GFAP (glial fibrillary acidic protein) promoter with mice that contain the IGF-1 transgene under the control of the tetracycline response element (pTRE). The double transgenic mice obtained from this cross, over express Igf-1 in brain astrocytes. To induce hypothyroidism, timed pregnant mice were treated with thyroid hormone disrupting chemicals in their drinking water starting at embryonic day 13.5 till pup sacrifice at P12. From each pup, blood and tail was collected, brains were dissected and cryoprotected. Defects in developmental processes in the cerebellum like granule cell proliferation, migration and purkinje cell differentiation were studied using immunohistochemistry. Our results showed no evidence of rescue in the defects caused by hypothyroidism in the presence of ectopic Igf-1 over expression in the brain.

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Introduction

Congenital hypothyroidism is a condition in which individuals are born with an improperly functioning thyroid gland that is unable to produce sufficient levels of thyroid hormone (Rastogi et al.2010). Thyroid hormone plays a critical role in development of the brain and insufficient levels during gestational and postnatal development causes neuroanatomical defects in the developing brain, which leads to neurological deficits (Ahmed. 2015).

Insulin like growth factor 1 (Igf-1), is a peptide hormone known to play an important role in cell proliferation and differentiation in the developing cerebellum. It has also been observed that low thyroid hormone induces a decrease in local brain levels of Igf-1(Fauquier et al. 2011; Morte et al. 2002). This relationship presents a possibility that some of the defects commonly associated with developmental hypothyroidism could be due to insufficient Igf-1 during development of the brain, including the cerebellum. This correlation presents the question of whether restoring Igf-1 expression has the potential to recover the defects in cerebellar development observed in hypothyroid mice. The focus/objective of this study was to examine the effect of ectopic Igf-1 over expression in cerebellar development, specifically in the purkinje cells, inner and outer granular layers, and investigate if ectopic Igf-1 over-expression in the brain can rescue the defects in cerebellar development in hypothyroid mice.

To investigate this, we utilized double transgenic mice that carry both the tTA^{GFAP} transgene and $Igf\text{-}1^{pTRE}$ transgene resulting in overexpression of Igf-1 in the brain (Figure 2.). Mice that carry tetracycline trans-activator under GFAP promoter (glial fibrillary acidic protein) were crossed with mice carrying Igf-1 transgene with the tetracycline response element ($Igf\text{-}1^{pTRE}$). This results in mice that ectopically express Igf-1 in the brain (Madathil et al.2013). **The working hypothesis is that defects in cerebellar development observed in hypothyroid animals are at**

least in part, the result of reduced brain Igf-1 levels and that ectopic expression of Igf1 can ameliorate developmental defects in the cerebellum associated with congenital hypothyroidism to some degree.

Literature Review

Cerebellum Structure and Development

1. Structure

The cerebellum is part of the brain that is located near the back of the brain stem at the junction between the midbrain and hindbrain (Faustino & Ortiga-Calvarho. 2014) This is an important structure that plays a role in motor function and neurocognition (Altman & Bayer. 1997).

The cerebellum is made up of a folded cerebellar cortex (folia) that covers the inner white matter and deep cerebellar nuclei (Sultan. 2015). The cortex is composed of three main layers: the outer most molecular layer, the middle purkinje cell layer and the inner most granular layer (Voogd & Glickstein. 1998). The outer most molecular layer is composed of axons of the cerebellar granule cells, the dendrites of purkinje cells, stellate, and basket cells (Voogd & Glickstein. 1998). The middle purkinje layer is composed of purkinje cell bodies that are in a single row at the border of the inner granular and molecular layer. The inner most granular layer is composed of densely packed cell bodies of granule cells (Hibi & Shimizu. 2012).

Purkinje cells are GABAergic neurons: neurons that produce the neurotransmitter GABA. The purkinje neurons exist in the cerebellum in a shape like a tree; commonly referred to as dendritic tree. The initial branching is relatively smooth with distal branches covered with spikes. The myelinated axons of the purkinje neurons terminate at nuclei of the cerebellum and brainstem (Voogd & Glickstein. 1998).

Purkinje cells express the calcium binding protein Calbindin-D28k, which appears early during the maturation process of Purkinje cells when they start to migrate and differentiate (Bastianelli E. 2003). Calbindin-D28k is a reliable marker for Purkinje cells (Whitney et al. 2008).

2. Development

Cerebellar development continues postnatally in mice, during which a fourth outer layer called the external granular layer (EGL) is present. This layer is composed of granule cell precursors that are proliferating. During the maturation process, these cells migrate along the radial fibers of the Bergmann glia (BG), a group of specialized astrocytes. The mature granule cells eventually move to the inner granular layer (IGL) (Fauquier et al. 2014).

The purkinje cells also undergo maturation during cerebellar development by differentiating an elaborated dendritic tree. This arborization is characteristic to cerebellar purkinje cells (Morte et al. 2002, Heuer et al. 2003).

GABAergic interneuron (GI) progenitors and oligodendrocyte precursor cells migrate from the white matter towards the molecular layer or the IGL. GI progenitors undergo terminal differentiation to become basket or stellate cells in the ML or Golgi cells in the IGL (Fauquier et al. 2014). It has been reported by various studies that thyroid hormone plays a role in these important developmental processes in the cerebellum including granule cell proliferation, migration and purkinje cell differentiation (Manzano et al. 2007; Morte et al. 2002; Ango et al. 2008).

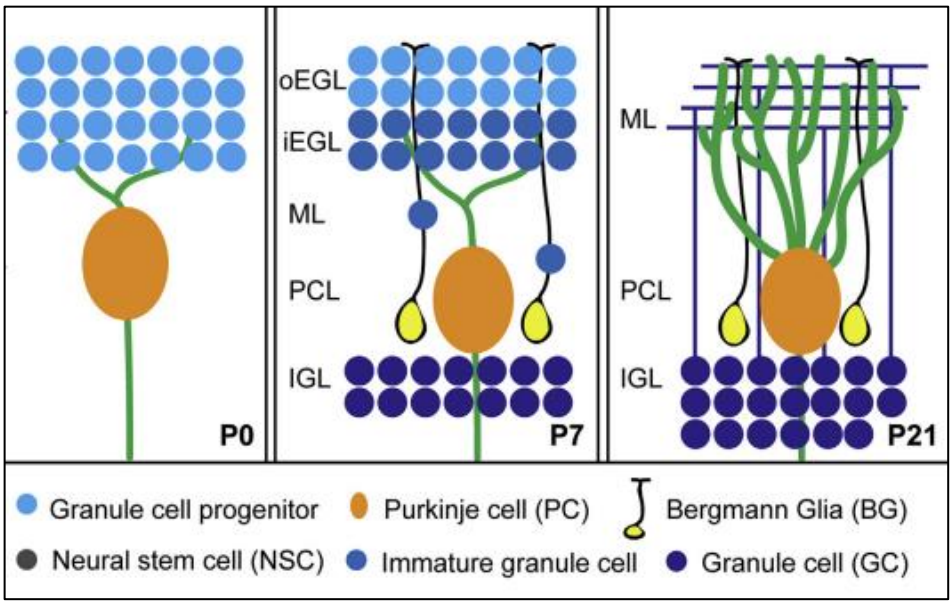

Figure 1. Schematic illustration of the development processes (purkinje cell differentiation and granule cell progenitor migration) in the mouse cerebellum (Ango et al. 2008).

Hypothalamic-Pituitary-Thyroid Gland Axis (HPT Axis)

1. Thyroid hormone production and regulation

Thyroid hormone (TH) is produced in the thyroid gland in two major forms, the prohormone thyroxine (T4) and triiodothyronine (T3) (Brent. 2012). T3 is the active form of the hormone and can be generated from T4 locally in tissues by enzymes (Morte et.al. 2002). These enzymes, known as the deiodinases, modify both T4 and T3.

Deiodinases are enzymes that contain amino acid selenocysteine in their active center. There are 3 types of deiodinases known as D1, D2 and D3. Type 1 deiodinase (D1) is mainly expressed in liver and kidney in vertebrates. In adult mammals, D1 transcripts are identified in thyroid, pituitary gland, intestine, placenta, and gonads. D1 mRNA is present at low levels during fetal development and increase at later life stages. However, D1 is undetectable in fetal brain tissue

(Maia et al. 2011). In thyroid hormone metabolism, D1 converts T4 to T3 by removing the iodine from the 5' or 3' position of the phenolic ring or the tyrosyl ring of iodothyronines (Bianco et al.2002).

Type 2 deiodinase (D2) is expressed in the pituitary gland, central nervous system, brown adipose tissue, uterus, placenta, and thyroid gland in humans. The highest levels of D2 expression in the brain are found in a type of glial cell called tanycytes. Astrocytes also contains D2 mRNA at lower levels. D2 is especially important in the brain due to its role in the conversion of T4 to T3 (Bianco et al. 2002).

Type 3 deiodinase (D3) is expressed in high levels in the pregnant uterus and placenta, and low levels in brain and skin. D3 is the enzyme that inactivates T4 by converting it to inactive iodothyronines rT3. D3 also inactivates T3 by converting it to 3,3'-diiodothyronine. It is considered the main thyroid hormone inactivating hormone (Visser et al.2008; Bianco et al. 2002).

