TISSUE BIOMARKERS OF THYROID STATUS AS CORRELATES OF NEURODEVELOPMENTAL IMPAIRMENT FOLLOWING GESTATIONAL AND LACTATIONAL EXPOSURE TO THYROID DISRUPTOR PROPYLTHIOURACIL

by

MATTHEW AARON TAYLOR

(Under the direction of Duncan C. Ferguson and Jeffrey Fisher)

ABSTRACT

From its influence on mitochondrial activity and basal metabolic rate to its complex interplay with other endocrine organs, the thyroid gland is vital for proper development and operation of the mammalian body. Neurological development is a special concern due to the relative sensitivity of gestating females and neonates to thyroid disruption. The studies described within investigate the effects of developmental exposure to antithyroid compounds on the neurodevelopment of rats. As peripheral tissues including the brain are capable of partial compensation in response to declining availability of thyroid hormones from serum, we hypothesized that measuring thyroid-responsive parameters such as tissue 5’-deiodinase enzyme activity thyroid hormone concentrations, and mRNA expression would provide data which would more closely correlate to neurological endpoints than serum thyroid hormone or thyrotropin (TSH) concentrations. We further hypothesized that younger animals would be more sensitive to thyroid disruption than older animals, due to diminished reserve of the compensatory mechanisms in the hypothalamic-pituitary-thyroid axis.

We used two antithyroid compounds to test these hypotheses. Propylthiouracil (PTU) at 0, 0.3, 1, 3, and 10 ppm in drinking water, and perchlorate at 150ppm in drinking water were administered from gestational day 2 until weaning. Serum and cerebrocortical thyroid hormone concentrations were measured at postnatal days 4, 14, and 21-32, as well as in dams and pups allowed to recover for 60 days post-weaning. Hippocampal in vitro electrophysiological measurements were performed on pups and
adults. Open field behavioral testing was performed on adults. Overall, we found that the chosen thyroid-responsive biomarkers were insufficient to consistently predict the presence of electrophysiological alterations. A severe reduction in serum total T4 concentration in developing pups was found to be a necessary condition for neurological impairment. Reductions in cortical T3 concentrations were not found to perfectly correlate with neurological outcomes.

INDEX WORDS: Rat, propylthiouracil, thyroid, hippocampus, perchlorate, neurodevelopment, electrophysiology, deiodinase, triiodothyronine, thyroxine
TISSUE BIOMARKERS OF THYROID STATUS AS CORRELATES OF NEURODEVELOPMENTAL IMPAIRMENT FOLLOWING GESTATIONAL AND LACTATIONAL EXPOSURE TO THYROID DISRUPTOR PROPYLTHIOURACIL

by

MATTHEW AARON TAYLOR

BS, University of Georgia, 2003

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2008
TISSUE BIOMARKERS OF THYROID STATUS AS CORRELATES OF
NEURODEVELOPMENTAL IMPAIRMENT FOLLOWING GESTATIONAL AND LACTATIONAL
EXPOSURE TO THYROID DISRUPTOR PROPYLTHIOURACIL

by

MATTHEW TAYLOR

Co-Major Professors: Duncan C. Ferguson
Jeffrey Fisher
Committee: Cham Dallas
Margarethe Hoenig
John Wagner

Electronic Version Approved:
Maureen Grasso
Dean of the Graduate School
The University of Georgia
August 2008
DEDICATION

Dedicated to those for whom the mundane mysteries of the universe are wonder enough for a lifetime without need of the supernatural.
ACKNOWLEDGEMENTS

I would like to thank all those who have helped me in my work. For Dr. Ferguson and my committee, thank you for your guidance and teachings. For former labmates, especially Nadia, thank you for your help and support. For my wife, thank you for helping me to stay on the correct side of insanity, and for everything else you’ve done for me. And for all who have encouraged my curiosity and love for science over the years, thank you.
# TABLE OF CONTENTS

**ACKNOWLEDGEMENTS** .......................................................................................................................... V

**CHAPTER**

1. **INTRODUCTION** ............................................................................................................................. 1
   REFERENCES ........................................................................................................................................... 2

2. **LITERATURE REVIEW** .................................................................................................................... 4
   THYROID HORMONE PRODUCTION AND METABOLISM ............................................................. 4
   PLASMA THYROID HORMONE BINDING AND PHARMACOKINETICS ............................................ 6
   EFFECTS OF THYROID HORMONES ................................................................................................. 13
   HYPOTHALAMUS-PITUITARY-THYROID AXIS DEVELOPMENT ..................................................... 16
   BRAIN DEVELOPMENT ....................................................................................................................... 17
   THYROID DISRUPTION ......................................................................................................................... 19
   ELECTROPHYSIOLOGY ....................................................................................................................... 27
   CONCLUSION ....................................................................................................................................... 31
   REFERENCES ......................................................................................................................................... 31

3. **LOWER THYROID COMPENSATORY RESERVE OF RAT PUPS FOLLOWING**
   MATERNAL HYPOTHYROIDISM: CORRELATION OF THYROID, HEPATIC, AND
   CEREBROCORTICAL BIOMARKERS WITH HIPPOCAMPAL
   NEUROPHYSIOLOGY ........................................................................................................................... 57
   ABSTRACT ............................................................................................................................................... 58
   INTRODUCTION ..................................................................................................................................... 58
   MATERIALS AND METHODS ............................................................................................................... 60
   RESULTS ............................................................................................................................................... 65
APPENDICES ................................................................................................................................. 211

A  LIST OF ABBREVIATIONS ........................................................................................................ 211

B  DIETARY COMPARISON ........................................................................................................... 213
CHAPTER 1
INTRODUCTION

As our understanding of endocrine physiology advances, so does the list of chemicals which may interfere with these regulatory systems. One endocrine organ currently of concern is the thyroid, which impacts function in virtually every tissue of the mammalian body. Although the thyroid was first identified about 500 years ago, new effects of thyroid hormones are still being identified, and along with them new implications of thyroid insufficiency. While widespread inclusion of iodine into table salt has virtually eliminated goiters and cretinism in developed nations, new environmental threats continue to emerge.

A major concern over chemicals which impact the thyroid axis is the growing body of literature which shows that thyroid hormones play a vital role in neural development in mammals including humans. Hypothyroidism has been shown to have effects ranging from outright cretinism to ones as subtle as altering the timing at which neuronal receptors switch from one isoform to another during development. While our understanding of the effects of thyroid insufficiency during development continues to grow, many of these experimental effects are demonstrated in the laboratory at doses that have no known environmental relevance. Some thyroid effects have been shown at current environmental levels of antithyroid chemicals, such as an increase in serum thyroid stimulating hormone (TSH) levels in American women with elevated perchlorate exposure.¹ However, the degree of exposure needed to induce adverse effects rather than merely compensatory effects is still poorly understood.

One of the biggest problems in translating exposure to antithyroid agents into detrimental effects is the large amount of compensation possible at the central (hypothalamic-pituitary-thyroid) and peripheral (target tissue) levels which help to ameliorate the effects of thyroid disruption caused by exposure to such agents. While it is quite common to measure serum TSH concentrations in screening procedures for hypothyroidism, serum hormone measurements alone do not necessarily explain what is
happening at the level of target tissues. Deiodinases in peripheral tissues such as the brain have cellular adaptive mechanisms by which to avoid local conditions of hypothyroidism or hyperthyroidism.²

We sought to fill in some of these knowledge gaps and devised hypotheses linking hormonal, biochemical, electrophysiological and behavioral assays in an effort to inform model development. Propylthiouracil (PTU) was chosen as the anti-thyroid agent for this project due to its prior use in several neurologically-focused studies. Specifically, it was hypothesized that

1) Thyroid hormone concentrations in serum do not provide sufficient information to predict the availability and activity of thyroid hormones to all tissues in the body, as alterations in peripheral metabolism help to counterbalance the effects of systemic hypothyroidism in critical tissues such as the brain.

2) Measurement of brain-specific biomarkers such as thyroid responsive gene expression, deiodinase activity and cerebrocortical T3 concentrations will be more reliable predictors of neurodevelopmental impairment than serum hormone concentrations due to their improved proximity to the developmental endpoint compared to serum effects.

3) The chosen biomarkers: cerebrocortical D2 activity, cerebrocortical T3 concentration, and expression of thyroid-responsive genes in the brain will provide data which will aid efforts to predict both temporary and permanent neurological dysfunction caused by any thyroid disruptive compound.

4) Younger animals are more sensitive to thyroid disruption than older animals due to their immature thyroid axis and lesser compensatory capabilities compared to adults.

5) The neurological impact of maternal hypothyroidism is modified by the nutritional status of developing pups, as impacted by the number of pups nursed by a single dam, as well as alterations in dietary contents such as soy isoflavones.

References

CHAPTER 2
LITERATURE REVIEW

Thyroid hormone production and metabolism

Basic Thyroid Physiology

The earliest recorded references to the thyroid date back to around 2700 BCE, with a reference to the use of seaweed to treat goiter.\(^1\) The name was associated with the gland in 1656 CE by Thomas Wharton, due to the shield-shaped cartilage associated with the gland in mammals. Understanding of the thyroid's purpose was hindered by the medical thinking of the era, as it did not fit into the traditional view of the balance of humors in the body. The importance of iodine was not elucidated until about 1820. Today, iodine's sole known role is as a micronutrient for synthesis of thyroid hormones (Figure 2.1), which have a variety of effects on most organs in the body.\(^2\)

The functional unit of the thyroid is the follicle, which consists of epithelial cells surrounding a compartment of viscous fluid known as colloid. The follicle contains all of the machinery necessary to take iodine from the serum, store it, produce hormones, and export the hormones back into the bloodstream. The follicular cells take up iodine from the blood via the sodium-iodine symporter (NIS). This iodine is then exported into the colloid, along with thyroglobulin (Tg), and a number of other compounds. Iodine is attached to Tg, and resorbed into the epithelial cells via endocytosis, where the Tg is digested and thyroid hormones released into the bloodstream.\(^2\)

Iodine Metabolism

The thyroid gland displays a remarkable ability to concentrate iodine from the blood, thanks to the actions of the sodium iodide symporter (NIS), located within the basolateral membrane of thyroid follicular cells. The gene for NIS was first cloned in 1996, and both the human and rat NIS genes were cloned that year.\(^3,4\) An 84% identity and 93% similarity was found between the NIS genes in these two
species. NIS contains 13 transmembrane domains, and several identified glycosylation sites. TSH stimulation is critical for proper targeting and function of NIS. TSH stimulates NIS activity through the cAMP pathway, as well as through upregulation of NIS mRNA. Under steady-state conditions, a 20-40 fold difference between thyroid free iodine concentrations and serum iodine concentrations can be maintained. NIS functions by utilizing the sodium gradient formed by the sodium-potassium ATPase. Two sodium ions are allowed through NIS to provide the energy needed to transport one iodine ion against its gradient. While NIS is essential to thyroid function, it should be noted that NIS is not exclusively localized to the thyroid, but exists elsewhere in the body including salivary and mammary glands.

After iodine is brought into the follicular cells by NIS, it is transported into the colloid. The colloid contains large amounts of iodine and protein, especially Tg. Colloid has been reported to contain protein concentrations as high as 100-400 mg/ml. The colloid is the location in which iodine is attached to Tg to create thyroid hormones. As such, it serves as a major site for hormone storage, and is responsible for maintaining constant circulating hormone levels despite periodic and erratic dietary consumption of iodine.

Organification of Iodide and Production of Thyroid Hormones

Mature Tg is a dimeric protein of approximately 660 kilodaltons (kD), and a sedimentation value of 19. The molecular weight of Tg depends in part upon the degree of iodination present. Iodination of Tg occurs only on tyrosine residues, and each tyrosine residue can form half of a completed thyroid hormone. Iodine is attached to Tg through a process involving thyroid peroxidase (TPO). TPO is a large heme-containing glycoprotein enzyme of approximately 105kD. The gene for TPO is located on human chromosome 2, and appears to have evolved separately from several other prominent human peroxidases, myeloperoxidase, eosinophil peroxidase, and lactoperoxidase.

Iodide must be oxidized before it can act as an effective iodinating agent. Only two known biological oxidizing agents, H$_2$O$_2$ and O$_2$, are potent enough to oxidize iodide. Thyroid peroxidase reacts with H$_2$O$_2$ to give an unstable oxoferryl porphyrin p-cation radical that reacts with protein tyrosine
or tryptophan and isomerizes to an oxoferryl protein radical. The porphyrin p-cation radical I catalyses a two-electron oxidation of iodide to hypoiodite, which iodinates thyroglobulin tyrosine to diiodotyrosine. The cation radical I then catalyses a one-electron oxidation of thyroglobulin diiodotyrosine to form phenoxy radicals that undergo coupling to form thyroglobulin thyroxine.\footnote{13}

In states of iodine sufficiency, the majority of thyroid hormones produced during iodination are T4, which is formed from the joining of two doubly-iodinated tyrosine residues. Tg is phagocytosed and returned to the follicular cells under TSH stimulation.\footnote{2} It is thought that this process results in “first in-first out” mechanics, as diffusion determines the position of a Tg molecule within the thyroid. As a result, the most recently iodinated Tg molecules are generally reabsorbed first. This phenomenon is of particular note when dealing with radioiodine, as it suggests ways to “flush” the radioactive material out of the thyroid through application of anti-thyroid agents such as methimazole (MMI), perchlorate, or several other compounds.

When Tg is reabsorbed by the follicular cells, the vesicles fuse with lysosomes. The lysosomal enzymes break down Tg and release mature thyroid hormones. The vesicles targeted for excretion from the cell send the mature hormones back into the bloodstream. A fraction of the T4 released undergoes deiodination prior to release, resulting in the T3/T4 ration of the secreted iodothyronines being higher than the ratio in the colloid. Once in the bloodstream, only a small fraction of the released hormones will circulate freely. Most will be bound to a number of serum proteins such as transthyretin (TTR), albumin, and thyroxine-binding globulin (TBG). The exact percentages usually bound to each protein vary from species to species. However, in most cases, albumin makes up the majority of the bound serum TH pool.\footnote{2} It should be noted that each iodothyronine type has different binding affinity for serum binding proteins which directly impacts their pharmacokinetic behavior.\footnote{14}

\textbf{Plasma Thyroid Hormone Binding and Pharmacokinetics}

As previously mentioned, three major thyroid hormone binding proteins exist in mammalian species. The relative concentrations and binding capacity of TBG, TTR, and albumin vary by species. As each of these three proteins have varying affinities for each thyroid hormone (affinities which again vary
by species), different species can have greatly differing serum clearance rates. Accordingly, the degree to which a species may tolerate insults to the thyroid gland is partially moderated by serum binding capacity and kinetics.

TTR was first identified from cerebrospinal fluid (CSF). TTR was originally known as prealbumin, and is also involved in Vitamin A binding by association with retinol binding protein. TTR is a 127-residue monomer which has been shown in human and chicken to function as tetramer of four TTR monomers. One TTR tetramer contains two identical hormone binding sites within the central channel. TTR is expressed in the liver and choroid plexus of eutherian mammals, marsupials, and birds, but only in the choroid plexus in reptiles. This has been interpreted as implying that the extrahepatic synthesis of TTR evolved first. However, TTR was found in the liver but not the choroid plexus of an amphibian and fish model. In humans, TTR is present in serum at approximately 300 mg/liter, with a binding capacity of 2300 µg/liter. TTR serves as the primary thyroid hormone binding protein in the CSF. TTR has different binding affinities between species, such as the preferential binding of T3 in birds and fish as opposed to T4 in human and rat. The preferential binding of TTR to thyroxine in eutherian mammals and its abundance in CSF combine to favor transport of T4 from blood to brain. There is an 82% identity and 92% similarity between human and rat TTR. In rats, TTR is the major T4 binding protein. The Kd for T3 and T4 are similar between rats and humans. In rats, the Kd for T3 is 67.2nM, and 8.0nM for T4. In humans, the Kd's are 56.6nM and 13.6nM respectively. The affinity and capacity would suggest that TTR would serve as a regulatory protein for free T4 fraction whenever thyroxine binding globulin is not present or present only in low quantities such as in rodents, or in the CSF of fish and mammals.

In humans, TBG binds approximately 75% of plasma T4. TBG is present in human serum at approximately 15 mg/liter, with a Kd of 0.1 nM. This small amount of protein binds approximately 600 nmol/l (200 µg/l) T4 in euthyroid adults. TBG is a member of the serpin family of protease inhibitors, but does not itself have inhibitory action. However, TBG retains a reactive site loop, which is
cleaved during sepsis. TBG mutations have been linked to several human thyroid disorders, with a prevalence of around 0.01% in newborn Caucasians.

The remaining major thyroid hormone binding protein is albumin. The gene encoding human albumin is located on Chromosome 4, mapped to regions q11-q13. The gene contains 15 exons, spanning over 16000 nucleotides on the chromosome. Albumin is a non-glycosylated globular protein, consisting primarily of alpha-helix structures. It makes up a significant portion of the total serum proteins, and is active in binding many classes of bioactive molecules, including steroids and thyroid hormones. Albumin has multiple distinct sites that bind TH, with varying binding kinetics. There is one primary binding site, and between 4-6 secondary binding sites. The primary binding site for T4 is shared by several other molecules, including L-tryptophan, oleate, and linoleate. In humans, the association constant for the primary site is $7.0 \times 10^5$ M$^{-1}$, and for the remaining sites, the constant is $4.8 \times 10^4$ M$^{-1}$. Many technical difficulties have been encountered while trying to determine the dissociation rate of TH from albumin.

In an average human (70kg), T3 secretion is approximately 33% that of T4, resulting from deiodination within the thyroid gland before secretion into the serum. However, the ratio in circulating serum pools is significantly different, with average values in humans around 1.35 ng/ml and 86 ng/ml T3 and T4 respectively. This is due to the much greater clearance rate for T3 than T4. These rates do not hold for all species, including rat. The turnover rate in rats is greater than that of humans, and the thyroidal reserve much smaller in proportion to the rat's needs.

In euthyroid rats, the average plasma T3 concentration is about 0.47 ng/ml and T4 about 36.4 ng/ml, about 40% of the concentrations in euthyroid humans. Rats have an average distribution volume of about 17 ml/100 g BW for T4 and 236 ml/100g BW for T3. Plasma clearance rates in rat are about 0.94 ml/h/100g BW for T4 and 21.3 ml/h/100g BW for T3. The T4 secretion rate is 38.3 ng/h/100g BW. The total body pool sizes for T4 and T3 are 702 and 100 ng/100g BW respectively. The rate of T3 production from T4 is about 6.77 ng/h/100 g BW. The tissue distribution of thyroid hormones varies between T3 and T4. 33.1% of T3 is found in the intestines, 2.3% in the kidneys, 8.7% in the liver, and
3.6% in the blood. 18.1% of T4 is found in the intestines, 1% in the kidneys, 8% in the liver, and 31.2%
in the blood.24

**Deiodinases**

While the majority of secreted thyroid hormones are in the form of T4, T4 has approximately
10% of the nuclear receptor-mediated activity of T3. Deiodination to T3 is required for full bioactivity
through this receptor-mediated mechanism. Recent studies have begun to show important roles for
3,5,3’-triiodothyronine (rT3) in neural development, such as in control of neuronal outgrowth and
migration, as well as actin polymerization.25 Deiodination is accomplished by three enzymes, known as
type 1, 2, and 3 deiodinase. Each of the three deiodinases have different affinities for the various thyroid
hormones, but all have the same basic action: removal of a single iodine molecule from a thyroid
hormone molecule. Thyroid hormone molecules contain two phenolic rings. Each ring contains up to
two iodine atoms. Type 1 deiodinase (D1) is primarily a 5’-deiodinase, but also has 5-deiodinase
potential. Type 2 deiodinase (D2) is exclusively a 5’-deiodinase. Type 3 deiodinase (D3) is exclusively a
5-deiodinase (Figure 2.2).

**D1**

Until the 1950’s, the only identified thyroid hormone was T4.26 After the identification of T3 by
Gross and Pitt-Rivers, it became clear that some process was able to remove iodine from T4, and that this
process was in a large part responsible for the circulating T3 observed.27,28 This process was soon found
to be inhibited by administration of the thionamide antithyroid drug propylthiouracil (PTU).29 Soon
thereafter, the enzyme was found to be localized to endoplasmic reticulum and plasma membrane
fractions.30 D1 has been determined to have primarily 5’-deiodinase activity, but also to have 5-deiodinase
activity under certain conditions.31,32 D1 has been localized to many tissues in the body,
including the liver, kidney, thyroid, placenta, and intestine. D1 has also been identified in the rat central
nervous system, but not that of the human.26 It should be noted that the deiodinases are selenoenzymes,
relying on a selenocysteine residue for much of their activity.26 D1 deiodination proceeds by ping-pong
kinetics involving the iodothyronine and a thiol cofactor.26 “Ping-pong” kinetics means that the
iodothyronine and thiol cofactor bind independently to D1, and each undergoes a half reaction. It is theorized that the first half-reaction deiodinates the iodothyronine and forms a selenoleyl iodide intermediate, which is subsequently reduced by the thiol cofactor. D1 has the greatest affinity for rT3, followed by 3', 5'-T2; 3, 3'-T2; 3'-T1; T4; and finally T3. At pH 7.2, the Km's for rT3 and 3', 5' – T2 are 0.1 and 0.77 µM respectively.

D1 is regulated by a variety of factors, including nutrition and thyroid hormones, such as fasting and the ratio of glucagon to insulin. D1 activity is increased at a transcriptional level by factors such as T3, retinoic acid, cyclic AMP, and TSH. D1 transcription is decreased by IL-1β, diabetes, and TNFα. In general, hypothyroidism reduces D1 activity whereas hyperthyroidism increases it. At a translational level, selenium deficiency reduces D1 activity due to the necessity of selenocysteine for proper functionality.

D2

The continued presence of 5'-deiodinase activity despite PTU inhibition in some brain preparations led to the identification of the Type 2- iodothyronine deiodinase. Like D1, D2 is a selenoenzyme, relying on selenocysteine for proper functionality of its active sites. D2 is a membrane-bound protein, attached to the endoplasmic reticulum, rather than the plasma membrane. Unlike D1, D2 has only been shown to have 5'-activity. D2 also contrasts with D1 in its relative affinities for the iodothyronines. D2 has a greater affinity for T4 than for rT3 with Km's of 0.5-3nmol/l and 1-10 nmol/l respectively. As with D1, D2 is found in a number of organs, including the CNS, pituitary, and brown adipose tissue. In the brain, D2 is found in astrocytes and tanyocytes. Interestingly, D2 also shows differences in expression patterns between rats and humans, with humans showing D2 activity in adult skeletal muscle, which was not expected after adult rat studies. D2 is especially important in the brain, where studies have shown it to be responsible for over 75% of the nuclear T3 in rat cerebral cortex. D2 activity peaks around PND15-20 in rats. In adult humans, D2 is the only remaining 5'-deiodinase present in the brain, again unlike the rat where both D1 and D2 remain expressed and active.
D2 has a relatively low Km for T4 of about 2nM, putting it several orders of magnitude lower than that of D1.\textsuperscript{42} D2 has a similar affinity for rT3 as for T4, though the overall kinetics favor T4 slightly.\textsuperscript{49} D2 also requires a thiol cofactor for activity. D2 deiodinase activity follows sequential reaction kinetics, which suggests that the TH substrate and thiol cofactor must be simultaneously bound to D2.\textsuperscript{42}

D2 is known to be regulated by thyroid status through both pre- and post-translational mechanisms, as well as by stress and other physiological alterations.\textsuperscript{50-56} D2 mRNA is upregulated under hypothyroid conditions, responding primarily to T3 mediated by nuclear receptors.\textsuperscript{37,54,45,58} However, photoperiod and other stress factors can also play a role in determining mRNA expression.\textsuperscript{59-61,52,62} On the posttranslational side, T4 is the driving force, though rT3 also plays a role.\textsuperscript{53} Posttranslational regulation includes targeting for ubiquitination, a process driven by the availability of T4 for D2.\textsuperscript{63,64,25} D2 is rapidly cycled under normal conditions, with destruction of D2 accelerated by availability of substrate.\textsuperscript{65-67}

The posttranslational regulation of D2 is essential to the rapid response of this enzyme to changing availability of thyroid hormones. Both \textit{in vivo} and \textit{in vitro} tests blocking gene transcription or protein synthesis with actinomycin D or cyclohexamide, respectively, have demonstrated rapid decreases in D2 activity when challenged with thyroid hormones.\textsuperscript{65,68,69} T4 and rT3 are far more potent than T3 in inactivating D2 in hypothyroid animals, further reinforcing the importance of the non-nuclear aspect of its regulation.\textsuperscript{68} One study looking at D2 mRNA and activity in rat cerebral cortex demonstrated an approximately 50% increase in D2 mRNA at the same time that the activity was increased by 500%.\textsuperscript{54}

Studies by Steinsapir et. al. of transiently expressed D2 in HEK-293 cells have helped to further elucidate the mechanisms underlying the posttranslational regulation of D2. When cyclohexamide and 50nM rT3 was added to cells transiently expressing D2, D2 activity dropped by 50% in four hours. However, in the presence of MG132, a specific inhibitor of the ubiquitin-proteasome pathway, this reduction was completely blocked. Further experimentation with 75-Se labeled D2 demonstrated that this reduction was in fact due to a 50% reduction in D2 protein, a reduction still blocked by administration of MG132. This data indicates that posttranslational regulation of D2 by rT3 occurs solely through
increased targeting for ubiquitin mediated proteolysis. A further experiment with a mutant D2 which changes one active site selenocysteine to alanine eliminates the deiodinative activity of D2, as well as its substrate-induced degradation. Overall, this data indicates that it is in fact the interaction with substrate which leads D2 to be targeted for ubiquitination and subsequent degradation.\textsuperscript{63}

**D3**

D3 is the primary 5-deiodinase, and is thought of primarily as an inactivating enzyme, though recent evidence is suggesting that rT3 has important physiological roles in neural development.\textsuperscript{70} D3 was first identified in a monkey hepatocarcinoma cell line.\textsuperscript{71} D3 is present in many organs, including the CNS, placenta, skin, and liver.\textsuperscript{72-76} It has been shown to be induced by TGFβ in a variety of human cell types, including fetal and adult fibroblasts from several tissues, hemangioma cells, fetal epithelia, and skeletal muscle myoblasts.\textsuperscript{77} The exact subcellular location of D3 is still uncertain, though it appears to join D1 and D2 as an integral membrane protein.\textsuperscript{78}

D3 catalyzes the 5-deiodination of T4, T3, and 3,3'-T2, though not the sulfoconjugates of these hormones.\textsuperscript{79} Similarly to D2, D3 requires an unknown thiol cofactor (though DTT works in vitro), and is insensitive to PTU inhibition.\textsuperscript{80} Another similarity to D2 is in the sequential kinetic pattern, as opposed to the ping-pong kinetics of D1, again suggesting coordinated and contemporaneous binding of the iodothyronine and thiol cofactor.\textsuperscript{81} D3 is upregulated by thyroid hormones, consistent with its perceived role as an inactivating enzyme.\textsuperscript{80,26,82} As with the other deiodinase enzymes, regulation is complicated and goes beyond only thyroid hormone stimuli.\textsuperscript{83-85} Like the other deiodinases, D3 is differentially regulated in different tissues.\textsuperscript{86,87}

**Conjugation and Other Modifications**

In addition to deiodination, peripheral metabolism of thyroid hormones also includes conjugation of the phenolic hydroxyl group of the outer ring with sulfate or glucuronic acid. This type of conjugation occurs primarily in the liver and kidney. The conjugation reactions inactivate the thyroid hormones, and prepare them for elimination.\textsuperscript{88,89} Glucuronides are stable conjugates, and are rapidly excreted in bile, and to some extent, urine.\textsuperscript{90} Glucuronidation is catalyzed by the enzyme UDP-glucuronosyltransferase, which
can be induced by a variety of compounds. Sulfation of the phenolic hydroxyl group blocks 5'-deiodination, but stimulates 5'-deiodination. Sulfates are rapidly deiodinated in the liver, and little are normally excreted intact in the bile or found in serum. T3 is a substrate for at least three phenol sulfotransferases. Thyroid hormones can also undergo deamination and decarboxylase reactions in the liver. This conversion results in deaminated acetic acid analogues of thyroid hormones. The analogues of T3 and T4 are known as triiodothyroacetic acid (Triac) and tetraiodothyroacetic acid (Tetrac) respectively.

