The Effects of Thyroid Hormone Insufficiency During Development On Cortical Morphology And The Behavioural Manifestations

by

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This manuscript has been read and accepted for the Graduate Faculty in Biology in satisfaction of the dissertation requirement for the degree Doctor of Philosophy.

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Abstract

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by

Susan Briffa-Mirabella

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The central hypothesis of this work is that developmental thyroid insufficiency impacts the development of the rat cerebral cortex by altering cortical volume and the number of cortical neurons. In addition, as these neuroanatomical changes caused by milder forms of developmental hypothyroidism or hypothyroxinemia may have both immediate and long term consequences on certain aspects of behaviour, the investigation sought to determine if the alterations in morphology, including the change in relative cortical volume, and the change in the number of cortical neurons in the rat brain, led to behavioral manifestations. Hypothyroidism was induced by the administration of graded levels of the antithyroid agent propylthiouracil (PTU) to suppress thyroid hormone production.

The number of neurons was estimated, using unbiased sampling techniques, to determine whether the cellular composition of cortex was altered following developmental TH insufficiency. To determine if these cortical alterations led to changes in behaviour, a battery of behavioural tests were performed which included maternal retrieval (PND 4), maternal separation anxiety (PND 6), Barnes maze (PND 48 and PND 86), social interaction social approach (PND 48-50), and open field (PND 46).

Taken together, the results presented here support the hypothesis that developmental hypothyroidism and hypothyroxinemia induced by chemical thyroid hormone suppression (PTU) cause alterations in the morphology of the cerebral cortex by altering cortical volume and changing the number of cortical neurons in the rat brain. Furthermore these alterations ultimately lead to changes in certain aspects of behaviour. These results have important clinical relevance because several studies suggest that developmental disabilities ranging from mild dyslexia to severe mental retardation can be attributed to alterations in cortical morphology resulting from abnormal cortical development.

This thesis is dedicated to my parents,

Gerald and Marian Briffa

And to my sister Marian Briffa

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TABLE OF CONTENTS

CHAPTER 1 – The Introduction……………………………………………………………….1

Chapter 2: An Overview: The Endocrine System Hypothalamus and Pituitary Gland…………………………………………………….…..................6

2.1. Endocrine Function of: Hypothalamus and Pituitary Gland… ………………………..…..6

2.1.1-Hypothalamus ………………………………………………………………….....6

2.1.2- Magnocellular Nuclei…………………………………………………………...........7

2.1.3 -Parvicellular Nuclei …………………………………………………….............8

2.1.4 - Pituitary Gland……………………………………………………………….……......8

2.1.5 - Posterior Pituitary……………………………………………………………….……..8

2.1.6 - Anterior Pituitary…………………………………………………………………....9

2.2 -The Adenohypophysis: Pars Distalis Regulates the Thyroid Gland……………………10

Chapter 3: Thyroid Hormone Production and Control of Release……………………….....12

3.1 - Thyroid Hormone Synthesis……………………………………………………………12

3.2 - Thyroid Hormone Release……………………………………………………….. 14

3.3 - Negative Feedback and Changing Needs………………………………….…..........14

3.4 - Hypothalamic-Pituitary-Thyroid Axis (HPT)……………………………………. 15

Chapter 4: Thyroid Hormone Receptors and the Deiodinases………………………………17

4.1 - Thyroid Hormone Receptors…………………………………………………………..17

4.1.1 - Mitochondrial Receptors……………………………………………………………17

4.1.2 - Characteristics of the Nuclear Receptors………………………………………….……...17

4.1.3 - Thyroid Hormones Transport to Nuclear Acceptor Sites…………………………….……..19

4.1.4 - Occupancy of Nuclear Receptor and Biological Response……………………….……..19

4.1.5 - Thyroid Hormone Receptor Structure and Expression…..………………………………21

4.1.6 - Thyroid Hormone Receptor Interaction with DNA……………………………………23

4.1.7 - Ligand- Free State of Receptor versus Ligand - Bound State of Receptor………………24

