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Temporal Localization of Insulin-Like Growth Factor 1 (Igf1) Positive Cells in Developing Hypothyroid Brain

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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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Dr. David Sharlin (Advisor)

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Dr. Rachel Cohen

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Dr. Michael Bentley

Abstract

Temporal Localization of Insulin-Like Growth Factor 1 (Igf1) Positive Cells in Developing Hypothyroid Brain

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Hypothyroidism has been known to result in numerous neuroanatomical defects in the developing brain. To investigate further into the mechanism in which these defects are obtained, local brain insulin-like growth factor 1 (Igf1) was investigated in relation to the hypothyroid brain. There are many similarities between hypothyroidism and the Igf1 deficient brain. Additionally, TH (thyroid hormone) is known to interact with Igf1. Therefore, the purpose of this study was to investigate the effect of induced congenital hypothyroidism on the temporal expression of Igf1-positive cells in the brain.

To investigate the relationship between TH and Igf1 in the brain, we inhibited TH production in C57BL6J mice by the administration of 1% $KClO_4$ and 0.05% MMI in drinking water from 14 (embryonic day 14) to P21 (postnatal day 21). Control and hypothyroid mice were sacrificed at select developmental time points (P7, P14, P21, P42). Serum was collected to measure Igf1 and T4 (thyroxine) by ELISA. Brain tissues were collected and cryosectioned to obtain the frontal motor cortex, hippocampus, and cerebellum, in which *in situ* hybridization was performed. From the *in situ* hybridization, Igf1-positive cells and relative Igf1 mRNA levels were analyzed.

Serum T4 and Igf1 data was consistent with previous literature that indicated low TH decreases Igf1 in the serum. Additionally, serum TH and serum Igf1 are positively

correlated and both peak in unison. Furthermore, also consistent with previous findings, low TH resulted in a decrease of Igf1 mRNA in the brain. A significant reduction of Igf1-positive cells was evident in the frontal motor cortex of hypothyroid treated animals, but no other region, demonstrating region specific expression of Igf1-positive cells. Additionally, we showed that the previously reported reduction of Igf1 mRNA in the hypothyroid brain was not due to a decrease in Igf1 mRNA synthesis in the cortex, hippocampus, or cerebellum, but potentially rather a total loss of Igf1-positive cells, like that of the cortex.

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Table of Abbreviations

Abbreviation	Definition
BSA	Bovine Serum Albumin
CC1	Allophyococyanin
Dio2	Deiodinase 2
Dio3	Deiodinase 3
E	Embryonic Day
ELISA	Enzyme Linked Immunosorbent Assay
GH	Growth Hormone
Igf1	Insulin-Like Growth Factor 1
IGF1r	Insulin-Like Growth Factor 1-Receptor
IR	Immunoreactivity
IRS	Insulin Receptor Substrate
ISH	*In Situ* Hybridization
MAG	Myelin Associated Glycoprotein
MB	Maleic Acid Buffer
MMI	Methimizole
NG2	Neural/Glial Antigen 2
OPC	Oligodendrocyte Progenitor Pool
P	Postnatal Day
PCN	Parvalbumin Containing Neurons
PCR	Polymerase Chain Reaction
PFA	Paraformaldehyde
PM	Paramedian Lobule
PTU	Proplythiouracil
PV	Parvalbumin
PVN	Paraventricular Hypothylamic Nuclei
RC3	Neurogranin
RT	Room Temperature
rT3	Reverse-T3
RTK	Receptor Tyrosine Kinase
RXR	Retinoid X Receptor
SSC	Saline-Sodium Citrate
T3	Triiodothyronine
T4	Thyroxine
TEA	Triethanolamine
TH	Thyroid Hormone
TMB	3,3',5,5'-Tetramethylbenzinidine
TR	Thyroid Receptor
TRE	Thyroid Receptor Element
UV	Ultraviolet

Introduction

Thyroid hormone (TH) is essential for the proper development of many tissues, including the brain. Low TH levels in development results in hypomyelination, disorganized neuronal migrations, altered neurogenesis, and permanent neurological defects. Although the lack of adequate TH at birth can now be detected and treated within days, residual, irreversible neurological effects remain (Zoeller and Rovet 2004).

There are numerous cell types that are the target of TH, including astrocytes, parvalbumin-containing interneurons, Purkinje cells, and oligodendrocytes. When TH levels are below a tissue's needs, the effects on these various cell types have a detrimental influence on brain development. However, it is unknown whether the effects of TH are direct, or indirect through another factor, such as local insulin-like growth factor-1 (Igf1).

Insulin-like growth factor-1 interacts with TH, and therefore, could act as an indirect regulator of TH signaling within the brain (Elder, Karayal et al. 2000). There are several similarities in neuroanatomical defects associated with hypothyroidism or Igf1 deficiency to support this claim. When occurring during development, both states show a significant reduction in the numbers of mature oligodendrocytes, ultimately leading to a decrease in myelination and density of axons (McMorris, Smith et al. 1986, Beck, Powell-Braxton et al. 1995, Gao, Apperly et al. 1998). Additionally, there is a total reduction of parvalbumin containing neurons (PCNs) in the hypothyroid and Igf1 null

brain (Beck, Powell-Braxton et al. 1995, Gilbert, Sui et al. 2007). Interestingly, Igf1 in serum is decreased in hypothyroid individuals (Miell, Taylor et al. 1993). However, it is largely unknown how changes in locally brain-produced Igf1 decrease during hypothyroidism. Considering the evidence described above and the lack of our current knowledge regarding Igf1 levels in the developing hypothyroid brain, our working hypothesis is that: **Low TH during development disrupts the temporal production of Igf1 positive cells within the developing frontal motor cortex, hippocampus, and cerebellum.** The corollary hypothesis is that TH-induced reductions in local brain-derived Igf1 result in neuroanatomical defects seen in mice lacking Igf1, suggesting that neuroanatomical defects associated with low TH in development are due, in part, to altered Igf1 signaling in the developing brain.

Literature Review

A. Regulations of TH Action

A.1—*Receptors*

Thyroid hormone action alters gene expression within the brain. Thyroid receptors (TR) are ligand-regulating receptors that bind to DNA in the absence or presence of T3 (triiodothyronine). TR act as heterodimers with retinoid X receptors (RXR). Together, these receptors, in conjunction with available TH, determine expression of specific target genes that direct development (Bernal 2002, Bernal 2007). Two receptors for thyroid hormone exist, alpha and beta, encoded by the Thra gene or the

Thrb gene respectively. The Thra gene produces two TR proteins through differential splicing: TRα1 and TRα2. TRα1 is a bonafide receptor that binds T3, whereas TRα2 is an isoform that binds DNA, but is unable to bind T3. The Thrb gene also produces two TR proteins: TRβ1 and TRβ2. Both TRβ isoforms bind T3.

The highest expressions of TRα1 and TRα2 were found in the hippocampus, cerebellar cortex, and the olfactory bulb, with TRα1 accounting for 70-80% of all the receptors located in the cerebellum. This observation suggests that most affects of TH in the brain are mediated by this receptor subtype (Bradley, Young et al. 1989, Bernal 2007). The greatest expression of TRβ1 was observed in the anterior pituitary and in the paraventricular hypothalamic nuclei (PVN), with lower levels in other brain regions. The expression of TRβ2 was highly restrictive, with expression largely restricted to the anterior pituitary (Bradley, Young et al. 1989). Within the cerebellum, TRβ receptors were expressed in Purkinje cells (Quignodon, Grijota-Martinez et al. 2007). Because of overlapping, yet distinct expression of TRα1 and TRβ, different functional roles for each of the corresponding receptors may exist (Bradley, Young et al. 1989).

A.2—*Deiodinase Enzymes*

The local availability of TH to bind TRs and regulate gene expression within given tissues is controlled, in part, by deiodinase enzymes. Two deiodinase enzymes are present in the murine brain: type II (Dio2) and type III (Dio3). The type II deiodinase enzyme converts prohormone T4 (thyroxine) to the biologically active hormone, T3

(triiodothyronine) (Figure 1). Dio2 is expressed primarily in two glial cells – tanycytes that line the 3rd ventricle, and astrocytes. Dio3 is expressed primarily in neurons and converts T4 to an inactive metabolite, reverse T3 (rT3), or T3 to the inactive metabolite T2 (Figure 1). Both of these enzymes are critical in the homeostasis of TH in the brain, and their spatial separation suggests a paracrine-like mechanism for TH functions within brain tissue (Freitas, Gereben et al. 2010).

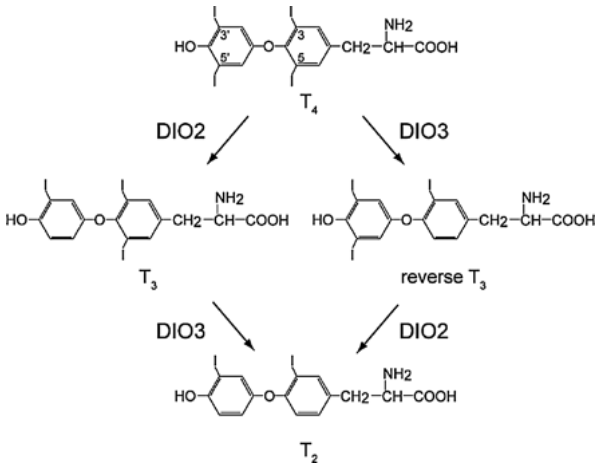


Figure 1. The conversion of iodothyronines by DIO2 and DIO3. The resulting products are dependent on the removal of one specific iodine from the appropriate specific location, which is dependent on either DIO2 or DIO3 (Nakao, Ono et al. 2008).

A.3—*Gene Transcription*

Gene regulation mediated by TH and TRs can possibly help explain developmental defects associated with hypothyroidism

(Quignodon, Grijota-Martinez et al. 2007). Although only a few direct targets of the TR have been identified in the brain, these genes functionally relate to developmental processes such as myelination, migration and differentiation of neuronal cells, and synaptogenesis (Bernal 2002, Bernal 2007). Binding of T3 to either TR represses or activates gene transcription, depending on multiple factors, such as the number of available co-factors and the cis-regulatory element that the TR are bound to (Yen 2001). The hormone-receptor complex binds specific DNA sequences called TH response elements (TREs), which directly regulate specific genes (Chatterjee, Lee et al. 1989). As stated, relatively few genes are direct targets of TH/TR; a subset of which are transcription factors that can modulate the expression of other genes. These latter genes do not directly interact with the TH-receptor complex and therefore lack TREs. These findings were significant in showing that TH can modulate the expression of numerous genes by indirect action (Gilbert and Zoeller 2010).