TH production and secretion is regulated by the HPT Axis. The hypothalamus produces and releases thyroid releasing hormone (TRH). TRH triggers the release of thyroid stimulating hormone (TSH) from the anterior pituitary into the blood stream. TSH then acts on the thyroid gland to release TH. Physiological levels of TH are maintained by negative feedback. TH inhibits the production of TRH and TSH by acting on the hypothalamus and anterior pituitary. (Calvaho-Ortiga et.al 2016).

2. Thyroid hormone receptors

The two thyroid receptor genes, Thra and Thrb encode the thyroid hormone receptor (TR) protein isoforms. Thra encodes receptors TRα1 and TRα2. Thrb encodes TRβ1 and TRβ2. Among these receptor types, only TRα1, TRβ1 and TRβ2 bind T3 (Chatonnet et. al 2011). T3 binding causes a conformational change in the receptor which initiates the process of gene transcription or

repression. T3 bound TR binds to the thyroid hormone response element (TRE) located on the regulatory region of a target gene and modifies transcription. Ligand bound TR can also repress gene expression by binding to the negative TRE on a target gene (Pascual et al. 2013). TRα1 is abundant in the cerebellum (about 80%) and binds T3 to activate or repress target genes to regulate cerebellar development (Morte et.al 2002).

The granular cells express TRα1 and TRα2 and the purkinje cells express TRβ. The relative and specific functions of these receptor types in cerebellar development is unknown and is a topic of interest (Morte et al. 2002).

Congenital Hypothyroidism and effects on brain development

Congenital hypothyroidism is a thyroid hormone deficiency condition in which an individual is born with a thyroid gland that is unable to produce enough thyroid hormone (Rastogi & LaFranchi. 2010). This condition leads to various neurological, cognitive, and developmental defects, including impaired motor skills, hearing loss, and growth retardation if left untreated (Chaker et al. 2017).

Numerous studies have shown that congenitally hypothyroid mice have abnormal cerebellar development. Specifically, the differentiation and maturation processes involved in the purkinje cells and the EGL (Fauquier et al. 2011). The EGL persists beyond post-natal day 21 due to the slowing down of granule cell precursor proliferation and radial migration along the Bergman glia. The maturation of GABAergic interneurons into stellate or basket cells is impaired and radial fiber development of Bergman glia is altered. The dendritic arborization of purkinje cells, which is induced by thyroid hormone via TRα1, is significantly reduced (Morte et al. 2004). These

alterations compromise the synaptogenesis in the molecular layer (Ango et al. 2008; Morte et al.2004; Manzano et al. 2007).

Myelination in the central nervous system is also known to be affected by neonatal hypothyroidism. Hypothyroid brains show a reduction of cholesterol, cerebrosides, glycolipids and gangliosides in the myelin sheath. mRNA studies on the expression levels of myelin-associated glycoprotein in rats have shown a decrease in expression in neonatal hypothyroid conditions (Rodriguez-Pena et al. 1993).

An abnormal cerebellum caused by hypothyroidism leads to various neurological deficits such as cognitive deficits and other neurological defects such as impaired motor skills and visual processing (Ahmed. 2015).

Insulin-like growth factors (IGF) in cerebellar development.

1. Insulin-like growth factors

Insulin-like growth factors are peptides that are similar in structure to proinsulin. There are two main types of Igfs: Igf-1 and Igf-2, both commonly discussed in central nervous system development studies (Morte et al. 2002). Igf-1 is released by the liver into the blood. It is also produced in muscles and neural cells locally and acts via autocrine (act on self) or paracrine (act on nearby cells) signaling (Croci et al. 2011). Igf-1 synthesis in the liver is regulated by growth hormone that is released from the anterior pituitary. Whether circulating growth hormone regulates local tissue Igf-1 is not clear. In serum and tissues, Igf-1 is tightly bound to Igf-1 binding proteins which modulates interactions between Igf-1 and its receptors and increases Igf half-life (Wrigley et al. 2015).

The bioactivity of Igf-1 is modulated by the binding proteins (IGFBP) and facilitate their stability in serum and extracellular matrices. There are 6 high affinity IGFBPs and their expression and distribution are dependent on factors like nutrition, exercise, and aging. IGFBP-2 is the predominant binding protein in serum. Only a few (5%) of IGFs are found freely in serum (Yakar & Adamo.2012).

Igf-1 promotes proliferation and differentiation of cells throughout the body, including the central nervous system. In the brain, Igf-1 is produced locally by nervous tissue and plays a role in differential and the survival of various neuronal cell types. In the cerebellum, the purkinje cells express Igf-1 and are a target of Igf-1 during development (Fukudome et al. 2003).

Studies have shown that the Igf-1 signaling in purkinje cells, and their presynaptic neurons increases around the 2nd postnatal week. The Igf-1 levels derived from these neurons have also been shown to constitute a significant fraction of cerebellar Igf-1 content during this postnatal stage (Kapoor et al.1988). Although various studies provide evidence for the involvement of Igf-1 in purkinje cell development, not much is known about the mechanism in which Igf-1 regulates purkinje cell differentiation during cerebellar development (Fukudome et al. 2003).

2. Insulin-like growth factor receptors and development

Igf-I binds to type 1- Igf receptor, type 2- Igf receptor and insulin receptor. Type 1-Igf receptor is abundant in the cerebellum and is expressed the most in purkinje cells. Igf-1 receptors are membrane bound receptor tyrosine kinases (RTK). The Igf-1 receptors are composed of either a homodimer or heterodimer with each dimer consisting of an extracellular alpha-chain and an intracellular beta-chain (Yakar & Adamo. 2012). RTKs signal via the 2 main signaling pathways; MAPK/Ras-Raf-ERk pathway, and PI3 pathway both of which lead to changes in gene expression (Hakuno et al.2018). The downstream activation of these cell signaling pathways is essential for

important functions like cell proliferation, cell survival as well as metabolism (Yakar & Adamo. 2012). It has been proposed that Igf-1 mRNA expression is at its peak in the cerebellum between birth and postnatal day 20. During this stage, purkinje cells establish synaptic contacts with other cerebellar fibers including those of the inner granular layer. Igf-1 has been shown to play a key role in sustaining these processes during cerebellar development. Igf-1 is also known as a neuroprotective factor due to its role in promoting neuronal survival by blocking apoptosis (Croci et al. 2011).

Considering this information, we hypothesized that ectopic over expression of Igf-1 in the brain rescues the defects caused by hypothyroidism in the developing cerebellum. This study investigates this idea.

Methods

1. Animal model

Hemizygous Igf-1^{pTRE} mice were crossed with hemizygous tTA^{GFAP} mice to obtain double transgenic mice that ectopically over express Igf-1 in the brain. tTA^{GFAP} mice carry the tetracycline trans activator gene linked to a GFAP (glial fibrillary acidic protein) promoter which codes for the tetracycline trans activator protein in glial cells. Igf-1^{pTRE} mice carry the Igf-1 transgene linked to a tetracycline response element. In the absence of doxycycline, the tTA protein binds to the tetracycline response element on the Igf-1 transgene and activates transcription. The double transgenic (dTG) mice obtained by breeding these two hemizygous mice, express both transgenes thus, over express Igf-1 in glial cells in the cerebellum (Figure 2). All mice were housed in cages with corn cob bedding with regular mouse chow diet. Appropriate breeding pairs were set up to

obtain pups. Each genotype and treatment were studied at P12. Euthyroid mice were studies at P45 as well.

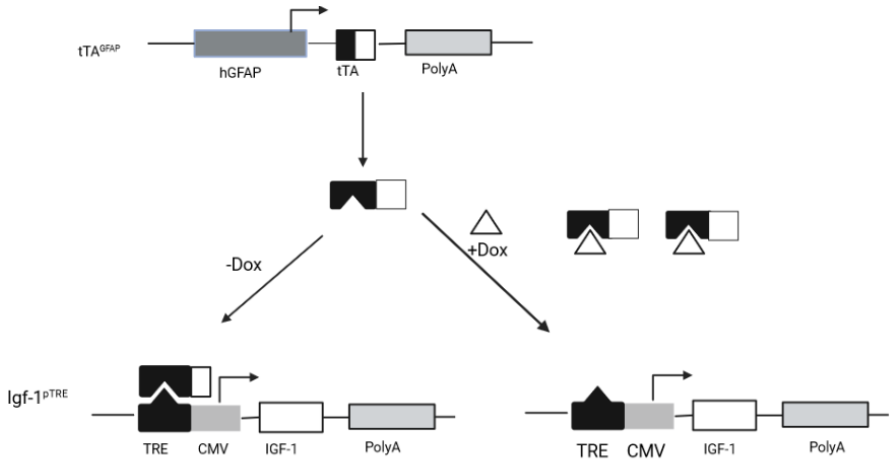

Figure 2. Diagram of the tTA^{GFAP} and Igf-1^{pTRE} transgenes and mechanism of interaction. Arrows indicate transcription site. Notched boxes represent the tTA protein and Dox.