4.2 - Deiodinases ……………………………………………………………………………..24

Chapter 5: Chapter 5: Background and Significance………………………………………..27

5.1 - Relative Importance of Thyroid Hormones to the Fetus………………………………27

5.2 - Hypothyroidism versus Hypothyroxinemia……………………………………………..31

5.3 - Iodine Deficiency………………………………………………………………………..34

5.4 - Thyroidal Economy in Pregnancy…………………………………………………………37

5.5 - Physiology of the Change Required by the Maternal Thyroid During Pregnancy…..39

5.6 - The Physiologic Consequences of Iodine Insufficiency During Pregnancy…………...40

5.7 - Metabolism of Iodine During Pregnancy………………………………………………..40

5.8 - Thyroid Hormones and Brain Development……………………………………………42

Chapter 6: Developmental Hypothyroidism Alters Neocortical Volume….………………..46

6.1 – Introduction………………………………………………………………………………47

6.2 – Materials & Methods……………………………………………………………………..49

6.2.1 - Developmental hormone insufficiency…………………………………………………49

6.2.2 - Brain sectioning and Nissl staining……………………………………………………50

6.2.3 - Cortical Measurements……………………………………………………………...50

6.3 – Results……………………………………………………………………………………..51

6.3.1 – Cortical and Whole Brain Volume Estimates PND23…………………………………...51

6.3.2 – Cortical Linear Measurements PND23……………………………….................51

6.3.3 **-** *Cortical and Whole Brain Volume Estimates PND86*……………………………….……52

6.3.4 - Cortical Linear Measurements PND86…………………………………………………......52

6.4 – Conclusion / Discussion……………………………………………………………..53

Chapter 7: Developmental Hypothyroidism Alters Cellular Composition: A Stereological Analysis……………………………………………………………..57

7.1 – Introduction………………………………………………………………………....58

7.2 – Materials & Methods…………………………………………………………………..61

7.2.1 - Developmental hormone insufficiency…………………………………………………61

7.2.2 - Brain sectioning ……………………………………………………………………61

7.2.3 – Immunohistochemistry………………………………………………………………...62

7.2.4 - Quantification of neuron number……………………………………………………...62

7.3 - Results……………………………………………………………………………….65

7.4 – Conclusion / Discussion…………………………………………………………….65

Chapter 8: Developmental Hypothyroidism Effects on Social Behaviour in the Rat……...72

8.1 – Introduction…………………………………………………………………………73

8.2 – Materials & Methods…………………………………………………………………..75

8.2.1 **-** *Perinatal Hypothyroidism Model Developmental hormone insufficiency*……………….75

8.2.2 - Maternal Retrieval……………………………………………………………….....76

8.2.3 - Ultrasonic Vocalizations……………………………………………………………76

8.2.4 - Barnes Maze Testing…………………………………………………………………..77

8.2.5 - Open Field Testing……………………………………………………………………78

8.2.6 - Three Chambered Social Approach Task…………………………………………......79

8.2.7 – Social Interaction……………………………………………………………………...82

8.3 – Results……………………………………………………………………………..88

8.3.1 – Maternal Retrieval………………………………………………………………….....88

8.3.2 - Ultrasonic Vocalizations……………………………………………………………88

8.3.3 - Barnes Maze Testing…………………………………………………………………..89

8.3.4 - Open Field Testing……………………………………………………………………90

8.3.5 - Three Chambered Social Approach Task…………………………………………...91

8.3.6 – Social Interaction…………………………………………………………………...92

8.4 – Conclusion / Discussion…………………………………………………………….92

Chapter 9: Global Conclusion/Discussion……………………………………………...112

9.1 - Developmental Hypothyroidism Alters Neocortical Volume………………………....112

9.2 - Developmental Hypothyroidism Alters Cellular Composition: A Stereological Analysis……………………………………………………………………113

9.3 - Developmental Hypothyroidism Effects on Social Behavior in the Rat……………..115

References..118

LIST OF FIGURES

Figure 6.1 -Developmental hypothyroid-induced changes in neocortical volume on PND23............55

Figure 6.2 - Developmental hypothyroid-induced changes in neocortical volume on PND 86……..56

Figure 7.1 – Methods………………………………………………………………………...64

Figure 7.2 – Percentage of neuronal volume (µ3)…………………………………………………...70

Figure 7.3 – Concentration of neurons (µ3) in cortex……………………………………………71

Figure 8.1 - Schematic RFID uses in tracking movement of individual animals in the colony……..85