B.—Regulation of Igf1 Action

Igf1 is a growth factor that is essential for normal brain development. It is a polypeptide genetically related to insulin, which also shares a similar tertiary structure and amino acid identity to insulin. Although Igf1 production from the liver is most well known, Igf1 is also produced locally in many tissues, including the brain. When synthesized in the brain, growth hormone (GH) plays no significant regulatory role in its synthesis, unlike Igf1 found in the serum

(Bondy and Cheng 2004, Aberg, Brywe et al. 2006). This growth factor's mRNA is found to be abundant in the brain, especially at times of early postnatal development (Bondy and Chin 1991). Expression of Igf1 in the murine brain begins around embryonic day 14 (E14), and declines after the peak expression at approximately two weeks postnatal, with expression, although lower, continuous throughout life (Bondy 1991, D'Ercole, Ye et al. 1996). Like TH, Igf1 is also essential for proper myelination, projection neuron growth, synaptogenesis, and dendritic arborization. During normal brain development, it also promotes neuronal survival, especially in areas that depend on postnatal neurogenesis, such as the hippocampus (Bondy and Cheng 2004).

B.1—*Igf1 Receptors*

Igf1 not only shows similarities to insulin in its structure, but also its receptor is a close homologue, both in structure and sequence (LeRoith 1996). The receptors for both insulin and Igf1 are receptor tyrosine kinases (RTK). Ligand binding to the Igf1 receptor (Igf1r) results in RTK autophosphorylation and association with insulin receptor substrate (IRS) adapter proteins with subsequent IRS phosphorylation. Insulin receptor substrate phosphorylation leads to a signaling cascade that ultimately leads to transcriptional changes. Igf1 receptors are concentrated in the brain in regions of neuron rich structures, suggesting that Igf1's actions are essential in modulating neuronal functio (Hill, Lesniak et al. 1986, Bohannon, Corp et al. 1988). The highest amounts of Igf1r were found in the olfactory bulb, dentate gyrus, cerebellum, piriform cortex, and the choroid plexus. The

lower levels of expression, as detected by *in situ* hybridization, were found in white matter tracts (Marks, Porte et al. 1991). This expression mimics that of Igf1 mRNA expression localization, where the highest expression was found in the cerebellum, olfactory bulb, and hippocampal complex, with expression rarely detected in white matter tracts (Bartlett, Li et al. 1991). This close proximity of Igf1 mRNA expression and its associated receptor expression goes to further support the theory of autocrine and/or paracrine signaling of Igf1.

C.—Potential Interaction between TH and Igf1 in Nervous System Development

Igf1 and TH are both essential for proper neurological development, and insufficiency of either factor results in permanent neurocognitive deficits. Interestingly, many of the detrimental effects associated with hypothyroidism are similar to those associated with Igf1 deficiency. Described below are examples of these effects, focusing on the role of each factor of neurological development.

C.1—*Neurogenesis*

TH plays an imperative role in the development of the central nervous system. Within the hippocampus, TH has been shown to increase the rate of neuroblast proliferation of the subgranular zone (Montero-Pedrazuela, Venero et al. 2006). In addition, an important function of TH in the brain is to regulate timing of differentiation and end neuronal proliferation at optimal times (Lauder 1977). Upon terminal differentiation and exit from the cell cycle, neurons migrate to their appropriate regions

of the brain in an orderly pattern (Porterfield 1994). Hypothyroidism prevents neurons from making reciprocal contacts by preventing migration and maintaining distances from predestined target cells during critical times of development. An example of TH's role in cortical lamination can be seen in hypothyroid rats that shown disorganization within their cerebellar cortex (Lauder 1977, Nunez 1984). This disorganization results from a retarded spatio-temporal program leading to altered connectivity (Nunez 1984). TH is also important for stimulating the formation and maturation of axons and dendrites, which is essential for synaptic survival (Nunez 1984, Heisenberg, Thoenen et al. 1992). Furthermore, the survival of neurons, at least *in vitro*, is dependent on adequate concentrations of active TH (Heisenberg, Thoenen et al. 1992).

Similar to the effects of TH in development, Igf1 expression is also necessary for proper neurogenesis and neuronal migrations. Igf1 has been shown to stimulate the proliferation of neuronal progenitor cells, induce oligodendrocyte differentiation, and increase the survival of oligodendrocytes and neurons (D'Ercole, Ye et al. 2002). In $Igf1^{-/-}$ mice, a 59% reduction in volume of the dentate gyrus granule cell body layer was observed when compared to controls. This further supports the neurogenic roll that Igf1 plays in the generation of cells, such as dentate granule neurons (Mozell and McMorris 1991, Beck, Powell-Braxton et al. 1995). Igf1 also promotes proper neuronal migrations in structures such as the olfactory bulb. Without Igf1 expression in mice, the layering of the olfactory bulb was altered, resulting in misplaced cells in the mitral and external

plexiform layer and fewer neurons in the glomerular layer (Hurtado-Chong, Yusta-Boyo et al. 2009).

C.2— *Oligodendrocytes, Myelination, & Brain Size*

Proliferation and maturation of oligodendrocytes begins in early postnatal life and continues throughout adulthood, unlike neurons (Rodier 1980). Myelination of central nervous system axons is completed by oligodendrocytes. Myelin electrically insulates and surrounds axons with multilamellar compacted membranes (Farsetti, Mitsuhashi et al. 1991). TH is necessary for proper brain myelination. Myelination is highly regulated and is dependent on timing and differentiation of oligodendrocytes. The myelin associated glycoprotein gene (MAG) is a key gene in the myelination process and the expression of MAG is delayed in developmentally hypothyroid animals (Rodriguez-Pena, Ibarrola et al. 1993). Furthermore, hypothyroidism significantly decreases the total amount of myelination, as well as total brain size and weight (Gravel, Sasseville et al. 1990). Previous findings have demonstrated that TH controls the accumulation of mature oligodendrocytes, in which case, hypothyroidism ultimately decreases the density of myelinated axons present in the corpus callosum (Berbel, Guadano-Ferraz et al. 1994, Schoonover, Seibel et al. 2004).

Similar to TH, Igf1 is also essential for myelination and oligodendrocyte development. Igf1 increases the survival and development of myelin creating oligodendrocytes, at least *in vitro* (Mozell and McMorris 1991). Differentiation of

oligodendrocytes involves processes such as increasing cell process branching and augmentation of myelin protein expression, along with myelin sheath production. Igf1 is thought to stimulate the differentiation process for oligodendrocyte progenitor cells (OPCs) by activating the MAPK and Akt pathways, and enhancing a regulator of the cell-cycle progression (Chesik, De Keyser et al. 2008). Consistent with this idea, a decrease in oligodendrocyte numbers is observed in Igf1 knockout mice (Beck, Powell-Braxton et al. 1995), whereas an overexpression of Igf1 in mice led to an increase in the number of oligodendrocyte markers (Chesik, De Keyser et al. 2008). Interestingly, developmental hypothyroidism also reduces the number of myelinating oligodendrocytes (Schoonover, Seibel et al. 2004, Sharlin, Tighe et al. 2008). Therefore, an altered temporal expression of Igf1 in the presence of low TH may decrease the numbers of mature oligodendrocytes.

Similar to the effect of developmental hypothyroidism, Igf1 inactivation results in a decrease in brain size compared to wildtype animals. Furthermore, in mice lacking Igf1, immunohistochemical staining found a significant decrease in myelin staining and an overall net loss in the number of present axons, with the majority of the remaining axons presenting unmyelinated characteristics. This decrease in myelin was more than twice as much as the general reduction in brain size. The total number of oligodendrocytes in the knockout mice was also significantly reduced. The corpus callosum, analyzed by electron microscopy, was one of the regions in which the density of myelination was measured, and showed a decrease when compared to wildtype subjects

(Beck, Powell-Braxton et al. 1995). Similar effects on myelination are also noted in developmentally hypothyroid animals (Gravel, Sasseville et al. 1990).

Treatment of primary brain cells with Igf1 following two days of culture resulted in an increase in myelination by 35-90% when compared to untreated samples. However, when treatment began after 20 days, a significant increase was not seen when compared to controls (Mozell and McMorris 1991). This alludes that there is a time sensitive period during development in which Igf1 is crucial to the myelination of axons in the developing brain. This timing mechanism is similar to TH action on myelination, where T3 stimulates myelination largely during the perinatal period (Schoonover, Seibel et al. 2004).

C.3—*Parvalbumin-Containing Neurons (PCNs)*

Parvalbumin (PV) is a calcium binding protein that can be found in inhibitory interneurons (McDonald and Betette 2001). Developmental hypothyroidism resulted in a profound loss in PV-immunoreactivity (IR). These persistent affects on PCNs were associated with functional deficits within inhibitory synaptic transmissions in the dentate gyrus. Furthermore, this loss of PV-IR was observed with low-dose Proplythiouracil (PTU; a thyroid hormone synthesis inhibitor) exposure, suggesting PV interneurons are highly sensitive to disruption in TH action. Moreover, when animals were returned to normal euthyroid status in adulthood, although some recovery of PV-IR ensued, a permanent loss of PV-IR remained. This observation suggests that some of the PCNs had

lost the full capacity to express PV in the absence of TH. Although there was some recovery of PV-IR, there still resulted a lasting reduction, indicating that there was also a total lost of PCNs. The absence of PV in the dendrites and soma of PCNs likely contributes to the physiological disruption of neuronal firing observed following developmental hypothyroidism (Gilbert, Sui et al. 2007).

Parvalbumin-containing neurons are not only dependent on TH, but also on Igf1. The numbers of striatal PCNs in Igf1 knockout mice were significantly reduced compared to wildtype animals (Beck, Powell-Braxton et al. 1995). Similarly, as discussed above, developmentally hypothyroid animals also showed a decrease in PV-IR (Gilbert, Sui et al. 2007). Considering this, it is tempting to speculate that altered Igf1 expression in hypothyroid animals is responsible for the dramatic changes in PV-IR observed.

D.—Igf1 Action in the Developing Brain and Potential Cross-Talk with TH

There are many similarities between the effects associated with low TH and the effects associated with low Igf1 within the developing mammalian central nervous system. We hypothesized that neurological defects found in hypothyroid animals are due, in part, from a decrease in local brain Igf1 production.

Additionally, we suggest that low TH alters brain development by shifting the temporal expression of local brain Igf1 to later in life. This delay in expression could potentially result in decreased progenitor pools of cells, and therefore decreased mature cells within brain tissue, such as can be seen with oligodendrocytes

(Beck, Powell-Braxton et al. 1995). It could also explain the disorganized migrations that have been observed, as timing is imperative to such mechanisms (Lauder 1977, Nunez 1984). The timing of expression of key factors in the brain, such as Igf1, is compulsory. If the timing mechanism of Igf1 were to be disrupted, and shifted to later in development, it could explain some of the irreversible defects found in hypothyroidism.

TH could cause the progenitor pools that produce Igf1 to develop, proliferate, and mature, which could be why a decrease is seen in local Igf1 in the brain under hypothyroid conditions. If there were a decrease in cells that are capable of producing Igf1, then there would result in a total reduction of local Igf1 mRNA expression. Alternatively, TH could also possibly act as a positive regulator of local Igf1 production through the TH receptor. Therefore, low TH would result in a direct decrease in local Igf1 expression, which could result in the observed defects associated with hypothyroidism and Igf1 deficiency. Which of these mechanisms contributes to the defects observed in animals with low TH or Igf1 remains to be determined, and is a goal of the proposed study.