**Effects of Thyroid Hormones**

**Overall Clinical Effects**

Clinical effects of thyroid hormones are best identified through the organismal changes observed in conditions of hyper- and hypothyroidism. In hypothyroidism, basal metabolic rate will decrease, meaning that overall energy production and consumption in the body are reduced. In humans, hair becomes coarse and sparse and the skin becomes dry and yellowish. Cold is poorly tolerated due to impaired thermogenesis. Mental function is slowed, and memory is impaired.

Hyperthyroidism, in contrast, leads an increase in basal metabolic rate, and weight loss is often seen. Nervousness results from increased sympathoadrenal output and secondary cardiovascular effects. Tremors can be found in extremities. Additionally, an intolerance to heat is noted, as thermogenesis is elevated even under warm conditions.

**Thyroid hormone receptors**

The first accepted cellular effect of thyroid hormones was action through nuclear receptors. The first demonstration of thyroid hormones stimulating RNA and protein synthesis came in the mid 1960's. Specific nuclear binding sites for T3 were first recognized in 1972. Further studies found a relationship between the amount of hormone present in nuclear preparations and the response generated. In the mid-1970's, it became apparent that there were more than one type of TH receptor (TR), though all types were believed to belong to the same family, and be conserved across species. Current understanding has evolved to recognize five current thyroid hormone receptor isoforms, belonging to two
primary isoforms and several variants. The genes were first identified in chick embryo and human placenta, and each species showed a different receptor.\textsuperscript{103,104} The gene coding for the chick isoform was designated Thra, and its product TR-\(\alpha\), and the human homologue was located on chromosome 17.\textsuperscript{105} The gene for the placental isoform was located on chromosome 3, and designated Thrb, with its product designated TR-\(\beta\).\textsuperscript{104} The thyroid hormone receptors have been classified into the nuclear hormone receptor superfamily, which includes receptors for vitamin D, retinoic acid, and several other hormones.\textsuperscript{106} Both of these genes have been identified in the rat as well.\textsuperscript{107,108} A second \(\alpha\) isoform was identified in humans in 1987.\textsuperscript{109} It was further found that there were two forms of the \(\alpha2\) isoform, which were denoted TR-\(\alpha2\)\(vI\) and TR-\(\alpha2\)\(vII\), or alternatively c-erbA\(\alpha\)-2.\textsuperscript{110,111,106} The \(\alpha2\) and \(\alpha1\) isoforms are created by alternative splicing of the TR\(\alpha\) mRNA, though the \(\alpha2\) form lacks the ability to bind T3 due to an alteration in the carboxy terminus.\textsuperscript{106,112,113} Interestingly, the noncoding strand of the Thra gene itself codes for Rev-Erb a related protein involved in adipogenesis.\textsuperscript{114,115} An additional \(\beta\) isoform, designated TR-\(\beta2\) was identified in a rat pituitary tumor line.\textsuperscript{116}

The thyroid hormone receptors act as ligand-regulatable transcription factors because they bind to both thyroid hormones and thyroid hormone response elements (TRE) in the genome.\textsuperscript{106} The TRs consist of several conserved domains. The central portion of the TR is a DNA-binding domain with two zinc finger motifs to intercalate with the major and minor grooves of the TRE.\textsuperscript{106} The TRs must dimerize to bind DNA, and have been shown to form heterodimers with related receptors, such as the retinoic acid receptor.\textsuperscript{117} In addition to the dimerization, co-activators and co-repressors can also bind to the TRs.\textsuperscript{118-122} The role of the various TR isoforms is not yet fully understood. In most cases, the effects seem to be interchangeable, with the exception of the \(\alpha2\) isoform.\textsuperscript{106} However, recent research has suggested that there may be some specificity for certain genes in the hypothalamus and pituitary, such as TRH, growth hormone, and TSH.\textsuperscript{123-125} Both the TR-\(\alpha1\) and TR-\(\beta1\) isoforms are expressed in almost all tissues, though the representation in rats skews towards the \(\beta1\) isoform in the brain, liver and kidney, and towards the \(\alpha1\) isoform in skeletal muscle and brown fat.\textsuperscript{126} Recent research has shown that unliganded TRs may play a
role in repression of basal expression of several genes, in conjunction with several corepressor proteins. 127-129

**Nongenomic actions of thyroid hormones**

In recent years, mechanisms of thyroid hormone action that do not involve transcriptional regulation have come to light. While these actions are still postulated to rely on receptors for mediation, the location and identity of these receptors varies from that of the genomic actions. 96 Many of these actions have been localized to specific tissues such as heart, brain, muscle, liver, and circulatory vessels. 130,25,131-138 It should be noted that the class of nongenomic actions does not preclude actions which may eventually have a genomic effect, such as the initiation of phosphorylation events which affect DNA-binding proteins. 139,140 Recent studies have even suggested the existence of thyroid hormone receptors among extracellular matrix proteins. 96

One of the first organ systems to earn consideration for nongenomic thyroid hormone effects was the cardiovascular system. 130 In the mid-1990's, it was shown that thyroid hormone could mobilize mitochondrial magnesium stores in the heart and liver of rat through interaction with the adenine nucleotide translocase. 137 T3 has been further shown to impact phosphorylation potential in sheep heart, in a mechanism involving a decrease in ADP concentrations. 141 T3 has been shown to have an acute effect on the inward rectifier potassium channel in guinea pig ventricular myocytes, decreasing the duration of action potentials. 142 This type of effect has also been shown in the rat, with effects including prolonged action potentials under hypothyroid conditions. 143 T3 has also been shown to increase the throughput of the Na+ channel in ventricular myocytes by increasing the fraction of channels in the open state at a given time. 144 T3 and T4 have been shown to have direct, rapid effects on arterial vasodilation in rat coronary artery. 138 Additionally, it has recently been shown that T3 can acutely increase the blood flow to some organs, such as the kidneys, through a vasodilative process. 145

Several mechanisms of nongenomic action have also been identified in the brain. Already mentioned is the effect of thyroid hormone on the degradation of D2. 25,65,146,147 Recently, another major mechanism has been elucidated regarding polymerization of actin, a mechanism which calls to light the
potential importance of rT3 in the brain. In hypothyroid PND14 rats, administration of rT3 or T4 served to rapidly decrease the proportion of G-actin in favor of F-actin, which may contribute to thyroid hormone's influence on arborization, axonal transport, and cell-cell contact in the developing brain. Notably, T3 did not have a similar effect. At the same time as the actin polymerization patterns were changing, D2 levels were rapidly returned to euthyroid levels within the span of only a few hours. It has been speculated that the cytoskeletal effects may help to regulate the degradation of D2 by attaching to the protein and removing it from its active location in the cell. A further nongenomic neurological effect of the thyroid hormones has also been identified in the hippocampus, where T4 and T3 have been shown to rapidly decrease the activity of NMDA receptors in hippocampal cultures. Additional effects have been found in the cerebral cortex, where T3 and T4 have been shown to have biphasic effects on the phosphorylation of several proteins in synaptosomal homogenates.

**Hypothalamus-Pituitary-Thyroid axis development**

It is important to consider not only the mature thyroid and its associated organs, but also to consider the development of the hypothalamic-pituitary-thyroid (HPT) axis. Due to the difference in developmental timescales between the rat and human, the absolute timing of events is far different between these species. The rat gestational period is approximately 21 days, and weaning traditionally occurs 21 days after birth. Thyroid hormones have been detected in rat embryos as early as GD9, refuting the old theory that thyroid hormones will not cross the placenta. Several studies have shown that the embryonic rat is highly sensitive to maternal thyroid disruption. Pituitary development begins around GD9.5, and secretory granules can be seen by GD16. The pituitary portal system is well developed by GD17. TSH mRNA can be found in the pituitary starting on GD15, and TSH protein can be isolated at GD17. TSH levels peak around PND11, and slowly decrease to adult levels between PND14 and PND40. The development of the hypothalamus takes place at a slower pace than that of the pituitary. The hypothalamus first becomes visible on GD12.5, but does not reach maturity, especially with regard to the vasculature, until about six weeks after birth. The rat thyroid is first visible on
GD9. In vitro cultures of rat thyroid cells are about to synthesize thyroid hormones at least as early as GD20. These developmental events are summarized in figure 2.3.

The human HPT axis develops at a much slower absolute rate than that of the rat. However, there are substantial parallels between the species, with events generally occurring at the same or similar relative developmental timepoints. The anterior pituitary begins to form from Rathke's pouch, which first appears in the fourth week of gestation. By the fifth week, the anterior pituitary connects to a derivative of the cerebrum which will become the posterior pituitary. Basophils, including thyrotropes and gonadotropes are detectable in the pituitary by the eighth week of gestation, and large numbers of thyrotropes are present by 12-13 weeks. The hypophyseal portal system develops around the same time, with capillaries detectable as early as the seventh week of gestation. TSH production is detectable by the twelfth week of gestation. Levels are relatively low until weeks 16-20, then peak around weeks 22-30, and decrease slightly until birth. The hypothalamus is visible by the fifth week of gestation. The hypothalamic nuclei are detectable at 12-14 weeks, and continue to mature through about week 35. The vasculature connecting the hypothalamus and pituitary is visible as early as 11.5 weeks. The thyroid gland begins to develop in the third week of gestation, visible around GD16. The thyroid gland tissues coalesce and migrate to their final location by GD45-50, and the gland is well-developed by the tenth week of gestation, by which time the gland accumulates iodine and produces thyroid hormones. These developmental events are summarized in figure 2.4.

**Brain Development**

Many aspects of neurological development rely at least in part upon thyroid hormone. During human pregnancy, there are several shifts in maternal thyroid regulation. Peripheral metabolism of thyroid hormones increase during pregnancy, and the thyroid compensates by increasing production. In most cases this results in increased total serum T3 and T4, but no alterations in serum free T4. During the early phases of development, the thyroid hormone requirement must be met from maternal hormone stores, with several negative consequences to maternal hypothyroidism. All of the thyroid hormone receptors have been identified in the human brain between 10 and 16 weeks of gestation, and
TRα1 and TRβ1 mRNA’s have been detected in the eighth week. In the rat, the TRβ1 and TRβ2 receptors have been detected at GD 12.5, and the alpha subtypes at GD14. After birth, receptor mRNA and protein levels increase until peaking at PND10 and PND15 respectively. This timing matches the time of eye opening in the rat, and is the time of full maturation of the HPT axis feedback loop.

The complexities of neural development are far from fully understood. However, certain portions of the developmental processes are better understood than others. This review will focus upon the basic development of the hippocampus. Much of the current knowledge regarding cell formation in the rodent hippocampus dates back to work in the 1960's and 1970's.

Stem cells for both the pyramidal neurons and granule cells originate from the ventricular germinal layers located below the ventricular wall along the CA1 area. Multiplying neurons directly migrate from the ventricular zone to their final target region. Pyramidal neurons in the rat are generated between day 16 of gestation and birth. CA3 pyramidal cell generation peaks at day 17, while CA1 generation peaks a day or two later. At birth, the pyramidal layer in the rat hippocampus is composed of 6-10 rows of neuronal somata, though in the larger adult hippocampus, this layer has thinned to two or three rows. In humans, the pyramidal layer is formed in the first half of pregnancy, with the CA1, CA2, and CA3 regions differentiated as early as gestational week 16. The formation of the granule cell layer of the dentate gyrus occurs later and lasts longer than the development of the pyramidal layer. In the rat dentate gyrus, the first granule cells appear a day after the first pyramidal neurons, and only 15% of the granule cells have developed by birth. This lengthened time frame also translates to human development. In humans, granule cell formation lasts more than 30 weeks, starting around the 13th week, and some evidence shows continued neurogenesis in the adult dentate gyrus. Disruption of the thyroid axis during any point of this developmental timeline will lead to abnormal development.
Thyroid Disruption

The hypothalamic-pituitary-thyroid axis is vulnerable to disruption at many points. A large number and variety of stress factors and exogenous compounds can disrupt the production, activity, and metabolism of thyroid hormones. Within the thyroid gland itself, there are several targets for disruption. Compounds such as perchlorate and thiocyanate directly inhibit the uptake of iodine into the thyroid.\textsuperscript{149} Amino acid deficiency or protein synthesis inhibitors can impair the production of Tg, thyroid peroxidase, or other vital proteins. Compounds like PTU, methylmercaptoimidazole (MMI), and iopanoic acid inhibit the incorporation of iodine into Tg. Disruption of the cytoskeleton can impair the phagocytic processes involved in reuptake of colloid, digestion of Tg, and export into the serum. Mutations in serum proteins can alter the binding kinetics of thyroid hormones, disrupting the normal equilibrium between bound and free hormone, altering the availability of hormones to peripheral tissues. Several other compounds will also compete with thyroid hormones for binding sites, such as lipids, polychlorinated biphenyls (PCB's), DDT, polybrominated biphenyls (PBB's), and many more. Disruption can also occur at the level of peripheral tissue uptake. Transporter proteins such as organic anion transporting polypeptide 1C1 (OATP1C1) and monocarboxylic acid transporter 8 (MCT8) are vital for proper uptake of thyroid hormones into the brain but transport more than just thyroid hormones. Even within the target tissues, interference with the thyroid hormone receptors will inhibit the activity of the produced hormones. Several agonists have been identified, including 3,5-dimethyl-4-(4-hydroxy-3-isopropylbenzyl)phenoxy acetic acid (GC-1), tetrac, triac, PCB105, PCB118, and many more.\textsuperscript{186,187} Several antagonists have also been identified or synthesized, including $O$-[4-hydroxy-3,5-diisopropylphenyl]-L-tyrosine, 3,5-dibromo-4-(3,5-diisopropylphenoxy)benzoic acid, {4-[4-hydroxy-3-isopropyl-5-(4-nitrophenyl)-benzyl]-3,5-dimethylphenoxy}acetic acid, and {4-[4-hydroxy-3-isopropyl-5-(4-nitrophenylethynyl)benzyl]-3,5-dimethylphenoxy}acetic acid, amiodarone, and bisphenol-A.\textsuperscript{188,189} Another form of disruption focuses on the metabolism of thyroid hormones. Compounds like propylthiouracil (PTU) and iopanoic acid will inhibit the action of some or all of the deiodinases. Several compounds including the PCB mixture Arochlor 1254, DDT, hexachlorobenzene, phenobarbital, and thiazopyrancan induce secondary metabolism
in the liver through mechanisms including interactions with the retinoic acid receptors, impairing sulfation and glucuronidation. 149

For most forms of disruption, the HPT axis has compensation mechanisms. The primary adjustment is the negative feedback pathway between thyroid hormones and the hypothalamus and the pituitary. A reduction in hormone availability to the hypothalamus and pituitary resulting in decreased negative feedback will result in increased synthesis and secretion of TRH, which will in turn trigger increased secretion of TSH, which will stimulate increased hormone production and export from the thyroid gland. The thyroid itself has been shown to respond to disruptions such as acute iodine excess. When plasma iodide concentrations are abnormally high, organification is reduced in the thyroid. 190 This phenomenon, known as the Wolff-Chaikoff effect, lasts for about two days before the thyroid escapes the effect through the downregulation of NIS mRNA and protein. 191 In conditions of prolonged hypothyroidism, the ratio of T3 to T4 produced by the thyroid will shift towards T3 as a result of both changes in the ratio in which these two hormones are produced as well as changes in deiodinase activity within the gland. 192 Different results are observed between various causes of hypothyroidism, such as thyroidectomy and iodine deficiency. In the peripheral tissues, deiodinase levels can be adjusted within only a few hours to compensate for increased or decreased availability of hormone. 54 There are limits to the amount of compensation possible by the thyroid and peripheral tissues. The thyroid is very efficient at sequestering iodine, even going so far as to drastically increase the size of the gland to the point of goiter, thus giving the original term for a thyroid disrupting compound, “goitrogen”. Even with glandular hypertrophy, the absolute iodine availability can still become insurmountable in extreme deficient situations. 193-196

Hypothyroidism and neural development

Proper functionality of the thyroid axis is essential for proper neural development. Iodine deficiency is recognized as being the most common preventable cause of mental retardation in the world. While the role of iodine was obscured for a time due to the inability of thyroid hormone supplementation after birth to reverse these changes, and the belief that thyroid hormones did not cross the placenta, it was
eventually understood that iodine's role came simply as a component of thyroid hormones.\textsuperscript{197} Developmental hypothyroidism caused by iodine deficiency has been shown to cause mental and growth retardation, rigid spastic motor disorders, and deaf mutism, and has affected millions of children around the world.\textsuperscript{1} It is estimated that more than a billion people in the world are at risk for consuming insufficient iodine.\textsuperscript{198}

**Thyroid Disruption and the Brain**

A substantial body of literature has developed around the investigation of the effects of hypothyroidism on the hippocampus and the rest of the brain. The hippocampus has received special focus because of its critical role in learning and memory.\textsuperscript{178} These investigations are made far more challenging by the varying regional responses to hypothyroidism, both in the impact on the concentrations of thyroid hormones, and the response to those hormones.\textsuperscript{199} Despite the currently primitive understanding of the complexities of neurological development, as steady stream of progress has been made in understanding the effects of developmental thyroid disruption, mainly in hypothyroid conditions. A wide range of effects have been observed, both transitory and permanent.

In 1979, Rabié demonstrated that the longitudinal growth, area, and volume of the hippocampus were reduced by hypothyroidism.\textsuperscript{200} In 1983, King found that the activities of 5'-nucleotidase and of 2',3'-cyclic nucleotide 3'-phosphohydrolase were reduced in hypothyroid rats, correlating with a delay in myelination.\textsuperscript{201} Dozin showed in 1984 that the hippocampus, cerebral cortex, olfactory bulb, and caudate nucleus had an increased nuclear binding capacity for T3, but a decrease in binding capacity with an increase in binding affinity in the pituitary.\textsuperscript{202} Rami found in 1986 that the arborization of the dendritic field of granule and pyramidal cells were impaired, and that not all of these changes could be overcome by thyroid hormone replacement.\textsuperscript{203} That same year, Rami also found that hypothyroidism did not change the production rate of granule cells, but that it did retard their migration, though this data was refuted in 1988, when Madeira demonstrated a reduction in granule cell numbers.\textsuperscript{204,205} Madeira further showed that hypothyroidism eliminated the bias towards greater granule cell numbers in male than female rats, suggesting that thyroid hormone levels may play a role in the sexual dimorphism shown in the rat
hippocampus. Madeira further found that the CA3 pyramidal cell layer was decreased in volume, combined with an increase in density and no change in cell numbers. In contrast, the CA1 region showed a decrease in pyramidal cell numbers between 13.2 and 22.5% at 30 and 180 days after birth for animals rendered hypothyroid from birth until sacrifice, a reduction that was not reversed after 150 days of recovery. Glutamatergic neurons were shown to be minimally responsive to neonatal thyroid deficiency in 1987, reinforcing the concept that thyroid hormone actions are cell-type specific. Neonatal hypothyroidism caused a marked retardation of the developmental patterns of choline acetyltransferase, a marker for cholinergic neurons, though the effect diminished with age in the hippocampus but not in the cerebral cortex and cerebellum. Glial cells were investigated by Rami in 1988, where it was found that hypothyroidism delayed the maturation of the radial glial fibers, as well as a reduction in glial fibrillary acidic protein (GFAP) labeled astrocytes later in development. This observation contrasts with observations in the cerebellum, where hypothyroidism leads to glial hypertrophy. Perinatal PTU administration was observed in 1988 to decrease the number of protoplasmic processes and end-feet in fibrous astrocytes in the cortical molecular layer, a reduction which remained even after a recovery period. Rami continued publishing in 1989, showing that hypothyroidism led to a delay in the arrival of cholinergic afferences, as well as a slight alteration in the early development of high-affinity vasopressin receptors. In 2003, Lavado-Autric investigated cell migration and cytoarchitecture in the somatosensory cortex and hippocampus of PND40 rats born to iodine deficient dams, and found a significant number of cells, including neurons, at abnormal locations. Farahvar took the morphometric analysis of the hippocampus to the next level, creating 2D foldout maps of the hippocampus. This analysis showed a decrease of 11% and 20% in hippocampal surface area in P25 and P90 hypothyroid rats, with the greatest reductions in the CA1 and CA4 regions. Rats allowed to recover for two months after weaning showed near complete return to normal sizes. This recovery was further confirmed by quantification of mitochondrial cytochrome oxidase staining.
synaptophysin immunostaining.\textsuperscript{215} The region-specific effects of hypothyroidism on GFAP were further elucidated in 1991, when Faive-Sarrailh demonstrated an increase of up to 78\% in GFAP mRNA in the cerebellum between birth and PND14, falling to 53\% of control levels by PND35.\textsuperscript{216} In contrast, the GFAP mRNA levels in the hippocampus were always reduced compared to control levels.\textsuperscript{216} Sandrini explored the responsiveness of neuronal receptors to thyroid status in the adult rat, and found that high doses of PTU for 35 days led to a decrease in prazosin, yohimbine, and dihydroalprenolol binding sites in the cerebral cortex.\textsuperscript{217} Imipramine binding was reduced in the cortex and hypothalamus, but increased in the hippocampus.\textsuperscript{217} In 1992, Savage demonstrated that 0.02\% PTU dosing through drinking water from GD18-PND31 led to a 75\% reduction in mossy fiber zinc density in the dorsal and ventral hippocampal CA3 regions at PND31, and a continued 33-45\% reduction after three months of recovery.\textsuperscript{218} Kulikov investigated the effect of hypothyroidism on the 5-HT1A and 5-HT2A receptors, as well as the serotonin transporter protein under an iodine deficient diet and with thyroxine replacement.\textsuperscript{219} Significant decreases were found in the binding of ketanserun to 5-HT2A receptors in the frontal cortex in the iodine deficient group, and the decrease was not present in the hormone replacement group.\textsuperscript{219} Vaidya studied the same receptors with an eye towards regulation of BDNF mRNA expression, and did not find any differences in basal expression under either hypothyroidism or chronic excess T3 conditions.\textsuperscript{220} However, acute T3 dosing did decrease dentate gyrus BDNF mRNA levels in a mechanism mediated by the 5-HT1A receptor.\textsuperscript{220}

In 1993, Rodriguez-Peña investigated the expression of myelin-associated glycoprotein (MAG) mRNA and protein.\textsuperscript{221} Expression of this protein is a biomarker of myelination of nerves. Hypothyroid rats demonstrated a delay of several days in the accumulation of MAG that was more severe in rostral regions of the brain, and recovered in later stages in more caudal regions.\textsuperscript{221} No differences in transcription of the MAG gene were observed, indicating that the mRNA differences found are a result of thyroid hormones affecting the stability of MAG mRNA.\textsuperscript{221} Íñiguez investigated RC3/neurogranin in 1993, finding that the normal accumulation of RC3 mRNA between PND5-12 was decreased by about 50-70\% in hypothyroid rats.\textsuperscript{222} This decrease could be quickly reversed by an administration of 10
micrograms of T4 on PND12, returning to normal levels or higher in 48 hours. Guadaño-Ferraz later showed that the differential sensitivity of some cells with respect to RC3 gene expression is not correlated with the distribution of thyroid hormone receptors in the brain. Navarro-Yubero reported on neuroserpin, a thyroid-responsive serine protease inhibitor shown to have a role in the regulation of anxiety in genetically modified mice. Neuroserpin mRNA levels were posttranslationally downregulated in hypothyroidism in the cortical II/III and V/VI layers, the hippocampus, the retrosplenial cortex, and the medial hemenular nucleus, but not in cortical layer V or other areas of the brain. Gilbert found that the PND21 offspring of dams treated from GD6-PND30 had diminished immunoreactivity of parvalbumin, a calcium binding protein in the hippocampus, which could be reversed by T4 administration. Additionally, a decrease in GABA-mediated inhibition of the perforant path-dentate gyrus synapse was found in adult offspring.

Alvarez-Dolado found in 1994 that hypothyroidism reduced nerve growth factor (NGF) mRNA by 20-50% in the cortex, hippocampus, and cerebellum at PND15 in neonatally hypothyroid rats, as well as in adult-onset hypothyroidism. Martínez-Galán investigated severe iodine deficiency and MMI administration with normal dietary iodine. Both treatments significantly decreased the proportion of glial cell fibers expressing GFAP, indicating impairment of maturation in cells involved in neuronal migration in the hippocampus. Vega-Núñez investigated the impact of hypothyroidism on mitochondria in the brain of neonatal rats, finding that there was a significant decrease in mitochondrial transmembrane potential in hypothyroid animals. This decrease could be reversed within 48 hours of administration of thyroid hormones. Electron microscopy revealed a decrease in the area of the inner membrane plus cristae in all areas studied, with the exception of the hippocampal CA1 neurons and nonneuronal cell types. Further investigations by Martínez showed a decrease in mitochondrial oxidative phosphorylation rate and mitochondrial transcription in the cerebral cortex and striatum, but not in the hippocampus, cerebellum, thalamus, mid brain, and brain stem.