Figure 8.2 - Place Preferences in the Housing Environment……………………………………...86

Figure 8.3 - Network Graph 1 day of data…………………………………………………...87

Figure 8.4 - Maternal Retrieval……………………………………………………………..…102

Figure 8.5 - Ultrasonic vocalizations…………………………………………………………..103/104

Figure 8.6 - Barnes maze PND28……………………………………………………...……105

Figure 8.7 - Barnes Maze PND48……………………………………………………...……106

Figure 8.8 - Open Field Test…………………………………………………………….…..107/108

Figure 8.9 - Social Approach…………………………………………………………………..109/110

Figure 8.10 – Social Interaction…………………………………………………………………111

ABBREVIATIONS

CH	congenital hypothyroidism
D1	deiodinase type 1
D2	deiodinase type 2
D3	deiodinase type 3
DIT	diiodotyrosine
E	embryonic day
FTF	fetal thyroid function
FT4	free T4
GD	gestational day
hCG	human chorionic gonadotropin
ID	iodine deficiency
µg/l	micrograms per liter
MGE	medial ganglionic eminence
MMI	methimazole
MIT	monoiodotyrosine
NBS	newborn screening
NIS	sodium iodide symporter
PII	pool of inorganic iodide
PMA	postmenstral age
PND	postnatal day
PTU	propylthiouracil
RFID	implanted radio frequency identification
S1	primary somatosensory cortex
TBG	T4- binding globulin
TH	thyroid hormone
TRH	thyroid releasing hormone

TRs	thyroid hormone receptors
TSH	thyroid stimulating hormone
T3	triiodothyronine
T4	thyroxine
US	United States
USVs	ultrasonic vocalizations

Chapter 1: Introduction

Thyroid hormones (THs) are essential to normal development of the brain (Bernal, 2007). Congenital hypothyroidism (CH) is considered the most common preventable cause of developmental disabilities (Glinoer and Delange, 2000). Although severe hypothyroidism during development is associated with profound mental retardation, motor and visual deficits(Gilbert and Zoeller, 2010), moderate alterations in thyroid function leads to IQ deficit and lower neuropsychological test scores in children (Haddow et al., 1999a). There are two conditions related to thyroid abnormalities: 1) subclinical hypothyroidism, a condition in which thyroid stimulating hormone (TSH) is elevated but thyroid hormone levels, thyroxine (T4) and triiodothyronine (T3) are normal, and 2) hypothyroxinemia, a first trimester condition during pregnancy in which TSH and T3 levels are normal while T4 levels are depressed by 50%. The majority of cases of hypothyroxinemia go undetected because current testing standards for hypothyroidism focus on circulating TSH levels. This can occur under conditions of low dietary iodine intake where auto regulatory mechanisms sensing the decrease in circulating iodide respond by favouring the synthesis and secretion of T3 over T4. As a result, circulating T4 decreases, T3 however, does not decrease and may in fact increase thereby preventing an increase in serum TSH and clinical detection of hypothyroidism (Missler et al., 1994; Vagenakis et al., 1973). Animal models of hypothyroxinemia demonstrated that brain development is dependent on adequate levels of TH. Alterations in neuronal migration (Goodman and Gilbert, 2007}, and cytoarchitectural abnormalities in the hippocampus and somatosensory cortex (Lavado-Autric et al., 2003) have been shown to be sensitive to TH levels during critical windows of development.

Moderate suppression of the active forms of thyroid hormone is achieved through pharmacological agents, such as propylthiouracil (PTU), and environmental factors including; xenobiotics and dietary iodine deficiency.

The current dissertation tested the central hypothesis that developmental thyroid insufficiency induced by the anti-thyroid agent propylthiouracil (PTU) alters the development of the cerebral cortex by altering cortical volume and changing the number of cortical neurons in the rat brain. The possible behavioural outcome of altered brain structures will be evaluated.