2. Hypothyroid treatments

To obtain hypothyroid mice, female mice (hemizygous tTA ^{GFAP} or Igf-1^{pTRE}) were examined daily during breeding periods for a vaginal plug to ensure copulation occurred and marked as embryonic day 0.5. These mice were weighed each day to determine pregnancy (a rapid increase in weight indicates pregnancy). Once pregnancy was confirmed by weigh gain, these mice were treated with drinking water containing 0.01% methimazole (a thyroid peroxidase inhibitor) and 1% potassium perchlorate (an inhibitor of the sodium iodide symporter), starting at embryonic day 13.5 until pup sacrifice at P12. Euthyroid animals were maintained on untreated drinking water.

3. Genotyping

PCR was used to determine the genotype of the breeders (age group 8 weeks-40 weeks) and pups (P12 and P45). Breeders/parent mice were anesthetized in a 1% isoflurane environment. Each parent mouse was given a numbered ear tag and a tail clip was obtained to acquire a DNA sample to be used for genotyping.

Each tail sample was digested in 500µL of tail lysis buffer (50mM Tris-Cl, 20mM NaCl, 1mM EDTA, 0.2% SDS, 200µg/mL Proteinase K) at 56ºC with continuous shaking for approximately 2 hours. The digested tail samples were centrifuged at 14,000 rpm for five minute to remove debris. 450µL of supernatant from each sample was placed in a 1.5ml centrifuge tube with 450µL of isopropyl alcohol. Tubes were inverted 10 times to ensure thorough mixing, and the precipitated DNA was scooped out using a pipette tip and transferred to a tube containing 500µL of ddH20. The tubes were placed at 56ºC for 10 mins with shaking to dissolve the DNA.

Tail DNA was genotyped using PCR. Each tTA^{GFAP} reaction received 1.0 µL DNA, 0.3 µL of the forward and reverse primer of 0.3 µM of human IGF-1 internal control (Integrated DNA Technologies), 0.3µL of forward and reverse primer of tTA (0.3µM final), 7.8µL of ddH2O and 10µL of GoGreen Master mix (Promega). Each $Igf\text{-}1^{pTRE}$ reaction received 1.0 µL DNA, 0.3 µL of the forward and reverse primer of 0.3 µM of human IGF-1 internal control, 0.3 µL of the forward and reverse human IGF-1 primer (0.5µM final), 7.0 µL ddH2O and 10µL of GoGreen Master mix. Each sample was mixed by vortex and centrifuged.

Gene	Primer sequence	Concentration (µg/µl)	Temperature (Celsius)
$Igf\text{-}1^{pTRE}$	F- GGACCGGAGACGCTCTGCGG R- CTGCGGTGGCATGTCACTCT	0.5	60.1
tTA^{GFAP}	F- CGCTGTGGGGCATTTTACTTTAG	0.3	56.5

	R- CATGTCCAGATCGAAATCGTC		

Table 1. Primer sequence for genotyping.

Both reactions were placed in a thermal cycler with the tTA^{GFAP} program under the following conditions:

94ºC for 3 minutes, 94ºC for 30 seconds, 57ºC for 1-minute, repeated steps for 35 cycles at 72ºC for 1 minute, 72ºC for 2 minutes, and 10ºC on infinite hold.

The samples were then loaded on a 2% agarose gel and visualized by gel electrophoresis.

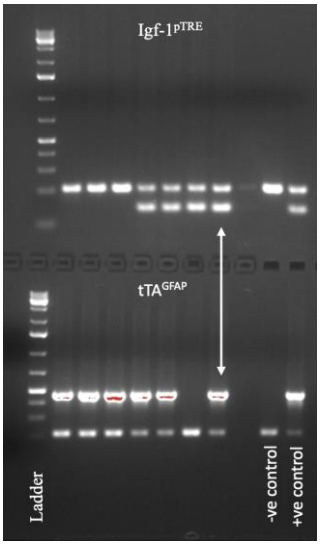

Figure 3. Gel electrophoresis representing double transgenic mouse genotyping. dTG mice marked by white double headed arrow show two bands in each PCR reaction. The single transgenic genotypes show two bands in one reaction only. The wildtype mice show a single band in both reactions.

4. Sacrifice and tissue collection.

Mice were euthanized at P12 and P45 using CO_2, followed by decapitation to obtain brain tissue and trunk blood. Blood from each mouse was placed on ice for 20-50 minutes to allow for clotting followed by centrifugation at 15000rcf for 10 minutes. The resulting serum was stored at -20ºC for further use. Tail cuttings from each pup were also stored at -20ºC for genotyping. The

brains removed from the cranial cavity were immersed in 4% PFA overnight at 4ºC with gentle rocking.

5. Brain fixation for microcopy and immunohistochemistry.

Brain tissue fixed overnight in 4% paraformaldehyde (as described above) were washed three times with 1x Phosphate-buffered saline (PBS). After third wash, each brain was cryoprotected in a 30% sucrose PBS solution, at 4°C, overnight. The brain tissue cryoprotected in sucrose were removed from sucrose solution and embedded in OCT (VWR; Clear Frozen Sections Compound) and stored at -80ºC for immunohistochemistry experiment.

6. Cryosectioning.

From each frozen brain, 30 μM sagittal sections were cut using a cryo-microtome and thaw-mounted on Superfrost Plus or Histobond slides. To confirm appropriate anatomical location, a single sagittal section (that includes the cerebellum and all lobes) was mounted on a slide, fixed in ethanol, stained with methylene blue, and visualized under a microscope. Slides with appropriate sections for analysis were collected, appropriately labelled, and stored at -80C until use.

7. Immunohistochemistry

Slides for each experiment were removed from freezer and allowed to warm to room temperature. A wax barrier was added to each slide. Each slide was washed with 1X PBS for 5mins, 3 times and blocked with 5% normal donkey serum, at room temperature for 2 hours in a humidity chamber to block non-specific binding sites. Slides were incubated overnight with a rabbit anti-mouse Calbindin primary antibody (Cell Signaling Technology) diluted 1:200 in dilution buffer (1% bovine serum albumin, 0.3% TritonX in PBS) at 4ºC.

The next day, slides were washed with 1X PBS three times for 5 minutes each and incubated for 2 hours with secondary antibody (donkey anti-rabbit Alexa 488, Jackson Immunoresearch Laboratories) diluted 1:500 in dilution buffer (1% bovine serum albumin, 0.3% TritonX in PBS) at room temperature, covered from light.

Slides were washed with diluted DAPI stain (1:2000 with 1X PBS) for ten minutes. Slides were then washed with 1X PBS for 5 minutes, twice. Slides were cover slipped with Antifade mounting media (VECTASHIELD PLUS) and stored at 4°C to prior to imaging under confocal microscope.

8. Confocal microscopy

Four tissues sections from each animal were visualized under the confocal microscope at 20X magnification, focused on lobule 4, at the juncture between lobules 3 and 4 (Figure 4.). Both a single image and Z stack was obtained for each section visualized on a single slide.

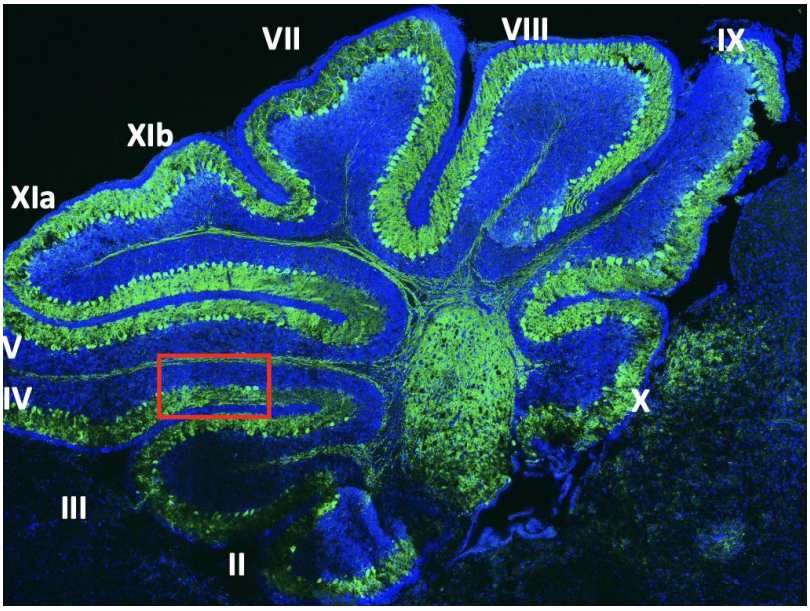

Figure 4. Sagittal section of Calbindin and DAPI stained whole cerebellum from wild type euthyroid mouse at P12. Red rectangle indicates the location where all relevant measurements of the study were taken.

9. Confocal Image analysis.

Five measurements of each cerebellar layer (EGL, IGL, and ML) from each section per mouse (average of 5 measurements equal 1 mouse) were acquired using the Fiji/Image J software. The total number of cells in the ML were also counted using cell counter in Image J software. Purkinje cell arborization was quantified by measuring the % area in the ML occupied by the purkinje cell dendrites. This was measured by assigning a threshold limit to each pixel in a selected area of the molecular layer. The minimum of the threshold limit was set at 50 and the maximum was set at 255. Every pixel in the selected area in the molecular layer that is within that threshold range was counted towards the % dendritic area. All measurement data collected from Image J were documented in MS excel.