Sawin investigated PTU doses from 5-25 ppm in drinking water from GD18- PND21 on the cholinergic neuronal system by measuring the activity of choline acetyltransferase (ChAT) and...
hemicholinium-3 binding to the high-affinity choline transmitter in the prefrontal cortex and hippocampus. ChAT activity was decreased in a dose-dependence manner, but hemicholinium-3 binding was elevated, though both returned to normal after a recovery period. In the same year, Alvarez-Dolado found that tenascin-C, an extracellular matrix glycoprotein involved in cell adhesion, migration, and neurite outgrowth, was upregulated in neonatal hypothyroid rats in several areas including the hippocampus, though downregulated in other areas such as the occipital and retrosplenial cortex. At the same time, the group investigated the reelin and dab1 genes, which are involved in neuronal migration and lamination. Hypothyroid rats showed decreased levels of reelin mRNA and protein between GD18 and birth. Reelin expression was increased in several areas by PND5, and returned to normal by PND15, though cells cultured from PND0 animals continued to demonstrate reduced reelin expression. In contrast, dab1 expression was increased at birth and decreased at PND5 in hypothyroid rats. Alvarez-Dolado investigated TAG-1, another cell adhesion molecule, and found an upregulation of TAG-1 mRNA and protein levels from GD20-PND15 in regions including the cerebral cortex, hippocampus, and olfactory bulb, and adding the cerebellum after PND15. Calloni investigated congenital neonatal hypothyroid rats, and found a 50% increase in ERK 1 and 2 phosphorylation, and a 50% decrease in p38 phosphorylation, showing significant impacts on the MAP kinase pathways.

In 2002, Vara reported on an investigation into short-term synaptic plasticity and neurotransmitter release in hippocampal slices. Hypothyroid rats displayed altered plasticity, and an increase in Ca\(^{2+}\)-dependent neurotransmitter release. Expression of synapsin I, synaptotagmin I, syntaxin, and alpha-Ca\(^{2+}\)/calmodulin kinase II was investigated in an attempt to elucidate the mechanism behind this alteration. Increased expression was found for synapsin I and synaptotagmin I in hypothyroid rats. In adult thyroidectomized rats, Lee found a decrease in the NR1 receptor mRNA in the hippocampus, as well as the NR2B receptor mRNA in the dorsal hippocampus. In 2004, Hoffman reported data on the effect of hypothyroidism on the Na\(^+\) current densities in cultured hippocampal and cortical cells. Treatment of these cells with thyroid hormones increases the rates of rise, amplitudes, and firing frequencies of action potentials. Kobayashi investigated synaptic gene expression and behavior in
hypothyroid rats with perinatal exposure to PTU, finding increased motor activity, decreased maze performance, and altered expression of GAP-43 and M1 mRNA during neuronal network formation.\textsuperscript{238} Oh-Nishi looked at dopamine D$_{2\text{-like}}$ receptor function in hippocampal slices, finding that the normal inhibitory effect upon glutamatergic neurons becomes an excitatory effect under hypothyroid conditions.\textsuperscript{239} In normal rats, this excitatory effect was seen in early ages, but transitioned to an inhibitory effect by adulthood.\textsuperscript{239} Kobayashi has shown a similar effect of hypothyroidism in delaying the switch from NR2B to NR2A subunits of the NMDA receptor in the rat cerebellum.\textsuperscript{240}

While many of these effects are transitory in nature, even an alteration in the timing of neurodevelopmental events can have devastating consequences. Even if no permanent alterations persist to adulthood, impaired neural function during the neonatal and juvenile phases pose significant problems to the ability to thrive in adverse conditions. Our understanding of neural development is still primitive, and the full consequences resulting from any minor alterations to this process are beyond our understanding. It is thus vital to focus even greater efforts on preventing exposure to thyroid disrupting compounds during the critical stages of neurological development.

**Persistent Effects of Developmental Hypothyroidism**

The impact of hypothyroidism continues to affect neurogenesis even in the adult rat. Ambrogini reports that the mitotic activity of the neural precursors is not decreased in adult-onset hypothyroidism, but that the survival of spawned cells was dramatically decreased, leading to a lower number of immature neurons being added to the granule cell layer, as well as a delay in differentiation of these new neurons.\textsuperscript{241} Desouza reinforces these observations by demonstrating that adult hippocampal progenitors expressed thyroid hormone receptors.\textsuperscript{242} Pacheco-Rosado demonstrated that adult-onset hypothyroidism generated with MMI decreased the activity of the Na$^+$/K$^+$ ATPase in the cortex, amygdala, and hippocampus, but not in cerebellum.\textsuperscript{243} Carageorgiou expanded upon this work with the inclusion of the Mg$^{2+}$ ATPase and acetylcholinesterase(AChE).\textsuperscript{244} In hyperthyroid adult rats, AChE activity was increased 22% in the hippocampus, while Na$^+$/K$^+$ ATPase activity was decreased by 47%.\textsuperscript{244} In hypothyroidism, AChE
activity was reduced by 23\% in frontal cortex, and increased 21\% in the hippocampus, while Na\textsuperscript{+}/K\textsuperscript{+} ATPase activity was decreased by 35\% and 43\% respectively.\textsuperscript{244}

Bruno investigated the effects of adult-onset hyper- and hypothyroidism on ATP, ADP, and AMP hydrolysis in rat hippocampal and cortical slices, finding that hyperthyroidism inhibited hydrolysis of all three molecules in hippocampal slices, but only of AMP in cortical slices.\textsuperscript{245} Hypothyroidism increased hydrolysis of all three molecules in both hippocampal and cortical slices, and these effects could be reversed by T4 replacement.\textsuperscript{245} Braganhol expands upon this work in a cell culture system in an attempt to determine the mechanism.\textsuperscript{246} Hippocampal, cortical, and cerebellar astrocyte cultures from neonatally hypothyroid rats were investigated.\textsuperscript{246} ATP and AMP hydrolysis are increased by 52 and 210\% respectively in cerebellar astrocytes, though T3 replacement reverses the increase in AMP hydrolysis.\textsuperscript{246} Studies have shown that ATP is directly involved in many neurological processes, including calcium wave propagation, promotion of association between neurons and glial cells, and induction of morphological differentiation and maturation of astrocytes.\textsuperscript{247,248} Constantinou investigated the effects of adult onset hypothyroidism on thyroid receptor isoform mRNA expression in the hippocampus, cerebral cortex, and cerebellum of the adult rat brain, finding a decrease of TR\textalpha\textsubscript{2} mRNA in all regions, an increase of TR\textalpha\textsubscript{1} mRNA in cortex and hippocampus, and no changes in the TR\textbeta isoforms.\textsuperscript{249}

A wide variety of neurological effects have thus been observed in gestational, neonatal, and adult-onset hypothyroidism. Whether the impact is on the structure of the brain, such as altered migration patterns; metabolism, such as ATP and AMP hydrolysis; or any other factor, it is clear that maintenance of normal thyroid hormone concentrations is vital to the proper development and function of the brain. Many impacts of hypothyroidism have been shown to have permanent effects, lending even greater urgency to the need to protect gestating females and their offspring from exposure to thyroid disrupting compounds.

**Electrophysiology**

The electrical nature of nerve signaling has been studied for the better part of a century.\textsuperscript{250} With the development of modern electronics, the electrical potentials generated by nerve cells can be monitored
in any scale from fractions of a cell to the entire brain. This capability has led to the use of electrophysiological recording to analyze the function of neurons. On a basic level, electrophysiology monitors the change in electrical potential either within a cell, across a membrane, or in the area surrounding a cell resulting from the opening of ion channels in membrane polarization or depolarization. When recording extracellular potential in multicellular samples, it is possible to determine the relative strength of responses to given stimuli. It has been shown that neural pathways can alter their responses within the course of an experiment, both in the positive and negative direction. These phenomena are known as long term potentiation (LTP) and long term depression (LTD) respectively. The term “synaptic plasticity” was coined in 1948, to describe the putative changes in synaptic efficacy thought by many to be the basis of information storage in the brain. A formal postulate of this putative phenomenon was advanced the following year by Donald Hebb.

Before the discovery of LTP, a prior model for learning known as post-tetanic potentiation (PTP) was discovered in 1947. PTP consisted of enhanced synaptic responses following a period of high frequency tetanization of afferent fibers. The main weakness of PTP was that it lasted only about seven minutes, making it far too short for permanent memory storage, though this was later increased to thirty minutes in 1966. That same year also saw the publication of data showing that the potentiation could be increased by increasing the stimulation frequency and using repeated tetani events. The discovery of true LTP, with a potential duration of weeks, occurred at the beginning of the 1970's. Originally called “long-lasting potentiation”, LTP gained its current name in 1975.

Electrophysiology in the Hippocampus

Two areas of the hippocampus are most commonly studied for electrophysiology. In vivo studies usually use the dentate gyrus, while slice studies most often use the CA1 region. Each type of experimentation allows testing of different parameters. Whole-brain studies have the distinct advantage of retaining all natural neural pathways for experimentation. In contrast, hippocampal slices isolate the regions of interest and remove external stimuli to the region of interest. Slice preparations also have the advantage of allowing rapid application of exogenous compounds to the slice during experimentation.
The usual pathways for studying LTP are the perforant path projection to granular cells of the dentate gyrus, and the Schaffer-commissural afferents to the CA1 area. LTP induced in the rat dentate gyrus in vivo has been observed to last for more than a year.178 

Quite a few prior studies have looked at alterations in LTP resulting from hypothyroidism induced in various ways and times, such as PTU, PCB's, MMI, and thyroidectomy. The results are somewhat confusing, and even contradictory at first glance. Pavlides studied adult rats that were made hypothyroid as neonates, and found that the previously hypothyroid animals had impaired LTP in the dentate gyrus, as well as impairment in spatial learning.259 Altman exposed rats prenatally to 3,3',3,3'-tetrachlorobiphenyl(TCB), and measured LTP in the visual cortex and hippocampus.260 LTP impairment was seen in the visual cortex, but not in the hippocampus.260 These observations were reinforced by further study with PCB-77 (coplanar) and PCB-47 (non-coplanar).261 PCB-77 replicated the impact of TCB, but PCB-47 had minimal effects.261 Gilbert examined Arochlor 1254, dosing dams with 6 mg/kg/day from GD6-PND21, and looking at in vivo electrophysiology in the dentate gyrus of 3-6 month old offspring, where a 50% reduction in LTP was seen in the dosed group.262 Further study was performed via behavioral assessment of the rats, with no obvious alterations.263 A later report also showed enhanced population spike and postsynaptic potential amplitudes in adult offspring.264 Altmann dosed rat dams with Arochlor 1254 starting 50 days prior to breeding.265 LTP was examined in cortical and hippocampal slices from PND10-20 pups, where a reduction in LTP was seen in the cortex, but not the hippocampus.265 Gerges looked at a combination of stress and thyroidectomy to alter in vivo early LTP (e-LTP) in adult rats.266 In the CA1 region, hypothyroidism and stress each impaired e-LTP, but the combination was sufficient to eliminate it, as opposed to the lack of effect seen in the dentate gyrus.266 Further study of thyroidectomized rats showed an impairment of LTP in the CA1, but not the dentate gyrus in in vivo electrophysiology.267 Still further study on these rats showed impaired performance in spatial memory paradigms.268 

Niemi looked at PTU dosage of rat pups starting at PND7, and continuing until sacrifice between the second and sixth week after birth.269 Serum T3 levels were reduced by over 80% after three weeks,
and LTP in the CA1 region of hippocampal slices was significantly reduced after two weeks. Vara used 0.02% MMI in drinking water from GD9 until weaning, finding a significant reduction in paired pulse facilitation, but no effect on LTP or LTD. Sui and Gilbert looked at 3 and 10ppm PTU from GD6-PND30, and found a decrease in paired pulse facilitation and LTP in CA1 slices. Another report under this paradigm showed enhanced LTP, increased baseline synaptic transmission, but reduced paired pulse facilitation, as well as increased phosphorylation of ERK1 and ERK2. The same dosing protocol led to impaired performance by adult offspring on the Morris water maze, as well as reduced LTP of the EPSP, but interestingly increased LTP of the population spike. A further study using 15ppm PTU from GD18-weaning found a decrease in in vivo fEPSP slope and population spike amplitudes, as well as impaired LTP. Additionally, there was a drastic decrease in circulating thyroid hormones and in pup growth. Dong used a gestational and neonatal iodine deficiency model, and found impaired in vivo population spike, fEPSP, and LTP, as well as decreased expression of c-fos and c-jun. Sui demonstrated that a 4-week dosing regimen of PTU in adult rats was sufficient to impair LTP and paired pulse facilitation of the dorsal hippocampo-medial prefrontal cortex pathway in in-vivo electrophysiological study. Goodman and Gilbert have found a cellular heterotopia in the corpus callosum of PND 23 rats from dams dosed with PTU from GD6, which persists to adulthood. The cells in this heterotopia were determined to have primarily originated as neurons between GD17-19.

Several studies have also looked at the acute application of thyroid active compounds to brain slices. Wong looked at acute slice application of PCB95 in adult rat, and found a decrease in CA1, as well as a decrease in population spike and EPSP potentials. Niemi followed suit in 1998, showing a decrease in LTP upon administration of Arochlor 1016, Arochlor 1254, and two specific congeners (the mono-ortho PCB congener 2,4,4,8-trichlorobiphenyl and the coplanar congener 3,3,4,4,8-tetrachlorobiphenyl, as well as a reduction in synaptic transmission at high doses of 1254. Gilbert also looked at acute slice exposure to Arochlor 1254, finding an increase in population spike amplitude in the CA1, and transient increase in EPSP amplitudes. Tang demonstrated the probable involvement of
thyroid hormone responsive protein (THRP) in LTP through the administration of a T3 injection into the dentate gyrus, which increased LTP and THRP mRNA.  

**Conclusion**

A wide range of detrimental neurological effects resulting from developmental hypothyroidism have been described. These effects range from severe physical and mental retardation to mild hyperactivity. It is clear that developmental hypothyroidism can cause permanent alterations in the functionality of the brain. At some dose/time combinations, clear alterations can be seen in the physical makeup of the brain, whereas in other combinations, subtle effects can be seen in biochemical analysis with no gross alterations. There is still no clear answer as to just how much of an insult can be tolerated by the developing thyroid axis before the onset of permanent damage. Further research is needed to probe the limits of compensation protecting the developing nervous system in order to understand the dangers of subtle changes in thyroid hormone metabolism and distribution, especially in the young and other sensitive subpopulations.

**References**


29. Oppenheimer JH, Schwartz HL, Surks MI. Propylthiouracil inhibits the conversion of L-thyroxine to L-triiodothyronine. An explanation of the antithyroxine effect of propylthiouracil and evidence supporting the concept that triiodothyronine is the active thyroid hormone. The Journal of clinical investigation. 1972 September;51(9):2493-7.

30. Hesch RD, Brunner G, Söling HD. Conversion of thyroxine (T4) and triiodothyronine (T3) and the subcellular localisation of the converting enzyme. Clinica chimica acta; international journal of clinical chemistry. 1975 March 10;59(2):209-13.


32. Fekkes D, Hennemann G, Visser TJ. One enzyme for the 5'-deiodination of 3,3',5'-triiodothyronine and 3',5'-diodothyronine in rat liver. Biochemical pharmacology. 1982 May 1;31(9):1705-9.


38. Jennings AS, Ferguson DC, Utiger RD. Regulation of the conversion of thyroxine to triiodothyronine in the perfused rat liver. The Journal of clinical investigation. 1979 December;64(6):1614-23.


76. Galton VA, Martinez E, Hernandez A, St Germain EA, Bates JM, St Germain DL. Pregnant rat uterus expresses high levels of the type 3 iodothyronine deiodinase. The Journal of clinical investigation. 1999 April;103(7):979-87.


98. Roodyn DB, Freeman KB, Tata JR. The stimulation by treatment in vivo with tri-iodothyronine of amino acid incorporation into protein by isolated rat-liver mitochondria. The Biochemical journal. 1965 March;94:628-41.


114. Lazar MA, Jones KE, Chin WW. Isolation of a cDNA encoding human Rev-ErbA alpha: transcription from the noncoding DNA strand of a thyroid hormone receptor gene results in a related protein that does not bind thyroid hormone. DNA and cell biology. 1990 March;9(2):77-83.


213. Farahvar A, Meisami E. Novel two-dimensional morphometric maps and quantitative analysis reveal marked growth and structural recovery of the rat hippocampal regions from early hypothyroid retardation. Experimental neurology. 2007 April;204(2):541-55.


263. Gilbert ME, Mundy WR, Crofton KM. Spatial learning and long-term potentiation in the dentate gyrus of the hippocampus in animals developmentally exposed to Aroclor 1254. Toxicological sciences: an official journal of the Society of Toxicology. 2000 September;57(1):102-11.


266. Gerges NZ, Stringer JL, Alkadhi KA. Combination of hypothyroidism and stress abolishes early LTP in the CA1 but not dentate gyrus of hippocampus of adult rats. Brain research. 2001 December 20;922(2):250-60.

267. Gerges NZ, Alkadhi KA. Hypothyroidism impairs late LTP in CA1 region but not in dentate gyrus of the intact rat hippocampus: MAPK involvement. 2004:40-5.


271. Sui L, Gilbert ME. Pre- and postnatal propylthiouracil-induced hypothyroidism impairs synaptic transmission and plasticity in area CA1 of the neonatal rat hippocampus. Endocrinology. 2003 September;144(9):4195-203.

272. Sui L, Anderson WL, Gilbert ME. Impairment in short-term but enhanced long-term synaptic potentiation and ERK activation in adult hippocampal area CA1 following developmental thyroid hormone insufficiency. Toxicological sciences : an official journal of the Society of Toxicology. 2005 May;85(1):647-56.


Figure 2.1 – Chemical Structures of T4, T3, and rT3
Figure 2.2 - Deiodination Pathways of T4, T3, and rT3

D1- Type 1 5'-Deiodinase
D2- Type 2 5'-Deiodinase
D3- Type 3 5-Deiodinase

T4- Thyroxine
T3- (3, 3', 5'-triiodothyronine)
rT3- (3, 5, 3'-triiodothyronine)
3, 3'-T2- (3, 3'-diiodothyronine)
Figure 2.3 – Timeline of Rat Neurological and Thyroid Axis Development

Reprinted with permission from Howdeshell 2002\textsuperscript{149}
Figure 2.4 – Timeline of Human Neurological and Thyroid Axis Development

Reprinted with permission from Howdeshell 2002¹⁴⁹
CHAPTER 3

LOWER THYROID COMPENSATORY RESERVE OF RAT PUPS FOLLOWING MATERNAL HYPOTHYROIDISM: CORRELATION OF THYROID, HEPATIC, AND CEREBROCORTICAL BIOMARKERS WITH HIPPOCAMPAL NEUROPHYSIOLOGY

__________________________

1Taylor MA, Swant J, Wagner JJ, Fisher JW, Ferguson DC. Published in Endocrinology 2008 Jul;149(7):3521-30

Copyright 2008, The Endocrine Society
Abstract

The developing central nervous system of the fetus and neonate are recognized as very sensitive to maternal or gestational hypothyroidism. Despite this recognition, there is still a lack of data concerning the relationship between thyroid-related biomarkers and neurological outcomes. We used propylthiouracil (PTU) administered at 0, 3, or 10 ppm in drinking water from gestational day 2 until weaning to create hypothyroid conditions to study the relationship between HPT axis compensation and impaired neurodevelopment. In addition to serum T3, T4, free T4, and TSH concentrations, cerebrocortical T3 concentration (cT3), hepatic Type I (D1) and cerebrocortical Type II (D2) 5'-deiodinase activity, and thyroidal mRNA for thyroglobulin (Tg) and sodium iodide symporter (NIS) were measured. Extracellular recordings from the CA1 region in hippocampal slices were obtained from both PND 21-32 (pups) and PND90-100 (adults) rats to assess neurophysiological effects. Thyroidal mRNA for Tg and NIS were increased in pups, but not in dams. Both PTU doses increased cerebrocortical D2 activity ~5-fold in pups, but only 10ppm increased D2 activity in dams. In dams, cT3 concentrations were maintained at 3ppm, but fell 75% at 10ppm. cT3 concentration in pups fell 50% at 3ppm, and over 90% at 10ppm. In both 3 and 10ppm pups, hippocampal baseline synaptic activity correlated negatively with cerebrocortical D2 activity. In 3ppm adults, impaired long-term potentiation was evident. In summary, during depletion of serum T4, D2 activity served as a sensitive marker of tissue thyroid status, an indicator of the brain's compensatory response to maintain cT3, and correlated with a neurophysiological outcome.

Introduction

The thyroid gland influences the function and development of many organ systems, including cardiovascular, skeletal, and nervous systems. Thyroid hormone receptors have been documented in the brain during the first trimester. Because of its many roles, maintenance of thyroid hormone levels during pregnancy is essential to the proper development of the offspring. A link between impaired maternal thyroid status and IQ decrement in human babies has been documented. Additionally, the recent increase in neurological disorders in the human population, such as autism and ADHD, has been
theorized to correlate with the increasing prevalence of endocrine (including thyroid) disrupting chemicals in the environment. Other well-documented outcomes of developmental hypothyroidism are hearing loss, altered migration of brain layers, delayed eye opening in rats, poor performance on maze tests, and impaired motor development. As the full range of detrimental neurodevelopmental effects of exposure to goitrogens is not yet understood, further investigation into the effects of well-characterized anti-thyroid compounds may assist the understanding of the impact of environmental goitrogens.

With respect to measures of impact on brain function, it is well established that disruption in the level of thyroid hormone during development can alter synaptic transmission in regions such as the hippocampal formation. Significant effects on long-term potentiation (LTP) have also been reported. LTP has been denoted as a cellular model for learning and memory processes; therefore, we and others have theorized that changes in LTP could provide a functional link between thyroid hormone deficits with deficiencies in cognitive function. In addition, prior studies have also demonstrated that inhibitory and excitatory pathways may be differentially impacted by thyroid insufficiency. Such complexities may contribute to the wide range of outcomes reported concerning the assessment of thyroid hormone deficiency on electrophysiological parameters measured in the hippocampus.

While significant animal research has been performed on thyroid toxicants, including studies of neurodevelopmental toxicity, thyroid status is most commonly determined by serum hormone concentrations such as total T4 and TSH. Many factors influence the relationship between serum and tissue levels of free and bound thyroid hormones, and the relationships may be tissue- and region-specific. PTU will have both dam-mediated and direct effects on the gestating pups, as it will cross the placental barrier. While PTU has been shown to have effects on the liver and immune systems, many of these effects have been demonstrated at doses well beyond those employed in this study. PTU has been routinely used to induce developmental hypothyroidism to study neurological impact.

The hypothalamic-pituitary-thyroid axis has a variety of mechanisms which would allow potential adaptation to a toxicological insult, including alteration of pituitary TSH secretion, alteration of intrathyroidal sodium-iodide symporter and thyroglobulin synthesis, change in deiodinase enzyme
activity, and altered thyroid receptor number or affinity. However, little is known about the threshold for triggering these changes nor about their compensatory limit. These issues become particularly critical to understand in subpopulations at greater risk from thyroid disruption, the developing fetus and neonate. The primary goal of the present study was to use gestational and lactational exposure to the mechanistically well described antithyroid drug, propylthiouracil (PTU) in the rat, to study serum, thyroid, and brain hormone markers of thyroid status in both offspring and dams. These parameters were then correlated with electrophysiology results measured in the CA1 region of hippocampal slices obtained from the offspring. This study identifies the biomarkers most tightly linking gestational and neonatal thyroid insufficiency with the altered neurophysiological outcomes measured in the hippocampus.

Materials and Methods

Animal acquisition, care, and dosing

Timed pregnant CD® IGS rats were obtained from Charles River Labs (Raleigh, NC). Rats were bred two days before arrival. Upon arrival, dams were housed individually and provided free access to Purina 5001 chow and tap water. Animals were maintained on a 12h/12h light/dark cycle. At approximately 1700h on the day of arrival, the water was replaced with either deionized water purified by Millipore RiOS 8 (Millipore, Milford, MA), or deionized water containing 3 or 10 ppm PTU (Sigma, St. Louis, MO). Doses of 0, 3, and 10 ppm PTU in DI drinking water were chosen to allow comparison of electrophysiological results with prior published studies (24). PTU dosing took place from GD2 until weaning, when pups selected for recovery were placed in standard housing with tap water and Purina 5001 chow.

Animals were weighed every other day, and water intake recorded. Water bottles were refilled every 2-4 days, depending on the amount remaining in the bottle. New PTU solutions were made at least every two weeks. Average water intake during gestation was approximately 200 ml/kg/day for the dams. Pups in the control and 3 ppm groups began to supplement their diet with food and water prior to weaning at varying times after PND14, but pups in the 10 ppm group were unable to do so due to reduced size and coordination. Pups were sacrificed beginning on PND21, with two males per litter sacrificed over the
next eleven days. Data from these animals is presented as “PND25” pups. Additionally, all female pups were culled at PND23. Remaining males were weaned at the end of this sacrifice period (on PND32) and followed to PND90-110 referred to hereafter as “adults.” In this study, with this weaning protocol, the weaned 10ppm pups were so physically and neurodevelopmentally delayed that it influenced their ability to independently seek adequate food and water. Despite attempted supportive measures, it was not possible to consistently maintain these animals for study in the PND90-110 adult grouping.

Animal studies were approved by the University of Georgia Institutional Animal Care and Use Committee and were in accordance with procedures outlined in the National Institutes of Health Guidelines for Care and Use of Laboratory Animals.

*Sacrifice protocol*

Male pups were weighed, anesthetized with halothane and sacrificed via decapitation. Blood, thyroid, and liver were collected. The brain was dissected for tissue collection and slices prepared for electrophysiology experiments (see below). In all pup and adult samples, the majority of the hippocampus was used for slice preparation. Cortex samples were taken from areas of the brain not impacted by dissection of the hippocampus, generally including prelimbic, motor, sensory, and cingulate regions, and brain tissue samples were divided equally between deiodinase and hormone assays. Liver and cortex samples intended for deiodinase assays were homogenized on the day of sacrifice in a 250mM sucrose, 20mM KH2PO4, 1mM EDTA, 20mM DTT buffer, then stored in aliquots at -80°C until assayed. Blood was allowed to clot and serum collected after centrifugation. All other tissues were flash-frozen and stored at -80°C until analysis.