Differentiation of cells in the brain during the period of corticogenesis appears to be affected by hypothyroidism, with glial-restricted precursors being guided away from an oligodendrocyte fate to become astrocytes (Sharlin et al., 2008). In addition ubiquitin-dependent programmed cell death during the period of cortical histogenesis appears to be regulated by TH (Pasquini et al., 2000), which suggests that developmental hypothyroidism may cause long-term changes in the number of cortical neurons. Together these findings indicate that developmental hypothyroidism alters neocortical development potentially leading to abnormalities in structural volume. However, no studies to date have specifically measured whether developmental hypothyroidism in the rat alters neocortical development potentially leading to abnormal neocortical volume.

In addition these neuroanatomical changes caused by mild developmental hypothyroidism and or hypothyroxinemia may have immediate and long term consequences on certain aspects of behaviour. Hence, we sought to determine if the alterations in morphology, including the change in relative cortical volume outlined in Chapter 6, and the change in the number of cortical neurons in the rat brain outlined in Chapter 7, led to behavioral alterations.

Kooistra and Colleagues (Kooistra et al., 2001) reported that children with a history of hypothyroidism during the perinatal period show a decrease in social behavior and an increased likelihood of introversion. Having this, we performed a battery of tests related to social behavior. Several nonsocial behaviors were examined; locomotion, working memory, repetitive behaviors as well as anxiety. It is however important to acknowledge that none of the battery of tests reported here directly measures cortical function. The battery included: maternal retrieval pups were tested at (PND4) to determine if the USVs results obtained at PND 6 were due to developmental hypothyroidism and not due to effects resulting from a decrease in maternal care caused by maternal exposure to PTU through gestation until weaning. Maternal separation anxiety, PND 6 was chosen as the age for testing because placing rat pups alone in novel surroundings reliably elicits USVs from 3 to 18 days of age (Hofer et al., 1993b). In addition differences in eye opening were observed with the hypothyroid rats opening their eyes later than euthyroid cohorts, as such results obtained at PND 11 were discarded (unpublished observations). Barnes maze (PND 28 and PND 48), these time points were selected as PND 28 represents a hypothyroid state while PND 48 represents a return to euthyroid status (Cooper et al., 1983). Social interaction social approach (PND 48-50) animals were weaned and transferred to the group housing environment at PND 31, data was analysed for PND 48-50 in order to allow for the animals to habituate to their environments. As PND 48-50 was chosen for analysis of social interaction the social approach assay was also run at the same time point as the three chambered social approach task was conducted in order to validate the findings of the social interaction assay. Finally, open field testing was conducted in order to rule out an effect of a drug/ treatment (PTU) on motor ability which would be an unwanted confound, PND 46 was chosen as it preceded the time points of other testing that relied on motor ability. This area of

investigation has important clinical relevance because undiagnosed subclinical hypothyroidism and hypothyroxinemia may account for some developmental disorders of unknown etiology like autism (Courchesne et al., 2001).

Background for the interpretation of this document will be outlined in Chapters 1 through 5, while chapters 6, 7 and 8 have been written in journal format and contain all relevant methodology and results obtained for this dissertation, followed by a global discussion in Chapter 9. Chapter I will provide a brief overview of the endocrine system and focuses on the hypothalamus and the pituitary glands two brain structures involved in regulating the release of the thyroid hormones. Both the synthesis and release of the THs, T4 and T3 as well as the regulatory mechanisms will be reviewed in chapter 3, in addition, the regulation of gene expression by the interaction of THs and their nuclear receptors will be covered. The focus of chapter 4 is the thyroid hormone receptors; their characteristics, structure, transport and the biological responses they induce. Additionally this chapter will include a review of the deiodinases, the peroxidase enzymes involved in the activation or deactivation of the thyroid hormones. Chapter 5 will discuss the metabolism of and the need for additional iodine during pregnancy as iodine deficiency (ID) is known to be the single most preventable cause of brain damage after starvation (Dunn and Delange, 2001; Glinoer and Delange, 2000). The role of ID in both hypothyroidism and hypothyroxinemia will be examined, as well as the deleterious consequences of TH dysregulation on brain function.