Materials and Methods

Mouse Strains

This study utilized C57BL/6J mice, a highly inbred strain, originally purchased from Jackson Laboratories. All animal procedures were approved by the MSU-Mankato IACUC.

Treatments

Timed-pregnant mice were divided into two groups: euthyroid and hypothyroid. The sample size for each group, at each timepoint to be investigated, was n=4-6. All treatments were administered via drinking water. The euthyroid group maintained normal drinking water throughout with the addition of 0.01% bovine serum albumin (BSA). Hypothyroid animals drinking water contained 0.02% methimizole (MMI), 1% potassium perchlorate and 0.01% BSA. Treatment of pregnant dams was initiated on embryonic day 14 (E14) and continued until weaning on postnatal day 21 (P21).

Sacrifice & Dissection

Sacrifice and tissue collection occurred at the following time-points: P7, P14, P21, and P42. Animals under the age of P10 were rapidly decapitated using sharp scissors (as per protocol). Animals after the age of P10 were sedated using CO_2 and then decapitated to obtain the brain tissue. Using sterile techniques and tools, the skin of the head was removed, followed by the skull. The brain was then removed from the cranial cavity and immediately frozen in liquid nitrogen vapor and stored at -80^{o}C until further use. If gender was unable to be determined at the time of sacrifice, a small tail clipping was collected at time of sacrifice and stored at -20^{o}C for analysis of the SRY gene through polymerase chain reaction (PCR) and gel electrophoresis.

Genotyping of Pup Sex

Tails were digested in 500 microliters of tail lysis buffer (50mM Tris-Cl (pH8.0), 20mM NaCl, 1mM EDTA, 0.2% SDS, 200ug/ml Proteinase K (10mM Tris-Cl (pH 8.0), 20mM $CaCl_2$, 50% Glycerol, ddH_2O). The tails were dissolved at 56^oC with constant shaking. Once dissolved, the samples were centrifuged for five minutes at 14000rpm to remove debris. Four hundred and fifty microliters of supernatant were placed into a clean tube labeled accordingly. Four hundred and fifty microliters of isopropyl alcohol were then added to each tube and inverted 8-10 times to mix. The samples were then quickly centrifuged to pellet DNA at the bottom. Decanting of each tube then occurred, followed by the addition of 200 μ l of ddH_2O. The tubes were then placed at 56^oC with constant shaking for 15 minutes to dissolve DNA.

For SRY specific PCR, each PCR reaction received 1 μ l DNA, 0.4 μ l of the forward and reverse SRY primer (10 μ M stock, IDT), 0.26 μ l of the forward and reverse primer of Interleukin3 (10 μ M stock, IL3), 8.68 μ l of ddH_20, and 10 μ l of 2x PCR master mix (GoGreen Matermix, Promega). Each sample was then briefly vortexed and centrifuged. Samples were then placed in a thermal cycler programmed for the following conditions: 95^oC for 4.5 minutes, followed by 33 cycles of 95^oC for 35 seconds, 50^oC for one minute, and 72^oC for one minute, finishing with 72^oC for five minutes. An agarose gel was prepared by mixing 25mls of 1xTBE (Tris/borate/EDTA) and 0.25g of agarose, and heated to dissolve. Once dissolved, one drop of ethidium bromide (0.625mg/ml) was

added and swirled to mix before pouring into the mold to set up. Once the gel had set, a ladder and each sample (post PCR) were added into their individual wells. Voltage of 80V was applied to the gel electrophoresis chamber and allowed to run for approximately one hour before stopping and analyzing results with ultraviolet (UV) light.

Collection of Trunk Blood & Analysis of Igf1 & T4

Immediately following decapitation, trunk blood was collected and held on ice for no less than 20 minutes and no more than one hour. Blood was then centrifuged at 0.8rcf for 10 minutes. Plasma was drawn off using a micropipette and placed into a clean, properly labeled plasma tube to be held at -20^{o}C until further use.

Cryosectioning

Frozen brain tissues were removed from the -80^{o}C freezer, equilibrated to cryostat temperature (-18 to -21^{o}C) for approximately 30 minutes, mounted appropriately in optimal cutting temperature medium (O.C.T.; Tissue-tek), and then sectioned at 12 microns. Three brain regions were collected and analyzed: frontal motor cortex, hippocampus, and cerebellum. The cortex and hippocampus were sectioned coronally and the cerebellum was sectioned in a sagittal direction. Sections were thaw mounted on Superfrost Plus/Colorfast Plus Microslides (75x25mm; Daigger). To confirm correct anatomic location, a single slide was fixed in ethanol, stained using methylene blue and

visualized under a microscope. Once appropriate location had been confirmed, collection continued. Six sections per animal per slide were mounted, and ten slides per region of age-matched euthyroid and hypothyroid animals were collected on a single slide. After collection was completed, the slides were stored appropriately at -80^{o}C until further use.

In situ Hybridization

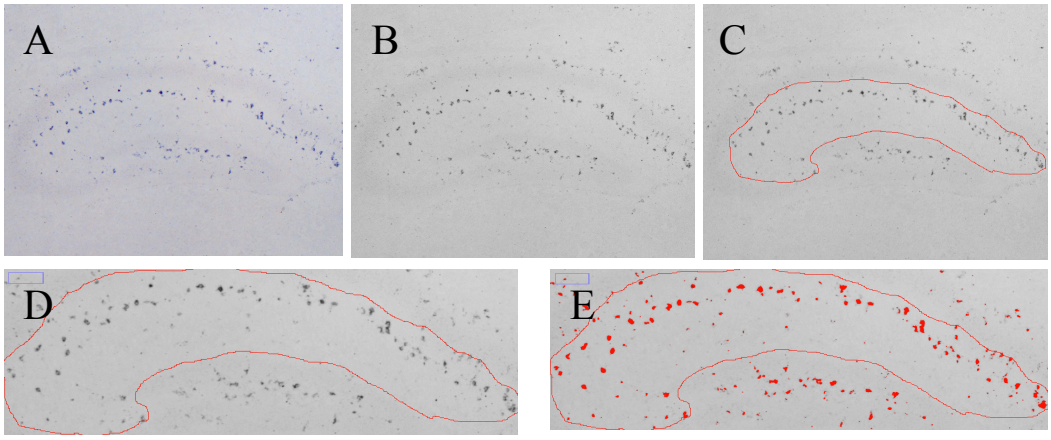
To elucidate the temporal and spatial pattern of Igf1 mRNA expression, *in situ* hybridization was performed as follows. The slides with brain sections were removed from the -80^{o}C storage and allowed to warm to RT (room temperature) for approximately 15 minutes before being placed in an RNAse-free coplin jar. The slides were fixed in 4% paraformaldehyde (PFA)/1x diethyl Pyrocarbonate treated (DEPC-Tx) PBS for 10 minutes followed by washing two times with 1xDEPC-Tx PBS for two minutes each time. The sides were then acetylated with 0.1M triethanolamine (TEA)/0.25% Acetic Anhydride for 10 minutes. After acetylation, the slides were washed three times in 1xDEPC-Tx PBS, each time for two minutes. The probe dilutions were heated to 80^{o}C for five minutes before immediate placement on ice. Approximately 250µl of the probe/hybridization buffer dilutions were added to the slides, followed by coverslips. The slides were placed in the hybridization chamber and allowed to sit overnight with 50% formamide/0.2X saline-sodium citrate (SSC) moistened paper towels placed in the bottom of the hybridization chamber. However, at this time, the chamber was also placed

in a temperature-controlled environment (55-58^{o}C). The next day, the coverslips were removed and the slides were washed in pre-warmed (70-78^{o}C) 0.2xSSC for about one hour at 70-78^{o}C. During this wash, the solution was changed after 5, 15, 15, and 15 minutes respectively. The slides were then allowed to cool to RT and washed again with RT 0.2xSSC for five minutes. Next, the slides were washed in maleic acid buffer (MB) for five minutes, at RT. Following the removal of the MB wash, thirty milliliters of 2% MBMB (Roche) blocking buffer was added to the coplin jars and allowed to equilibrate for one hour at RT. Each slide was individually removed from the chamber and covered with 500µl of anit-digoxigenin antibody (anti-DIG-AP) (diluted to 1:2000 2% MBMB) and allowed to incubate overnight. On day three, the slides were washed two times in MB for 30 minutes each. Then they were washed twice in color reaction buffer for 30 minutes each to allow the pH to adjust. Five hundred microliters of color reaction mix (color reaction buffer with the addition of 200µl nitro-blue tetrazolium and 5-bromo-4-chloro-3'-indolyphosphate (NBT/BCIP) per 10ml of color reaction buffer) were pipetted onto the slides in a hybridization chamber. The chamber also encased wetted (with molecular grade water) paper towels, and it was protected from light over night (approximately 18 hours). On the last day, the slides were washed in 1xPBS for five minutes. Glass coverslips (24x50) were mounted to the slides using Aqua/Poly mount. The slides were allowed to dry for no less than 30 minutes before examining.

Quantification of Igf1-Positive Cells

Using ImageJ, a RGB photomicrograph was converted to a gray scale image. Then, a standard threshold was applied to all images such that the *in situ* signal was selected with little background. Then, the integrated density (gray value x thresholded pixel area) was quantified. Specific brain regions were quantified as follows: For the cortex, a 550um x 550um (302.5um^2) box was drawn in the first cortical layer, directly adjacent to the midline. The threshold was set to 65-255 grey values and anything smaller than 25um was not counted as a cell. For the analysis of the molecular layer of the hippocampus, the entire hippocampal molecular layer region was outlined and brought to a threshold of 65-255 grey values, with anything smaller than 25um being excluded from the count (Figure 2). For the cerebellum, the paramedian lobule (PM) was analyzed. A line was drawn directly over the Igf1-positively stained cells in the PM between 1000-2000um. Only the cells along that line were analyzed (representing purkinje layer).

Figure 2 demonstrates the analysis process of the hippocampus.