10. Statistical data Analysis.

Graph Pad Prism was used for all statistical analysis. Grouped analysis (Two-way ANOVA) was used to determine any differences between male and female data for all measurements in all euthyroid genotypes at P12. Since there were no significant differences, all female and male data were pooled for the remaining analyses. Two-way ANOVA with multiple comparisons tests (Tukey's multiple comparisons test) were used to detect and analyze any significant difference of treatment, genotype, and interaction between variables.

For analysis of all euthyroid data sets, ordinary One-way ANOVA was used to detect any significant differences between genotypes. Tukey's multiple comparisons test was used to analyze the significant differences between genotypes.

11. Measurement of T4 in serum

Serum T4 levels were measured using an Enzyme-linked Immunosorbent Assay (ELISA) according to manufacturer's (Diagnostic Automation) protocol. Each standard was run in triplicate. The serum samples were run in duplicate with groups evenly distributed across each plate. The concentrations were calculated based on the standard curve created.

Results

1. TH disrupting treatment significantly reduced pup body weight at P12, at sacrifice.

In WT, Igf-1^{pTRE}, tTA^{GFAP} groups, thyroid hormone disrupting treatment induced a statistically significant decrease in pup body weight at P12 compared to their respective euthyroid genotype group. A statistically significant reduction in body weight in hypothyroid dTG mice compared to its control group was not observed. The hypothyroid pup weights of all genotypes were not significantly different from each other. The euthyroid dTG pups weighed significantly lower compared to all other euthyroid genotype groups. The amount of drinking water consumed per day by each mouse was monitored for both control and treatment groups. We found no significant difference between the amount of regular drinking water consumed per day and the amount of treatment water consumed per day.

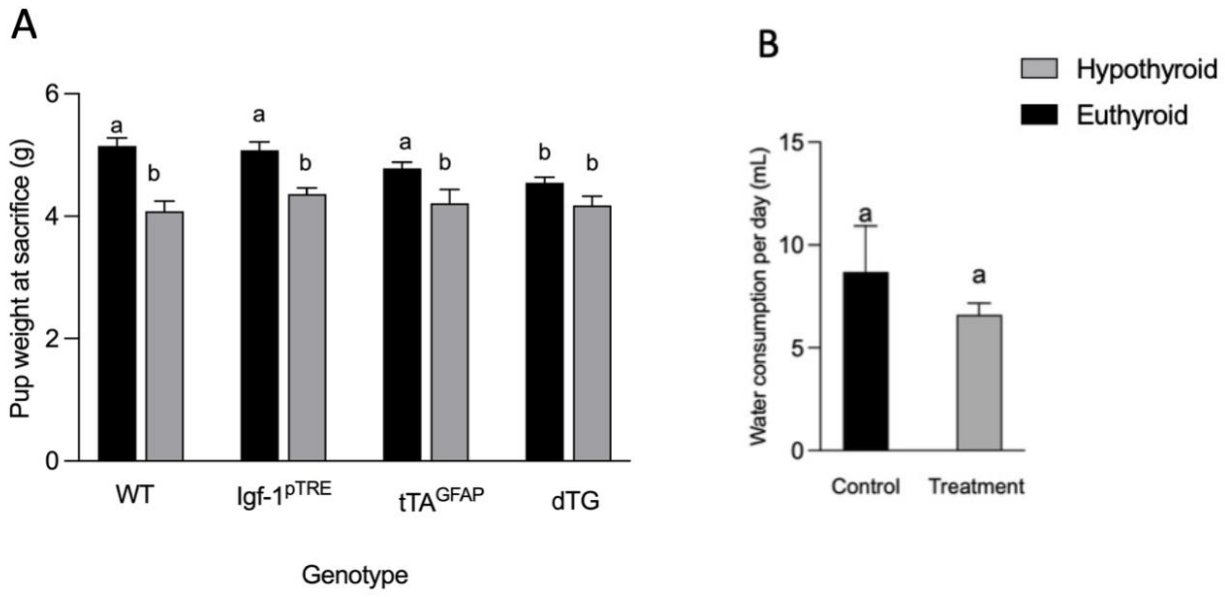

Figure 5. (A)The effect of hypothyroidism on pup weights at sacrifice on post-natal day 12. Bars represent mean weights ± standard error. Different letters denote significant statistical differences between groups with a p value greater than 0.05. n=8-20. No significant difference between mean weight of euthyroid and hypothyroid dTG mice. Mean weight of hypothyroid pups of WT, tTA^{GFAP} and $Igf\text{-}1^{pTRE}$ significantly less than respective euthyroid groups. No significant difference between mean weight of euthyroid dTG and hypothyroid mice of all genotypes. (B) Average water consumption per day in control and TH disrupting treatment groups. No significant difference in daily water consumption between control and treatment group. n=5-8

2. Thyroid hormone disrupting treatment significantly reduced serum T4.

To confirm that the MMI/Perchlorate treatment induced hypothyroidism in the treatment group, an ELISA assay was performed to quantify T4 levels in serum. The results demonstrated that the treatment significantly reduced T4 levels in serum in mice of all genotypes in the treatment group compared their respective control group.

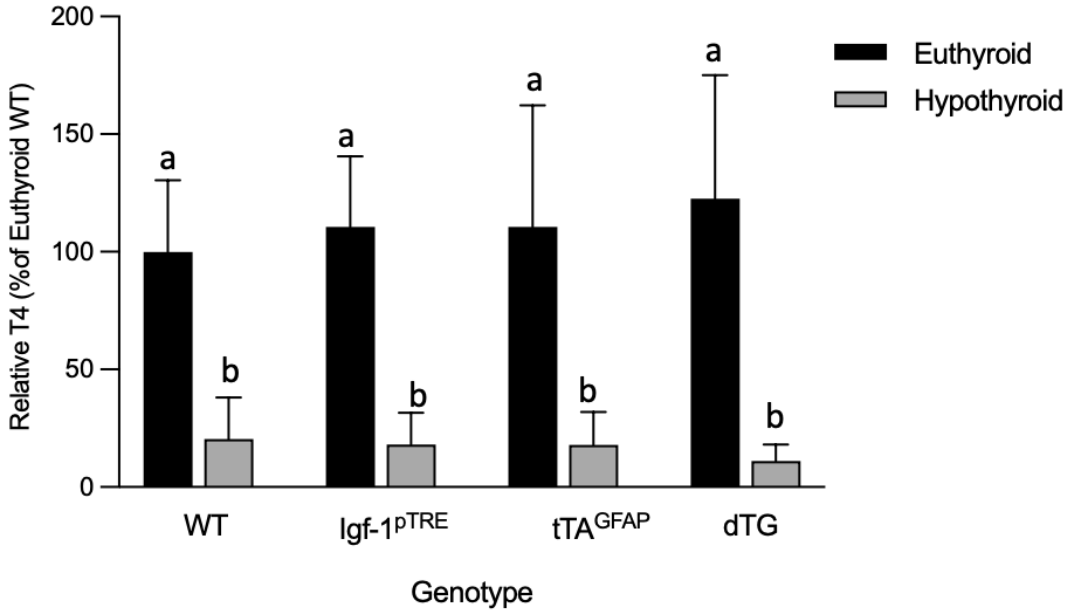

Figure 6. The effect of hypothyroidism on serum T4 concentration. The graph represents the serum T4 concentration in treatments group compared to their respective control groups. in Bars represent mean serum T4 ± standard error. Different letters denote significant statistical differences between groups with a p value less than 0.05. n=8-20.

3. Effect of ectopic Igf-1 over expression on external granular layer thickness at P12 and P45 under euthyroid conditions.

To test effects of Igf-1pTRE over expression on the EGL in euthyroid conditions, we measured the thickness of the EGL in euthyroid pups at P12 and P45. Staining of the external granular layer (EGL) using DAPI staining showed that the EGL had completely disappeared by post-natal day 45 in all genotypes in euthyroid conditions. The external granular layer was clearly visible in all genotypes at P12 and there were no significant differences in the EGL thickness between the genotypes under euthyroid conditions.