Whether an additional consequence of developmental TH insufficiency is an alteration in neocortical volume due to a change in the cellular composition of cortex will be reviewed in Chapters 6 and 7. Finally, as several studies suggested that developmental disabilities ranging from mild dyslexia's to severe mental retardation could be attributed to alterations in cortical

morphology due to abnormal cortical development (Crome, 1960; Galaburda et al., 1985; Humphreys et al., 1990), Chapter 8 discusses if the alterations in cortex we reported had either immediate or long-term consequences on certain aspects of behaviour in rats developmentally exposed to graded levels of PTU.

Chapter 2: An Overview: The Endocrine System

Hypothalamus and Pituitary Gland

This chapter will provide a brief overview of the endocrine system, highlighting the endocrine functions of the hypothalamus and pituitary glands, which regulate the release of the thyroid hormones T3 and T4.

All members of a group of functionally related yet anatomically discrete organs that synthesize hormones and release them into the blood stream are considered endocrine glands. They include the pituitary, parathyroid, thyroid and adrenal glands as well as the testes, ovaries, pancreatic islets and the pineal gland. However, because more hormones are produced outside these structures than within them, and some hormones are produced extracellularly, formulating a definition of an endocrine gland that takes into account / accommodates all the sites of biosynthesis is impossible. When reviewing endocrinology the hypothalamic pituitary axis must be considered as it is the feedback system that coordinates the activity of major peptide hormones. Critical to endocrine physiology is the process of auto regulation, which is responsible for detecting and adjusting for changes within the system, allowing for the maintenance of hormones within a target range. This process is essential as hormones function properly only when present in optimum concentrations (Dot-ton, 2000; Fuxe et al., 1979; Hadley, 1996).

2.1 - Endocrine Function of: Hypothalamus and Pituitary Gland

2.1.1 - Hypothalamus

The hypothalamus, is one of the richest sites of hormones, that act as neuromodulators in the brain including oxytocin (affects the uterus and mammary gland), vasopressin (acts on the kidney for water conservation and the pituitary) as well as several hormones that maintain the functions of the glandular portion of the pituitary/adenohypophysis. At one site hypothalamic secretions act as hormones and at another as neuromodulators /neurotransmitters (Fuxe et al., 1979).

2.1.2 - Magnocellular Nuclei

Above the infundibulum or stalk from which the pituitary gland is suspended clusters of perikarya (neuronal soma) enter into the formation of parvicellular nuclei while large cell bodies are grouped into the magnocellular nuclei (Reichlin et al., 1978)

The magnocellular nuclei include the supraoptic nuclei located by the crossing of the optic nerves (optic chiasma), and the paraventricular nuclei which lie close to the third ventricle and have bundles of long axons (hypothalamo-hypophysial nerve tracts) that connect the nuclei with the neural lobe of the pituitary gland (posterior pituitary). The neurons whose cell bodies reside in the hypothalamus synthesize thyrotropin releasing hormone (TRH) (Segersen et al., 1987a; Segersen et al., 1987b). TSH neurons resides in the Paraventricular nucleus and project to the median eminence (Ishikawa et al., 1988; Merchenthaler and Liposits, 1994). The median eminence is a neurohemal organ, i.e. an organ in which an intimate relationships between axons and terminal blood vessels is established), that is attached to the anterior portion of the pituitary gland by the hypothalamic-pituitary-portal vessels (Martin and Reichlin, 1987). In mammals, Oxytocin and vasopressin (two anti-diuretic hormones) are synthesized within the magnocellular neurosecretory cells, packaged into large granules along with the neurophysins proteins (Seif

and Robinson, 1978; Walter, 1975), released into the systemic blood vessels through the neural lobe capillaries (Leng et al., 1999).

2.1.3 - Parvicellular Nuclei

Synthesis of the hormone takes place in the neuronal soma, the hormones, which are sometimes called "release factors" (Martin, 1985) travel down short fine axons to the median eminence terminating near blood vessels of the hypothalamohypophysial portal system (specialized compartment of the circulatory system). The hormones include but are not limited to TRH and travel through these blood vessels to the pars distalis of the pituitary gland (Squire et al., 2003).