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	Label	Area	Mean	Min	Max	IntDen	RawIntDen
1	hip_P7_C00007.tif	44	155.523	121	169	6843	6843
2	hip_P7_C00007.tif	160	143.131	100	169	22901	22901
3	hip_P7_C00007.tif	50	153.740	130	169	7687	7687
4	hip_P7_C00007.tif	191	117.131	42	169	22372	22372
5	hip_P7_C00007.tif	384	125.927	45	169	48356	48356
6	hip_P7_C00007.tif	444	135.890	74	169	60335.000	60335.000
7	hip_P7_C00007.tif	366	107.298	43	169	39271	39271
8	hip_P7_C00007.tif	88	138.989	96	169	12231	12231
9	hip_P7_C00007.tif	136	146.485	124	169	19922	19922
10	hip_P7_C00007.tif	239	141.741	84	169	33876	33876
11	hip_P7_C00007.tif	105	150.733	126	169	15827.000	15827.000
12	hip_P7_C00007.tif	58	160.328	140	169	9299	9299
13	hip_P7_C00007.tif	30	156.433	137	169	4693	4693
14	hip_P7_C00007.tif	42	150.333	122	169	6314	6314
15	hip_P7_C00007.tif	112	129.241	67	169	14475.000	14475.000
16	hip_P7_C00007.tif	61	141.607	94	169	8638	8638
17	hip_P7_C00007.tif	257	114.451	56	169	29414	29414
18	hip_P7_C00007.tif	309	126.900	44	169	39212	39212
19	hip_P7_C00007.tif	78	151.718	126	169	11834	11834
20	hip_P7_C00007.tif	71	161.014	144	169	11432	11432

Figure 2. Data analysis process of the hippocampus. (A) RGB colored photomicrograph (B) the same photomicrograph converted to gray scale. (C) The hippocampal area to be analyzed was then outlined (D) and cropped and zoomed in on. (E) The threshold was set to 65-255 gray values. (F) The outlined area was then analyzed, with anything smaller than 25um not being included in the analysis, resulting with the total Igf1-positive cell numbers and integrated density values.

To determine whether there was significant affect of treatment on Igf1-postive mean cell numbers, a two-way ANOVA was used to analyze differences of treatment, time, and an interaction between variables for ages P7-P21. A post-hoc Bonferroni's t-test was used to identify significant differences between treatments. An affect of treatment on Igf1-postive cell numbers at P42 was determined using an unpaired t-test.

Quantification of Relative Igf1 mRNA Levels

To determine whether there was significant affect of treatment on relative cellular Igf1 mRNA levels, the *in situ* signal was quantified as an integrated density. For all regions analyzed, the integrated density was calculated from the same cropped, thresholded images that were used for the cellular count analysis. For integrated density data in the cerebellum, a 25um x 25um circle was drawn and then placed over identified cells and the integrated density was measured of each cell. A two-way ANOVA was used to analyze differences of treatment, time, and an interaction between variables for ages P7-P21. If significance was determined, a Bonferroni's post-hoc t-test was used to identify significant differences between treatments. For animals aged P42, an unpaired t-test was used to determine significant differences between treatments.

Enzyme-Linked Immunosorbent Assay (ELISA)

To measure Igf1 and T4 serum levels, ELISA was performed on collected serum. First, the desired number of coated wells were secured into the holder. A data sheet was made with sample identification.

T4 ELISA.

Twenty-five microliters of standards, experimental samples, and controls were dispensed into the appropriate wells. Next, 100µl of the working (1:10) Enzyme Conjugate Reagent were dispensed into each well. Each well was thoroughly and

completely mixed for 10 seconds. The samples were then incubated at RT for 60 minutes. The incubation mixture was then removed by flicking the plate contents into the waste container. The microtiter wells were rinsed and flicked five times with washing buffer (1X). The wells were then struck sharply onto an absorbent paper to remove all residual water droplets. Next, 100µl of TMB solution was dispensed into each well and gently mixed for five seconds and then incubated in the dark at RT for 20 minutes. Adding 100µl of Stop Solution to each well, and gently mixing for thirty seconds stopped the reaction. The results were read by optical density at 450nm with a microtiter well reader (AccuDiag, Diagnostic Automation/Cortez Diagnostics Inc.).

Igf1 ELISA

One hundred microliters of standards, experimental samples (1:100 dilution with Assay Diluent A), and controls were dispensed into the appropriate wells and covered while gently shaking for two and a half hours. Next, the solution in each well was decanted and washed with 300 microliters of 1xWash Buffer four times, decanting after each successive wash. The wells were then struck sharply onto an absorbent paper to remove all residual water droplets. Next, 100µl of the prepared biotin antibody (mouse Igf1) was added to each well and incubated for one hour. The solution in each well was decanted and washed with 300 microliters of 1xWash Buffer four times, decanting after each successive wash. The wells were struck sharply onto an absorbent paper to remove all residual water droplets. Next, 110 microliters of prepared Streptavidin solution was

added to each well and allowed to incubate for 45 minutes. The solution in each well was decanted and washed with 300 microliters of 1xWash Buffer four times, decanting after each successive wash. The wells were struck sharply onto an absorbent paper to remove all residual water droplets. Next, 100 microliters of 3,3',5,5'-Tetramethylbenzidine (TMB) One-Step Substrate Reagent was added to each well and allowed to incubate in the dark for 30 minutes with gentle shaking. Adding 50µl of Stop Solution to each well and gently mixing for thirty seconds stopped the reaction. The results were immediately read by optical density at 450nm with a microtiter well reader (RayBio, Mouse IGF-1 ELISA kit).

ELISA Analysis

Total serum T4 or Igf1 levels were determined using the standard curve method. Corresponding mean absorbance values from experimental samples were then interpolated. The statistical significance of interpolated concentrations for P7-P21 was analyzed using a two-way ANOVA to detect significant differences of treatment, time, and an interaction between variables. A Bonferroni's post-hoc t-test was then used to identify significant differences between treatments. The interpolated concentrations for P42 were analyzed by an unpaired t-test to determine significant differences between treatments.

Linear Regression Analysis

To determine whether a correlation between total serum T4 and total serum Igf1 existed on our experimental animals, serum from each individual animal was used to investigate Igf1 and T4 levels. The matched serum values from animals at each age were then plotted and analyzed by linear regression to determine significant correlation between Igf1 and T4 serum levels.

Results

Pup Weights

Figure 3 summarizes mean pup body weights at time of sacrifice. No significant effect of treatment was detected at P7 or P42. However, hypothyroid pup body weights were significantly reduced compared to controls at P14 and P21.

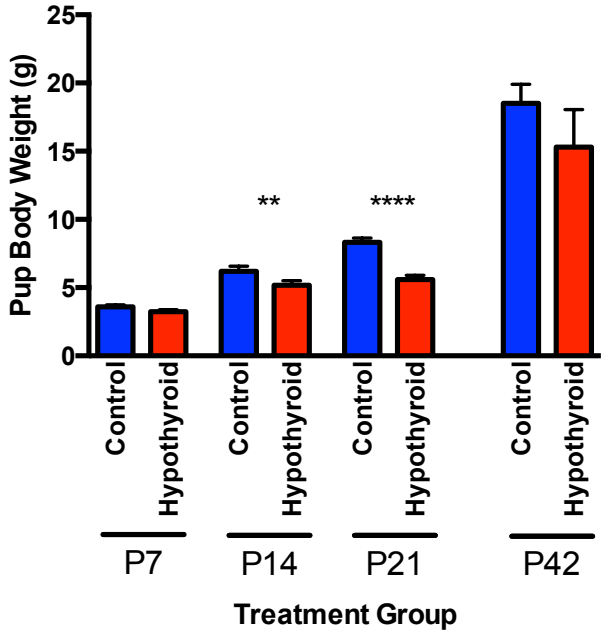


Figure 3. The effect of developmental hypothyroidism on pup body weights. Bars represent mean weight ± standard error at indicated developmental age. (P) postnatal day. Two-way ANOVA indicated significant effect of treatment ($p<0.0001$), time ($p<0.0001$), and an interaction between the variables ($p<0.001$). Unpaired t-test indicated no significant effect of treatment at P42. Bonferroni's post hoc, *$p<0.01$ between control and hypothyroid animals, ****$p<0.0001$ between control and hypothyroid animals.

Serum T4

Figure 4 summarizes mean serum T4 at the time of sacrifice. Serum T4 was significantly lower in hypothyroid animals at P14 and P21 when compared to their respective control animals. No significant differences were observed between control and treated animals at P7 or P42.

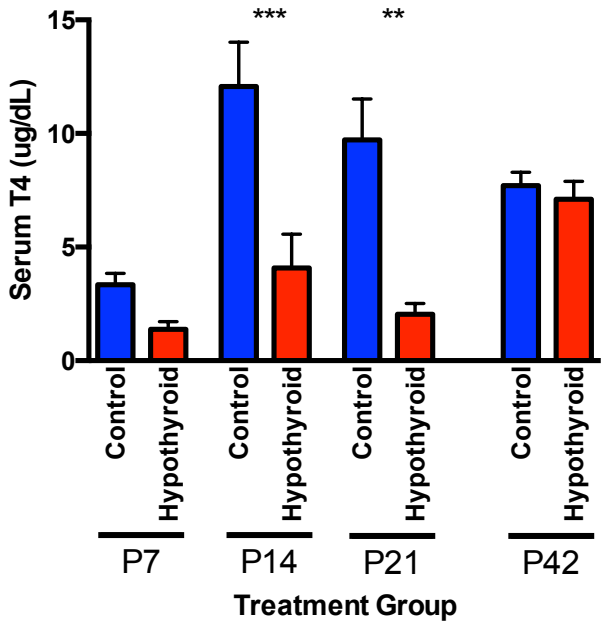


Figure 4. The effect of developmental hypothyroidism on serum T4 concentrations as measured by ELISA. Bars represent mean serum T4 ± standard error at indicated developmental ages. (P) postnatal day. Two-way ANOVA indicated significant effect of treatment ($p<0.0001$), time ($p<0.01$), and no interaction between the variables. Unpaired t-test indicated no significant effect of treatment at P42. Bonferroni's post hoc, ***$p<0.001$ between control and hypothyroid animals, **$p<0.01$ between control and hypothyroid animals.

Serum Igf1

Figure 5 summarizes mean serum Igf1 at the time of sacrifice. Serum Igf1 was significantly lower in hypothyroid animals when compared to their respective controls at P14 and P21. No significant differences were observed between control and treated animals at P7 or P42.

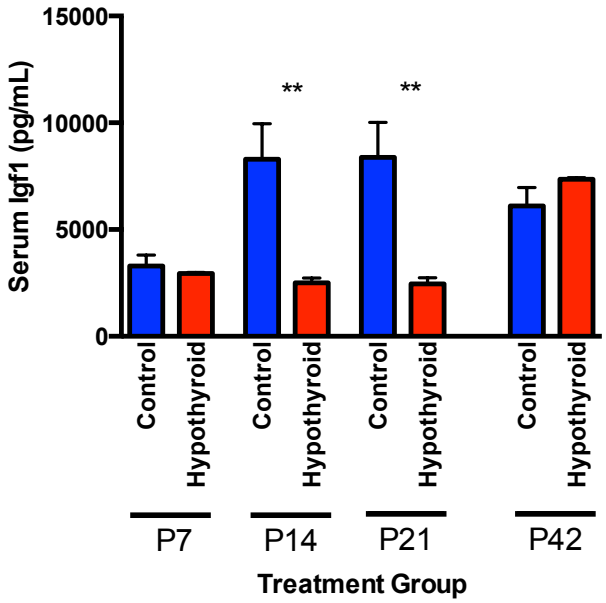


Figure 5. The effect of developmental hypothyroidism on serum Igf1 as measured by ELISA. Bars represent mean serum Igf1 ± standard error at indicated developmental ages. (P) postnatal day. Two-way ANOVA indicated significant effect of treatment ($p<0.0001$), time ($p<0.05$), and an interaction between the variables ($p<0.05$). Unpaired t-test indicated no significant effect of treatment at P42. Bonferroni's post hoc, **$p<0.01$ between control and hypothyroid animals.