Biochemical Assays:

*Serum Thyroid Hormone and TSH Radioimmunoassays:*

T3 and T4 radioimmunoassays were validated for rat serum as previously described, with CV’s of approximately 12% and 5% respectively. TSH was measured in dams with a commercial immunoassay kit (MP Biomedicals, Solon, OH). Free T4 concentrations were measured by use of a two-step direct dialysis commercial radioimmunoassay kit (Nichols Institute, San Juan Capistrano, CA) with CV
approximately 5%. All samples for serum hormone quantification were run in a single assay for each hormone.

**Thyroid RNA extraction and analysis:**

Thyroglobulin (Tg) and the Sodium Iodide Symporter (NIS) were selected for mRNA analysis because they are, at least partly, TSH-dependent. Thyroids were extracted by use of the Stratagene Absolutely RNA kit (Stratagene, La Jolla, CA). After extraction, real-time RT-PCR was performed for NIS and Tg at the UGA Functional Genomics Resource Facility using Taq-Man primers and probes (see Table 3.1).

**Protein assays:**

All deiodinase samples were homogenized on the day of collection in 250mM sucrose, 20mM KH2PO4, 1mM EDTA, pH 7, and frozen in aliquots at -80°C until assay. Protein concentrations were determined by the Bio-Rad protein assay reagent (Hercules, CA) using BSA as a standard.

**Cerebrocortical Type II 5'-Deiodinase Activity:**

This protocol was based on a modification of that of Leonard. Cerebrocortical Type II 5’D activity was assayed using 2nM rT3, including 1.9MBq [125I]-3',5'-3'-triiodothyronine (reverse T3) in 100mM KH2PO4, 1mM EDTA, 20mM DTT, pH 7 with and without 1mM PTU. Triplicate tubes were incubated at 37°C for one hour with 50µg protein for pups, or 200µg for dams and adults, in a total volume of 320µl, along with tissue-free blanks. The reaction was stopped by addition of 150µl ice cold 10% BSA. Tubes were then incubated 30 minutes at 4°C before addition of 500µl of 20%TCA. The tubes are spun at 2600xg at 4°C for 45 minutes. 400µl of supernatant is then added to a 1.5ml column of 50WX2-200 resin (Sigma, St. Louis, MO) and eluted with 2ml 70% acetic acid. Tubes were then counted on a gamma counter (Wallac Wizard 1470) along with tubes for total counts. The percentage of free iodide was calculated by comparison of the elution fractions to the total counts. The radioactivity in the eluted fractions minus that of the blanks was used to calculate the total deiodinase activity. The percent free iodine generated in the samples containing PTU was multiplied by 2 to account for the equal
possibility of liberating a radioactive or nonradioactive iodide, then divided by the time and protein amount and then multiplied by the total rT3 concentration in the assay to arrive at the final activity measurements. The D2 activity was shown to be inhibited by iopanoic acid and excess of rT3 (data not shown).

**Hepatic Type I 5′-Deiodinase Activity:**

Liver type 1 deiodinase (D1) assays were carried out using ~2nM rT3 in 200mM KH2PO4, 1mM EDTA, 2mM DTT, pH 7 with and without 1mM PTU. Triplicate tubes were incubated at 37°C for 2 and 12 or 62 minutes (PTU-treated animals) with 2-3 μg protein in a total volume of 120μl. The reaction was stopped by addition of 500μl ice cold 10% calf serum and then incubated for 30 minutes on ice. 500μl of ice cold 10% TCA was then added to precipitate the reaction tubes. The tubes were centrifuged at 2590xg 4°C for 10 minutes. 500μl of supernatant was placed into a separate tube and counted alongside the pellet. The percent free iodine was calculated by multiplying by a factor of 2 to account for liberation of nonradioactive iodine, dividing the supernatant activity by that of the pellet and multiplying by 2.24 to account for volume. The percentage of free iodine above that of the 2-minute point was used to calculate the total deiodinase activity.40

**Cerebrocortical T3 extraction:**

Frozen cerebrocortical tissue was homogenized in cold 100% methanol containing 1mM PTU with ~0.001 μCi [125I]3,5,3’-T3 tracer. The homogenate was centrifuged for 15min at 2000xg and the supernatant fluid was collected into another tube and kept on ice. The pellet was resuspended in methanol and centrifuged again. The supernatants were pooled and passed over an AG 1-X2 column (Bio-Rad, Hercules, CA). The columns were washed and then eluted with 3ml 70% acetic acid.23 The elution fractions were lyophilized and resuspended in 50mM KH2PO4, 0.25% bovine serum albumin before counting in a gamma counter to determine recovery. Average recovery for this procedure was 57%. T3 concentrations in the extracts were determined by specific RIA as with the serum T3 assay. The
determined concentration was divided by the product of the extraction efficiency, the proportion of the extract tested, and the initial mass of the tissue to give the final tissue concentration.

**Electrophysiology:**

In general, the electrophysiological procedures and analysis were conducted as previously reported, except as noted. 42

**Hippocampal Slice Preparation:**

Freshly prepared hippocampal slices (500µm) were obtained following anesthesia and decapitation. Horizontally cut slices were dissected in ice cold, oxygenated (95% O2/5% CO2) dissection artificial cerebrospinal fluid (aCSF) containing (mM): NaCl (120), KCl (3), MgCl2 (4), NaH2PO4 (1), NaHCO3 (26), and glucose (10). Slices recovered for one hour in an oxygenated interface holding chamber with standard aCSF containing (mM): NaCl (120), KCl (3), MgCl2 (1.5), NaH2PO4 (1), CaCl2 (2.5), NaHCO3 (26), and glucose (10)). Slices were then transferred to a submerged recording chamber and recovered for an additional hour at 30° C with continuously perfused standard aCSF saturated with 95% O2/5% CO2 at approximately 1ml/min before experiments were begun.

**Extracellular recording:**

Extracellular recording electrodes were placed in the stratum radiatum of CA1. Field excitatory post-synaptic potential (fEPSP) responses were evoked with a bipolar stimulating electrode placed on either the CA3 or the subicular side of the recording electrode in the stratum radiatum. Stimulus pulses consisted of a single square wave of 270 µsec duration delivered at 60-160 µA.

**Data acquisition and analysis:**

Data were digitized at 10 kHz, low pass filtered at 1 kHz, and analyzed with pCLAMP9.2 software (Molecular Devices, Sunnyvale, CA). The initial slope of the population fEPSP was measured by fitting a straight line to the first millisecond of the fEPSP immediately following the fiber volley. Stimulus response curves were performed at the beginning of each experiment. Baseline stimulation pulses of an intensity that gave 40-60% of the maximum response were given at a frequency of .05 Hz for
the entire length of the experiment. Synaptic responses were normalized by dividing all slopes by the average of the 15 fEPSP slopes 5 minutes pre-tetanus.

*Long Term Potentiation induction:*

The LTP high frequency stimulation protocol consisted of a single 100 Hz train of 1 second duration.

*Statistics:*

All statistical analysis was performed using SAS (The SAS group, Cary, NC). All group comparisons were performed using ANOVA, followed by Duncan’s multiple range test. \( p<0.05 \) was taken as the level of significance.

**Results**

1) **Serum hormones (Table 3.2)**

*Dams*

Serum total T4 concentrations were reduced by 60% at 3ppm and over 90% at the 10ppm dose in the dams (\( p=0.001 \)). Free T4 concentrations were also reduced by a similar amount, and were undetectable at the 10ppm dose (\( p=0.001 \)). Serum T3 concentrations were unchanged at 3ppm, but dropped to about 25% of control values at 10ppm (\( p=0.03 \)). TSH concentrations were greatly increased at both 3 and 10ppm doses (\( p=0.001 \)).

*PND25 pups*

Serum total T4 concentrations were reduced by 75% at 3ppm and over 90% at the 10ppm dose in the pups, with most of the 10ppm samples below the limit of detection (\( p<0.0001 \)). Serum T3 levels were reduced by 20% at 3ppm and by 50% at 10ppm (\( p=0.005 \)). Individual free T4 concentrations were not obtained due to limited serum volumes available from pups, but pooled samples indicated a dose-dependent decline in free T4 concentration, with undetectable levels at 10ppm. This change paralleled that of total T4 concentrations.
**PND100 adults**

In 3 ppm dosed pups allowed to recover for approximately two months after weaning, all serum concentrations of T3 and T4 returned to control levels.

2) **Body weight (Figure 3.1)**

Almost from birth, the mean body weights of the 10ppm pups were significantly reduced in comparison to the undosed pups. In addition to the low body weights, a delay in eye opening and decrease in overall physical coordination was observed (data not shown). At weaning, the 10ppm pups were unable to reach food and water in standard rat cages, so special housing arrangements were provided. While the 3ppm pups were not as affected as the 10ppm pups, they also weighed less than controls, with the difference becoming significant around PND18, and expanding as the pups aged until weaning.

3) **Liver Type 1 5’-deiodinase activity (Figure 3.2)**

Hepatic Type 1 5’-deiodinase activity was evaluated to confirm relative bioavailability and activity of orally administered PTU. In both the dams and pups, liver D1 activity was significantly reduced at both 3 and 10ppm. In the pups, D1 inhibition was more significant at the lower dose with 90% reduction at 3ppm, and a near-complete reduction at 10ppm (p<0.0001). The dams were less affected, with a 60% reduction at 3ppm, and a 95% reduction at 10ppm (p=0.0001).

4) **Thyroid mRNA (Figure 3.3)**

* Sodium Iodide symporter (NIS)*

In the pups, thyroid NIS mRNA levels were increased to about 250% of control at both the 3 and 10ppm groups (p=0.0001). No significant differences were noted in the dams, though there was a trend towards an elevation at 3ppm (p=0.09).

*Thyroglobulin (Tg)*

In the pups, thyroid Tg mRNA levels were increased significantly at both the 3 and 10 ppm doses (p=.014). The levels in the 3ppm dosed animals were about 300% of controls, whereas the levels in the
10ppm dose group rose to almost 1400% of control values. No significant alterations in Tg levels were detected in dams.

5) **Cerebrocortical thyroid parameters (Figure 3.4)**

**D2 activity**

In the pups, cerebrocortical D2 activity was significantly increased at both 3 and 10ppm, increasing to about six-fold that of control values (p<0.0001) with no significant difference between doses. In the dams, a slightly different pattern was observed. There was a tripling in D2 activity at 3ppm, and a further increase to 10 times control values at 10ppm (p<0.0001). However, the observed baseline and maximal activity in the dams was about 10% of the activity in the pups. D2 activity was normalized in 3ppm adults.

**Cerebrocortical T3 concentration**

In the pups, cerebrocortical T3 (cT3) levels were significantly reduced at both 3 and 10ppm (p=.0004). At 3ppm, the levels dropped by about 50%. At 10ppm, cerebrocortical T3 levels were about 12% of control levels. As with D2, the dams were less affected by 3ppm PTU, with no significant changes in T3 levels. However, at 10ppm, the cT3 of the dams was reduced to about 20% of control levels. Cortical T3 concentrations were normalized in 3ppm adults.

6) **Baseline synaptic transmission is impaired in hypothyroid pups (Figure 3.5)**

Extracellular recordings of the field excitatory postsynaptic potential (fEPSP) response were evoked from the stratum radiatum layer of the CA1 region of the hippocampus. The magnitude of the baseline synaptic transmission was significantly reduced (p=0.0002) in hippocampal slices obtained from pups (Fig. 5A&D). The maximal slope of the fEPSP was reduced by approximately 50% in both the 3 and 10 ppm dose groups, with no significant difference between these two conditions.

A normalized graph of the stimulus-response data shows no significant shift in the sensitivity of the synaptic response at either dose group (Fig. 5B). Paired-pulse analysis of the fEPSP indicated that there was no effect of PTU exposure on presynaptic function, as the paired-pulse facilitation ratios were not affected. The combined results from Figs 5A and 5B suggest that while the absolute magnitude of the
synaptic response was decreased by PTU treatment, the sensitivity of this response was not impacted, nor was the function of the presynaptic nerve terminals (Fig. 5C).

7) **Long-term potentiation is impaired in recovered adults (Figure 3.6)**

Unlike the results observed in PND21-32 animals, hippocampal slices prepared from littermates allowed to mature in the absence of PTU from weaning until PND90-110 did not exhibit any persisting change in baseline synaptic transmission (Fig. 6A). However, a significant impairment in the magnitude of long-term potentiation was observed in the 3ppm adult group (Fig. 6B), suggesting that there may be some consequences of PTU exposure during development that persist following recovery to the euthyroid state.

8) **Correlation between cerebrocortical D2 activity and serum total T4 concentration (Figure 3.7)**

In both the dams and pups, a highly significant exponential correlation was observed between cerebrocortical D2 activity and serum total T4 concentrations. As there was no evidence for an alteration in the fractional free T4 percentage with this dosing paradigm, the correlation of cerebrocortical D2 is most likely to be with serum free rather than total T4 concentrations. As D2 is known to be both pre- and post-translationally regulated by thyroid hormones, this correlation was not unexpected.

9) **Correlation of synaptic response to cerebrocortical D2 activity, cerebrocortical T3 concentrations, and serum total T4 (Figure 3.8)**

Several prior studies have shown links between thyroid status and electrophysiological activity. We identified correlations between the maximal synaptic response in pups and three thyroid-related parameters. Linear correlations were observed between synaptic response and cortical T3, as well as serum total T4 concentrations. Interestingly, and never before reported, a significant correlation was observed between cerebrocortical D2 enzymatic activity and maximal synaptic response, supporting the use of D2 activity as a marker of tissue thyroid status in the brain.

**Discussion**

Developmental thyroid disruption with PTU was initiated at gestational day 2 and maintained until weaning. This protocol was designed to ensure that maternal thyroid hormone production was
compromised prior to the development of fetal thyroid function around GD17. This window of dosing began earlier than that of most prior developmental studies performed in the rat with this compound.

16,17,19,21,24,29,30,45

It is noteworthy that cerebrocortical D2 activity increased in PND25 pups to maximal levels (~6-fold) even at the 3ppm dose, a point at which cerebrocortical T3 concentrations had fallen by 50%. No further increase was seen in D2 at the 10ppm dose, yet cerebrocortical T3 concentration fell further to 12% of control levels. In the dams, a tripling of cerebrocortical D2 appeared to allow maintenance of cerebrocortical T3 concentrations at the 3ppm dose, but despite a ten-fold increase in D2 at the 10ppm dose, the cerebrocortical T3 concentrations fell to 20% of control. Therefore, based upon a maximal stimulation of D2 activity at 3ppm PTU, and reduction of cerebrocortical T3 at this dose, the brains of pups were more severely impacted than those of the dams which were not impacted until the 10 ppm dose. Recordings of the fEPSP response in the CA1 region of hippocampal slices revealed significantly decreased basal synaptic transmission in both the 3 and 10ppm pups, indicating the presence of a functional impairment in neuronal signaling as compared with 0ppm control animals. Although we cannot point to a specific deficit based on extracellular recording results alone, the lack of effect on either the sensitivity of the fEPSP or on paired-pulse facilitation suggests that neither postsynaptic responsiveness nor presynaptic release were primary underlying factors. As the fEPSP recorded in the stratum radiatum reflects a “population synaptic response”, one remaining possibility is that the synaptic density was decreased in these PND25 pups. A prior report consistent with this speculation describes a decrease in CA1 pyramidal cell density in hypothyroid rats.43

Cerebrocortical D2 activity was found to be a sensitive respondent to reductions in serum T4 concentrations. While this study focused on total T4, showing parallel changes in free T4 concentration in pooled samples from pups, the true correlation is undoubtedly to the unbound free T4 concentration. Both the dams and pups had a strong exponential correlation between declining T4 and increasing cerebrocortical D2 activity (Figure 3.7). D2 is known to be regulated both pre- and post-translationally
by T3 and T4 respectively. One recognized method of post-translational regulation by T4 is control of the rate of ubiquitination, and thus degradation of D2.\(^{44}\)

In addition to a correlation with serum T4 concentrations, D2 activity was also found to correlate with an important functional endpoint. A strong correlation between cerebrocortical D2 activity and the maximal synaptic response in pups was observed (Figure 3.8). Synaptic response also correlated positively with serum T4 and cerebrocortical T3 concentrations, although neither of these relationships were as strong as the correlation with D2 activity (Figure 3.8). Whether representative of a mechanistic link or reflective of a co-dependence, the correlation between D2 activity and synaptic potential is not fully understood at this time, but D2 and cT3 concentrations did recover in 3 ppm pups as they returned to euthyroidism at PND 90-110, along with the baseline fEPSP response. Nonetheless, D2 activity did reflect the degree of availability of T4 from serum to cerebrocortical tissue at the time of sacrifice.\(^{37}\)

Analysis of thyroid mRNA levels for Tg and NIS, chosen to be markers of serum TSH bioactivity, further demonstrated the greater sensitivity to PTU in pups compared to their dams. The upregulation of these genes reflect an attempt to compensate for diminished hormone production. The lack of significant upregulation in the dams, compared to the pronounced upregulation in pups at both doses, further reinforces the hypothesis that the developing rat is more sensitive to thyroid disruption than is the adult rat. If this observation is paralleled in other mammalian systems, it reinforces the need to consider juveniles as a hypersensitive subpopulation with regards to thyroid disruption.

Although the serum and tissue thyroid hormone concentrations returned to normal after weaning, the LTP data obtained from littermates approximately 60 days after weaning demonstrated that some of the effects of treatment could not be reversed by the removal of PTU at weaning. This observation supports the conclusion that persisting changes can occur in the brain following a developmental insult, possibly involving alterations in myelination and migration patterns of neurons.\(^{14,23,25-27}\) An interesting aspect of our results was that the deficit in baseline synaptic transmission recovered concomitantly with the hormone levels, as expected from the relationships illustrated in Figure 3.8. Despite this, our LTP data suggest that the PTU-treated rats retained a neuronal deficiency from which they did not recover over
the time frame assessed in our studies. Thus in contrast to LTP of the population spike response recorded from the dentate gyrus, an impaired capacity for synaptic plasticity of the fEPSP in the CA1 region may be predictive of the well established cognitive impairments which persist following recover to the euthyroid state.\textsuperscript{15}

While PTU exposure is not a major environmental concern, we have attempted to use this drug as a model goitrogenic compound. PTU targets thyroid secretion and tissue Type I 5’-deiodination, with mechanistic effects common to dietary and environmental goitrogens, including apigenin and luteolin, and mimics the T4 lowering effects of some PCBs, PBDEs and atrazine.\textsuperscript{46} Therefore, the results from this study should be applicable to other compounds which disrupt the thyroid axis. Data gathered from this study provide a correlative if not causative link between serum (free) T4, cerebrocortical D2 activity and a neurophysiological consequence in hypothyroid rats. Such relationships could become the basis for a predictive model of developmental thyroid disruption with regard to neurological outcomes.

References

2. Howdeshell KL 2002 A Model of the development of the brain as a construct of the thyroid system. Environ Health Persp 110:337-348
7. Colborn T 2004 Neurodevelopment and endocrine disruption. Environ Health Persp 112:944-949

10. Porterfield S 1994 Vulnerability of the developing brain to thyroid abnormalities: environmental insults to the thyroid system. Environ Health Persp 102:125-130


15. Gilbert ME, Sui L 2006 Dose-dependent reductions in spatial learning and synaptic function in the dentate gyrus of adult rats following developmental thyroid hormone insufficiency. Brain Res 19:10-22

16. Gilbert ME, Mundy WR, Crofton KM 2000 Spatial learning and long-term potentiation in the dentate gyrus of the hippocampus in animals developmentally exposed to Aroclor 1254. Toxicol Sci 57:102-11


19. Gilbert ME 2004 Alterations in synaptic transmission and plasticity in area CA1 of adult hippocampus following developmental hypothyroidism. Dev Brain Res 31:11-8

20. Gilbert ME, Crofton KM 1999 Developmental exposure to a commercial PCB mixture (Aroclor 1254) produces a persistent impairment in long-term potentiation in the rat dentate gyrus in vivo. Brain Res 850:87-95


24. Sui L, Gilbert ME 2003 Pre- and postnatal propylthiouracil-induced hypothyroidism impairs synaptic transmission and plasticity in area CA1 of the neonatal rat hippocampus. Endocrinology 144:4195-4203


30. Sui L, Anderson WL, Gilbert ME 2005 Impairment in short-term but enhanced long-term synaptic potentiation and ERK activation in adult hippocampal area CA1 following developmental thyroid hormone insufficiency. Toxicol Sci 85:647-56


46. Zoeller RT 2007 Environmental chemicals impacting the thyroid: targets and consequences. Thyroid 17:811-817
Table 3.1: Primers and Probes for Real Time-Reverse Transcriptase PCR analysis of thyroidal mRNA

<table>
<thead>
<tr>
<th></th>
<th>Forward</th>
<th>Reverse</th>
<th>Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tg</td>
<td>TGAGTGGTGCC</td>
<td>GCAATAGAACGTA</td>
<td>TCCTGCCCACCCA</td>
</tr>
<tr>
<td></td>
<td>AGATGGGATAT</td>
<td>GGAGTCCAGAGT</td>
<td>GAATCAAGGAAC</td>
</tr>
<tr>
<td>NIS</td>
<td>CAGCCTCGCT</td>
<td>ACCGGCTCC</td>
<td>CCGGATCAACCTG</td>
</tr>
<tr>
<td></td>
<td>CAGAACCATT</td>
<td>GAGGATCA</td>
<td>ATGGACTTTTGACC</td>
</tr>
</tbody>
</table>
Table 3.2: Serum Hormone Concentrations

Serum total T4 was significantly decreased at all doses in both the dams and pups. All but one total T4 concentration in the 10ppm pups were below the limit of detection of 0.2 µg/dl. The limit of detection for the free T4 assay was 0.25 ng/dl. Serum total T3 concentrations were reduced in 3 and 10ppm pups, and in 10ppm dams. Serum Free T4 concentrations paralleled those in total T4, leaving open the possibility that free T4 concentration may be the true underlying serum biomarker. Serum TSH concentrations rose significantly (p<0.05 in both doses) in the dams. No statistics are available on free T4 concentrations in the pups, which were measured in pooled samples of serum from each male pup in a dose group.

(*p<0.05, ND = Not Determined)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>3 ppm</th>
<th>10 ppm</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Dams</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T3 (ng/dl)</td>
<td>51 ± 14</td>
<td>52 ± 15</td>
<td>12 ± 6.7</td>
</tr>
<tr>
<td>T4 (µg/dl)</td>
<td>4.1 ± 0.8</td>
<td>1.6 ± 0.7*</td>
<td>0.30 ± 0.05*</td>
</tr>
<tr>
<td>Free T4 (ng/dl)</td>
<td>2.4 ± 0.5</td>
<td>0.33 ± 0.1*</td>
<td>&lt;0.25*</td>
</tr>
<tr>
<td>TSH (ng/ml)</td>
<td>3.5 ± 1.1</td>
<td>19 ± 10*</td>
<td>46 ± 8*</td>
</tr>
<tr>
<td><strong>Pups</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T3 (ng/dl)</td>
<td>63 ± 15</td>
<td>51 ± 15*</td>
<td>31 ± 25*</td>
</tr>
<tr>
<td>T4 (µg/dl)</td>
<td>3.2 ± 0.7</td>
<td>0.8 ± 0.5*</td>
<td>0.3 ± 0.1*</td>
</tr>
<tr>
<td>Free T4 (pooled) (ng/dl)</td>
<td>1.6</td>
<td>0.41</td>
<td>&lt;0.25</td>
</tr>
<tr>
<td><strong>Adults</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T3 (ng/dl)</td>
<td>36 ± 10</td>
<td>39 ± 10</td>
<td>ND</td>
</tr>
<tr>
<td>T4 (µg/dl)</td>
<td>4 ± 0.</td>
<td>4.1 ± 1.0</td>
<td>ND</td>
</tr>
<tr>
<td>Free T4 (ng/dl)</td>
<td>2 ± 0.3</td>
<td>1.9 ± 0.5</td>
<td>ND</td>
</tr>
</tbody>
</table>
Figure 3.1: Mean body weight of A) offspring and B) dams

Almost from birth, the mean body weights of the 10ppm pups were significantly reduced in comparison to the 0ppm controls. In addition to the low body weights, a delay in eye opening and decrease in overall physical coordination was observed (data not shown). At weaning, the 10ppm pups were unable to reach food and water in standard rat cages, so special housing arrangements were provided. While the 3ppm pups were not as affected as the 10ppm pups, they also weighed less than controls, with the difference becoming significant around PND18, and expanding as the pups aged until weaning. There was no significant difference between the weights of the 0 and 3 ppm dams at any age, but the body weight of the 10ppm dams averaged significantly less than control on days 30-36 and 40 (p<0.05).
Offspring Weight Gain

Age (Post Natal Days)

Average pup weight (g)

Control
3ppm
10ppm
### Dam Weight Gain

<table>
<thead>
<tr>
<th>Days since mating</th>
<th>Average dam weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>200</td>
</tr>
<tr>
<td>10</td>
<td>250</td>
</tr>
<tr>
<td>20</td>
<td>300</td>
</tr>
<tr>
<td>30</td>
<td>350</td>
</tr>
<tr>
<td>40</td>
<td>400</td>
</tr>
<tr>
<td>50</td>
<td>450</td>
</tr>
</tbody>
</table>

- **Control**
- **3ppm**
- **10ppm**

---

*Graph showing changes in dam weight gain over days since mating for different treatments.*
Figure 3.2: Liver D1 activity in dams and pups

Liver D1 activity in dams and pups decreases with PTU dosage. D1 activity is directly inhibited by PTU administration. Significant reductions in liver D1 activity are noted at both doses in both dams and pups. Error bars represent standard deviation of the means.

*p<0.05 relative to control.
Figure 3.3: Thyroid Tg and NIS mRNA levels in dams and pups

A) Tg and B) NIS have different response patterns to PTU administration in pups, with NIS increasing at 3ppm and further increasing at 10ppm, as opposed to the maximal increase of Tg at 3ppm with no further increase at 10ppm. No significant changes in mRNA levels for these two genes were detected in dams. Error bars represent standard deviation of the means.