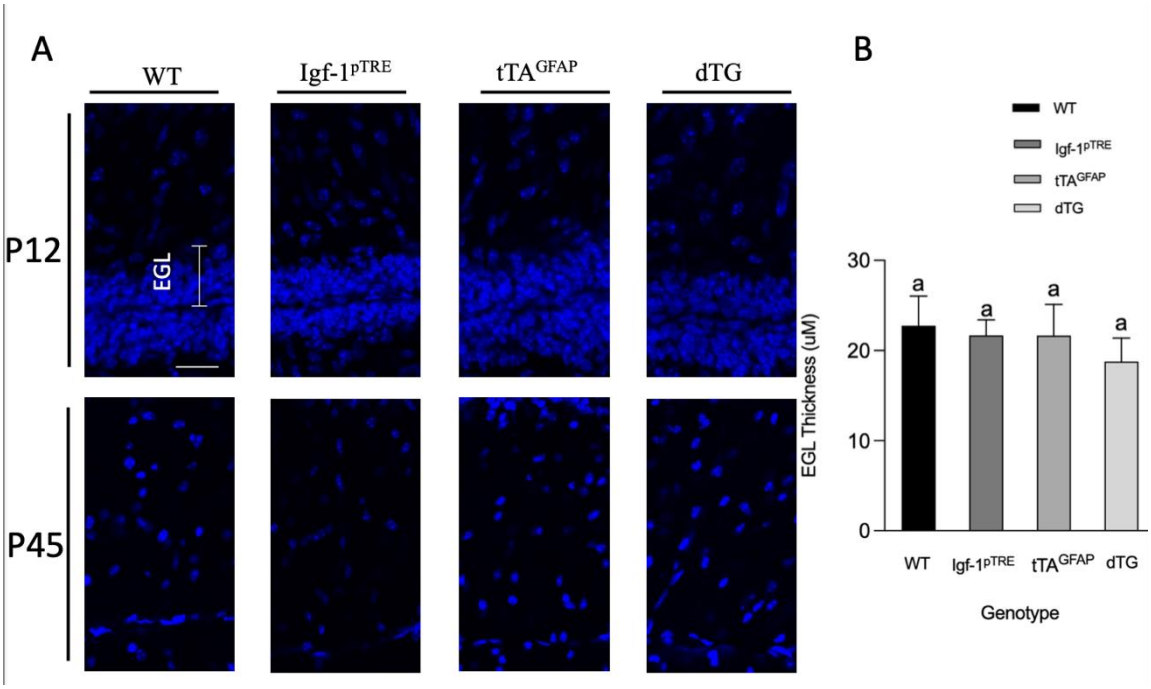

Figure 7. A) The effect of ectopic Igf-1^{pTRE} over expression on EGL thickness at sacrifice on post-natal day 12 and 45 under euthyroid conditions. White vertical line indicates the EGL. No presence of EGL in any genotype at P45. B) Graph representing EGL thickness measurements at P12. No significant difference in EGL thickness between all genotypes at P12, under euthyroid conditions. Bars represent mean weights ± standard error. Different letters denote significant statistical differences between groups with a p value greater than 0.05. n=5-8 per group. Scale bar= 50μm.

4. Effect of ectopic Igf-1 over expression on Inner granular layer thickness at P12 and P45 under euthyroid condition.

Staining of the inner granular layer (IGL) using DAPI stain showed that the thickness of the IGL was significantly increased in the euthyroid double transgenic mice when compared to the other genotypes at P12 ($P<0.05$). However, at P45 no significant differences in IGL thickness between any genotypes were observed, under euthyroid conditions (Figure 8).

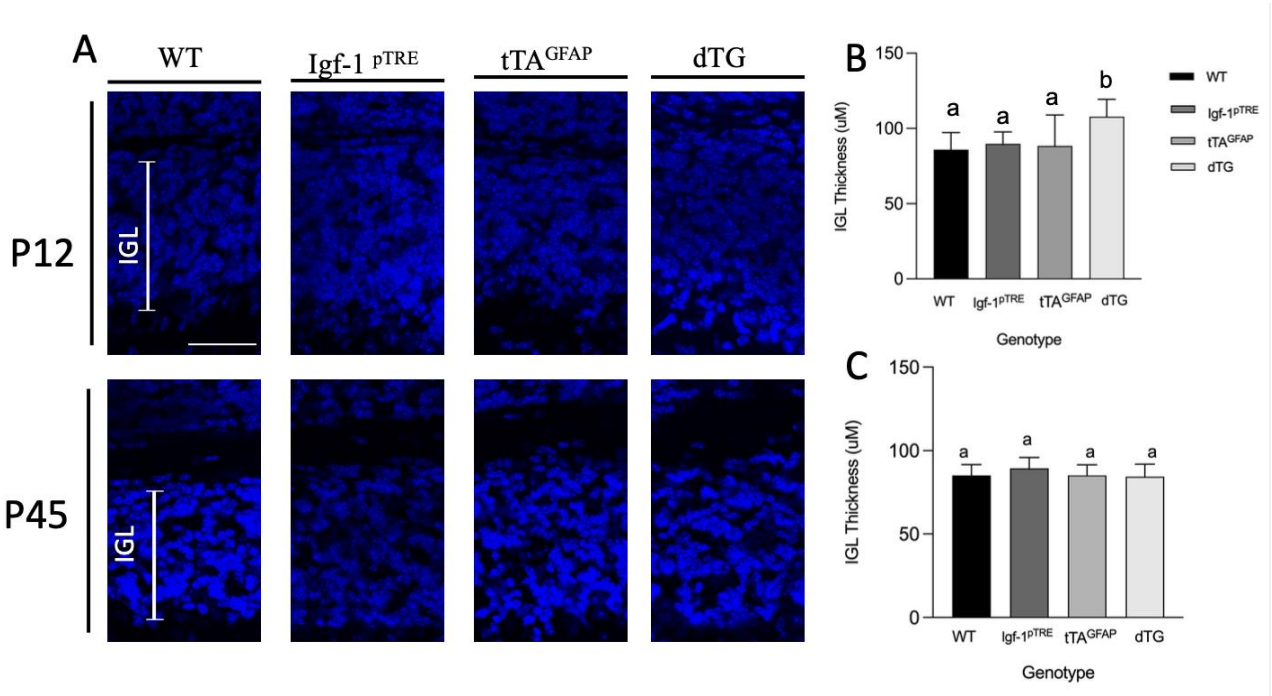

Figure 8. A) The effect of ectopic Igf-1^{pTRE} over expression on IGL thickness at sacrifice on post-natal day 12 and 45 under euthyroid conditions. White vertical line indicates the IGL. B), C) Graphs representing IGL thickness measurements at P12 and P45 respectively. Mean IGL thickness of dTG mice was significantly higher than that of all other genotypes under euthyroid conditions at P12. No significant difference in IGL thickness between all genotypes at P45, under euthyroid conditions. Bars represent mean weights ± standard error. Different letters denote significant statistical differences between groups with a p value greater than 0.05. n=5-8 per group. Scale bar= 50μm.

5. Effect of ectopic Igf-1 over expression on Molecular layer thickness at P12 and P45 under euthyroid conditions.

Immunostaining of the Purkinje cells in the ML using Calbindin indicated that the thickness of the ML of the euthyroid dTG pups at P12 was significantly larger than that of the wildtype and Igf-1^{pTRE} pups. Interestingly in the P45 group, the molecular layer thickness of the dTG group was significantly larger compared to the wildtype group under euthyroid conditions (P>0.05). There was no significant difference in ML thickness between WT, Igf-1^{pTRE} and tTA^{GFAP} genotype groups at P12 and P45 under euthyroid conditions.

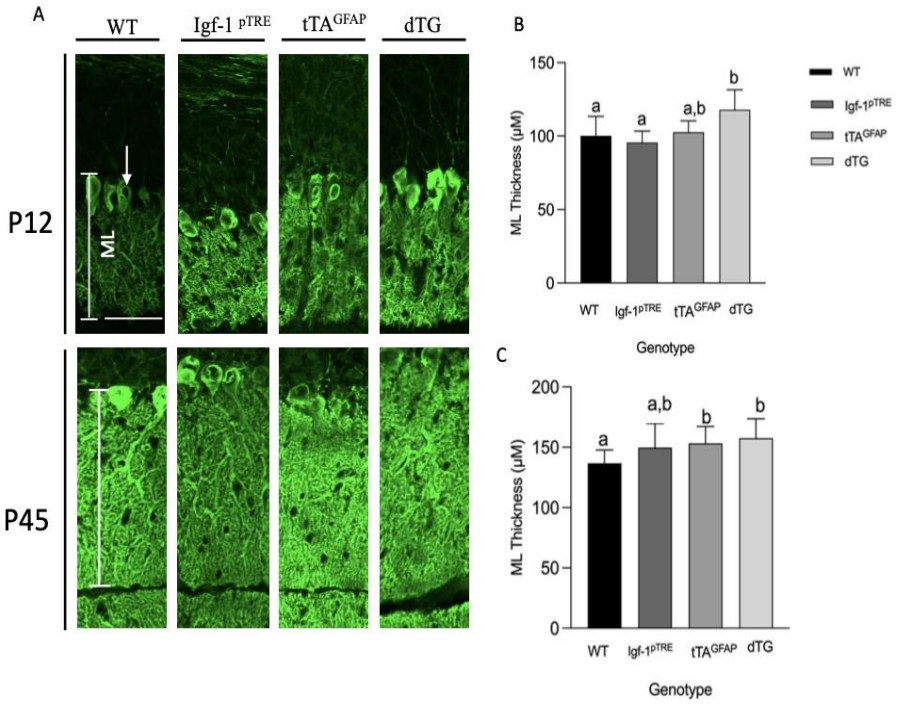

Figure 9. A) The effect of hypothyroid treatment on ML thickness at sacrifice on post-natal day 12 and 45. Vertical white line indicates to the molecular layer. White arrow points to a purkinje cell body. B, C) Graphs represent the ML thickness measurements at P12 and P45 respectively, under euthyroid conditions. The mean ML thickness of euthyroid dTG mice is significantly larger than that of euthyroid wild type and Igf-1^{pTRE} groups at P12. The mean ML thickness of the dTG mice was significantly higher than that of the wildtype group at P45 under euthyroid conditions. No significant difference in ML thickness between WT, Igf-1^{pTRE} and tTA^{GFAP} groups at P12 and P45. Bars represent mean weights ± standard error. Different letters denote significant statistical differences between groups with a p value greater than 0.05. n=5-8 per group. Scale bar=50 μm

6. Effect of hypothyroidism on external granular layer thickness at P12 and rescue potential of ectopic Igf-1 over expression.