Correlation Between Serum T4 and Serum Igf1

Figure 6 demonstrates the relationship between matched serum T4 and serum Igf1 in animals at all ages tested (P7, P14, P21, and P42). A significant positive correlation between total serum T4 and serum Igf1 was observed.

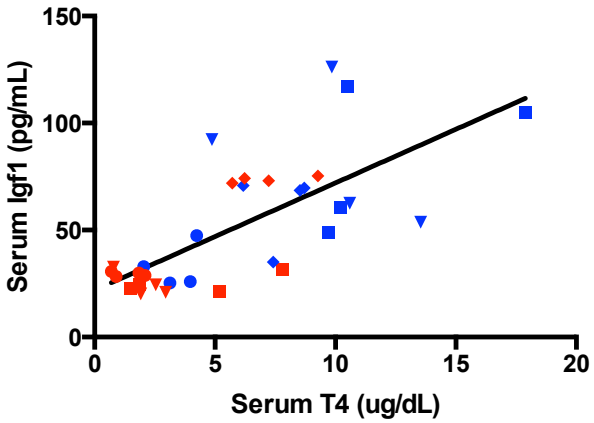
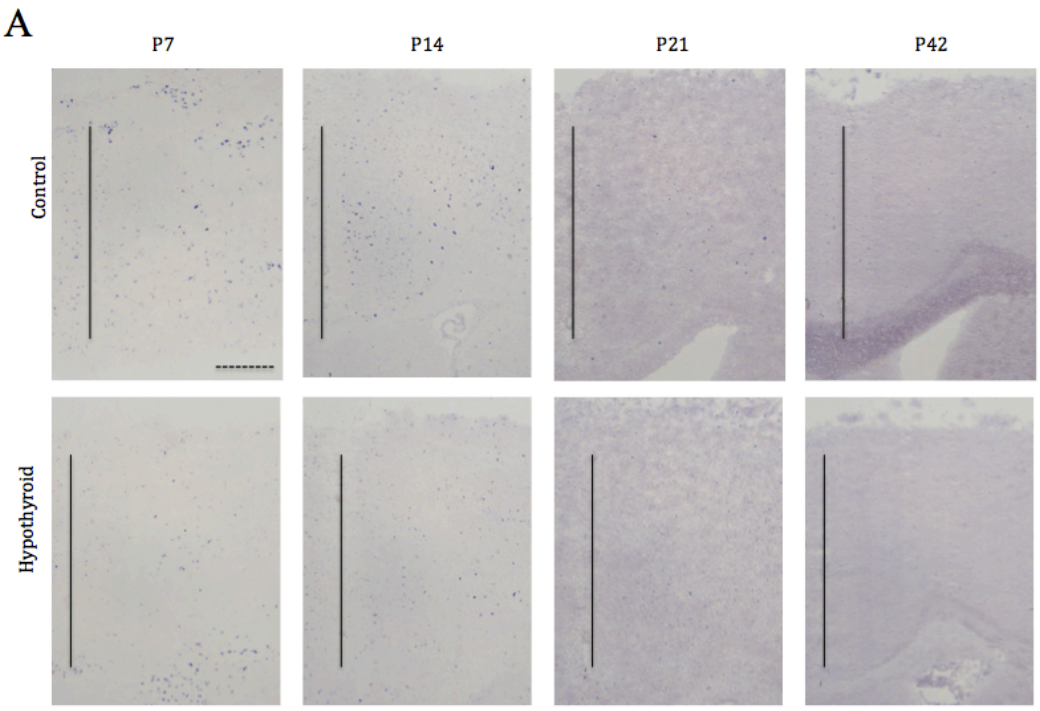


Figure 6. The correlation between serum Igf1 and serum T4. Data compiled from animal matched serum samples of control (blue) and hypothyroid (red) animals at P7 (circle), P14 (square), P21 (triangle), and P42 (diamond). Linear regression showed a significant deviation of the slope from zero ($p<0.0001$; $R^2=0.4951$).

In Situ Hybridization

Figure 7 demonstrates Igf1-positive cell distribution observed in the three identified regions of the brain analyzed at each developmental age.



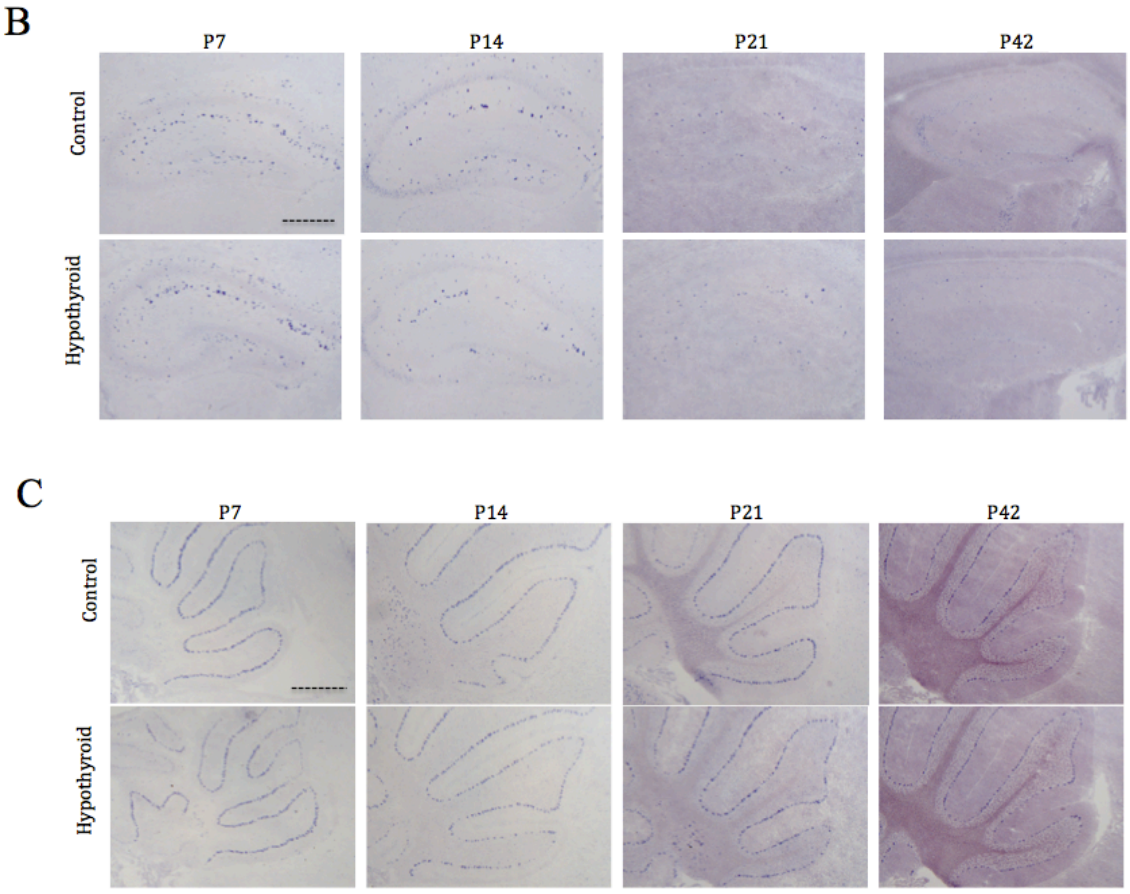
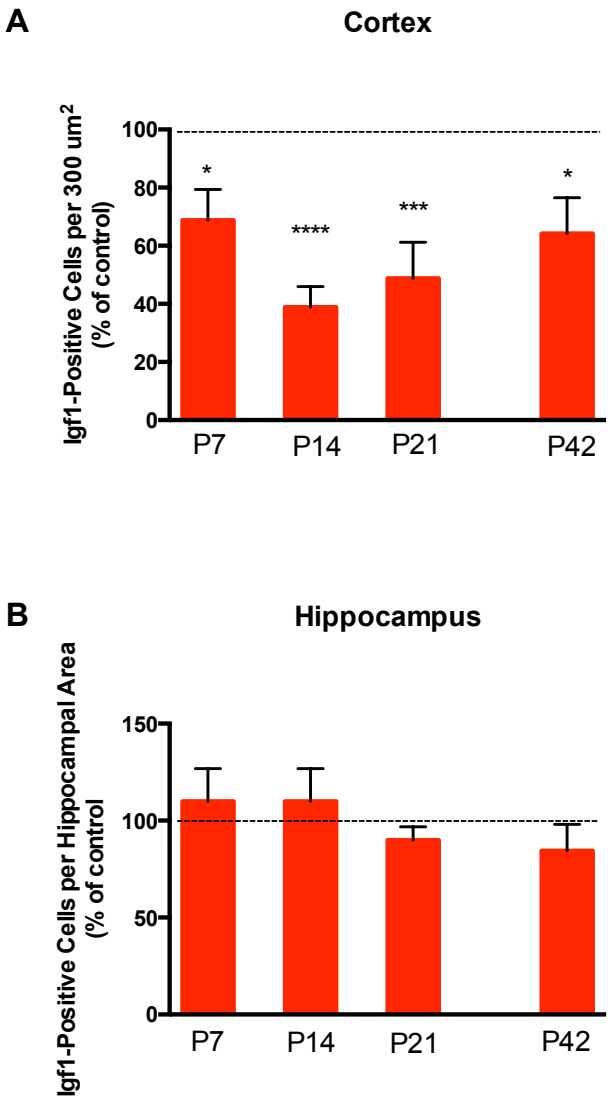


Figure 7. Distribution of Igf1 mRNA in the developing mouse (A) frontal motor cortex, (B) hippocampus, and (C) cerebellum. (A) Positively stained Igf1 cells are apparent throughout the cortex, peaking around P14 and declining thereafter, with control animals consistently showing higher levels of mRNA when compared to hypothyroid animals at all ages. Solid black bar indicates midline (longitudinal fissure). (B) Igf1-positively stained cells are most apparent in the strata oriens, radiatum, and lacunosum-molecular regions, along with the dentate gyrus. Igf1 mRNA levels appear to peak around P7-P14 declining thereafter, and show no visual differences in expression between treatments. (C) A strong Igf1 mRNA signal is located within the purkinje cell layer (PCL), with expression appearing to peak around P7-P21, declining thereafter. For all images, dashed black bar represents 250um.

Igf1-Postive Cell Numbers

Figure 8 summarizes the mean number of Igf1-positive cells in the cortex, hippocampus, and cerebellum. Hypothyroid treated animals had significantly fewer Igf1-positive cell numbers in the cortex at all developmental ages investigated compared to

controls. No significant differences in the numbers of Igf-1 positive cells between control and treated animals were detected in the hippocampus or cerebellum.