* p<0.05 relative to control.
Thyroid Tg mRNA

Dose (ppm PTU)

Relative amount

PND25

Dam

*
Thyroid NIS mRNA

Dose (ppm PTU)

Relative amount

PND25
Dams

*
Figure 3.4: Cerebrocortical T3 concentration and D2 activity

A,B) Cerebrocortical T3 concentrations and C) cerebrocortical D2 activity levels are affected by both PTU doses in the pups. Dams are significantly affected at the 10ppm dose level. Error bars represent standard deviation of the means. * p<0.05 relative to control for that age.
Pup Cortical D2 Activity

D2 activity (fmol l⁻¹/hr/mg)

Dose (ppm PTU)

0 3 10

* *
Dam Cortical D2 Activity

D2 activity (fmol l⁻¹/hr/mg)

Dose (ppm PTU)

0 3 10

*
Cortical T3 Concentration

Dose (ppm PTU)

Cortical T3 (pg/g)

PND25
Dams

* * *
Figure 3.5A
Figure 3.5: Basal synaptic transmission in hypothyroid pups

A) EPSP stimulus-response curves for the indicated treatment groups. B) Normalized stimulus-response curves for the indicated treatment groups. C) Paired-pulse fEPSP sweeps for the indicated treatment groups. No significant difference in the ratio of paired-pulse facilitation was observed. D) The mean fEPSP slope values (error bars indicate SEM) at the maximal stimulus intensity of 140 mA. Results from 2-3 slices/animal were averaged, yielding n values (animals(litters)) of 9(5); 9(5); and 8(4) for the 0 ppm, 3 ppm, and 10 ppm, treatment groups, respectively. *p<0.05 compared to controls.
stimulus intensity (μA)

normalized fEPSP slope

0 ppm
3 ppm
10 ppm

○ 0 ppm
● 3 ppm
△ 10 ppm
C

0 ppm:
PPF Ratio=1.15

3 ppm:
PPF Ratio=1.20

10 ppm:
PPF Ratio=1.17
Figure 3.6: Basal synaptic transmission and LTP in euthyroid adult littermates

A) fEPSP stimulus-response curves for the indicated treatment groups in adult (PND 90-100) animals. No significant differences were noted in the weaned adults. B) Long-Term Potentiation was induced via a single, 100 Hz/1 sec stimulus train delivered at the test pulse intensity at time=30 minutes. The relative increase in the fEPSP response at 30, 60, and 90 minutes post tetanus are indicated. Results from 2-3 slices/animal were averaged, yielding n values (animals(litters)) of 9(5) and 10(5) for the 0 ppm and 3 ppm treatment groups, respectively (error bars indicate SEM). *p<0.05 compared to controls.
Figure 3.7: Correlation between cerebrocortical D2 activity and serum total T4 concentration

Cerebrocortical D2 activity was found to have a significant exponential correlation with serum total T4. This correlation is consistent with the prior knowledge that cerebrocortical D2 activity directly responds to serum T4 concentrations.

A – Dams: \[ y = 15.1 + 161.8 \exp(-1.22 \times x) \quad R^2 = 0.92 \]

B – Pups: \[ y = -34.8 + 1355.8 \exp(-0.60 \times x) \quad R^2 = 0.89 \]
Serum T4 (ug/dl)

D2 Activity (fmol I/mg/h)
Figure 3.8: Correlation analysis of synaptic response vs. A) cerebrocortical D2 B) cerebrocortical T3, and C) serum T4

A linear correlation was observed between cerebrocortical D2, cerebrocortical T3, and serum T4 with maximal synaptic response, suggesting a possible mechanistic link between these parameters. A more negative synaptic response is indicative of stronger neural activity. Lines shown are linear regression with 95% confidence intervals.

A – Vs. D2: \( y = -2.5 - (1.295 \times 10^{-3})x \) \( R^2 = 0.578 \)

B – Vs. T3: \( y = 1.269 + (2.756 \times 10^{-3})x \); \( R^2 = 0.188 \)

C – Vs. T4: \( y = 1.109 + 0.351x \); \( R^2 = 0.362 \)
CHAPTER 4
DECREASED LITTER SIZE AMELIORATES THE THYROID DISRUPTIVE AND
NEURODEVELOPMENTAL EFFECTS OF GESTATIONAL AND LACTATIONAL EXPOSURE TO
PROPYLTHIOURACIL

\(^1\)Taylor MA, Stramiello M, Leluti N, Wagner JJ, Sharlin D, Zoeller T, Ferguson DC. In Preparation
Abstract

The developing central nervous system of the fetus and neonate are recognized as very sensitive to maternal or gestational hypothyroidism. A previous study from this laboratory identified 0, 3 and 10 ppm propylthiouracil resulted in significant maternal hypothyroidism, as well as hypothyroidism and impaired electrophysiological performance in the brains of pups. Type II 5’-deiodinase (D2) activity was elevated at both PTU doses and correlated tightly with the diminution in synaptic potential in hippocampus of weanling rats. In the current study, lower degrees of thyroid insufficiency were sought to explore the threshold for compensation, as well as to evaluate the effect of a relatively high dose of perchlorate as thyroid disruptor with a different mechanism of action. We used propylthiouracil (PTU) administered at 0, 0.3, 1, or 3 ppm or perchlorate at 150 ppm in drinking water from gestational day 2 until weaning to induce graded levels of maternal hypothyroidism. In addition to serum T3, T4, free T4, and TSH concentrations, cerebrocortical T3 concentration (cT3), cerebrocortical Type II (D2) 5’-deiodinase activity, and thyroidal mRNA for thyroglobulin (Tg) and sodium iodide symporter (NIS) were measured. All assays were performed on serum or tissues from pups sacrificed on postnatal day 4 (PND4), 14, and 21-25, as well as from dams and weaned pups allowed to mature to adulthood. Extracellular recordings from the CA1 region in hippocampal slices were obtained from both PND 21-32 (pups) and PND90-100 (adults) rats to assess neurophysiological effects, and adults were observed in open-field chambers to investigate basic behavioral changes such as habituation and activity. Younger pups were shown to be more sensitive to maternal thyroid insufficiency than older pups, but the overall impairment seen in this study was less severe than that seen in the first study. While serum free and total T4, and T3 concentrations were reduced in the pups, there was no alteration of cortical T3 concentrations in the PND21-25 pups, and no electrophysiological or behavioral changes were noted. Based on the findings in our first study, the lack of cortical T3 impairment at PND21-25 may help to explain the lack of electrophysiological alterations.
Introduction

The thyroid gland influences the function and development of many organ systems, including cardiovascular, skeletal, and nervous systems.\textsuperscript{1-5} Thyroid hormone receptors have been documented in the brain during the first trimester in humans, and gestational day 12.5 in rats.\textsuperscript{6} Because of its many roles, maintenance of thyroid hormone levels during pregnancy is essential to the proper development of the offspring. A link between impaired maternal thyroid status and IQ decrement in human babies has been documented.\textsuperscript{7, 8} Other well-documented outcomes of developmental hypothyroidism are hearing loss, altered migration of brain layers, delayed eye opening in rats, poor performance on maze tests, and impaired motor development.\textsuperscript{9-17} As the full range of detrimental neurodevelopmental effects of exposure to goitrogens is not yet understood, further investigation into the effects of well-characterized antithyroid compounds may assist the understanding of the impact of environmental goitrogens.

With respect to measures of impact on brain function, it is well established that disruption in the level of thyroid hormone during development can alter synaptic transmission in regions such as the hippocampal formation. Significant effects on long-term potentiation (LTP) have also been reported.\textsuperscript{18-27} LTP has been denoted as a cellular model for learning and memory processes; therefore, we and others have theorized that changes in LTP could provide a functional link between thyroid hormone deficits with deficiencies in cognitive function.\textsuperscript{28} A prior study in our lab showed impaired synaptic function in PND25 rats, and impairments in LTP persisting into adulthood even after normalization of serum thyroid hormone concentrations.\textsuperscript{29} In addition, prior studies have also demonstrated that inhibitory and excitatory pathways may be differentially impacted by thyroid insufficiency.\textsuperscript{30, 31} Such complexities may contribute to the wide range of outcomes reported concerning the assessment of thyroid hormone deficiency on electrophysiological parameters measured in the hippocampus.\textsuperscript{15,19,24}

While significant animal research has been performed on thyroid toxicants, including studies of neurodevelopmental toxicity, thyroid status is most commonly determined by serum hormone concentrations of T4 and TSH.\textsuperscript{32-36} Many factors influence the relationship between serum and tissue levels of free and bound thyroid hormones, and the relationships may be tissue- and region-specific.\textsuperscript{37-39}
The antithyroid drug propylthiouracil (PTU) was chosen for this and a prior study because this antithyroid drug, by crossing the placental barrier, will impact thyroid hormone production and deiodinative metabolism of both the dam and the gestating pups. PTU has been routinely used to induce developmental hypothyroidism to study neurological impact. Perchlorate was also used in an attempt to expand prior PTU studies to include an environmental antithyroid chemical. Perchlorate acts by blocking iodine uptake into the thyroid through competitive inhibition of the sodium-iodide symporter (NIS).

The present study was designed to expand upon a prior study in our laboratories which evaluated maternal exposure to 0, 3, and 10 ppm PTU in the drinking water. Since the neurological effects at 3 and 10 ppm in the prior study were virtually identical at postnatal day 21, we sought to produce a graded response in this study by expanding the PTU dose range to include 0.3 and 1 ppm PTU doses, as well as 150 ppm perchlorate, and to evaluate serum and tissue parameters in pups from a wider range of developmental ages. Measurement of serum and tissue thyroid markers at postnatal days 4 and 14 were added to evaluate pups just after birth, and during the crucial period for establishment of hypothalamic-pituitary negative feedback, eye opening, and thermogenic autonomy. The primary goal of the present study was to expand the dose range while continuing to evaluate serum, thyroid, and brain hormone markers of thyroid status in both offspring and dams. As with the prior study, these parameters were then correlated with electrophysiology results measured in the CA1 region of hippocampal slices obtained from the offspring. We expanded our evaluation to include brain in situ hybridization studies for known thyroid-responsive genes including RC3/Neurogranin. Additionally, we included basic habituation and activity observations in an open field chamber at postnatal day 90-100 to assess for behavioral impacts of persistent neurological effects after serum thyroid parameters had normalized post weaning.

Potential adaptation to a thyroid disrupting compound may occur by a variety of mechanisms which include alteration of pituitary TSH secretion, alteration of intrathyroidal sodium-iodide symporter and thyroglobulin synthesis, change in deiodinase enzyme activity, and altered thyroid receptor number or affinity. However, little is known about the threshold for triggering these changes nor about their compensatory limit. These issues become particularly critical to understand in subpopulations at greater
risk from thyroid disruption, the developing fetus and neonate. Our prior study quantified compensatory mechanisms in the thyroid with adaptation of NIS and Tg mRNA levels, and in the brain by alteration of D2 activity.

Materials and Methods

Animal acquisition, care, and dosing

Timed pregnant CD® IGS rats were obtained from Charles River Labs (Raleigh, NC). Rats were bred two days before arrival. Upon arrival, dams were housed individually and provided free access to Purina 5008 chow and tap water. Animals were maintained on a 12h/12h light/dark cycle. At approximately 1700h on the day of arrival, the water was replaced with either deionized water purified by Millipore RiOS 8 (Millipore, Milford, MA), or deionized water containing PTU or sodium perchlorate (Sigma, St. Louis, MO). Doses of 0, 0.3, 1, and 3ppm PTU, as well as 150ppm sodium perchlorate in DI drinking water were chosen to expand the dose-response curve from our first study. Litters were culled to 8 pups on PND4, and to 6 pups on PND 14, each time retaining the maximal number of surviving males. Dosing took place from GD2 until weaning, when pups selected for recovery were placed in standard housing with tap water and Purina 5001 chow. Five dams were received per week, and each dose was replicated once per week. Sacrifices were scheduled through an extended Latin square design to eliminate bias in sacrifice day selection.

Animals were weighed three times per week, and water intake recorded. Water bottles were refilled every 2-4 days, depending on the amount remaining in the bottle. New PTU dosing solutions were made at least every two weeks from a stock solution, which was validated by analysis by HPLC. Perchlorate solutions were made in a similar timeframe. Average water intake during gestation was approximately 200 ml/kg/day for the dams. Pups in the control and 3ppm groups began to supplement their diet with food and water prior to weaning at varying times after PND14, but pups in the 10ppm group were unable to do so due to reduced size and coordination. Pups were sacrificed on PND4, PND14, and PND21-PND25. On PND4, only pups in litters larger than 8 were sacrificed. On PND14, two pups per litter were sacrificed. Starting at PND21, pups were sacrificed for electrophysiological
analysis, with one male per litter sacrificed over a period of five days. A randomized block design was used to ensure a lack of bias in sacrifice day selection. Data from these animals is presented as “PND25” pups. Remaining males were weaned at the end of this sacrifice period (on PND25) and followed to PND90-110 and are referred to hereafter as “adults.”

Animal studies were approved by the University of Georgia Institutional Animal Care and Use Committee and were in accordance with procedures outlined in the National Institutes of Health Guidelines for Care and Use of Laboratory Animals.

**Sacrifice protocol**

In an effort to better understand the impact of hypothyroidism on the development of pups, tissue was collected at PND4, PND14, PND25, and around PND90. Data from the PND4 and PND14 timepoints should help to explain the observed effects on their older littermates.

**PND4**

On PND4, litters were culled to 8 remaining pups. Pups selected for culling were euthanized by CO2 administration followed by decapitation. After decapitation, trunk blood was collected and the brain was removed. The brain was assigned to one of four procedures. Brains selected for histological analysis were removed from the skull and placed in a paraformaldehyde solution. Brains selected for in-situ hybridization were placed in a container and snap frozen as whole heads in liquid nitrogen. Brains selected for deiodinase assays were removed from the skull, and cerebral cortex subdisected, and kept on ice until homogenization. Brains selected for thyroid hormone quantification were removed from the skull, placed in a container, and snap frozen in liquid nitrogen.

**PND14**

On PND14, two additional pups were sacrificed. Pups were anesthetized with halothane or isofluororane and sacrificed by decapitation. After decapitation, trunk blood was collected and the brain was removed. One brain was subdisected to provide cerebral cortex tissue for deiodinase assays and thyroid hormone quantification. These were processed as above. The remaining brain was assigned
either to histological analysis or in-situ hybridization. These were processed as above, except that the brain was removed from the skull in both cases.

_PND21-25_

Male pups were weighed, anesthetized with halothane or isofluorane and sacrificed via decapitation. Blood, thyroid, and liver were collected. The brain was dissected for tissue collection and slices prepared for electrophysiology experiments (see below). In all pup and adult samples, the majority of the hippocampus was used for slice preparation. Cortex samples were taken from areas of the brain not impacted by dissection of the hippocampus, generally including prelimbic, motor, sensory, and cingulate regions, and brain tissue samples were divided equally between deiodinase and hormone assays. Liver and cortex samples intended for deiodinase assays were homogenized on the day of sacrifice in a 250mM sucrose, 20mM KH2PO4, 1mM EDTA, 20mM DTT buffer, then stored in aliquots at -80°C until assayed. Blood was allowed to clot and serum collected after centrifugation. All other tissues were flash-frozen and stored at -80°C until analysis.

**Biochemical assays**

*Serum Thyroid Hormone and TSH Radioimmunoassays:*

Serum T3 was measured by radioimmunoassay based on the method of Galton. This assay entailed a five day process. On the first day, 20 μl of serum is added to 180 μl of GAB buffer (0.2M Glycine, 0.13M Sodium acetate trihydrate, 0.02% bovine serum albumin BSA, pH 8.6.) Next, 100 μl of GAB is added containing 2mg/ml of 8-anilino-1-naphthalene-sulfonic acid (ANS). Finally, 100 μl of a 1:32,000 dilution of a polyclonal rabbit anti-T3 antibody(Cat#20-TR45, Fitzgerald Industries International, Concord, MA) was added in GAB/EDTA (0.1M EDTA in GAB). Tubes were incubated for two days at 4°C, before addition of 100 μl of GAB containing approximately 14 000 dpm (~0.006 μCi) of 125-I T3. The tubes were then incubated at 4°C for an additional two days. After the final 48 hour incubation, 100 μl of a donkey anti-rabbit immunoglobulin secondary antibody coated to magnetic beads was added (Donkey anti-rabbit magnetic separation reagent for immunoassay, Amersham, Piscataway,
NJ) and tubes were incubated at room temperature for 1 hour. 1 ml of phosphate buffered saline (0.01 M NaCl, 0.01 M NaH$_2$PO$_4$, pH 7.5) (PBS) was then added to each tube, and tubes were centrifuged for 15 minutes at 2600xg at 4C. The supernatant fluid was then aspirated and pellets were counted in a gamma counter (Wallac Wizard 1470). Each rat age group was always run in a single assay. This assay had a limit of detection of 5 ng/dl with 20μl of serum, or 25 ng/tube. Intraassay coefficients of variation (CV) averaged 23.2 ± 20%(n=3) at 50ng/dl and 16.8 ± 6%(n=3) at 60 ng/dl.

Serum T4 was measured by radioimmunoassay based on the methods of Galton. The process was similar to the T3 assay with the following modifications. 5μl of serum was assayed per tube and added to 200μl of GAB. The primary antibody used was a polyclonal rabbit anti-T4 antibody (Cat#20-TR40, Fitzgerald Industries International, Concord, MA). Approximately 0.006μCi of $[^{125}\text{I}]$-T4 was added on the third day. On the fifth day, 50 μl of 200μg/ml (10μg) solution of rabbit immunoglobulin was added, followed by 100 μl of a GAR secondary antibody solution (Cat#R0881, Sigma) prepared at 60% of the manufacturers recommended volume for a final dilution of approximately 1:8. Tubes were incubated at room temperature for 30 minutes before addition of 1 ml of a 25% wt/vol solution of PBS/PEG. Tubes were then centrifuged, aspirated, and counted as in the T3 assay. Each age group was always run in a single assay. The serum T4 assay had a limit of detection of 0.08 μg/dl with 10μl of serum, or 0.8μg per tube. The average intraassay CV at 3.6 μg/dl (35 μg/tube) was 15%.

The cortical T3 assay was also performed similarly to the serum T3 assay with the following modifications. On the first day, 50μl of cortex extract was added to 150μl GAB. On the final day, the assay was terminated as with the serum T4 assay. Each age group was always run in a single assay. The limit of detection was about 3.5 pg T3/tube, corresponding to 70 pg per extract, or 1 μg/g for a 100 mg sample at 70% extraction efficiency. The average intraassay CV at 7 pg/tube was about 11%. The T4 radioimmunoassay for cerebrocortical tissue extracts was also performed similarly to the serum T4 assay with the following modifications. On the first day, 100 μl of extract was added to 100ul of GAB. On the final day, the assay was terminated as with the serum T3 assay. Each age group was always run in a
single assay. The limit of detection was about 1.9 pg T4/tube, corresponding to 19 pg T4 per extract, or about 500 pg/g for a 100 mg sample at 40% extraction efficiency. The average intraassay CV at 60 pg/tube averaged 9%.

The serum TSH assay was performed using reagents provided by Dr. A. F. Parlow (National Hormone & Peptide Program, Harbor-UCLA Medical Center, Torrance, CA) 100 µl of serum was added to a tube along with 100 µl of a PBS/BSA solution. Horse serum was used as the blank serum for the standard curve. About 14,000 dpm (0.006 µCi) of 125-I TSH was then added to the tubes in PBS, followed by 100 µl of a 1:1 500,000 dilution of the antibody. Tubes were incubated overnight at room temperature. The next day, 100 µl of Donkey Anti-Rabbit bead solution was added to the tubes and tubes were then incubated for an hour at room temperature. 900 µl of PBS was then added, and tubes were centrifuged for 15 minutes at 2400xg. The supernatant was aspirated, and tubes were counted on a gamma counter. Each age group was always run in a single assay. The limit of detection was about 0.6 ng/ml, and the average CV at 1.2 ng/ml and 13 ng/ml were about 18% and 3% respectively.

**Free T4 fraction by Equilibrium Dialysis:**

Serum free T4 fractions were determined by tracer equilibrium dialysis. The protocol was a modification of that of Sterling to allow for smaller sample volumes. Briefly, 65ul of serum was spiked with approximately 1.4 x 10^6 dpm (0.64 µCi) [125I]T4. 50µl of serum was transferred into one side of a microdialysis chamber (Harvard Apparatus, Holliston, MA) with two 100 µl chambers separated by a 5,000 molecular weight cut off semipermeable cellulose membrane(The Nest Group, Inc., Southborough, MA). 100µl of dialysis buffer (0.15M NaH₂PO₄, 20 mM HEPES pH 7.4) was added to the other side. After an overnight dialysis at 37C, the serum and dialysate were removed, with care to precoat all surfaces of pipettes with a T4 carrier solution. The dialysate was precipitated with a magnesium solution (500mM MgCl₂*6H₂O, 100mM NaCl, 50mM Trizma base, 0.27mM NaI, pH 9.3) and washed as previously described but with volumes adjusted proportionally (80 µl precipitation, 200µl wash). The interassay coefficient of variation (CV) using a pooled sample was approximately 27%. 

113
**Thyroid RNA extraction and analysis:**

Thyroglobulin (Tg) and the Sodium Iodide Symporter (NIS) were selected for mRNA analysis as markers which, in part, reflect TSH bioactivity. RNA extraction and analysis were extracted and analyzed as previously reported.²⁹

**Protein assays:**

All deiodinase samples were homogenized on the day of collection in 250mM sucrose, 20mM KH₂PO₄, 1mM EDTA, pH 7, and frozen in aliquots at -80°C until assay. Protein concentrations were determined by the Bio-Rad protein assay reagent (Hercules, CA) using BSA as a standard.

**Cerebrocortical Type II 5’-Deiodinase Activity:**

This assay was performed as previously described, but with the following alteration. Triplicate tubes were incubated at 37°C for one hour with 50µg protein for PND25 pups, 100µg for PND 4 and 14 pups, or 200µg for dams, in a total volume of 320µl, along with tissue-free blanks.²⁹

**Cerebrocortical Thyroid Hormone extraction:**

The extraction of thyroid hormones from the brain was performed as previously described.²⁹

**Electrophysiology:**

The electrophysiological procedures and analysis were conducted as previously reported.²⁹

**Habituation and Activity Behavioral Tests:**

Two months following termination of PTU dosing at weaning, rats underwent behavioral tests. This data was designed to complement hippocampal electrophysiology as a test of functional neurological alterations resulting from developmental hypothyroidism. Rats were handled individually for approximately two minutes each weekday preceding the week they were scheduled for analysis. Behavior was observed using an open field chamber (ENV-515 Test Environment, Med Associates, St. Albans, Vermont) into which rats were placed and monitored for thirty minutes per day over a period of five days. Infrared beams in the cage recorded position and motion of the rats, and this data was analyzed for habituation and activity levels using the Activity Monitor software package.
In-situ hybridization:

In-situ hybridization of brain sections for RC3 Neurogranin, a thyroid hormone-responsive gene, was performed and analyzed as previously described.43

Statistics:

All statistical analysis was performed using SAS (The SAS group, Cary, NC). All group comparisons were performed using ANOVA, followed by Duncan’s multiple range test. p<0.05 was taken as the level of significance.

Results

1) Serum hormones (Table 2)

Dams

Serum total T3 and T4 concentrations were unchanged in any dose. TSH concentrations were tripled at the 3ppm dose level (p= 0.03). No significant alterations were seen in the perchlorate dosed animals.

PND25 pups

Serum total T4 concentrations were reduced by 30% at 1ppm and 70% at the 3ppm dose in the pups (p<0.0001). No significant changes were noted in serum T3 concentrations. No changes were observed in the free T4 percentage which averaged 0.15% ± 0.1% across all dose groups. Serum TSH concentrations were about 3-fold at 1ppm, and increased to 9-fold of control at 3ppm (p<0.0001). Again, no changes were seen in the perchlorate dosed animals.

PND14 pups

Serum total T4 concentrations were reduced by 60% at 1ppm, and 95% at 3ppm compared to controls (p<0.0001). Again, no changes were seen in the perchlorate-dosed animals.

PND4 pups

Serum total T4 concentrations were reduced by 50% at 1ppm, and over 90% at 3ppm compared to controls. (p<0.0001).
**PND100 adults**

In 3 ppm dosed pups allowed to recover for approximately two months after weaning, all serum concentrations of T3 and T4 returned to those of the 0 ppm group.

2) **Body weight (Figure 4.1)**

The weight of all pups track closely together until the end of the pre-weaning period. On PND14, the body weight of 1ppm pups was slightly greater than the control pups, and on PND23, the average body weight of 3ppm pups was less than all other pups (p<0.01). At all other times, there was no statistical difference from control values. Additionally, no significant delay in eye opening was detected. At all times, the body weights of PTU-treated dams were similar to control values.

3) **Thyroid mRNA (Figure 4.2)**

**(2A) Sodium Iodide symporter (NIS)**

In the PND25 pups, NIS levels were unchanged after PTU dosing, but increased by about 1000% in the perchlorate dosed animals. The dams demonstrated a similar response pattern, with a 5-fold increase in NIS in the perchlorate dosed animals, and no change in the PTU dosed animals.

**(2B) Thryoglobulin (Tg)**

In the PND25 pups, Tg levels were doubled at the 3ppm PTU dose, with no change in the perchlorate dose. In the dams, no Tg changes were noted.

4) **Cerebrocortical thyroid parameters (Figure 4.3)**

**(3A) D2 activity**

In the dams, cerebrocortical D2 activity was increased by 50% at 3ppm (p=0.0087). In the PND25 pups, cerebrocortical D2 activity was tripled at 3ppm (p<0.0001). The PND14 pups demonstrated even greater sensitivity, with a 5-fold increase at 1ppm, and a 10-fold increase at 3ppm (p<0.0001). The PND4 pups showed a 5-fold increase in D2 activity at 3ppm but not at lesser doses (p<0.0001). No changes in D2 activity were observed in the perchlorate animals at any age.
(3B) Cerebrocortical thyroid hormone concentrations

No significant change in cerebrocortical T3 concentrations were noted in the dams, PND25 pups, or adults. In the PND14 pups, cortical T3 concentrations were reduced by 60% at 3ppm. No significant changes in T3 were detected in PND4 animals. However, significant variability was seen in all age groups, reducing the resolution of the assay.

5) Hippocampal Electrophysiology

No significant alterations of basal synaptic activity were observed in either PND25 pups or recovered adults. Additionally, no LTP alterations were noted.

6) Behavioral studies

No significant differences in adult behavior in the open field tests were noted.

7) In-situ (Figure 4.4)

In-situ hybridization analysis of the PND25 pups indicated an approximately 10% decline in RC3 mRNA concentration in the cerebral cortex at 3ppm PTU (p<0.05). A potential decline in RC3 mRNA in the lower dentate gyrus was noted, though this fell short of statistical significance (p=0.064). No alterations were noted in the upper dentate gyrus.