Staining of the external granular layer using DAPI stain indicated that hypothyroidism induced a persistence of the external granular layer at postnatal day 12 in all four genotype groups compared to their respective control groups (Figure 9. A, B)

The layer thickness measured using Fiji Image J software indicated a statistically significant increase in the layer thickness in the hypothyroid groups of all genotypes compared to their respective euthyroid groups, indicating that ectopic Igf-1^{pTRE} over expression makes no change to the effects of hypothyroidism in the EGL.

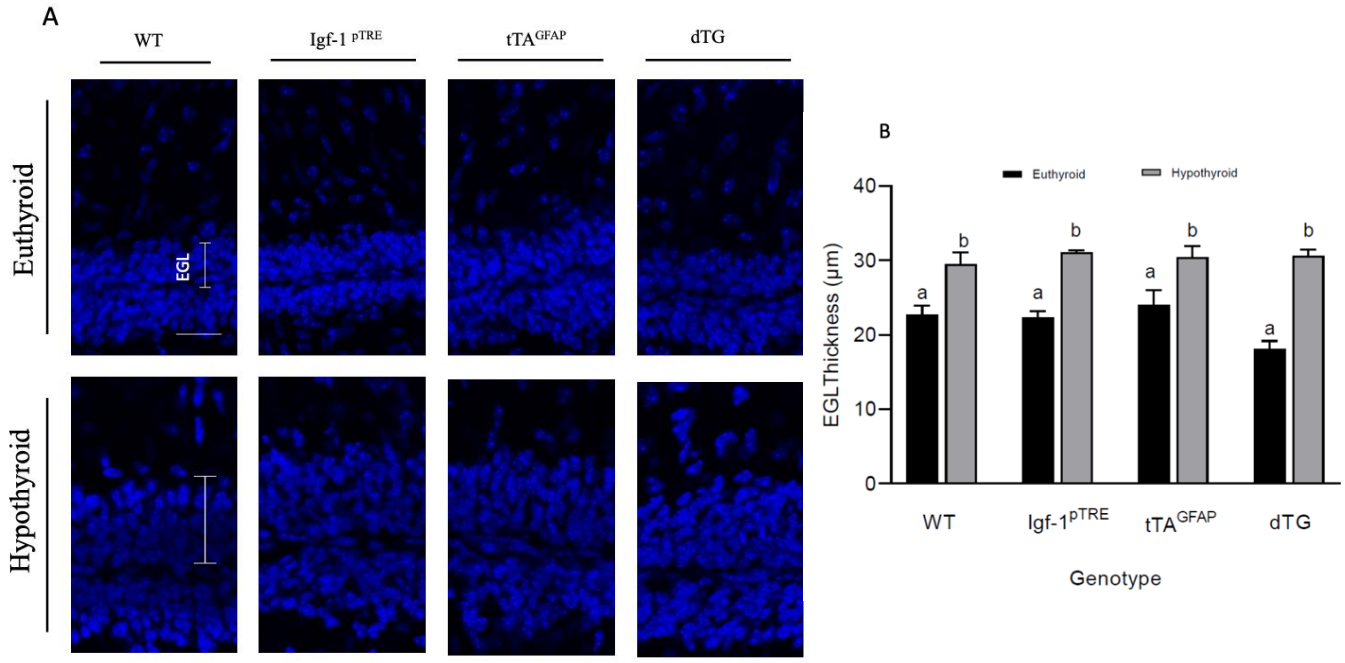

Figure10. The effect of hypothyroid treatment on EGL thickness at sacrifice on post-natal day 12. Vertical white line indicates to the EGL. B) Graph representing the EGL thickness measurements at P12 in euthyroid and hypothyroid conditions. The EGL thickness is significantly higher in the hypothyroid groups of all genotypes compared to their respective euthyroid groups (P>0.05). Bars represent mean weights ± standard error. Different letters denote significant statistical differences between groups with a p value less than 0.05. n=5-8 per group. Scale bar= 50µm.

7. Effect of hypothyroidism on Inner granular layer thickness at P12 and rescue potential of ectopic Igf-1 over expression.

Staining of the inner granular layer using DAPI stain indicated a significantly larger thickness in the euthyroid double transgenic mice compared to the euthyroid wild type mice. However, there was no significant difference in inner granular layer thickness in the hypothyroid groups compared to their respective euthyroid groups.

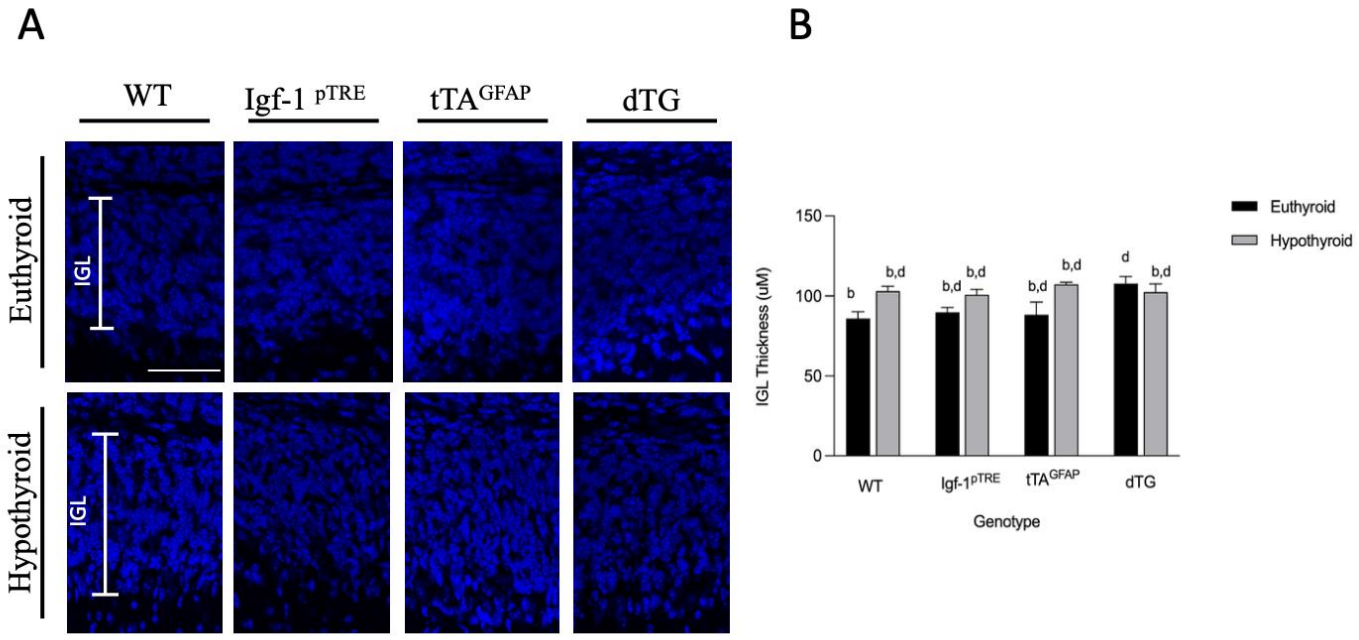

Figure 11. The effect of hypothyroid treatment on IGL thickness at sacrifice on post-natal day 12, under euthyroid and hypothyroid conditions. Vertical white line indicates to the IGL. B) Graph representing the IGL measurements of all genotypes under euthyroid and hypothyroid groups at P12. Mean IGL thickness of euthyroid dTG mice is significantly higher than that of the euthyroid wildtype mice. No significant difference in IGL thickness between the hypothyroid groups of all genotypes compared to their respective euthyroid groups. Bars represent mean weights ± standard error. Different letters denote significant statistical differences between groups with a p value greater than 0.05. n=5-8 per group. Scale bar=50μm.

8. Effect of hypothyroidism on molecular layer thickness at P12 and rescue potential of ectopic Igf-1 over expression.

Immunostaining of the Purkinje cells using Calbindin, indicated a significantly larger thickness in the euthyroid double transgenic mice compared to the euthyroid wild type, euthyroid Igf-1 ^{pTRE} and all hypothyroid groups (P>0.05). mice. However, there was no significant difference in ML thickness in the wildtype, Igf-1^{pTRE} and tTA^{GFAP} hypothyroid groups compared to their respective euthyroid groups.