2.1.4 - Pituitary Gland

The pituitary gland has two major subdivisions that differ in their embryological origin, structure and function. The (1.) neurohypophysis; An extension of the hypothalamus, the neurohypophysis, stores regulators formed in the magnocellular nuclei and releases the peptides into systemic circulation, but does not produce hormones and the 2.) adenohypophysis; The glandular portion of the pituitary, the adenohypophysis, is distinct from the hypothalamus but makes functional connections with it via the portal blood vessels, and synthesizes and secretes hormones (Dot-ton, 2000).

2.1.5 - Posterior Pituitary

The neurohypophysis or posterior pituitary develops from the floor of the embryonic brain, receives hormones produced in the hypothalamus. The neurohypophysis is actually not a gland but a collection of axonal projections from the hypothalamus. The axons release peptide

hormones into the capillaries of the hypophyseal circulation. Contained within the posterior pituitary are specialized glial cells resembling astrocytes called pituicytes. The neurohypophysis is an extension of the hypothalamus and is comprised of three regions (1) the neural lobe which stores oxytocin and vasopressin and includes pituicytes ; (2) Median eminence some sources exclude it from the pituitary; and the infundibular stalk which is the connection between the hypothalamus and posterior pituitary (Dot-ton, 2000; Hadley, 1996; Martin, 1985; Moore and Persaud, 1998).

2.1.6 - Anterior Pituitary

Developed from Rathke's pouch, part of the oral embryonic ectoderm (Martin, 1985), the adenohypophysis has three subdivisions. The pars distalis is the first subdivision and has resident cells that synthesize and release hormones associated with the anterior pituitary and each cell type is named for its secretory products. The specialized compartment of the circulatory system that transports hormones directly from the hypothalamus to the anterior pituitary through a specialized set of blood vessels is known as the hypothalamic-hypophyseal portal system (a portal system is composed of two capillary beds connected in series by a set of blood vessels). It allows endocrine communication between the two structures. As it relates to thyroid function the cell type of the pituitary is the thyrotrope which synthesizes thyroid-stimulating hormone (TSH) in small granules and the function is secretion of thyroid hormones T4 and T3 and growth of the thyroid gland. Although associated with the posterior pituitary and not part of thyroid endocrinology the next division of the pituitary is the pars tuberalis, which forms a collar around the median eminence (Dot-ton, 2000; Hadley, 1996; Martin, 1985; Moore and Persaud, 1998). Portal blood vessels pass through this region and hypothalamic and pituitary hormones travel through them in both directions (Bergland and Page, 1978). Finally the pars intermedia plays a

role in water and electrolyte balance (Glickman et al., 1979), and in development of the adrenal cortex. A more simplified version is the anterior pituitary consists of the pars distalis and some of the pars tuberalis and the posterior pituitary include the pars intermedia, the neural lobe and the remainder of the pars tuberalis.

The adenohypophysis has great physiologic importance as it has been shown that hypophysectomized animals not receiving replacement therapy have compromised ability to adjust to changing internal needs as well as external environments. These animals are unable to adjust to situations that are easily tolerated by control animals with healthy endocrine systems. Compromised abilities include inability to adjust to marked temperature changes, brief periods of food and water deprivation are intolerable, they are unable to perform tasks that require skeletal muscle strength and endurance in addition these animals are not capable of either reproduction or lactation (Hamburg et al., 1964).

2.2 - The Adenohypophysis: Pars Distalis Regulates the Thyroid Gland

The hormones released from pars distalis regulate the following endocrine glands; the adrenal cortex, gonads and thyroid gland. The thyroidal economy is maintained by the hormones T4 and T3 which are secreted by thyroid follicular cells (albeit only small quantities of T3). A conversion of T4 to the active form of the hormone T3 (Wirth et al., 2009) is accomplished by most of the target organs. Thyroid hormones (THs) are essential to the normal development of the central nervous system (Dussault and Ruel, 1987; Myant, 1971) and for the maturation of both the reproductive and skeletal systems (Longcope, 1991; Shao et al., 2006). In addition TH's regulate metabolic rate and affect the heart, liver and kidney (Capasso et al., 1999;Hyyti et al., 2005; Malik and Hodgson, 2002). Hood (Hood et al., 1999) found in the rat that small increases

in serum TSH can be sufficient to stimulate thyroid follicular cell proliferation. If not stimulated by TSH, the follicular cells will not produce adequate amounts of the hormones and will eventually undergo atrophy (reviewed in Martin, 1985).