Discussion

Developmental thyroid disruption with PTU and perchlorate was initiated at gestational day 2 and maintained until weaning on PND25. This protocol was designed to ensure that maternal thyroid hormone production was compromised prior to the development of fetal thyroid function around GD17. Based on our studies with adult rats, we projected that hypothyroidism was induced by GD9, prior to the first appearance of thyroid hormone receptors in the rat brain at GD12. This window of dosing began earlier than that of most prior developmental studies performed in the rat with PTU. Analysis of thyroid mRNA levels for Tg and NIS, chosen in part to be markers of serum TSH bioactivity on the thyroid gland, once again demonstrated the greater sensitivity to PTU in pups compared to their dams. Additionally, the striking upregulation of NIS mRNA in the perchlorate dosed animals
despite the lack of effect on serum thyroid hormone levels, including TSH, suggests a powerful role for thyroid iodide availability and autoregulation in response to perchlorate exposure.\(^{46}\)

We note that the serum and tissue hormonal, biomarker and electrophysiological changes in dams and post-weaning pups (PND25) from this study at the dose of 3ppm PTU were essentially nonexistent and inconsistent with our findings in our prior work. In our prior study, both serum and cortical thyroid hormone levels were decreased in pups exposed to 3ppm PTU, and the synaptic potential in the hippocampus was decreased.\(^{29}\) It is notable, however, that the chosen biomarkers largely predicted the reduced neurological consequence to animals at this dose in this study. The alterations made in the current experimental protocol involved in this change are as follows. First, litters were culled to 8 pups at PND4, with a further decrease to six pups at PND14 which represent a significant decrease in litter size from the first study, which had average litter sizes around 12 pups. Prior studies have demonstrated an alteration in the makeup and nutritional value of maternal lactation dependent on litter size, leading us to believe that the nutritional status of the pups in this study was superior to that of the first study.\(^{47}\) Secondly, the diet for the dams was changed to the Purina 5008 chow, with a higher percentage of fat than the 5001 diet, with a corresponding 10% increase in calories per gram of food. However, this diet contains slightly less iodine than the 5001 diet.

This study provides a glimpse at the effect of PTU dosing on pups younger than those used for electrophysiology at PND21-25. In almost all measured parameters, pups at PND4 and PND14 were more affected by a given dose of PTU than their PND25 littermates, and were affected at lower doses as well. Of special note in this regard were the relative activities of cortical D2 observed at each age point. PND4 pups had a reduced D2 activity compared to their elder littermates, further demonstrating the heightened sensitivity to thyroid disruption at this age. While no electrophysiological or behavioral changes were noted in this study, the data gained from the younger pups should help to understand any such changes in future studies. Given the postulated relationship between serum and cortical thyroid hormone concentrations and electrophysiological functionality from our prior work, it is not unexpected that a lesser neurological effect is observed in this study, corresponding to the decreased dose effect on
these concentrations. However, the RC3 decline in cortex without a corresponding decline in the dentate gyrus could indicate that the impact of hypothyroidism is less severe in the hippocampus than the cortex.

It should be noted that the prior relationship observed between serum T4 and cerebrocortical D2 activity held true in this study, though the exact regression curve was slightly different. However, the prior relationship between D2 activity and synaptic potential in the pups was no longer observed, possibly because the severity of serum T4 depression from the lowest to the highest PTU dose did not produce the same spread in data. It seems likely that the lack of electrophysiological changes reflected the reduced effectiveness of 3ppm PTU in this study to diminish thyroid hormone production as compared to the first, as indicated by the lack of reduction in cortical T4 concentrations in the PND21-25 pups, with no detectable decline in serum T3 concentrations in PND21 pups from this study. However, the noted decline in serum T4 concentrations was similar, with approximately a 70% reduction in this study compared to a 75% reduction in the first at 3ppm in PND25 pups. However, the addition of TSH data in this study indicates a pituitary perception of hypothyroidism, indicating that some regions of the brain may still lack adequate supplies of thyroid hormones despite the lack of reduction in serum T3 concentrations. The results of this second study prompted the following third study which would span all PTU doses used in the first and second studies, along with a culling protocol intermediate to that of the first and second studies. The goal for the third study was clarification of the dose-response curve ranging from no effect to marked developmental retardation, with a graded response between these extremes.

References

2. Howdeshell KL 2002 A Model of the development of the brain as a construct of the thyroid system. Environ Health Persp 110:337-348


7. Colborn T 2004 Neurodevelopment and endocrine disruption. Environ Health Persp 112:944-949


10. Porterfield S 1994 Vulnerability of the developing brain to thyroid abnormalities: environmental insults to the thyroid system. Environ Health Persp 102:125-130


15. Gilbert ME, Sui L 2006 Dose-dependent reductions in spatial learning and synaptic function in the dentate gyrus of adult rats following developmental thyroid hormone insufficiency. Brain Res 19:10-22

16. Gilbert ME, Mundy WR, Crofton KM 2000 Spatial learning and long-term potentiation in the dentate gyrus of the hippocampus in animals developmentally exposed to Aroclor 1254. Toxicol Sci 57:102-11


19. Gilbert ME 2004 Alterations in synaptic transmission and plasticity in area CA1 of adult hippocampus following developmental hypothyroidism. Dev Brain Res 31:11-8

20. Gilbert ME, Crofton KM 1999 Developmental exposure to a commercial PCB mixture (Aroclor 1254) produces a persistent impairment in long-term potentiation in the rat dentate gyrus in vivo. Brain Res 850:87-95


24. Sui L, Gilbert ME 2003 Pre- and postnatal propylthiouracil-induced hypothyroidism impairs synaptic transmission and plasticity in area CA1 of the neonatal rat hippocampus. Endocrinology 144:4195-4203


31. Sui L, Anderson WL, Gilbert ME 2005 Impairment in short-term but enhanced long-term synaptic potentiation and ERK activation in adult hippocampal area CA1 following developmental thyroid hormone insufficiency. Toxicol Sci 85:647-56


42. Schneider MJ, Firing SN, Thai B, Wu SY, St. Germain E, Parlow AF, St. Germain DL, Galton VA 2006 Targeted disruption of the type 1 selenodeiodinase gene (Dio1) results in marked changes in thyroid hormone economy in mice. Endocrinology 147:580-9


Table 4.1: Serum Hormone Concentrations

Serum total T4 is reduced at both 1 and 3 ppm PTU in all three pup age groups. No significant changes in serum total T4 are seen in the dams. No alterations in serum T3 concentrations are seen at any dose in any age group. Serum TSH concentrations are elevated at 1 and 3 ppm PTU in PND25 pups, but only at 3ppm in the dams.

* =p<0.05

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>0.3 ppm</th>
<th>1 ppm</th>
<th>3ppm</th>
<th>Perchlorate</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Dams</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T3 (ng/dl)</td>
<td>46.4 ± 8.8</td>
<td>48.3 ± 8.6</td>
<td>43.5 ± 5.6</td>
<td>47 ± 9.7</td>
<td>52.2 ± 17</td>
</tr>
<tr>
<td>T4(µg/dl)</td>
<td>6.5 ± 1.7</td>
<td>7.4 ± 1.7</td>
<td>8.7 ± 4.2</td>
<td>4.2 ± 1.34</td>
<td>4.6 ± 1.2</td>
</tr>
<tr>
<td>TSH(ng/ml)</td>
<td>1.8 ± 1</td>
<td>1.8 ± 0.8</td>
<td>2.8 ± 0.5</td>
<td>5.2 ± 4.4*</td>
<td>2.3 ± 0.8</td>
</tr>
<tr>
<td><strong>PND25</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T3 (ng/dl)</td>
<td>53.6 ± 7</td>
<td>49 ± 8.7</td>
<td>58.4 ± 7.3</td>
<td>52.5 ± 5.2</td>
<td>59.8 ± 12.1</td>
</tr>
<tr>
<td>T4 (µg/dl)</td>
<td>6.4 ± 1.8</td>
<td>5.9 ± 0.3</td>
<td>4.3 ± 0.5*</td>
<td>1.88 ± 0.3*</td>
<td>5.4 ± 0.8</td>
</tr>
<tr>
<td>TSH (ng/ml)</td>
<td>0.6 ± 0.4</td>
<td>0.9 ± 0.4</td>
<td>1.6 ± 0.8*</td>
<td>4.7 ± 1*</td>
<td>0.3 ± 0.3</td>
</tr>
<tr>
<td>%Free T4</td>
<td>0.12 ± 0.1</td>
<td>0.17 ± 0.12</td>
<td>0.18 ± 0.11</td>
<td>0.18 ± 0.12</td>
<td>0.21 ± 0.14</td>
</tr>
<tr>
<td><strong>PND14</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T3 (ng/dl)</td>
<td>75.1 ± 13.6</td>
<td>82 ± 3.7</td>
<td>89.1 ± 17.2</td>
<td>60.2 ± 32.5</td>
<td>76.4 ± 6.9</td>
</tr>
<tr>
<td>T4 (µg/dl)</td>
<td>7.1 ± 2.7</td>
<td>6 ± 2.2</td>
<td>2.5 ± 0.9*</td>
<td>0.5 ± 0.4*</td>
<td>8 ± 2.6</td>
</tr>
<tr>
<td><strong>PND4</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T4 (µg/dl)</td>
<td>2.1 ± 0.5</td>
<td>1.8 ± 0.3</td>
<td>1 ± 0.2*</td>
<td>0.2 ± 0.1*</td>
<td>2.4 ± 0.5</td>
</tr>
</tbody>
</table>
Figure 4.1: Mean body weight of A) offspring and B) dams

Minimal weight differences were observed in the pups prior to weaning, with the 3ppm animals lighter only at PND23. This minor weight gap closed during the post-weaning period. No differences in mean body weights between dose groups were seen in the dams.
Dam Weight

Days since arrival
0 10 20 30 40 50

Weight (grams)
180
200
220
240
260
280
300
320
340
360
380

Control
0.3ppm
1ppm
3ppm
Perchlorate
Figure 4.2: Thyroid Tg and NIS mRNA levels in dams and pups

A) Tg and B) NIS mRNA levels. Error bars represent standard deviation of the means. NIS was increased in the dams and pups under perchlorate dosing, but Tg was increased only in 3ppm pups.

* p<0.05 relative to control.
Thyroid TG mRNA

Dose
Control 0.3ppm 1ppm 3ppm Perchlorate

Relative Quantity
0.0
0.5
1.0
1.5
2.0
2.5
3.0

PND25
Dam*
Thyroid NIS mRNA

Dose
Control 0.3ppm 1ppm 3ppm Perchlorate

Relative Quantity
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5

PND25
Dam *

Thyroid NIS mRNA

- PND25
- Dam

Dose
Control 0.3ppm 1ppm 3ppm Perchlorate

Relative Quantity
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5

*
Figure 4.3: Cerebrocortical T3 concentration and D2 activity

A) Cerebrocortical D2 activity levels were increased at 3ppm in all pups and dams, as well as at 1ppm in PND14 pups. The greatest relative increase occurred in the PND14 pups, with an approximately 9-fold increase in activity at 3ppm.

B) No changes in cerebrocortical T3 concentrations were seen in dams, PND25 pups, PND4 pups, or adults. A cerebrocortical T3 reduction of approximately 50% was seen at 3ppm in PND14 pups.

* p<0.05 relative to control for that age.
Figure 4.4: in-situ data

In-situ hybridization analysis of the PND25 pups indicated a decline in RC3 mRNA concentration in the cerebral cortex at 3ppm PTU. No changes in RC3 expression levels were detected in the dentate gyrus at any dose.

* p<0.05 relative to control
RC3 mRNA

Dose

Control 0.3ppm 1ppm 3ppm Perchlorate

Arbitrary Units

0
50
100
150
200

Dentate Gyrus
Cortex

*
CHAPTER 5

GESTATIONAL AND NEONATAL HYPOTHYROIDISM LEADS TO HYPERACTIVITY IN ADULT RATS: BIOCHEMICAL AND NEUROLOGICAL OUTCOMES FROM GRADED THYROID INSUFFICIENCY

Abstract

The developing nervous system is highly sensitive to thyroid disruption. To better understand the observed outcomes in our two prior studies of the effect of graded doses of propylthiouracil (PTU) provided to pregnant rat dams, we initiated a third study spanning the entire PTU dose range of the two prior studies. PTU administration occurred from GD2 until weaning, and litter sizes were culled to 10 pups at PND4, and 8 at PND14. Serum thyroid hormone concentrations were measured along with cerebrocortical T3 concentrations and D2 activity at PND4, 14, 21-25, 90, and in dams. Electrophysiological analysis of hippocampal function was performed at PND21-25 and PND90. Behavior in an open field test chamber was observed in adult pups. Thyroid NIS and Tg mRNA were measured in PND21-25 pups and adults. In most measurements, younger pups were found to be more sensitive to disruption than older pups or their dams, with decreases in serum T4 seen at 10ppm in dams and PND21-25, 3 and 10ppm in PND14, and 1, 3, and 10ppm at PND4. Cerebrocortical D2 activity was increased at 10ppm in dams and PND21-25, and 1, 3, and 10ppm in PND14. Maximal synaptic potential was impaired at 3 and 10ppm at PND21-25, but not in adults. LTP alterations in PND21-25 pups were noted at all doses except 10ppm, but not in adults. Adults from litters dosed with 10ppm PTU were found to be hyperactive in open field testing. In summary, dose effects were found at lower doses in younger animals, and despite the lack of persistent electrophysiological alteration, hyperactivity was seen in adults previously exposed to 10ppm PTU.

Introduction

The thyroid gland influences the function and development of many organ systems, including cardiovascular, skeletal, and nervous systems.1-5 Thyroid hormone receptors have been documented in the brain during the first trimester.6 Because of its many roles, maintenance of thyroid hormone levels during pregnancy is essential to proper development of the offspring. A link between impaired maternal thyroid status and IQ deficit has been documented in infants.7,8 Additionally, the recent increase in neurological disorders in the human population, such as autism and attention deficit hyperactivity disorder (ADHD), has been theorized to correlate with the increasing prevalence of endocrine (including thyroid)
disrupting chemicals in the environment. Other well-documented outcomes of developmental hypothyroidism are hearing loss, altered migration of layers of the cerebral cortex, delayed eye opening in rats, poor performance on maze tests, and impaired motor development. As the full range of detrimental neurodevelopmental effects of goitrogen exposure is not yet understood, further investigation into the effects of well-characterized anti-thyroid compounds may assist the understanding of the impact of environmental goitrogens.

With respect to measures of impact on brain function, it is well established that disruption in the level of thyroid hormone during development can alter synaptic transmission in regions such as the hippocampal formation. Significant effects on long-term potentiation (LTP) have also been reported. LTP has been denoted as a cellular model for learning and memory processes; therefore, we and others have theorized that changes in LTP could provide a functional link between thyroid hormone deficits with deficiencies in cognitive function. In addition, prior studies have also demonstrated that inhibitory and excitatory pathways may be differentially impacted by thyroid insufficiency. Such complexities may contribute to the wide range of outcomes reported concerning the assessment of thyroid hormone deficiency on electrophysiological parameters measured in the hippocampus.

While significant animal research has been performed on thyroid toxicants, including studies of neurodevelopmental toxicity, thyroid status is most commonly determined by serum hormone concentrations of total T4 and TSH. Many factors influence the relationship between serum and tissue levels of free and bound thyroid hormones, and the relationships may be tissue- and region-specific. PTU has been routinely used to induce developmental hypothyroidism to study neurological impact. PTU, the chosen thyroid disruptor in this study, has both maternal and direct effects on the gestating pups, as it will cross the placental barrier.

The hypothalamic-pituitary-thyroid axis has a variety of mechanisms which would allow potential adaptation to thyroid disruption, including alteration of pituitary TSH secretion, alteration of intrathyroidal sodium-iodide symporter and thyroglobulin synthesis, change in deiodinase enzyme activity, and altered thyroid receptor number or affinity. However, little is known about the threshold for
triggering these changes nor about their compensatory limit. These issues become particularly critical to understand in subpopulations at greater risk from thyroid disruption, the developing fetus and neonate.

The investigators have conducted two prior studies of gestational and lactational propylthiouracil exposure on behavioral and electrophysiological outcomes in the rat. At the highest common dosage of 3 ppm PTU in the drinking water, study 1 demonstrated significant reduction of hippocampal synaptic potential in PND21 animals with impaired LTP in the adults. In the second study, no such detriment was seen at this dose, which was the highest examined in that study. The present study was designed to replicate the dose ranges used in both our first and second studies to better understand the entirety of the observed dose-response curve. All assays performed in the second study were repeated in this third study, though slight modifications were made to the behavioral testing protocol.

**Materials and Methods**

*Animal acquisition, care, and dosing*

Timed pregnant CD® IGS rats were obtained from Charles River Labs (Raleigh, NC). Rats were bred two days before arrival. Upon arrival, dams were housed individually and provided free access to Purina 5008 chow and tap water. Animals were maintained on a 12h/12h light/dark cycle. At approximately 1700h on the day of arrival, the water was replaced with either deionized water purified by Millipore RiOS 8 (Millipore, Milford, MA), or deionized water containing 3 or 10ppm PTU (Sigma, St. Louis, MO). Doses of 0, 0.3, 1, 3, and 10ppm PTU in DI drinking water were chosen to complete the dose-response curve from our first and second studies. Litters were culled to 10 pups on PND4, and to 8 pups on PND 14, each time retaining the maximal number of surviving males. Dosing took place from GD2 until weaning, when pups selected for recovery were placed in standard housing with tap water and Purina 5001 chow.

Animals were weighed 3 times per week, and water intake recorded. Water bottles were refilled every 2-4 days, depending on the amount remaining in the bottle. New PTU dosing solutions were made at least every two weeks. Average water intake during gestation was approximately 200 ml/kg/day for the dams. Pups in the control and 3ppm groups began to supplement their diet with food and water prior to
weaning at varying times after PND14, but pups in the 10ppm group were unable to do so due to reduced size and coordination. Pups were sacrificed for electrophysiological analysis beginning on PND21, with one male per litter sacrificed over a period of five days. A randomized block design was used to ensure a lack of bias in sacrifice day selection. Data from these animals is presented as “PND25” pups. Remaining males were weaned on PND25 and allowed to recover until approximately PND90, and are referred to hereafter as “adults.”

Animal studies were approved by the University of Georgia Institutional Animal Care and Use Committee and were in accordance with procedures outlined in the National Institutes of Health Guidelines for Care and Use of Laboratory Animals.

Sacrifice protocol

In an effort to better understand the impact of hypothyroidism on the development of pups, tissue was collected at PND4, PND14, PND25, and around PND90. Data from the PND4 and PND14 timepoints should help to explain the observed effects on their older littermates. The study design was nearly identical to that described in chapter 4, with the exception of the dose range and two extra pups retained in each litter.

PND4

On PND4, litters were culled to 10 remaining pups. Pups selected for culling were euthanatized by CO2 administration followed by decapitation. After decapitation, trunk blood was collected and the brain was removed. The brain was assigned to one of four procedures.Brains selected for histological analysis were removed from the skull and placed in a paraformaldehyde solution as previously described.28 Brains selected for in-situ hybridization were placed in a container and snap frozen as whole heads in liquid nitrogen. Brains selected for deiodinase assays or thyroid hormone quantification were removed from the skull, placed in a container, and snap frozen in liquid nitrogen for later processing.

PND14

On PND14, two additional pups were sacrificed. Pups were anesthetized with halothane or isofluorane and sacrificed by decapitation. After decapitation, trunk blood was collected and the brain
was removed. One brain was dissected to provide cerebral cortex tissue for deiodinase assays and thyroid hormone quantification. These were processed as above. The brain from the second animal was assigned either to histological analysis or in-situ hybridization. These were processed as above, except that the brain was removed from the skull in both cases.

**PND21-25**

Male pups were weighed, anesthetized with halothane or isoflurane and sacrificed via decapitation. Blood, thyroid, and liver were collected. The brain was dissected for tissue collection and slices prepared for electrophysiology experiments (see below). In all pup and adult samples, the majority of the hippocampus was used for slice preparation. Cerebral cortex samples were taken from areas of the brain not impacted by dissection of the hippocampus, generally including prelimbic, motor, sensory, and cingulate regions, and brain tissue samples were divided equally between deiodinase and hormone assays and immediately snap-frozen. Blood was allowed to clot and serum collected after centrifugation. All other tissues were flash-frozen and stored at -80°C until analysis.

**Biochemical assays**

*Serum Thyroid Hormone Radioimmunoassays:*

Serum thyroid hormone assays were performed as described in chapter 4.

*Thyroid RNA extraction and analysis:*

Extraction and analysis of thyroid mRNA was performed as previously described. 28

*Protein assays:*

Protein concentrations of homogenates for deiodinase activity assays were determined by the Bio-Rad protein assay reagent (Hercules, CA) using BSA as a standard.

*Thyroid iodine quantification:*

Thyroid glands were collected from pups sacrificed at weaning, flash frozen, and submitted to Dr. Braverman for analysis by a modification of the previously reported ashing technique of Wilson and Van Zyl. 41

*Cerebrocortical Type II 5'-Deiodinase Activity:*
Analysis of cerebrocortical D2 activity was performed as described in chapter 4.

**Cerebrocortical T3 extraction:**

Extraction of thyroid hormones from cerebrocortical tissue was performed as previously described. The intraassay coefficient of variation was approximately 20% at 23.8 pg T3 per tube. All samples were run in a single assay.

**Electrophysiology**

Electrophysiological analysis of PND21 pups and adults was performed as previously described.

**Behavior assay:**

Rats allowed to recover from gestational and lactational PTU exposure for two months after weaning were used for behavioral analysis. Each day for three days prior to analysis, rats were taken in their home cage into a new room, placed in an empty cage cleaned between each animal with diluted Windex™, a commercial multisurface cleaning agent, left in the cage for approximately two minutes, then taken back to their home room in their home cage. Behavior was observed using an open field behavior chamber (Med Associates, St. Albans, VT) into which rats were placed and monitored for twenty minutes per day over a period of three days. Infrared beams in the cage recorded position and motion of the rats, and this data was analyzed for habituation and activity levels using the associated Activity Monitor software.

**Serum isoflavone analysis:**

Serum samples from PND14 pups were submitted to Dr. Daniel Doerge for analysis according to a previously established protocol using LC/MS. Concentrations of genistein, diadzein, and the genistein metabolite equol were determined in the serum samples.

**Statistics:**

All statistical analysis was performed using SAS (The SAS group, Cary, NC). All group comparisons except electrophysiology were performed using ANOVA, followed by Duncan’s multiple
range test. Electrophysiology results were analyzed as pairwise comparisons with the control group. p<0.05 was taken as the level of significance.

Results

1) Serum hormones (Table 5.1)

Dams

Serum total and free T4 concentrations were reduced by 80% at 10ppm (p=0.007). Serum total T3 concentrations were reduced by 30% at 10ppm (p=0.033).

PND25 pups

Serum total and free T4 concentrations were reduced by approximately 95% at 10ppm (p<0.0001). Serum total T3 concentrations were reduced by 50% at 10ppm (p<0.0001).

PND14 pups

Serum total T3 concentration was increased at 1ppm and decreased at 10ppm (p<0.0001). Serum total T4 concentration was reduced at 3 and 10ppm (p=0.007).

PND4 pups

Serum total T4 concentration was decreased by 30% at 1ppm, 75% at 3ppm, and over 90% at 10ppm (p<0.0001).

PND100 adults

Serum total T4 concentration was increased by 0.5-1.2 µg/dl in adults previously exposed to 0.3, 1, and 3, and 10ppm PTU (p=0.0031) No changes were seen in any TH concentrations in adults.

2) Body weight (Figure 5.1)

On all days starting at the first recorded weight at PND4, the body weight of the 10ppm pups were significantly decreased compared to all other dose levels. On PND16 and PND18, the body weights of 1ppm pups were higher than control pups. No significant differences were found in the weights of the dams.

3) Thyroid mRNA (Figure 5.2)

Sodium Iodide symporter (NIS)
NIS mRNA levels in PND25 pups were significantly increased at 3 and 10ppm (p<0.0001). No changes were observed in adults.

**Thyroglobulin (Tg)**

Tg mRNA levels in PND25 pups are significantly increased at 10ppm (p=0.0028). No changes were observed in adults.

4) **Cerebrocortical thyroid parameters (Figure 5.3)**

**D2 activity**

In the dams, cerebrocortical D2 activity was increased approximately 300% at 10ppm (p=0.0013). In the PND25 pups, cerebrocortical D2 activity also increased approximately 300% at 10ppm (p<0.0001). The PND14 pups demonstrated even greater sensitivity, with an approximate tripling at 1ppm, 800% increase at 3ppm, and 1200% increase at 10ppm (p<0.0001).

**Cerebrocortical T3 concentrations**

No change in cerebrocortical T3 concentrations were seen in the dams or adults. In the PND25 pups, cortical T3 was reduced by about 40% at 10ppm (p=0.026). In the PND 14 pups, cerebrocortical T3 concentrations were reduced by 60% at 10ppm (p=0.003). No changes were seen in cortical T3 concentrations at PND4, and high variability was observed in this age group.

5) **Electrophysiology (Figure 5.4)**

In the PND25 pups, several electrophysiological alterations were noted. Maximal synaptic potential was reduced by approximately 25% at 3 and 10ppm. 30 minute LTP (weak LTP) was impaired by approximately 10% at 1 and 3ppm (though 10ppm was nearly significant), while 90 minute LTP (strong LTP) was impaired by approximately 10% at 0.3 and 1ppm PTU. None of these changes were observed to persist in adults.

6) **PND25 Thyroid iodine content (Figure 5.5)**

The average iodine content of thyroids from the control group averaged 1.48 µg per gland. No significant change was observed at the 0.3ppm dose. Concentrations in the 1ppm dose were reduced about 35%. Concentrations in the 3ppm dose were reduced about 65%. Concentrations in the 10ppm
dose were reduced nearly 90%. 3(control) or 4(dosed) pups per litter for one litter of each dose group were studied.

7) Serum Isoflavones

Serum from control PND14 pups from this study and the prior study were submitted to Dr. Daniel Doerge for analysis of soy isoflavones and metabolites. Serum concentrations of genistein, daidzein and equol were determined. Concentrations of genistein and daidzein were both unchanged between the studies. Concentrations of equol, the primary metabolite of genistein, approximately 0.19+/−0.09 µM (n=7) in the second study, and 0.45+/−0.19 (n=8) µM in the third (p=0.006), supportive of reduced dietary isoflavone exposure of the dam and/or pup bioavailability of the maternal metabolite.