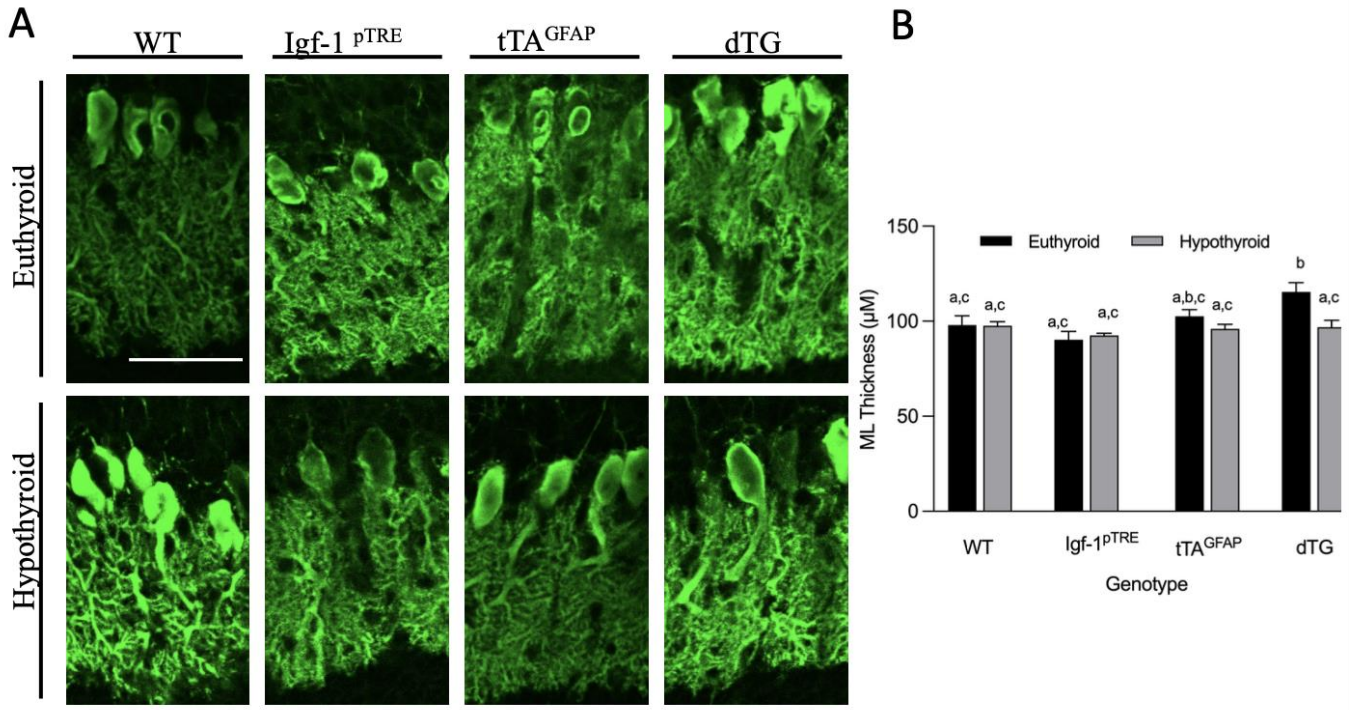

Figure 12. A) The effect of hypothyroid treatment on ML thickness at sacrifice on post-natal day 12. B) Graph representing ML thickness of each genotype in euthyroid and hypothyroid conditions. Mean ML thickness of euthyroid dTG mice is significantly higher than that of euthyroid wildtype and Igf-1 ^{pTRE} mice at P12. Mean ML thickness of hypothyroid dTG mice is significantly lower than the euthyroid dTG mice at P12. Bars represent mean weights ± standard error. Different letters denote significant statistical differences between groups with a p value greater than 0.05. n=5-8 per group. Scale bar=50μm.

9. Effects of hypothyroidism on Purkinje cell arborization and rescue potential of ectopic Igf-1 over expression.

Purkinje cell arborization was quantified by measuring the degree of dendritic arborization in the molecular layer as a % area occupied by purkinje dendrites. The results showed a decrease in the % area of the ML occupied by dendrites, in the hypothyroid groups on all genotypes compared to their respective euthyroid groups. A statistically significant decrease was observed only in the hypothyroid group of tTA^{GFAP} genotype compared to their respective euthyroid group. The % area occupied by dendrites in the ML of hypothyroid Igf-1^{pTRE}, tTA^{GFAP} and dTG mice were significantly less compared to WT, euthyroid mice.

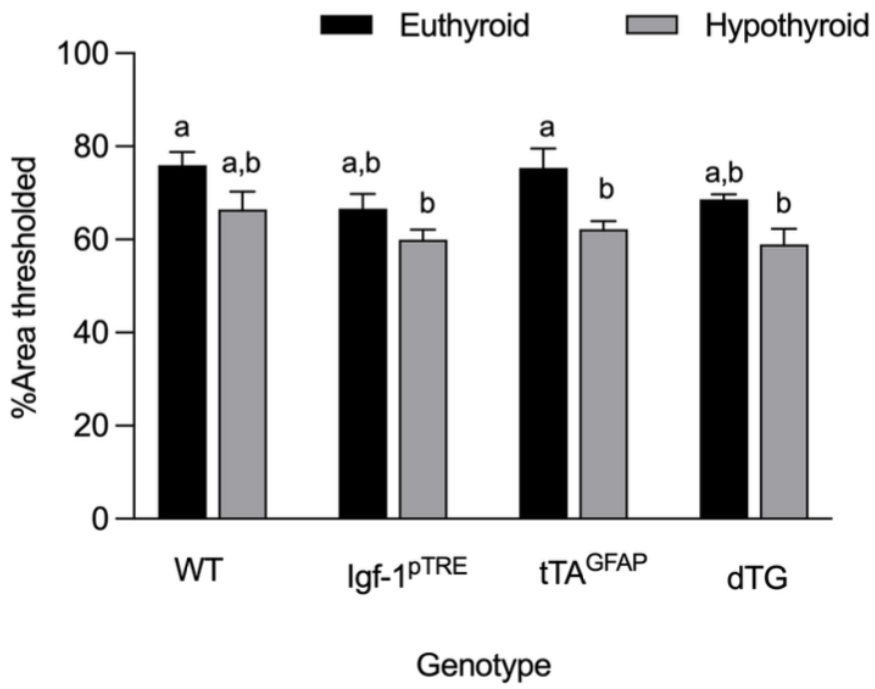

Figure 13. The effect of hypothyroid treatment on Purkinje cell arborization at sacrifice on post-natal day 12. Mean % dendritic area is significantly decreased in the hypothyroid group of tTA^{GFAP} compared to its respective euthyroid group. Bars represent mean % dendritic area ± standard error. Different letters denote significant statistical differences between groups with a p greater than 0.05. n=5-8 per group.

10. Effects of hypothyroidism on total granule cell number in the molecular layer and rescue potential of ectopic Igf-1 over expression.

Granule cell distribution in the molecular layer was measured by counting the total number of cells using a cell counter tool on Image J software. There was a significant decrease in the total cell number in the ML in hypothyroid WT, Igf-1^{pTRE} and tTA^{GFAP} mice compared to their respective euthyroid groups at P12. No significant difference in the total cell number in the ML between euthyroid and hypothyroid dTG mice.

The total number of cells in the ML of euthyroid dTG mice is significantly less compared to the other genotypes under euthyroid conditions at P12.

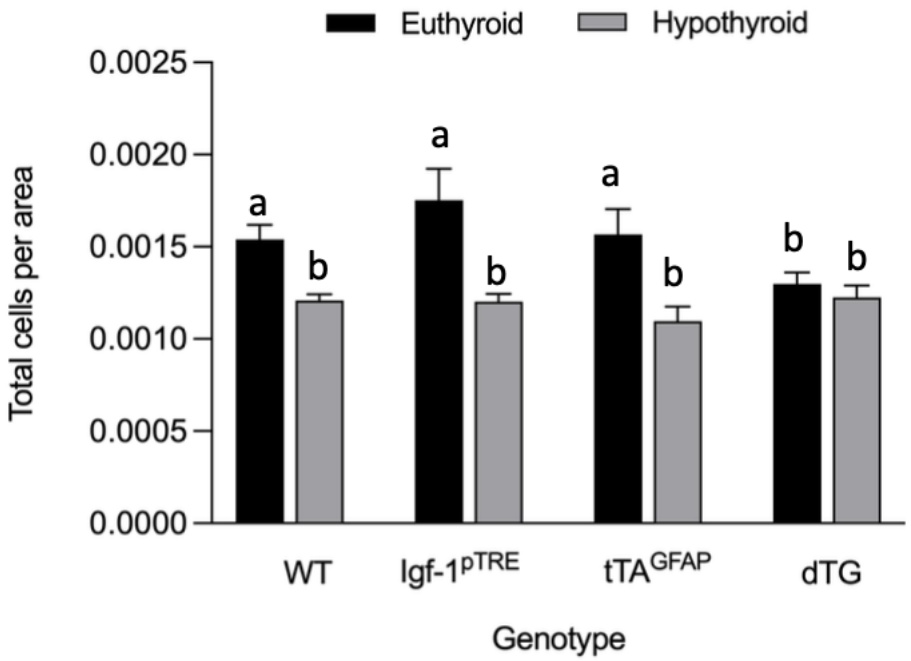

Figure 14. The effect of hypothyroid treatment on cell total cells per area in the ML at sacrifice on post-natal day 12. Mean total cells per area in the ML of dTG mice is significantly lower than all other euthyroid groups. Mean total cells per area in the ML of hypothyroid tTA^{GFAP}, Igf-1^{pTRE} and WT mice at P12 was significantly lower compared their respective euthyroid group. Bars represent mean total cells per area ± standard error. Different letters denote significant statistical differences between groups with a p greater than 0.05. n=5-8 per group.