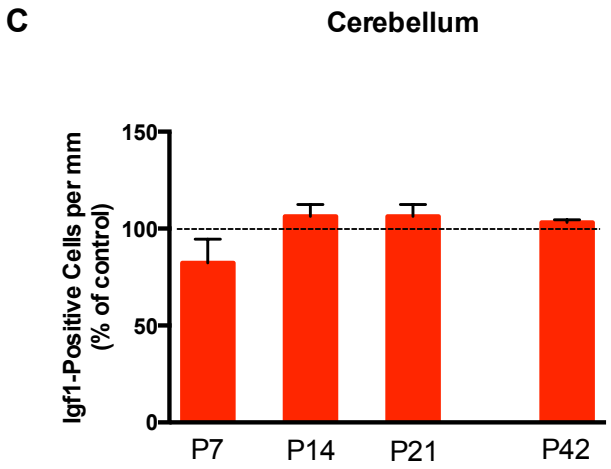
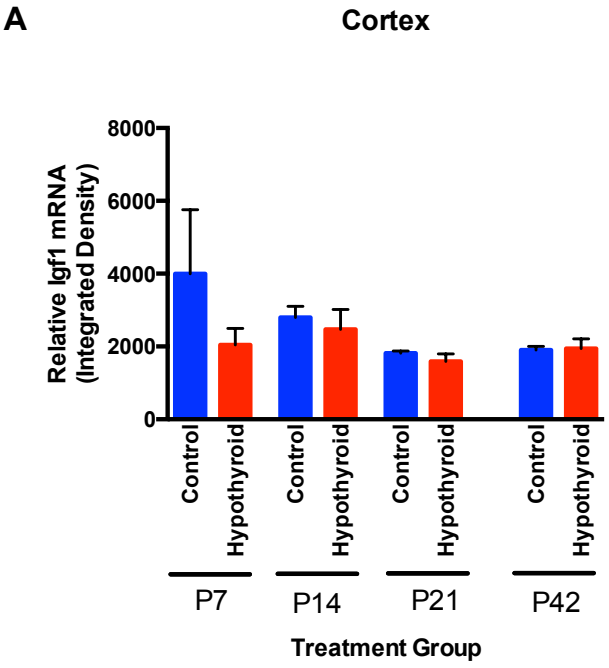
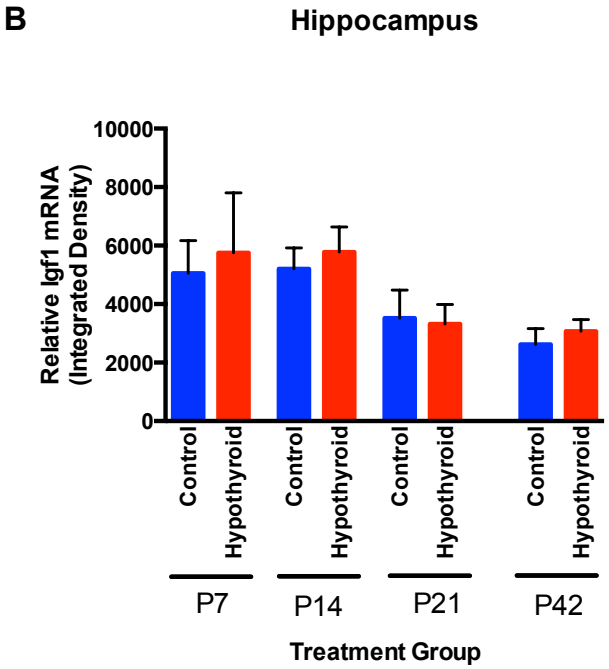


Figure 8. The effect of developmental hypothyroidism on the mean number of Igf1-positive cells in the (A) cortex, (B) hippocampus, and (C) cerebellum. Bars represent mean number of Igf1-positive cells ± standard error at indicated developmental ages. (P) postnatal day. Red bars represent hypothyroid treatment compared to percent of control. Dotted horizontal line represents control treatment as percent control value. Two-way ANOVA indicated significant effect of treatment ($p<0.0001$) for the cortex (A) and no other region. Unpaired t-test revealed no significant effect of treatment at P42. Bonferronis post hoc, *$p<0.05$ between control and hypothyroid animals, ***$p<0.001$ between control and hypothyroid animals, ****$p<0.0001$ between control and hypothyroid animals.

Integrated Density

Figure 9 summarizes relative cellular Igf1 mRNA levels determined by quantifying the *in situ* hybridization signal as a gray scale and area of the signal (integrated density). No significant differences between control and treated animals were measured in the cortex, hippocampus, or cerebellum.


A
Cortex
Relative Igf1 mRNA (Integrated Density)
8000
6000
4000
2000
0
Control
Hypothyroid
P7
P14
P21
P42
Treatment Group


B
Hippocampus
Relative Igf1 mRNA (Integrated Density)
10000
8000
6000
4000
2000
0
Control
Hypothyroid
P7
P14
P21
P42
Treatment Group

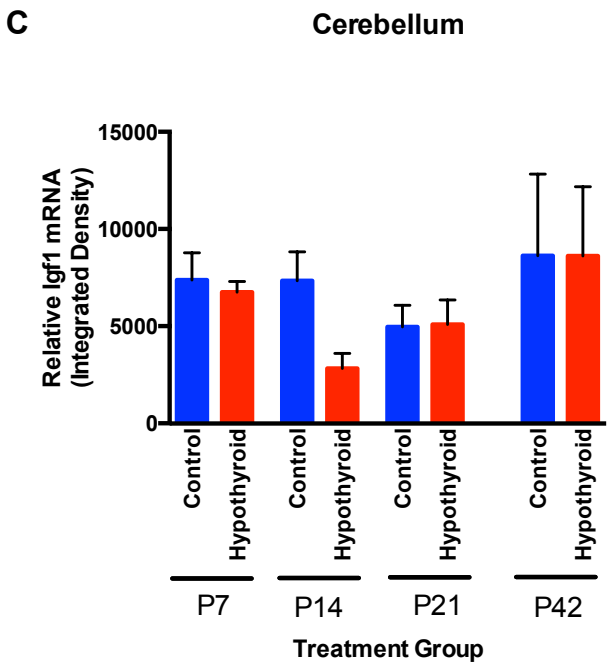


Figure 9. The effect of developmental hypothyroidism on Igf1-positive cells integrated density measured by gray scale values in the (A) cortex, (B) hippocampus, and (C) cerebellum. Bars represent mean integrated density ± standard error at each indicated developmental age. (P) postnatal day. Two-way ANOVA indicated no effect of treatment (p=0.0959), time (p=0.2287), or an interaction (p=0.2569). Unpaired t-test revealed no significant effect of treatment at P42.

Discussion

In the mammalian brain, growth factors are the main components that control cell proliferation, differentiation, and survival (Abe 2000). Out of the many growth factors contributing to the development of the central nervous system, thyroid hormone is known to interact with Igf1, a vital growth factor for the developing central nervous system (Elder, Karayal et al. 2000). It is also known that TH is essential for proper Igf1 levels in the serum and the brain

(Elder, Karayal et al. 2000, Iglesias, Bayon et al. 2001, Poguet, Legrand et al. 2003). However, the mechanistic relationship that TH and Igf1 share is poorly defined.

During the development of the central nervous system inadequate levels of TH or Igf1 results in similar neuroanatomical defects. Considering this, we sought to test the hypothesis that some of the developmental defects observed between low TH and Igf1 might be due to an altered temporal expression of Igf1 in the brain of hypothyroid animals as compared to control animals. To test this, we rendered pregnant dams hypothyroid through the administration of two well-known goitrogens - methimizole and potassium perchlorate – and then quantified Igf1-positive cells and relative Igf1 mRNA levels.

Effects of Treatment on Serum TH and Igf1 Levels

Induction of a hypothyroid state was confirmed by measuring serum T4 concentrations by ELISA (Figure 4). Furthermore, growth was severely retarded in treated animals; an effect consistent with hypothyroidism and Igf1 deficiency (MacGillivray, Aceto et al. 1968, Woods, Camacho-Hubner et al. 1996). However, once goitrogen treatment was withdrawn, serum T4 levels normalized (Figure 4) and body weight returned to normal (Figure 3), suggesting a return to euthyroidism.

Hypothyroidism has been previously shown to cause a reduction in serum Igf1 (Holder and Wallis 1977). Our results support this finding, such that hypothyroid treated animals, when compared to controls, resulted in a significant reduction in serum Igf1 at ages P14 and P21, when T4 was also significantly reduced (Figures 4 and 5). This observation likely resulted from a disruption of the GH-axis induced by low TH. Thyroid hormone directly regulates pituitary GH secretion, which in turn regulates Igf1 production in the liver (Nanto-Salonen, Glasscock et al. 1991). In addition, once serum T4 recovered after the withdrawal of goitrogens, Igf1 levels in the serum also recovered (Figures 4 and 5). Furthermore, our results indicate that there is a significant positive correlation between circulating levels of T4 and Igf1. That is, as TH levels decrease in the serum, Igf1 also decreases (Figure 6). This observation further supports that idea that reductions in serum Igf1 described above are due to the indirect effect of TH on pituitary GH secretion.

As stated previously, the regulation of serum Igf1 differs from local brain Igf1 in that GH plays no regulatory role brain synthesis (Bondy and Cheng 2004, Aberg, Brywe et al. 2006). Additionally, Igf1 does not easily cross the blood brain barrier; suggesting that serum Igf1 is separate from locally brain derived Igf1 (Russo, Gluckman et al. 2005). In the present study, we found that low TH was associated with a significant reduction in Igf1-positive cells in the frontal motor neocortex at all developmental ages investigated, but not in the hippocampus or cerebellum. Notably, a significant reduction was seen at

P42, after goitrogen withdrawal, and TH levels were restored to normal; suggesting a permanent loss in Igf1-positive cells (Figure 8). Considering that brain Igf1 levels appear to not be regulated by GH, this observation suggests that the observed changes in Igf1-positive cells is likely not due to altered GH signaling in TH deficient animals. Interestingly, developmental and region specific reductions in brain Igf1 mRNA have been previously recorded in hypothyroid rats (Elder, Karayal et al. 2000). However, it is not clear from the previous study whether the observed decrease in Igf1 mRNA was due to changes in the transcriptional levels of Igf1 or a loss of Igf1-positive cells.

The survival of neurons has previously been shown to depend on TH (Nunez 1984, Heisenberg, Thoenen et al. 1992). Additionally, neurons have also been identified as the principle sites of Igf1 mRNA expression (Bartlett, Li et al. 1991). These reports support the idea that TH has the potential to alter the total number of Igf1-positive cells in the cortex, with hypothyroidism resulting in decreased Igf1-positive cells. Thyroid hormone has also been shown to increase neuroblast proliferation and neuronal proliferation (Montero-Pedrazuela, Venero et al. 2006). Furthermore, TH is responsible for regulating the differentiation and maturation of astrocytes and stimulating growth factor production, including Igf1 (Rodier 1980, Trentin 2006). Considering these reports and the similarity in neuroanatomical defects between low TH or Igf1 in development, it is reasonable to suggest that the negative effects hypothyroidism has on the developing

nervous system are, in part, due to low TH preventing progenitor pool proliferation, maturation, and/or survival of Igf1-positive cells. Loss of neuronal progenitors that expand the Igf1-positive population would ultimately result in decreased levels of Igf1 mRNA in the brain.