8) Behavior (Figure 5.6)

Adult rats monitored in the open field chamber demonstrated significant behavioral alterations at the 10ppm dose. Rats in this dose group demonstrated a significantly greater amount of ambulatory activity over each 20 minute session. No alterations were seen in the number of vertical counts or central zone entries, indicating hyperactivity without a modification of anxiety.

8) Cortical D2 correlations (Figure 5.7)

Cortical D2 continues to display a significant correlation with serum total T4 in pups. (PND25: y=597.2-223*ln(x) R^2=0.88, PND14: y=789.1-323*ln(x) R^2=0.89) where y=D2 activity (fmol/hr/mg) and x=serum total T4 concentration (µg/dl)

Discussion

As in the prior two studies, developmental thyroid disruption with PTU was initiated at gestational day 2 and maintained until weaning. This protocol was designed to ensure that maternal thyroid hormone production was compromised prior to the initiation of fetal thyroid hormone secretion around GD17.² Serum and brain data obtained at the PND4 timepoint confirmed the effectiveness of our dosing routine in inducing neonatal as well as gestational hypothyroidism. The doses employed in this study demonstrated a variety of effects, including neurological changes of varying degree and nature at all
doses. The presence of LTP impairment in PND25 pups from the 0.3ppm dose group is confusing, as no decline in thyroid hormone concentrations have been detected in any age group exposed to 0.3ppm PTU except for elevated T4 concentrations in the adult. Further studies on the gestational period are warranted at this dose, to determine if any perturbations in thyroid hormone concentrations are caused prior to our first sacrifice point at PND4.

As was seen in our second study, serum total thyroid hormone concentrations were affected to a greater extent in younger pups than in older pups or dams. It is worth noting that the threshold for serum effects was only 1ppm at PND4, whereas reductions in thyroid hormones were only seen starting at 3ppm at PND14, and at 10ppm in the PND25 pups and dams. It is clear from this data that the ability of the pups to compensate for insults to the thyroid system improved with maturity. However, interpretation of the potential for permanent effects requires a complete understanding of the timing of thyroid hormone-dependent neurodevelopmental effects. As this data shows, younger animals are more likely to be affected by a given level of insult to the thyroid system, creating the potential for neurological damage that may persist after serum thyroid hormone concentrations normalize.

The 3ppm dose effects on most of the observed parameters in this study were less severe than those observed in the first study, but were more severe than the second study. At the same time, the overall increase in body weight of the pups was increased in the second and third studies compared to the first, with control pups averaging about 40 grams at PND21 in the first study, versus about 57 grams in the second and 53 grams in the third. This alteration in growth patterns extended even to the 10ppm dose, averaging about 25 grams at PND21 in the first study, and 35 in the third. Considering this data, it is likely that the basic nutritional profile of the pups varied significantly between the three studies. Between the first and second studies, the alteration in diet from Purina 5001 to 5008 may play a part, but the same diet was used in the second and third studies. However, serum equol levels in PND14 pups were significantly higher in the third study than in the second. As equol is a metabolite of soy isoflavones, this indicates a greater isoflavone content of the diet in the third study, which may have increased the insult to the thyroid axis in this study.\textsuperscript{42-46} Soy isoflavones have been shown to impact the thyroid axis at many
levels, including nuclear receptors and the retinoic acid system.\textsuperscript{42-46} For example, interactions with the retinoic acid receptor in the liver can cause hypertriglyceridemia.\textsuperscript{46} Additionally, soy proteins were shown to regulate the expression of genes for fatty acid synthase (FAS), malic enzyme (ME), acetyl-CoA carboxylase (ACC), hydroxymethylglutaryl- CoA reductase, LDL receptor, and CYP7A1 in humans, animals, and cultured human hepatoma cells.\textsuperscript{45} In this light, we interpret that litter size likely played a significant role in resulting in a difference in nutritional plane for the pups in study 1 compared with 2 and 3. In study 1, no pups were culled until at least PND21 resulting in average litter sizes around 13. The second study had the lowest average litter size (8 pups at PND4 and 6 at PND14,) while study 3 had two more pups than the second, but still fewer than the first (10 pups at PND4, 8 at PND14). Studies by other investigators have shown that the nutritional content of milk varies with the size of the litters being nursed.\textsuperscript{47} This data emphasizes the need to carefully consider litter size and dietary constituents when comparing data across studies.

Cerebrocortical Type II 5'\textsuperscript{-}deiodinase (D2) enzymatic activity was again found to be a sensitive respondent to reductions in serum T4 concentrations. The correlation was similar at PND25 and PND14, with differences mainly arising from the variations in normal serum T4 concentration and basal D2 activity between the two age groups (Figure 6). While the observed correlations are not exactly identical to those observed in prior studies, a strong similarity is still observed, and normalized data from each study overlap, suggesting that the differences in D2 alterations observed in each study relate mainly to the relative efficacy of PTU in reducing serum T4 concentrations. D2 is known to be regulated both pre- and post-translationally by T3 and T4 respectively. One recognized method of post-translational regulation by T4 is control of the rate of ubiquitination, and thus degradation of D2.\textsuperscript{48} Accordingly, the observed D2 activities are highly informative as to the immediate availability of thyroid hormones to the brain at the time of sacrifice. The correlation of D2 to serum total or fT4 remains the strongest observed correlation of D2.

Surprisingly, serum T4 concentrations were found to be elevated by about 20\% in all doses in the mature (adult) rats having been exposed to high doses of PTU. Prior studies have shown a restoration of
normal serum T4 concentrations in all doses, and we are unable to explain this surprising finding.

Behavioral data obtained from littermates approximately 60 days after weaning demonstrated that some of the effects of the 10ppm treatment could not be reversed by restoration of euthyroidism after weaning. This observation of altered behavior supports the conclusions made previously by other researchers that persisting changes can occur in the brain following a developmental insult, possibly involving alterations in myelination and migration patterns of neurons.\textsuperscript{14,23,25-28} Electrophysiological alterations were not shown to persist, to adulthood unlike the first study.\textsuperscript{28} However, even in the absence of electrophysiological alterations, significant hyperactivity was seen in the 10ppm pups. This effect was observed on every day of testing, and provides evidence that some neurological alterations were still present in the adults, despite the lack of electrophysiological alterations. While the exact cause of the hyperactivity is unclear, this finding suggests the need for expanded behavioral testing in future studies. This behavioral outcome parallels recent studies on the relationship between thyroid disruption and hyperactivity in human populations.\textsuperscript{49}

While electrophysiological changes were again observed in this study, the magnitude of the impacts on synaptic potential were half that of our first study.\textsuperscript{28} Impairments in either 30-minute or 90-minute LTP were observed in all doses except 10ppm. Unlike our first study, these alterations are not observed in adults.

While there are quantitative and qualitative differences between the results obtained in our three studies, a coherent picture emerges when all three are considered. First, litter size can moderate the effects of insults to the thyroid axis in neonatal pups, probably through nutritional status. Second, younger animals are more sensitive to insults to the thyroid axis than their older littermates. Finally, sufficient neurological hypothyroidism in developing pups leads to irreversible neurological dysfunction, indicated by electrophysiology in the first study and behavior in the third. Unfortunately, no significant linear correlations between any of the observed biomarkers and electrophysiological outcome have been consistently observed across the three studies. Therefore, the chosen electrophysiological markers are unlikely to be the most sensitive possible indicators of neurodevelopment. It is also unfortunate that
histological data on physical brain structure is currently unavailable, as alterations in physical structure would provide valuable data to explain the observed electrophysiological and behavioral results. More sensitive functional measurements of neurodevelopmental impairment would be helpful in overcoming this hurdle in future studies. Further studies to elucidate the cause of the LTP impairment in the PND25 pups exposed to 0.3ppm PTU would be especially helpful in understanding the true nature of the observed changes. Traditional maze testing in particular may aid in detecting subtle changes in neurological function which were undetectable through in vitro electrophysiology in the hippocampus. With the exception of the puzzling reduction in LTP noted in the 0.3ppm dosed pups in this study, all electrophysiological changes were accompanied or preceded by measurable increases in cerebrocortical D2 activity, providing support for the thyroid-dependent nature of the observed neurological alterations. However, the increased concentrations of isoflavones detected in the serum of this third study may help to explain some of the differences between the first and second studies, as genistein may be modulating the effects of thyroid hormones on the brain. Any future studies should strongly consider the need to control and characterize isoflavone content for consistency across studies.

References
2. Howdeshell KL 2002 A Model of the development of the brain as a construct of the thyroid system. Environ Health Persp 110:337-348
7. Colborn T 2004 Neurodevelopment and endocrine disruption. Environ Health Persp 112:944-949


10. Porterfield S 1994 Vulnerability of the developing brain to thyroid abnormalities: environmental insults to the thyroid system. Environ Health Persp 102:125-130


15. Gilbert ME, Sui L 2006 Dose-dependent reductions in spatial learning and synaptic function in the dentate gyrus of adult rats following developmental thyroid hormone insufficiency. Brain Res 19:10-22

16. Gilbert ME, Mundy WR, Crofton KM 2000 Spatial learning and long-term potentiation in the dentate gyrus of the hippocampus in animals developmentally exposed to Aroclor 1254. Toxicol Sci 57:102-11


19. Gilbert ME 2004 Alterations in synaptic transmission and plasticity in area CA1 of adult hippocampus following developmental hypothyroidism. Dev Brain Res 31:11-8

20. Gilbert ME, Crofton KM 1999 Developmental exposure to a commercial PCB mixture (Aroclor 1254) produces a persistent impairment in long-term potentiation in the rat dentate gyrus in vivo. Brain Res 850:87-95


24. Sui L, Gilbert ME 2003 Pre- and postnatal propylthiouracil-induced hypothyroidism impairs synaptic transmission and plasticity in area CA1 of the neonatal rat hippocampus. Endocrinology 144:4195-4203


31. Sui L, Anderson WL, Gilbert ME 2005 Impairment in short-term but enhanced long-term synaptic potentiation and ERK activation in adult hippocampal area CA1 following developmental thyroid hormone insufficiency. Toxicol Sci 85:647-56

32. Morreale de Escobar G, Obregón MJ, Escobar del Ray F 2004 Is Neuropsychological development related to maternal hypothyroidism or to maternal hypothyroxinemia?. J Clin Endocrinol Metab 85:3975-3987


the offspring of mothers exposed to mild-moderate iodine deficiency: a possible novel iodine deficiency disorder in developed countries. J Clin Endocrinol Metab 89(12):6054-60.

Table 5.1 - Serum total T4, free T4, and total T3 Concentrations

Serum total T4 concentrations in PND4 pups are reduced by approximately 30% at 1ppm, 80% at 3ppm and over 90% at 10ppm. In PND14 pups. The reduction is approximately 85% at 3ppm, and over 95% at 10ppm. In PND25 pups, the reduction is approximately 90% at 10ppm, and 80% at 10ppm in the dams. (p<0.05) No alterations in the fraction of free T4 were seen at any dose.

Serum total T3 concentrations in PND14 pups are increased approximately 30% at 1ppm and decreased approximately 80% at 10ppm. In PND25, serum T3 concentrations are reduced approximately 50% at 10ppm, and approximately 30% at 10ppm in the dams. (p<0.05)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>0.3 ppm</th>
<th>1 ppm</th>
<th>3ppm</th>
<th>10ppm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dams</td>
<td>T3 (ng/dl)</td>
<td>52.8 ± 8.9</td>
<td>50.8 ± 8.7</td>
<td>52.3 ± 11.6</td>
<td>51 ± 3.7</td>
</tr>
<tr>
<td></td>
<td>T4(µg/dl)</td>
<td>7.2 ± 2.6</td>
<td>7.9 ± 2</td>
<td>9.7 ± 6.6</td>
<td>7.8 ± 2.2</td>
</tr>
<tr>
<td></td>
<td>%Free T4</td>
<td>0.07 ± 0.02</td>
<td>0.06 ± 0.01</td>
<td>0.07 ± 0.01</td>
<td>0.06 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>Free T4</td>
<td>4.8 ± 1.9</td>
<td>4.9 ± 1.4</td>
<td>6.3 ± 4.7</td>
<td>4.6 ± 1.3</td>
</tr>
<tr>
<td>PND25</td>
<td>T3(ng/dl)</td>
<td>51.7 ± 8.4</td>
<td>53.2 ± 7.7</td>
<td>51.7 ± 8.2</td>
<td>50 ± 14.2</td>
</tr>
<tr>
<td></td>
<td>T4(µg/dl)</td>
<td>4.7 ± 1</td>
<td>5.4 ± 2</td>
<td>5.6 ± 2.9</td>
<td>3.5 ± 2.1</td>
</tr>
<tr>
<td></td>
<td>%Free T4</td>
<td>0.1 ± 0.01</td>
<td>0.09 ± 0.01</td>
<td>0.1 ± 0.04</td>
<td>0.1 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>Free T4</td>
<td>4.9 ± 1.4</td>
<td>4.6 ± 1.7</td>
<td>6.3 ± 5.5</td>
<td>3.6 ± 2.6</td>
</tr>
<tr>
<td>PND14</td>
<td>T3(ng/dl)</td>
<td>27.4 ± 11.3</td>
<td>35.1 ± 5.4</td>
<td>40.7 ± 11.5*</td>
<td>28.8 ± 17.4</td>
</tr>
<tr>
<td></td>
<td>T4(µg/dl)</td>
<td>7.8 ± 4.5</td>
<td>6.4 ± 2.5</td>
<td>3.8 ± 2.4</td>
<td>0.9 ± 0.8*</td>
</tr>
<tr>
<td>PND4</td>
<td>T4(µg/dl)</td>
<td>1.0 ± 0.2</td>
<td>0.9 ± 0.1</td>
<td>0.7 ± 0.2*</td>
<td>0.2 ± 0.02*</td>
</tr>
<tr>
<td>Adult</td>
<td>T4(µg/dl)</td>
<td>4.2 ± 0.4</td>
<td>5.5 ± 0.8</td>
<td>5.0 ± 0.8</td>
<td>5.5 ± 0.8</td>
</tr>
</tbody>
</table>
Figure 5.1) A) Pup and B) Dam weight gain

Pups exposed to 10ppm PTU were significantly lighter than all other doses starting from the first recorded weights at PND4. 3ppm pups became significantly lighter than controls only at PND23. No weight differences were noted in dams.
Dam Weights

Days since arrival
0 10 20 30 40 50

Dam Weight (g)
180
200
220
240
260
280
300
320
340
360
380

Control
0.3ppm
1ppm
3ppm
10ppm
Figure 5.2) Thyroid RNA

NIS mRNA was slightly upregulated in PND25 pups exposed to 3 ppm PTU, and increased by approximately 5-fold at 10 ppm. Tg mRNA concentrations were unchanged below 10 ppm, and only slightly upregulated at 10ppm.
Figure 5.3) Cortical A)D2 B)T3

A) Cerebrocortical D2 activity in PND14 pups was increased approximately 200% at 1ppm, 600% at 3ppm, and 1000% at 10ppm. Cerebrocortical D2 activity in PND25 pups increased approximately 400% only at 10ppm. Cerebrocortical D2 activity was increased approximately 300% in dams exposed to 10 ppm PTU. B) In PND14 pups, cerebrocortical T3 concentrations were reduced approximately 50% at 10 ppm. In PND25 pups, cerebrocortical T3 concentrations were reduced approximately 40% at 10ppm. No significant changes in cerebrocortical T3 concentrations were seen at any dose in PND4 pups or dams. No significant alterations remained until adulthood in weaned pups.
Figure 5.4) PND25 Electrophysiology: A) Baseline Synaptic Response, B) Maximal Synaptic Response, C) LTP, D) wLTP, E)sLTP

Maximal synaptic potential was decreased by approximately 20% at 3 and 10 ppm. Weak LTP was impaired by approximately 10% at 1 and 3 ppm, and strong LTP was impaired by approximately 10% at 0.3 and 1 ppm PTU. (p<0.05)
Synaptic response at 140 µA

max fEPSP (% control)

control 0.3 ppm 1 ppm 3 ppm 10 ppm
Long-Term Potentiation

HFS: 100Hz *3

Time (min)

normalized fEPSP slope

0 10 20 30 40 50 60 70 80 90 100 110

0 0.5 1 1.5 2 2.5

HFS: 100Hz *3
LTP at 30 minutes post HFS

% control potentiation

control 0.3 ppm 1 ppm 3 ppm 10 ppm

*
90 minute LTP (% of control)

- Control
- 0.3ppm
- 1ppm
- 3ppm
- 10ppm

* *
Figure 5.5) PND25 Thyroid iodine content

The iodine content was reduced in pups dosed with 1, 3, and 10ppm PTU by approximately 30%, 60%, and 90% respectively. Only a single litter from each dose group was analyzed, so error bars represent standard deviation within the litter. (p<0.05)
Figure 5.6) Open Field Behavior

Adult rats previously exposed to 10ppm PTU were found to be hyperactive in a 20 minute open field test session. Data shown is total activity per 20 minute session. One session was performed each day for three days. No significant alterations were found in the proportion of this activity in the center of the open field vs. the periphery indicating no change in the levels of anxiety.
Ambulatory Activity

Behavior day
1 2 3

Ambulatory Counts
0
500
1000
1500
2000
2500
3000

Control
0.3ppm
1ppm
3ppm
10ppm

*
Cortical D2 (Y) (fmol I/mg/h) demonstrates a tight and highly significant correlation to (x) serum total T4 concentrations (µg/dl)

PND25: \[ Y = 597.2 - 223 \cdot \ln(x) \] \( R^2 = 0.88 \)

Cortical D2 (Y) (fmol I/mg/h) demonstrates a tight and highly significant correlation to (x) serum free T4 concentrations (ng/dl)

PND25: \[ Y = 571.4 - 212.7 \cdot \ln(x) \] \( R^2 = 0.89 \)

Cortical D2 (Y) (fmol I/mg/h) demonstrates a tight and highly significant correlation to (x) serum total T4 concentrations (µg/dl)

PND14: \[ Y = 789.1 - 323 \cdot \ln(x) \] \( R^2 = 0.89 \)
CHAPTER 6
SUPPLEMENTAL AND COLLABORATIVE STUDIES

1) PTU Timecourse study

Materials and Methods:

Young adult male CDS rats (Charles River Labs, Raleigh NC) were maintained on standard diet and water until onset of study. Animals were weighed and transferred to single cages immediately preceding the onset of study. Weights and water intake were recorded daily. PTU doses of 0, 0.3 and 3ppm were employed in DI drinking water with 4 rats per group.

On Day 0, animals were lightly anaesthetized and bled via retroorbital sinus. Approximately 600ul of blood was collected. This blood was allowed to clot, spun down for serum collection, and frozen until assay. After the initial bleed, the animals were given dosed water for their cage. Subsequent bleeds occurred at 1, 2, 4, and 7 days after the onset of dosing, each of approximately 600ul. On day 10, the animals were terminated by decapitation under anesthesia. Trunk blood was collected at this time. Additionally, cerebral cortex was collected and used for deiodinase assays.

Serum T4 concentration and cortical D2 were measured as previously described (Chapter 3).

Results:

Serum T4 Concentration (Figure 6.1)

There were no significant changes from initial serum T4 concentrations on the first day after dosing, but a 25% decrease in all three groups was noted on the second. This decrease was maintained in the control and 3ppm groups on the fourth day, but the 0.3ppm rats returned to initial levels on that day. On the seventh day, an elevation of approximately 15% was seen in the 0.3ppm group, while the control group was still reduced by about 20%, and the 3ppm group was further reduced to 50% of initial values. On the tenth and final day, the 0.3ppm group remained elevated, while the control group had returned to normal and the 3ppm group remained depressed by 50%.
Cortical D2 (Figure 6.2)

Cortical D2 activity was measured after the terminal bleed. An 80% reduction in D2 activity was seen in the 3ppm adults treated for 10 days.

Discussion:

Evaluating the control group, it is clear that frequent repeat bleeds on days 1, 2, and 4 were likely associated with stress or an anesthetic drug effect which resulted in lowering serum T4. Regardless, it seems clear from this study that a clear decrease in serum T4 levels in adult rats is achieved after no more than seven days of dosing with 3ppm PTU in drinking water. Strangely, D2 levels are not elevated in the dosed animals, suggesting that the repeated stress of bleeding impacted D2 regulation in these subjects, as has been described in nonthyroidal illness.

2) Deiodinase activity of perchlorate treated rat pups

Materials and Methods:

CDS Rats were treated in Dr. Zoeller’s lab at the University of Massachusetts. Timed pregnant rat dams were exposed to perchlorate at doses of 0, 10, 100, 250, 500, and 5000 ppb in drinking water from GD6 until weaning. Brains from PND21 animals were collected, flash frozen, and shipped to UGA. Cerebral cortex was removed from the frozen brains and homogenized for cortical deiodinase measurement. D2 assays were performed as previously described (Chapter 3).

Results:

No significant changes were noted in cortical D2 activity across any of the measured doses.

Discussion:

This data indicates that continued exposure to up to 5ppm perchlorate in the drinking water of gestational and neonatal rats provides an insufficient insult to the thyroid axis to trigger a deiodinase response in the brain. We would predict from this data that there are no alterations in thyroid hormone availability to the brain under these treatments, at least at PND21.
3) Deiodinase activity and cerebrocortical T3 concentrations of PTU dosed Long-Evans rats

Materials and Methods:

Pregnant Long-Evans rats were obtained from Charles River (Raleigh, NC) on gestational day (GD) 2 and housed individually in standard plastic hanging cages in an Association Assessment and Accreditation of Laboratory Animal Care-approved animal facility at the USEPA laboratories in RTP, NC. All animal treatments were in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize the number of animals and their suffering. Animal rooms were maintained on a 12-h light, 12-h dark schedule, and animals were permitted free access to food (rat chow, Purina; St. Louis, MO) and tap water.

Beginning on GD 6 and continuing until PND23, dams were rendered hypothyroid by addition of 0, 1, 2, or 3 ppm of the thyroid hormone synthesis inhibitor propylthiouracil (PTU; Sigma, St. Louis, MO) to the drinking water. The day of birth was designated PN0 and all litters were culled to 10 pups on PN4, retaining the maximal number of males per litter. On PND23, the offspring were weaned, transferred to plastic hanging cages (two to four/cage) and were permitted free access to food and tap water. A subset of animals were sacrificed for tissue collection on PND4, PND14, PND15, PND21, and PND22. Cortical tissue from dams, PND14-15 and PND21-22 animals were frozen and shipped to UGA for analysis of cortical D2 activity and cortical T3 concentrations. These assays were performed as previously described (Chapter 3). Serum thyroid hormone data was provided for correlation with these data.

Results:

D2 (Figure 6.3)

In PND21 animals, cortical D2 activities were increased by about 250% and 650% at 2 and 3ppm respectively (p<0.0001). In the PND 14 animals, this upregulation is about 600% at both 2 and 3ppm(p=0.0005). In dams, D2 activities were increased by about 250% at 3ppm, with no change at 2ppm(p<0.0001).

Cortical T3 Concentration

Cortical T3 concentrations were unchanged at PND14, PND21 and in dams.
Cortical T4 (Figure 6.4)

Cortical T4 concentrations were reduced by about 40% and 70% at 2 and 3ppm respectively in PND21 animals (p=0.0043). Cortical T4 concentrations were reduced by about 55% in all three doses at PND14 (p=0.0084). Cortical T4 concentrations were reduced by about 30% at 1 and 2ppm, and about 60% at 3ppm in dams (p=0.0005).

Correlations of D2 Activity with Serum Concentration Data (Gilbert lab)

Cortical D2 was again found to correlate tightly with serum total T4 concentration. Each age group was found to fit a logarithmic curve. The relationship in the PND14 animals is somewhat obscured by a larger number of serum T4 levels below the limit of detection than the other ages.

PND14 (Figure 6.5)

\[ Y = 1677 - 390 \times \ln(X) \quad R^2 = 0.58 \]

PND21 (Figure 6.6)

\[ Y = 2159 - 533 \times \ln(X) \quad R^2 = 0.90 \]

Dams (Figure 6.7)

\[ Y = 219 - 51 \times \ln(X) \quad R^2 = 0.76 \]

Where x = serum T4 concentration in ng/ml and Y = cortical D2 activity in fmol I- hr/mg

Discussion:

Despite the differences in dosing protocol and rat strain between the animals studied in Dr. Gilbert’s lab and those in this laboratory, cortical D2 data correlates with serum T4 concentrations in a very similar fashion. This correlation provides further evidence that the regulation of D2 in the brain is qualitatively identical between the CDS and Long-Evans strains of rats dosed with PTU. As D2 is regulated both pre- and post-translationally by thyroid hormones, this similarity is not unexpected, but its similarity should aid in modeling D2 response to serum T4 across rat strains. As PTU has not been shown to change the free T4 fraction, this relationship should hold for both free and total T4.
Figure 6.1 – Serum T4 Concentration in PTU Timecourse Study

A significant (20-30%) reduction in serum total T4 concentration is seen in all three dose groups at day 2 of testing. This reduction is fully reversed by day 4 in the 0.3ppm group, but the reduction remains until day 10 in the control group. A further reduction to about 50% of control levels is seen in the 3ppm group at day 7, and persists at termination on day 10.

*=P<0.05 compared to control for that day
PTU Timecourse Serum T4

Days since onset of PTU dosing

Serum Total T4 (% initial)

Control
0.3ppm
3ppm

*
Figure 6.2 – D2 in PTU Timecourse Study

Cortical D2 activity is reduced by approximately 80% at the 3ppm dose level after 10 days of dosing.

*=p<0.05
Figure 6.3 – Cortical D2 in Gilbert Study

At PND14, cerebrocortical D2 concentrations are increased approximately 800% at both the 2 and 3ppm doses. At PND21, an approximately 150% increase is seen at 2ppm, and approximately a 900% increase at 3ppm. In the dams, an approximately 200% increase is seen at 3ppm.*=p<0.05
Cerebrocortical T4 concentrations are reduced by approximately 50% at all three doses in PND 14 pups. At PND21, an approximately 50% reduction is seen at 2ppm, and an 80% reduction at 3ppm. In dams, a 50% reduction is seen at 2ppm, and an approximately 75% reduction at 3ppm.*=p<0.05
Figure 6.5 – Cortical D2 vs. Serum T4 at PND14 in Gilbert Study

Cortical D2 and serum T4 displayed a strong correlation.

\[ Y = 1677 - 390 \ln(x) \quad R^2 = 0.58 \]

*=p<0.05
Cortical D2 activity and serum total T4 concentrations displayed a strong logarithmic correlation.

\[ Y = 2159 - 533 \ln(x) \quad R^2 = 0.90 \]

\[ *=p<0.05 \]
Serum T4 vs. Cortical D2 - PND21

Serum T4
0 20 40 60 80

Cortical D2 activity (fmol/min/mg)
-200
0
200
400
600
800
1000
1200
1400
1600
1800
6.7 – Cortical D2 vs. Serum T4 in Dams in Gilbert Study

Cortical D2 activity and serum total T4 concentrations displayed a strong logarithmic correlation.

\[ Y = 219 - 51 \ln(x) \quad R^2 = 0.76 \]

* = p < 0.05
CHAPTER 7
SUMMARY AND DISCUSSION

Review of hypotheses:

1) Thyroid hormone concentrations in serum do not provide sufficient information to predict the availability and activity of thyroid hormones to all tissues in the body, as alterations in peripheral metabolism help to counterbalance the effects of systemic hypothyroidism in critical tissues such as the brain.