Discussion

Effects of ectopic Igf-1 over expression and thyroid disrupting treatment on pup body weight.

As expected, hypothyroid mice of WT, Igf-1^{pTRE} and tTA^{GFAP} genotypes showed significantly reduced body weights compared to their respective euthyroid group at P12; a well reported consequence of insufficient thyroid hormone (Ahmed 2015; Moreno et al 2006). Interestingly, the euthyroid dTG mice showed a significantly lower body weight compared to the other genotypes at sacrifice. This is a result that was observed in previous projects in our lab. It is hypothesized that this reduction in body weight in euthyroid conditions is due to the negative feedback exerted on the pituitary by the over expressed Igf-1 in the brain, that leads to a decrease in growth hormone secretion and release of serum Igf-1 from the liver (Al-Samerria S and Radovick S. 2021). Since both GH and Igf-1 play a role in normal body growth, the negative regulation of these hormones caused by Igf-1 over expression in the brain, lead to disrupted body growth in the euthyroid double transgenic mice.

The hypothyroid dTG pup weights were not significantly different from their euthyroid group due to having a decreased body weight, even in euthyroid conditions.

Effect of ectopic Igf-1 over expression on EGL thickness under euthyroid conditions.

Our results show that there is no significant effect of ectopic Igf-1^{pTRE} over expression on the thickness of the external granular layer at P12 under euthyroid conditions (Figure 7). However, it is interesting to note that even though not statistically significant, the dTG mice have the thinnest EGL compared to all other genotypes. This correlates to another important result in our study; a significantly larger inner granular layer in the euthyroid dTG mice at P12 compared to all other genotypes. A group (P. Ye et al) that studied the role of Igf-1 in the cerebellum using an IGF-2

promoter driven IGF-1 transgenic mouse model, showed that Igf-1 promotes cell proliferation of the EGL. This information supports our presumption that more cells could be proliferating and migrating to the IGL in the dTG which is consistent with the significantly thicker IGL in the euthyroid dTG mice at P12 compared to the other genotypes. The group also showed localization of a IGF binding protein (IGFBP)-5 in proliferating external granular layer cells. This binding protein is capable of potentiating Igf-1 actions. We speculate that this is causing an increased rate of cell proliferation and migration from the EGL to the IGL in the euthyroid dTG mice (P. Ye et al.1996)

At P45, our results show that the EGL has completely disappeared; an established and documented observation (Morte et al.2002).

Effects of Igf-1 over expression on ML thickness and purkinje cell arborization, under euthyroid conditions.

Our results showed that ectopic over expression of Igf-1 in the brain caused a significant increase in the ML thickness of euthyroid dTG mice at P12 compared to the wildtype and Igf-1^{pTRE} single transgenic mice (Figure 9). This result further confirms previous studies that report a role of Igf-1 in promoting purkinje cell development and differentiation (P. Ye et al. 1996, R. Peeters et al. 2013, Croci. L et al. 2011, Fukudome et al.2003)

The significantly greater thickness of the ML in euthyroid dTG mice compared to the wildtype we observed at P12, was also seen at P45. This result suggests that the role of Igf-1 in promoting purkinje cell development is sustained through adulthood. A previous study done on human brains reported intense positive staining of Igf-1 in the purkinje cell soma, dendrites, and

dendritic spines (F. Aguado et al. 1994). We speculate that the thicker ML in the dTG mice is due to specific role Igf-1 plays in growth of primary dendrites of Purkinje cells.

Effects of Igf-1 over expression on IGL thickness under euthyroid conditions.

Our results represented in figure 8 show that the thickness of the IGL is significantly higher in the euthyroid dTG mice at P12 compared to the other genotypes. We presume that this result is due to an increase in the rate of cell migration, supported by previously reported evidence of higher cell proliferation and migration associated with Igf-1 (P. Ye et al. 1996). Igf-1 is also known to protect granule cells from apoptosis according to a study that showed a decrease in caspase 3 activity in mice with an Igf-1 transgene compared to weaver mice. (Zhong et al.2005). The significantly thicker IGL in the euthyroid dTG mice we saw at P12 could also be due to the role of Igf-1 in promoting granule cell survival in the IGL.

The significant difference of the thicker IGL observed in the euthyroid dTG mice observed at P12, was normalized by P45 (see Figure 8C). Normal cerebellar development is complete by P28 (White et al. 2013), so the normalization seen by P45 was likely corrective. We assume that the effects of Igf-1 on granule cell migration seen at P12 in the euthyroid dTG mice, have normalized by P45. It is also presumable that the excess cells in the IGL of dTG mice eventually die by apoptosis.

Effects of ectopic Igf-1 over expression on the defects caused by hypothyroidism in the EGL.

As represented by figure 10, hypothyroid groups of all genotypes show a significantly larger EGL compared to their respective euthyroid groups; a result that supports the idea that hypothyroidism disrupts normal cerebellar development by causing a persistence of the EGL (Morte et al 2002). We hypothesized that the increased cell proliferation observed with Igf-1 over

expression may be able to ameliorate the effect of hypothyroidism on the EGL. However, this was not the case as no normalization was observed in hypothyroid dTG mice. Morte et al. have provided evidence that TRα1; a TH receptor, is upregulated in the granule cells at the time they undergo the last cell divisions and become prepared to migrate. This suggests that there is a specific role of TH hormone in the EGL that is independent of Igf-1 action.

Effect of hypothyroidism on ML thickness, arborization and granule cell distribution.

Our results demonstrated in figure 12 show that the ML was altered in the hypothyroid groups of all genotypes at P12 compared to their respective euthyroid groups. In the hypothyroid mice, there is a visible lack of alignment of the cell bodies and an arching of the primary dendrites of the purkinje cells compared to the euthyroid mice, upon close qualitative analysis (Figure 12, A). These defects we observed are consistent with other groups that have looked at the purkinje cell defects in TR knockout models (Morte et al. 2002, Ortiga Carvalho and Faustino. 2014). This allows us to speculate that TH and Igf-1 play separate roles in normal purkinje cell development, and Igf-1 over expression cannot replace thyroid hormone's role to rescue the defects caused in its absence. It is also important to note that under normal conditions, Igf-1 is expressed in the purkinje cells. In our dTG mice, the over expression is in the Bergman glial cells, which may not be sufficient to ameliorate the defects in the purkinje cells.

In hypothyroid dTG mice, the ML thickness was significantly decreased when compared to their euthyroid groups. This indicates that hypothyroidism blocked the ability of ectopic Igf-1 to enhance the ML thickness which suggests that the effect of Igf-1 is dependent on TH.

As shown in figure 14, the total number of cells per area in the ML of euthyroid dTG mice is significantly less compared to the other genotypes. We presume that this reduction is due to the

role of Igf-1 in increasing granule cell migration from the EGL to the IGL, thus decreasing the total cells in the ML. This presumption is based on previous studies that support the idea that Igf-1 plays a role in increasing granule cell migration in the cerebellum (Komuro et al. 2012).

Figure 14 also shows a significant decrease in the total number of cells per area in the ML in hypothyroid WT, Igf-1^{pTRE} and tTA^{GFAP} mice at P12 compared to their respective euthyroid groups. It is known that hypothyroidism causes a reduction in granule cell migration (Morte et al. 2002). We presume that the reduction in total number of cells per area in the ML is due to the lack of TH. The lack of a statistically significant reduction in total cells in the ML in hypothyroid dTG mice is presumed to be the role of Igf-1 in enhancing granule cell migration from EGL to the IGL.

Effect of ectopic Igf-1 over expression on the defects caused by hypothyroidism on IGL thickness.

Figure 11 shows the effects of hypothyroidism on the IGL at P12. We saw no significant difference in IGL thickness between any of the genotypes under hypothyroid condition when compared to their respective euthyroid groups.

Conclusion

It is well known that the developmental roles of TH and Igf-1 and the defects caused by their deficiency are similar in the developing cerebellum. However, if these roles are independent of each other is not fully known. The results from our study show that the defects caused by low TH are apparent, even in the presence of excess Igf-1 in the brain, with no evidence that ectopic Igf-1 over expression can ameliorate the effects of hypothyroidism on cerebellar development. We presume that this because the over expression of Igf-1 in our mouse model is in the Bergman glia,

not the purkinje cells, thus insufficient to ameliorate the defects in purkinje cell development. The level of Igf-1 over expression and the temporal pattern of over expression are also unknown in the dTG model used in our study. We conclude that, the level of TH insufficiency induced in this study is too severe to be rescued with ectopic over expression of Igf-1. The effects of Igf-1 over expression could perhaps be measured with a more moderate decrease in TH.

Future directions

1. Cerebellum plays a role in motor function and learning. A behavioral assay to investigate the effects of ectopic Igf-1 over expression in behavior and any rescue in behavioral defects of hypothyroidism on mice. Ex: Rotar rod.
2. Another closer expansion of the study to examine the cell physiology /cell cycle stage and cell number in the IGL can give us an insight to specific cellular mechanism or pathways that igf-1 may be playing a role in, using cell staining like Caspase-4 or TUNEL staining.

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