Igf1 is reported to stimulate neural progenitor cell proliferation and differentiation into oligodendrocytes (D'Ercole, Ye et al. 1996, Hsieh, Aimone et al. 2004). In Igf1 knockout mice, the percentage of OPCs and mature oligodendrocytes was reduced in all regions of the brain (Ye, Li et al. 2002). Similarly, in mice lacking the Igf1r, a reduction in neural/glial antigen 2 (NG2)-positive and allophycocyanin (CC1)-positive oligodendrocytes was reported. This reduction was assumed to be a result from reduced cell survival and growth, especially considering Igf1's protective role against apoptosis and its positive influence on proliferation, differentiation, and maturation of oligodendrocytes and neurons (Chesik, De Keyser et al. 2008). Conversely, the addition of exogenous Igf1 administration to rats resulted in an increase of progenitor cell proliferation within the dentate gyrus (Dempsey, Sailor et al. 2003). Interestingly, similar defects in myelination are reported in the hypothyroid brain (Baas, Bourbeau et al. 1997). In cases of hypothyroidism, the total amount of myelination is decreased compared to controls, most likely a result of delayed expression of MAG, a differentiated property of oligodendrocytes (Gravel, Sasseville et al. 1990, Rodriguez-Pena, Ibarrola et al. 1993). Thyroid hormone has been identified in controlling the accumulation of mature

oligodendrocytes as well (Billon, Jolicoeur et al. 2002). These studies, along with the present results, suggest that specific neuroanatomical defects observed between low TH and low brain Igf1 may be due to a reduction in Igf1-positive cells, which could result in an overall reduction in brain Igf1 mRNA levels.

In addition to the effects on oligodendrocytes, low TH or Igf1 in development are known to reduce PCNs (Beck, Powell-Braxton et al. 1995, Gilbert, Sui et al. 2007). Considering our observations, it would be interesting to investigate whether the PCNs that are lost in the cortex and hippocampus are also responsible for synthesizing Igf1, with their loss ultimately decreasing the amount of Igf1mRNA and parvalbumin in the brain. This phenocopy between TH and local brain Igf1 on PV-IR could therefore be a result from the TH-induced reduction of Igf1-positive cells in the brain.

To determine whether neuroanatomical defects associated with hypothyroidism are due to a loss of Igf1 mRNA, Igf1 could be infused into the hypothyroid brain, or, alternatively, a transgenic model could be used to exogenously drive Igf1 during hypothyroidism.

Spatially Specific Effects of Low TH on Igf1-Positive Cells

Our results indicate the effect that hypothyroidism has on Igf1-positive cells occurs in a region dependent matter, with no reductions of Igf1-positive cell numbers found in the hippocampus or cerebellum, unlike the cortex (Figure 8). Differential

sensitivity to TH has previously been found in RC3 (neurogranin), with no relation to differential expression of TR isoforms. Hypothyroid rats had decreased expression of RC3 in regions such as the dentate gyrus, but not the pyramidal layers of the hippocampus. Decreased expression was also seen in layer six of the cerebral cortex, unlike the upper layers of the cerebral cortex. In terms of the regulation of gene expression, this leads us to believe that TR interacting factors, such as co-activators or co-repressors, have input in determining whether a population of neurons will be sensitive or not to TH (Guadano-Ferraz, Escamez et al. 1997).

Distribution of Igf1-Positive Cells in the Developing Brain

Cells positive for Igf1 mRNA in the neocortex appeared scattered with no discernible layer enrichment. Qualitatively, Igf1 mRNA peaked between P7-P14, with Igf1 mRNA declining thereafter (Figure 7). Although the present study is unable to define which cell type is positive for Igf1, reports from others has suggested that Igf1-postive cells in the cortex are neuronal in nature (Bartlett, Li et al. 1991). In the hippocampus, Igf1-positive cells were identified in the strata oriens, radiatum and lacunosum-molecular regions with peak expression occurring at approximately P7 (Figure 7). These hippocampal Igf1-positive cells have been previously described as being large polymorphic neurons (Bartlett, Li et al. 1991). In the cerebellum, Igf1-positive cells were restricted to the Purkinje cell layer (PCL) with peak expression of Igf1

mRNA occurring from P7-P21 (Figure 7). Within the cerebellum, the majority of proliferation and differentiation of migrating granule cells occurs after birth. Considering this, sustained postnatal Igf1 may play a role in this process, as proposed by Bartlett and Li (Bartlett, Li et al. 1991). This may explain why expression does not as rapidly decline after P14, like in other regions discussed. These observed patterns of Igf1 mRNA are consistent with those previously reported in the hippocampus and cerebellum (Bartlett, Li et al. 1991).

Due to the variability within the ISH (*in situ* hybridization) assay and the need to normalize to percent of control, it was impossible to detect temporal changes. However, previous findings have found that local brain Igf1 expression occurs in a time dependent matter, with its peak expression occurring in early postnatal life, declining thereafter (Bondy 1991, D'Ercole, Ye et al. 1996). In the rat brain, peak Igf1 expression occurs in late gestation through weaning, matching the time in which TH is essential for normal brain development (Bach, Shen-Orr et al. 1991, Porterfield 1994). This can also be seen in serum Igf1, with peak serum T4 correlating to peak Igf1 in the serum (Figures 4 and 5). When hypothyroidism is induced in adult mice, Igf1mRNA does not differ between control and hypothyroid animals, indicating that during development is when it is most critical for the nervous system (Elder, Karayal et al. 2000).

Neurons have previously been implicated as being the principle sites of Igf1mRNA expression, with some evidence also suggesting that neuroglia in some brain

regions may also express Igf1, like that of astrocytes found in forebrain fiber tracts (Bartlett, Li et al. 1991). However, further investigation through dual labeled immunohistochemistry is needed in identifying the precise cells that synthesize Igf1 in the brain.

Treatment Effects on Cellular Igf1 mRNA Levels

Our results indicated no effect of treatment on relative Igf1 mRNA per Igf1-positive cell in any of the investigated brain regions (Figure 9). This observation suggests that the decrease seen in Igf1 mRNA in the brain measured by others (Elder, Karayal et al. 2000) is not from a reduction in TH-mediated regulation of Igf1 mRNA synthesis through its receptors. This further suggests that hypothyroidism does not inhibit brain cells capacity to synthesize Igf1, but rather there is a loss of Igf1 secreting cells, which would result in a decrease in total brain Igf1 mRNA. However, in the current study, we cannot exclude the possibility that Igf1-positive cells are present in the cortex, but Igf1 mRNA levels were below the detection of the assay, rather than completely absent. Furthermore, the levels of expression of local brain Igf1 mRNA in the current study did not significantly differ from when treatment was being administered, to when it was halted, further concluding that TH does not inhibit the synthesis of Igf1, but rather the accumulation of Igf1-positive cells (Figure 9). However, the possibility exists that the Igf1-positively stained cells had reached saturation in the enzymatic reaction of the ISH

assay, which would have made detection of small changes in Igf1 mRNA synthesis impossible to observe, and potentially could have masked differences of Igf1 mRNA between treatments. Qualitative-PCR could be used in future investigation to examine the possibility of small changes and differences in Igf1 mRNA over time and between treatments, which may not have been detectable by the current studies utilized assay.

Thyroid hormone acts on its associated nuclear receptors to effect gene transcription (Bernal 2002, Bernal 2007). Although many of TH affects are indirect, a TRE in intron 1 of the Igf1 gene has been identified and reported to directly regulate Igf1 gene transcription in bone (Xing, Govoni et al. 2012). In the present study, we observed a significant permanent loss in Igf1-postive cells following developmental hypothyroidism (Figure 8). However, Igf1-positive cells that remained appeared to have normal levels of Igf1 mRNA (Figure 9). One possible explanation for this finding is that in the developing brain, TH and its receptors do not control the rate of Igf1 transcription. This conclusion would imply that another, unidentified mechanism controls Igf1 transcription. Consistent with this idea, in the other regions analyzed, neither the numbers of Igf1-positive cells or relative amounts of Igf1 mRNA we reduced (Figures 8 and 9); suggesting TH-independent regulation in the cerebellum and hippocampus.

Concluding Remarks and Future Studies

Thyroid hormone negatively alters the amount of Igf1-positive cells within specific regions of the developing brain (Figure 8). Additionally, we show that the synthesis of Igf1 mRNA from brain cells is most likely not directly dependent on TH (Figure 9). Furthermore, a region specific expression of Igf1-positive cells is shown (Figure 8), demonstrating that Igf1-positive cells in the cortex may be directly regulated by TH, whereas in regions such as the hippocampus and cerebellum, there is the potential for additional regulatory TR interacting factors controlling Igf1 mRNA synthesis. The evidence presented previously, along with these findings, make it tempting to suggest that the loss of TH causes a reduction of local brain Igf1 by decreasing the amount of Igf1 synthesizing cells, resulting in hypothyroid associated neuroanatomical defects. Based on preliminary data, a rescue group, consisting of the hypothyroid treatment with the addition of exogenous T4 resulted in control-like levels of serum T4, indicating that the reductions in serum TH, serum Igf1, and Igf1-positive cells in the frontal motor cortex are a result of the induced hypothyroidism, not the drug. However, more investigation is needed to further solidify this evidence.

References:

Aberg, N. D., K. G. Brywe and J. Isgaard (2006). "Aspects of growth hormone and insulin-like growth factor-I related to neuroprotection, regeneration, and functional plasticity in the adult brain." ScientificWorldJournal **6**: 53-80.

Bartlett, W. P., X. S. Li, M. Williams and S. Benkovic (1991). "Localization of insulin-like growth factor-1 mRNA in murine central nervous system during postnatal development." Dev Biol **147**(1): 239-250.

Beck, K. D., L. Powell-Braxton, H. R. Widmer, J. Valverde and F. Hefti (1995). "Igf1 gene disruption results in reduced brain size, CNS hypomyelination, and loss of hippocampal granule and striatal parvalbumin-containing neurons." Neuron **14**(4): 717-730.

Berbel, P., A. Guadano-Ferraz, A. Angulo and J. Ramon Cerezo (1994). "Role of thyroid hormones in the maturation of interhemispheric connections in rats." Behav Brain Res **64**(1-2): 9-14.

Bernal, J. (2002). "Action of thyroid hormone in brain." J Endocrinol Invest **25**(3): 268-288.

Bernal, J. (2007). "Thyroid hormone receptors in brain development and function." Nat Clin Pract Endocrinol Metab **3**(3): 249-259.

Bohannon, N. J., E. S. Corp, B. J. Wilcox, D. P. Figlewicz, D. M. Dorsa and D. G. Baskin (1988). "Localization of binding sites for insulin-like growth factor-I (IGF-I) in the rat brain by quantitative autoradiography." Brain Res **444**(2): 205-213.