   This hypothesis is partially supported, at least for the PTU model of hypothyroidism. Specifically, no clear correlation was found between serum thyroid hormone concentrations and cortical T3 concentrations. This is due in large part to the sensitive upregulation of D2 in response to declining T4. It should be noted that the relationship between serum T4 and cortical D2 activity is very close, and the serum T4 concentrations in an animal from each tested age group is sufficient to predict the cortical D2 activity with a fair degree of precision. Further study may help to better bridge the gap between serum and tissue availability and effectiveness of thyroid hormones. This study presents no data to clarify the effects of any alterations of serum or receptor binding kinetics caused by some other antithyroid agents. Future studies comparing the relative impact of these alternative pathways of disruption would help to clarify the degree to which deiodinase regulation can compensate for generalized insults to the thyroid axis.

2) Measurement of brain-specific biomarkers such as thyroid responsive gene expression, deiodinase activity and cerebrocortical T3 concentrations will be more reliable predictors of neurodevelopmental impairment than serum hormone concentrations due to their improved proximity to the developmental endpoint compared to serum effects.

   Again, this hypothesis is partially supported. Cortical D2 levels seem to correlate very well with serum T4 concentrations, potentially allowing accurate modeling of cortical D2 regulation in response to
serum T4 perturbations. However, cortical D2 upregulation seems to be limited to a single order of magnitude. Cortical T3 concentrations are maintained despite moderate serum T4 depletion, but will fall under severe T4 depletion. However, this data does not address any potential changes that would result from mechanistically dissimilar anti-thyroid agents. An additional strike against this hypothesis lies in the lack of any consistent correlation between serum or tissue biomarkers and observed electrophysiological results, despite the strong correlation between synaptic potential and D2 activity noted only in the first study. This does not mean that no such correlations exist, and further investigation of thyroid hormone availability to the brain at varying timepoints during development may help to clarify the exact mechanisms responsible for hypothyroidism’s effect on electrophysiological parameters of neural development.

3) The chosen biomarkers: cerebrocortical D2 activity, cerebrocortical T3 concentration, and expression of thyroid-responsive genes in the brain will provide data which will aid efforts to predict both temporary and permanent neurological dysfunction caused by any thyroid disruptive compound. No biomarkers were observed in this study which consistently correlated with electrophysiological outcomes. Expanding future studies to include new biomarkers and more sensitive neurological outcomes may provide the missing data. It is likely that data from late prenatal and early postnatal periods may provide the critical link, as many structures of the brain are still developing during these times. Further analysis during periods of critical development in specific brain regions, combined with sensitive testing of morphology and functionality of these regions in mature animals would help to understand which markers of thyroid hormone availability to the target tissues are most critical for proper development.

Apart from the puzzling impairment of 90-minute LTP in the third study, a measurable decline in at least serum T4 levels was required before any neurological alterations were noted. It is possible that an elevation of available T4 at an inappropriate developmental period might also lead to adverse neurophysiological events. Given the potential perturbations caused by the varying levels of soy isoflavones from lot to lot of the same diet, even the tissue thyroid hormone concentrations may not be providing the full picture of thyroid hormone availability in the brain.
4) Younger animals are more sensitive to thyroid disruption than older animals due to their immature thyroid axis and lesser compensatory capabilities compared to adults.

This hypothesis is well supported by the data obtained in these studies. In almost every case, thyroid hormone reductions were seen both at lower doses, and to a greater extent at equivalent doses than their older littermates or dams. The period of greatest impact seems to be PND14, though the limited sample size at PND4 complicates analysis of that age group. Based on this data, it is possible that doses of antithyroid compounds which are insufficient to cause noticeable effects in mature animals may still have detrimental consequences for younger animals. It is thus critical to investigate effects on the gestational and neonatal periods when considering the true impact of exposure to thyroid disrupting compounds. Measureable

5) The neurological impact of maternal hypothyroidism is modified by the nutritional status of developing pups, as impacted by the number of pups nursed by a single dam, as well as alterations in dietary contents such as soy isoflavones.

The data obtained by the three UGA studies supports, but does not confirm this hypothesis. The severity of results obtained in the three studies ranks in the same order as litter sizes, but further analysis would be necessary to confirm the nutritional status of the pups. The best direct support for this hypothesis comes from the weight gain of control pups. The pups from the second and third studies were significantly heavier than those from the first, and the pups in the second study were slightly heavier than those in the third. This would be expected if litter size were truly important, as litters were largest in the first study, smaller in the third, and smallest in the second. While significant differences in isoflavone content were noted between the second and third study, the lack of parallel data from the first study makes it impossible to declare how strongly the varying isoflavone content of the diet impacted the results noted in the study.

**Study 1 results (Table 7.1):**

The first study employed doses of 0, 3, and 10ppm PTU to induce gestational and neonatal hypothyroidism. The PND25 pups proved to be more sensitive to disruption than their dams.
Specifically, serum T3 levels were reduced at 3ppm in pups, but not in dams. Also, the pups showed an increase in thyroidal NIS and Tg mRNA, which was not seen in the dams. Both the 3 and 10ppm doses served to decrease the weight gain of dosed pups, and both led to impaired synaptic functionality at the PND25 timepoint. A striking correlation was noted between synaptic potential and cortical D2 activity, suggesting a link between immediate thyroid status and synaptic function. The 10ppm dose further caused gross physical retardation, and left the affected pups unable to survive in standard housing after weaning. No significant differences were found in the degree of electrophysiological impairment in the PND25 pups between the doses, so a second study was designed to use lower doses of PTU to produce a graded effect. Only 3ppm and control pups were maintained to adulthood, and the 3ppm pups demonstrated a reduction in LTP at PND90, confirming a permanent effect of the gestational and neonatal dosing.

**Study 2 results (Table 7.1):**

The second study employed doses of 0, 0.3, 1, and 3 ppm PTU, as well as 150ppm perchlorate. The perchlorate dose chosen had been previously shown to induce chronic depression of serum thyroid hormone levels. To harmonize the study design with collaborators at the University of Massachusetts and the US EPA, culling was implemented to reduce litter sizes to eight pups at PND4. Tissue collection was also initiated at PND4 and PND14 to explore the impact of hypothyroidism on several points in neonatal development. Only one pup per litter was used for electrophysiological analysis, as opposed to two pups per litter in the first study. As a result, weaning was moved to PND25 instead of PND32.

No serum thyroid hormone alterations were noted in the perchlorate dosed animals, but a striking increase in NIS mRNA in the thyroids of PND25 pups and dams was noted. This large increase was not accompanied by an increase in Tg mRNA, unlike the response observed to PTU in the first study. Additionally, only a Tg response was seen in the PTU doses in the second study, with no NIS response mirroring that seen in the first study. When combined with the lack of serum thyroid hormone alterations, this suggests that NIS is autoregulated by the thyroid in response to iodine availability, even in the
absence of TSH modulation. Autoregulation of genes in the thyroid in the absence of TSH stimulation has been previously observed by other investigators.2,3

The data obtained from pups in the second study further supported our hypothesis that younger pups would be more impacted by insults to the thyroid axis. Serum T4 concentrations were reduced at both 1 and 3ppm dose levels in PND4, PND14, and PND25 pups, though the reduction was smaller in the PND25 pups. Conversely, serum T4 concentrations were unchanged in dams. This differential sensitivity translated into the brain, where cortical D2 continued to correlate nicely with serum T4. No alterations in serum T3 were noted in this study, nor were any electrophysiological alterations observed in either PND25 pups or adults. When considering the decline in cerebrocortical RC3 expression which is not mirrored in the hippocampus, it is possible that the hippocampus is more highly protected against hypothyroidism than the cortex. Analysis of adult behavior in an open field chamber over five testing sessions of 30 minutes each also showed no significant alterations. Additionally, pups in the second study had higher body weights than their counterparts in the first study.

Overall, the effects of the 3ppm dose in the second study were substantially reduced from the effects in the prior study. The major differences in animal handling between the first and second studies were culling and alteration of the pre-weaning diet from Purina 5001 to Purina 5008. Comparison of the nutritional profiles of the two diets showed a slightly higher fat content in the 5008 diet (6.5% in 5008 vs. 4.5% in 5001), but a 20% decrease in the iodine concentration. See Appendix B for a more detailed comparison. Accordingly, litter sizes emerged as the likely candidate for the leftward shift of the PTU dose-response curve. A third study was designed to verify the shift of the dose response curve, as opposed to a qualitative alteration in the effects of PTU dosing.

**Study 3 results (Table 7.1):**

The third study employed PTU doses of 0, 0.3, 1, 3, and 10ppm PTU, replicating all PTU doses used in the first two studies. The litters were culled in a similar fashion to the second study, but the litter sizes were two pups larger. The behavioral analysis was refined to three 20-minute sessions. Overall, the electrophysiological results obtained in the third study were intermediate between those seen in the prior...
two studies. Unfortunately, the lack of LTP data in the PND25 pups from the first study does not allow the full comparison of electrophysiological results between all studies.

Once again in the third study, younger animals were observed to be more sensitive to thyroid insults than their older littersmates and dams. Serum T4 was reduced starting at 1ppm at PND4, 3ppm at PND14, and 10ppm at PND25 and in dams. Serum T3 was reduced starting at 3ppm at PND14, and 10ppm at PND25 and in dams. The weights of 10ppm pups were reduced starting on the first recorded weight at PND4, though they were still heavier than the 10ppm pups from the first study.

Measurements of thyroid hormone concentrations and deiodinase activity in the brain also showed age-related differences. Cortical D2 activities were increased starting at 1ppm in the PND14 pups, and only at 10ppm in dams and PND25 pups. Little data of this nature is available in the literature, and previous studies have shown lower cortical D2 activity in PND12 pups than PND22, so the greater activity noted in PND14 pups than PND21-25 in our studies is somewhat surprising. Cortical T3 concentrations were reduced only at 10ppm in PND14 and PND25 pups, and were unchanged in the dams. As in the first study, synaptic potential in the PND25 pups was reduced at 3ppm and 10ppm. Changes in LTP were also seen in the PND25 pups, with impaired weak LTP at the 1 and 3ppm dose levels, and impaired strong LTP at the 0.3 and 1ppm dose levels. The effects on the 0.3ppm dose level are especially surprising, as no thyroid hormone concentrations in serum or brain were observed to be altered at that dose in any age group. Given the generally lesser effect of dosing in the second study, it is possible that fluctuations in thyroid hormone concentrations earlier on in development may have impacted the 0.3ppm pups in the third study but not in the second. An additional study further expanding the sampling of early developmental timepoints may help to explain this effect. Unlike the first study, a change in LTP magnitude was not observed in the recovered adults in the third study. However, behavioral observation in an open field chamber revealed that the adults previously exposed to 10ppm PTU were hyperactive. No changes were observed in habituation patterns or activity in the central zone, indicating no impairment in simple place recognition or anxiety.

**Supplemental and collaborative results:**
Between the first and second UGA studies, Dr. Zoeller at the University of Massachusetts conducted an experiment dosing timed pregnant rats with perchlorate doses up to 5ppm in drinking water. Brains from these animals were sent to UGA, where cortical D2 activity levels were determined. No changes were seen in cortical D2 activity in any of these animals. This data, with the consideration of prior literature demonstrating persistent hypothyroidism in rats exposed to 30mg/kg/day of perchlorate was used to arrive at the 150ppm dose used in the second UGA study, which still showed no D2 effects.

Also between the first and second UGA studies, cortex sections were sent to UGA from rats dosed with 0, 1, 2, and 3 ppm PTU by Dr. Gilbert at the US EPA. Cortex D2 activity, as well as cortex T3 and T4 concentrations were determined at UGA. This data was correlated with serum hormone data obtained elsewhere. The correlation between cortical D2 activity and serum total T4 concentrations was found to be nearly identical to that found in the UGA studies, further validating the quantitative relationship between these two biomarkers of thyroid status as it was observed in two strains of rats studied in 2 laboratories. Preliminary analysis of the in-vivo electrophysiology data from Gilbert’s study does not indicate a correlation between D2 activity and synaptic potential in vivo.

Contemporaneously with the second study, a timecourse study was conducted to verify the time at which serum thyroid hormones would be impacted by drinking water administration of 0.3 or 3ppm PTU. This study showed a slight increase in serum T4 levels after 7 days of dosing with 0.3ppm PTU, and a significant decrease in serum T4 under 3ppm PTU at the same time. However, this data was somewhat clouded by the effects of frequent bleeding under anesthesia of these animals, as a slight reduction in serum T4 levels were observed even in the controls after two days of dosing and bleeding. Overall, this data suggests that normal adult rats will be impacted by our PTU dosing protocol after no more than seven days of treatment. This would further imply that dams dosed beginning on day 2 of gestation would be significantly affected well before thyroid hormone receptors are first detected in the developing rat brain around GD12. If this is the case, then it confirms that rats born to dams dosed starting at either GD6 (Gilbert and Zoeller) or GD2 (UGA) will develop under hypothyroid conditions at all relevant times during neural development.
Overall:

The studies of this dissertation confirmed some of the hypotheses in one of the studies, while refuting it in others. In general, the first study best fit the hypotheses, whereas the lesser effects of dosing in the second and third study did not fit the patterns observed in the first study. While several striking findings arose, such as the correlation between deiodinase activity and hippocampal synaptic activity found in the first study, no linear correlations were found to exist when looking at all three datasets. Of all the studied data, the only completely consistent finding was the relationship between cortical D2 activity and serum total T4 concentrations, with a doubling of D2 for each 30-35% reduction in serum T4 concentrations below control levels. However, given the presence of electrophysiological alterations in doses without concomitant T4 reductions or D2 elevations at PND25, it becomes clear that perturbations which subside before PND25 may still leave a lasting impact on neurological function. Based on our observations of PND4 and PND14 animals in the second and third studies, it seems clear that any future studies of hypothyroidism and neurodevelopment must focus on identifying the critical gestational and neonatal timepoints associated with abnormalities in the weanling and/or adult. Pups in these two younger age categories were more strongly affected by each tested PTU dose than their dams or PND25 littermates. In the thyroid, either Tg, NIS, or both of the mRNA’s were upregulated at both 3 and 10ppm in all studies. In general, a decline in cortical T3 concentrations at PND25 was linked with neurological impairment, though impairment was also seen in the absence of cortical T3 changes at PND25. Each of the three studies helped to refine our understanding of the effects of thyroid insufficiency on the hippocampal neurodevelopment of the rat, but our experiments failed to definitively link any tested thyroid related parameters such as deiodinase activity or hormone concentrations to neurological outcome, though with the exception of the 0.3ppm LTP impairment in the third study, measurable declines in serum T4 in at least preweaning age group were linked to adverse neurological effects. New endpoints capable of measuring function in other critical brain regions, such as maze testing, startle responses, and other common developmental toxicity tests should be considered for any future work. In addition, the prioritization of histological analysis of the brain will assist in detecting physical
malformations not impacting the chosen neurofunctional assays. By expanding brain measurements beyond the hippocampus, many more changes in the brain may be detected, greatly expanding our understanding of the neurodevelopmental impacts of hypothyroidism. Further characterization of regional concentrations of thyroid hormones in the brain should also help to understand the true relationship between thyroid hormone depression and adverse neurological alterations. Except for study 1 which showed a relationship between D2 and srmax and cortical T3 and srmax in PND25 pups, the other studies did not replicate these findings, suggesting that the larger litter size and/or a reduced nutritional plane may have been key to this relationship. The isoflavone concentrations in the pups were not measured in study 1 so variation relative to studies 2 and 3 cannot be confirmed. Further studies would be necessary to confirm this possibility of dietary effects.

References

4. Bates JM, St. Germain DL, Galton VA 1999 Expression profiles of the three iodothyroinine deiodinases, D1, D2, and D3, in the developing rat. Endocrinology 140(2)844-51
Table 7.1 - Overall findings

□: Not Studied; ═: No Change; ▲: Increased; ▼: Decreased

<table>
<thead>
<tr>
<th></th>
<th>Dose 0.3</th>
<th>1</th>
<th>3</th>
<th>10</th>
<th>Dose 0.3</th>
<th>1</th>
<th>3</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Study</td>
<td>1/2/3</td>
<td>1/2/3</td>
<td>1/2/3</td>
<td>1/2/3</td>
<td>1/2/3</td>
<td>1/2/3</td>
<td>1/2/3</td>
</tr>
<tr>
<td></td>
<td>PND4</td>
<td>Dam</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T4</td>
<td>□/□/□</td>
<td>□/▼/▼</td>
<td>□/▼/▼</td>
<td>□/▼/▼</td>
<td>□/□/□</td>
<td>□/□/□</td>
<td>□/□/□</td>
<td>□/□/□</td>
</tr>
<tr>
<td>D2</td>
<td>□/□/□</td>
<td>□/□/□</td>
<td>□/▲/□</td>
<td>□/□/□</td>
<td>T4</td>
<td>□/□/□</td>
<td>□/□/□</td>
<td>□/□/□</td>
</tr>
<tr>
<td>cT3</td>
<td>□/□/□</td>
<td>□/□/□</td>
<td>□/□/□</td>
<td>□/□/□</td>
<td>TSH</td>
<td>□/□/□</td>
<td>□/□/□</td>
<td>□/□/□</td>
</tr>
<tr>
<td>PND14</td>
<td>D2</td>
<td>□/□/□</td>
<td>□/□/□</td>
<td>□/□/□</td>
<td>□/□/□</td>
<td>□/□/□</td>
<td>□/□/□</td>
<td>□/□/□</td>
</tr>
<tr>
<td>T3</td>
<td>□/□/□</td>
<td>□/□/□</td>
<td>□/□/□</td>
<td>□/□/□</td>
<td>□/□/□</td>
<td>□/□/□</td>
<td>□/□/□</td>
<td>□/□/□</td>
</tr>
<tr>
<td>T4</td>
<td>□/□/□</td>
<td>□/□/□</td>
<td>□/□/□</td>
<td>□/□/□</td>
<td>□/□/□</td>
<td>□/□/□</td>
<td>□/□/□</td>
<td>□/□/□</td>
</tr>
<tr>
<td>cT3</td>
<td>□/□/□</td>
<td>□/□/□</td>
<td>□/□/□</td>
<td>□/□/□</td>
<td>□/□/□</td>
<td>□/□/□</td>
<td>□/□/□</td>
<td>□/□/□</td>
</tr>
<tr>
<td>TSH</td>
<td>□/□/□</td>
<td>□/□/□</td>
<td>□/□/□</td>
<td>□/□/□</td>
<td>□/□/□</td>
<td>□/□/□</td>
<td>□/□/□</td>
<td>□/□/□</td>
</tr>
<tr>
<td>PND25</td>
<td>wLTP</td>
<td>□/□/□</td>
<td>□/□/□</td>
<td>□/□/□</td>
<td>□/□/□</td>
<td>□/□/□</td>
<td>□/□/□</td>
<td>□/□/□</td>
</tr>
<tr>
<td>T3</td>
<td>□/□/□</td>
<td>□/□/□</td>
<td>□/□/□</td>
<td>□/□/□</td>
<td>□/□/□</td>
<td>□/□/□</td>
<td>□/□/□</td>
<td>□/□/□</td>
</tr>
<tr>
<td>T4</td>
<td>□/□/□</td>
<td>□/□/□</td>
<td>□/□/□</td>
<td>□/□/□</td>
<td>□/□/□</td>
<td>□/□/□</td>
<td>□/□/□</td>
<td>□/□/□</td>
</tr>
<tr>
<td>sLTP</td>
<td>□/□/□</td>
<td>□/□/□</td>
<td>□/□/□</td>
<td>□/□/□</td>
<td>□/□/□</td>
<td>□/□/□</td>
<td>□/□/□</td>
<td>□/□/□</td>
</tr>
<tr>
<td>cT3</td>
<td>□/□/□</td>
<td>□/□/□</td>
<td>□/□/□</td>
<td>□/□/□</td>
<td>□/□/□</td>
<td>□/□/□</td>
<td>□/□/□</td>
<td>□/□/□</td>
</tr>
<tr>
<td>NIS</td>
<td>□/□/□</td>
<td>□/□/□</td>
<td>□/□/□</td>
<td>□/□/□</td>
<td>□/□/□</td>
<td>□/□/□</td>
<td>□/□/□</td>
<td>□/□/□</td>
</tr>
<tr>
<td>TG</td>
<td>□/□/□</td>
<td>□/□/□</td>
<td>□/□/□</td>
<td>□/□/□</td>
<td>□/□/□</td>
<td>□/□/□</td>
<td>□/□/□</td>
<td>□/□/□</td>
</tr>
</tbody>
</table>

204
Figure 7.1-Serum T4 vs. Cortical D2 – Dams

When all studies were adjusted to calculate T4 and D2 alterations on a percent of control basis, all studies clustered tightly into a common relationship between serum total T4 and cortical D2 activity. No major differences are noted in this relationship across studies. In the following equation, Y is the percentage of control cortical D2 activity, and x is the percentage of control serum T4 concentration

\[ Y = 94.5 + 880e^{-0.05x} \quad R^2 = 0.7 \]
Figure 7.2-Serum T4 vs. Cortical D2 - PND21

When all studies were adjusted to calculate T4 and D2 alterations on a percent of control basis, all studies clustered tightly into a common relationship between serum total T4 and cortical D2 activity. No major differences are noted in this relationship across studies. In the following equation, Y is the percentage of control cortical D2 activity, and x is the percentage of control serum T4 concentration

\[ Y = -8 + 747 \times e^{(-0.02 \times x)} \quad R^2 = 0.68 \]
Serum T4 vs. Cortical D2 - PND21

![Graph showing the relationship between Serum T4 (% control) and Cortical D2 (% control)]
When all studies were adjusted to calculate T4 and D2 alterations on a percent of control basis, all studies clustered tightly into a common relationship between serum total T4 and cortical D2 activity. No major differences are noted in this relationship across studies. In the following equation, Y is the percentage of control cortical D2 activity, and x is the percentage of control serum T4 concentration:

\[ Y = 93 + 1134 e^{-0.04x} \]

\[ R^2 = 0.78 \]
Serum T4 vs. Cortical D2 - PND14
# APPENDIX A

## LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BCE</td>
<td>Before Common Era</td>
</tr>
<tr>
<td>BW</td>
<td>Body weight</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td>CE</td>
<td>Common Era</td>
</tr>
<tr>
<td>D1</td>
<td>Type 1 deiodinase</td>
</tr>
<tr>
<td>D2</td>
<td>Type 2 deiodinase</td>
</tr>
<tr>
<td>D3</td>
<td>Type 3 deiodinase</td>
</tr>
<tr>
<td>GD</td>
<td>Gestational day</td>
</tr>
<tr>
<td>kD</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>LTP</td>
<td>Long term potentiation</td>
</tr>
<tr>
<td>MMI</td>
<td>Methimazole</td>
</tr>
<tr>
<td>NIS</td>
<td>Sodium/iodide symporter</td>
</tr>
<tr>
<td>NOAEL</td>
<td>No Observed Adverse Effect Level</td>
</tr>
<tr>
<td>PND</td>
<td>Postnatal day</td>
</tr>
<tr>
<td>ppm</td>
<td>Parts per million</td>
</tr>
<tr>
<td>PTU</td>
<td>Propylthiouracil</td>
</tr>
<tr>
<td>rT3</td>
<td>3,3’,5’-triiodothyroinine</td>
</tr>
<tr>
<td>T3</td>
<td>3,5,3’-triiodothyronine</td>
</tr>
<tr>
<td>T4</td>
<td>Thyroxine</td>
</tr>
</tbody>
</table>

211
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBG</td>
<td>Thyroxine-binding globulin</td>
</tr>
<tr>
<td>Tg</td>
<td>Thyroglobulin</td>
</tr>
<tr>
<td>TH</td>
<td>Thyroid hormone</td>
</tr>
<tr>
<td>TPO</td>
<td>Thyroid peroxidase</td>
</tr>
<tr>
<td>TSH</td>
<td>Thyroid stimulating hormone</td>
</tr>
<tr>
<td>TTR</td>
<td>Transthyretin</td>
</tr>
</tbody>
</table>
## APPENDIX B

### DIETARY COMPARISON

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Diet 5008</th>
<th>Diet 5001</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>23.5%</td>
<td>23.9%</td>
</tr>
<tr>
<td>Fat (ether extract)</td>
<td>6.5%</td>
<td>5.0%</td>
</tr>
<tr>
<td>Fat (acid hydrolysis)</td>
<td>7.5%</td>
<td>5.7%</td>
</tr>
<tr>
<td>Starch</td>
<td>34.9%</td>
<td>31.9%</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.22%</td>
<td>0.22%</td>
</tr>
<tr>
<td>Fructose</td>
<td>0.24%</td>
<td>0.30%</td>
</tr>
<tr>
<td>Sucrose</td>
<td>2.57%</td>
<td>3.70%</td>
</tr>
<tr>
<td>Lactose</td>
<td>0.39%</td>
<td>2.01%</td>
</tr>
<tr>
<td>Calories from Protein</td>
<td>26.849%</td>
<td>28.507%</td>
</tr>
<tr>
<td>Calories from Fat (ether extract)</td>
<td>16.710%</td>
<td>13.496%</td>
</tr>
<tr>
<td>Calories from Carbohydrates</td>
<td>56.441%</td>
<td>57.996%</td>
</tr>
<tr>
<td>Gross Energy, kcal/gm</td>
<td>4.15</td>
<td>4.07</td>
</tr>
<tr>
<td>Metabolizable Energy, kcal/gm</td>
<td>3.31</td>
<td>3.02</td>
</tr>
<tr>
<td>Iodine (ppm)</td>
<td>0.8</td>
<td>1.0</td>
</tr>
</tbody>
</table>