Bondy, C. and E. Chin (1991). "IGF-I mRNA localization in trigeminal and sympathetic nerve target zones during rat embryonic development." Adv Exp Med Biol **293**: 431-437.

Bondy, C. A. (1991). "Transient IGF-I gene expression during the maturation of functionally related central projection neurons." J Neurosci **11**(11): 3442-3455.

Bondy, C. A. and C. M. Cheng (2004). "Signaling by insulin-like growth factor 1 in brain." Eur J Pharmacol **490**(1-3): 25-31.

Bradley, D. J., W. S. Young, 3rd and C. Weinberger (1989). "Differential expression of alpha and beta thyroid hormone receptor genes in rat brain and pituitary." Proc Natl Acad Sci U S A **86**(18): 7250-7254.

Chatterjee, V. K., J. K. Lee, A. Rentoumis and J. L. Jameson (1989). "Negative regulation of the thyroid-stimulating hormone alpha gene by thyroid hormone: receptor interaction adjacent to the TATA box." Proc Natl Acad Sci U S A **86**(23): 9114-9118.

Chesik, D., J. De Keyser and N. Wilczak (2008). "Insulin-like growth factor system regulates oligodendroglial cell behavior: therapeutic potential in CNS." J Mol Neurosci **35**(1): 81-90.

D'Ercole, A. J., P. Ye, A. S. Calikoglu and G. Gutierrez-Ospina (1996). "The role of the insulin-like growth factors in the central nervous system." Mol Neurobiol **13**(3): 227-255.

D'Ercole, A. J., P. Ye and J. R. O'Kusky (2002). "Mutant mouse models of insulin-like growth factor actions in the central nervous system." Neuropeptides **36**(2-3): 209-220.

Elder, D. A., A. F. Karayal, A. J. D'Ercole and A. S. Calikoglu (2000). "Effects of hypothyroidism on insulin-like growth factor-I expression during brain development in mice." Neurosci Lett **293**(2): 99-102.

Farsetti, A., T. Mitsuhashi, B. Desvergne, J. Robbins and V. M. Nikodem (1991). "Molecular basis of thyroid hormone regulation of myelin basic protein gene expression in rodent brain." J Biol Chem **266**(34): 23226-23232.

Freitas, B. C., B. Gereben, M. Castillo, I. Kallo, A. Zeold, P. Egri, Z. Liposits, A. M. Zavacki, R. M. Maciel, S. Jo, P. Singru, E. Sanchez, R. M. Lechan and A. C. Bianco (2010). "Paracrine signaling by glial cell-derived triiodothyronine activates neuronal gene expression in the rodent brain and human cells." J Clin Invest **120**(6): 2206-2217.

Gao, F. B., J. Apperly and M. Raff (1998). "Cell-intrinsic timers and thyroid hormone regulate the probability of cell-cycle withdrawal and differentiation of oligodendrocyte precursor cells." Dev Biol **197**(1): 54-66.

Gilbert, M. E., L. Sui, M. J. Walker, W. Anderson, S. Thomas, S. N. Smoller, J. P. Schon, S. Phani and J. H. Goodman (2007). "Thyroid hormone insufficiency during brain development reduces parvalbumin immunoreactivity and inhibitory function in the hippocampus." Endocrinology **148**(1): 92-102.

Gilbert, M. E. and R. T. Zoeller (2010). Thyroid Hormones-Impact on the Developing Brain: Possible Mechaniss of Neurotoxicity. Neurotoxicology, Third Edition. J. A. T. A. Wallace Hayes, Donald E. Gardner, CRC Press. **28**.

Gomes, F. C., C. G. Maia, J. R. de Menezes and V. M. Neto (1999). "Cerebellar astrocytes treated by thyroid hormone modulate neuronal proliferation." Glia **25**(3): 247-255.

Gravel, C., R. Sasseville and R. Hawkes (1990). "Maturation of the corpus callosum of the rat: II. Influence of thyroid hormones on the number and maturation of axons." J Comp Neurol **291**(1): 147-161.

Heisenberg, C. P., H. Thoenen and D. Lindholm (1992). "Tri-iodothyronine regulates survival and differentiation of rat cerebellar granule neurons." Neuroreport **3**(8): 685-688.

Hill, J. M., M. A. Lesniak, C. B. Pert and J. Roth (1986). "Autoradiographic localization of insulin receptors in rat brain: prominence in olfactory and limbic areas." Neuroscience **17**(4): 1127-1138.

Hurtado-Chong, A., M. J. Yusta-Boyo, E. Vergano-Vera, A. Bulfone, F. de Pablo and C. Vicario-Abejon (2009). "IGF-I promotes neuronal migration and positioning in the olfactory bulb and the exit of neuroblasts from the subventricular zone." Eur J Neurosci **30**(5): 742-755.

Lauder, J. M. (1977). "The effects of early hypo- and hyperthyroidism on the development of rat cerebellar cortex. III. Kinetics of cell proliferation in the external granular layer." Brain Res **126**(1): 31-51.

LeRoith, D. (1996). "Insulin-like growth factor receptors and binding proteins." Baillieres Clin Endocrinol Metab **10**(1): 49-73.

Liu, X., D. L. Yao, C. A. Bondy, M. Brenner, L. D. Hudson, J. Zhou and H. D. Webster (1994). "Astrocytes express insulin-like growth factor-I (IGF-I) and its binding protein, IGFBP-2, during demyelination induced by experimental autoimmune encephalomyelitis." Mol Cell Neurosci **5**(5): 418-430.

Madathil, S. K., S. W. Carlson, J. M. Brelsfoard, P. Ye, A. J. D'Ercole and K. E. Saatman (2013). "Astrocyte-Specific Overexpression of Insulin-Like Growth Factor-1 Protects Hippocampal Neurons and Reduces Behavioral Deficits following Traumatic Brain Injury in Mice." PLoS One **8**(6): e67204.

Marks, J. L., D. Porte, Jr. and D. G. Baskin (1991). "Localization of type I insulin-like growth factor receptor messenger RNA in the adult rat brain by in situ hybridization." Mol Endocrinol **5**(8): 1158-1168.

McDonald, A. J. and R. L. Betette (2001). "Parvalbumin-containing neurons in the rat basolateral amygdala: morphology and co-localization of Calbindin-D(28k)." Neuroscience **102**(2): 413-425.

McMorris, F. A., T. M. Smith, S. DeSalvo and R. W. Furlanetto (1986). "Insulin-like growth factor I/somatomedin C: a potent inducer of oligodendrocyte development." Proc Natl Acad Sci U S A **83**(3): 822-826.

Mendes-de-Aguiar, C. B., R. Alchini, J. K. Zucco, B. Costa-Silva, H. Decker, M. Alvarez-Silva, C. I. Tasca and A. G. Trentin (2010). "Impaired astrocytic extracellular matrix distribution under congenital hypothyroidism affects neuronal development in vitro." J Neurosci Res **88**(15): 3350-3360.

Miell, J. P., A. M. Taylor, M. Zini, H. G. Maheshwari, R. J. Ross and R. Valcavi (1993). "Effects of hypothyroidism and hyperthyroidism on insulin-like growth factors (IGFs) and growth hormone- and IGF-binding proteins." J Clin Endocrinol Metab **76**(4): 950-955.

Montero-Pedrazuela, A., C. Venero, R. Lavado-Autric, I. Fernandez-Lamo, J. M. Garcia-Verdugo, J. Bernal and A. Guadano-Ferraz (2006). "Modulation of adult hippocampal neurogenesis by thyroid hormones: implications in depressive-like behavior." Mol Psychiatry **11**(4): 361-371.

Morreale de Escobar, G., R. Pastor, M. J. Obregon and F. Escobar del Rey (1985). "Effects of maternal hypothyroidism on the weight and thyroid hormone content of rat embryonic tissues, before and after onset of fetal thyroid function." Endocrinology **117**(5): 1890-1900.

Mozell, R. L. and F. A. McMorris (1991). "Insulin-like growth factor I stimulates oligodendrocyte development and myelination in rat brain aggregate cultures." J Neurosci Res **30**(2): 382-390.

Nakao, N., H. Ono and T. Yoshimura (2008). "Thyroid hormones and seasonal reproductive neuroendocrine interactions." Reproduction **136**(1): 1-8.

Nunez, J. (1984). "Effects of thyroid hormones during brain differentiation." Mol Cell Endocrinol **37**(2): 125-132.

Porterfield, S. P. (1994). "Vulnerability of the developing brain to thyroid abnormalities: environmental insults to the thyroid system." Environ Health Perspect **102 Suppl 2**: 125-130.

Quignodon, L., C. Grijota-Martinez, E. Compe, R. Guyot, N. Allioli, D. Laperriere, R. Walker, P. Meltzer, S. Mader, J. Samarut and F. Flamant (2007). "A combined approach identifies a limited number of new thyroid hormone target genes in post-natal mouse cerebellum." J Mol Endocrinol **39**(1): 17-28.

Rodier, P. M. (1980). "Chronology of neuron development: animal studies and their clinical implications." Dev Med Child Neurol **22**(4): 525-545.

Rodriguez-Pena, A., N. Ibarrola, M. A. Iniguez, A. Munoz and J. Bernal (1993). "Neonatal hypothyroidism affects the timely expression of myelin-associated glycoprotein in the rat brain." J Clin Invest **91**(3): 812-818.

Schoonover, C. M., M. M. Seibel, D. M. Jolson, M. J. Stack, R. J. Rahman, S. A. Jones, C. N. Mariash and G. W. Anderson (2004). "Thyroid hormone regulates oligodendrocyte accumulation in developing rat brain white matter tracts." Endocrinology **145**(11): 5013-5020.

Sharlin, D. S., D. Tighe, M. E. Gilbert and R. T. Zoeller (2008). "The balance between oligodendrocyte and astrocyte production in major white matter tracts is linearly related to serum total thyroxine." Endocrinology **149**(5): 2527-2536.

Trentin, A. G. (2006). "Thyroid hormone and astrocyte morphogenesis." J Endocrinol **189**(2): 189-197.

Trentin, A. G., M. Alvarez-Silva and V. Moura Neto (2001). "Thyroid hormone induces cerebellar astrocytes and C6 glioma cells to secrete mitogenic growth factors." Am J Physiol Endocrinol Metab **281**(5): E1088-1094.

Trentin, A. G. and V. Moura Neto (1995). "T3 affects cerebellar astrocyte proliferation, GFAP and fibronectin organization." Neuroreport **6**(2): 293-296.

Ye, P., G. J. Popken, A. Kemper, K. McCarthy, B. Popko and A. J. D'Ercole (2004). "Astrocyte-specific overexpression of insulin-like growth factor-I promotes brain overgrowth and glial fibrillary acidic protein expression." J Neurosci Res **78**(4): 472-484.

Yen, P. M. (2001). "Physiological and molecular basis of thyroid hormone action." Physiol Rev **81**(3): 1097-1142.

Zoeller, R. T. and J. Rovet (2004). "Timing of thyroid hormone action in the developing brain: clinical observations and experimental findings." J Neuroendocrinol **16**(10): 809-818.