Chapter 3: Thyroid Hormone

Production and Control of Release

A brief overview of the thyroid hormones T3 and T4 and their synthesis will be provided in this chapter, along with a review of the regulatory mechanisms involved in maintaining the concentrations of T3 and T4 within optimal ranges. Negative feedback is one such mechanism that maintains fairly constant levels of T4 in circulating blood in the thyroid releasing hormone (TRH) – thyroid stimulating hormone (TSH) system. In addition, feedback loops of the Hypothalamic – Pituitary - Thyroid Axis will be reviewed; these mechanisms are responsible for monitoring and adjusting the levels of hormone according to an ideal set point.

3.1 - Thyroid Hormone Synthesis

The thyroid gland is an endocrine gland with the primary function of producing the hormones, thyroxine (T4) and triiodothyronine (T3) which are amines derived from the amino acid tyrosine; in addition it produces the hormone calcitonin. Circulating triodothyronines are formed largely from peripheral deiodination of T4 which is the major product released from the thyroid gland (Leonard and Koehrle, 1996). T4 is mainly secreted but it is primarily converted by target cells to T3. Although both hormones are bound by the same receptor protein located in the cell nucleus, the receptor has a greater affinity for T3, and T3 is three to five times more active biologically. Therefore, T3 is the active hormone in the target cells and many processes during mammalian brain development are T3 dependent (Gilbert and Zoeller, 2010 ;Wirth et al., 2009; Zoeller et al., 2002)

The thyroid gland is located in the lower part of the neck in front of the trachea. Within the gland there are small structures that consist of a ring of cells surrounding a ball of fluid. The fluid portion in the lumen of the cell is called colloid. Such cells are referred to as thyroid follicular cells and the entire structure is known as a thyroid follicle. Thyroid follicular cells produce a glycoprotein called thyroglobulin (Taurog, 1996), which is secreted into the lumen of the cell by exocytosis. Thyroglobulin is a large molecule with a long peptide chain that has many tyrosine side chains/rings on it. The synthesis of thyroid hormones in the thyroid follicles is dependent on dietary iodide and iodide is sequestered by iodide trapping and is brought into cells by the NIS transporter called the sodium iodide symporter (reviewed in Gilbert and Zoeller, 2010). Once inside, the follicular cell it is then transported into the lumen via a transport protein called pendrin. Once inside the lumen, iodide gets converted to atomic iodine by the enzyme thyroperoxidase, which oxidizes iodide (Dupuy et al., 2000;Pachucki et al., 2004;Vigone et al., 2005; Wang et al., 2005). Iodine then attaches to the tyrosine rings on thyroglobulin in one of two ways. Iodine can attach to one spot on the tyrosine molecule and this is called monoiodotyrosine (MIT) or iodine can attach to two areas on the tyrosine rings called diiodotyrosine (DIT). The tyrosine molecules can join together and form DIT + MIT which produces a molecule with 3 iodines called triiodothyronine (T3) or DIT + DIT which give us a molecule with 4 iodines called tetraiodothyronine/thyroxine (T4). Thyroglobulin with its attached iodinated tyrosine rings gets taken back inside the follicular cells by endocytosis and is degraded by lysosomes. The free thyroglobulin is released into the blood stream by exocytosis. Free T4 and T3 diffuses back into the blood stream, while the remaining iodotyrosines are

converted back to iodide by deiodinases and recycled by the cell (Campbell and Reece, 2005; Kandel et al., 2000; Silverthorn, 2004).

3.2 - Thyroid Hormone Release

Neurons in paraventricular nucleus of the hypothalamus releases the hormone TRH into the portal vein (Segersen et al., 1987a; Segersen et al., 1987b). TRH travels down the portal vein and diffuses into the anterior pituitary where endocrine cells called thyrotropes are located. In response to TRH, thyrotrope cells produce TSH and release it into systemic circulation (Haisenleder et al., 1992). The thyroid gland receives pituitary TSH from the bloodstream. TSH binds to receptors on the surface of the thyroid follicle cells and stimulates their activity culminating in the release of the thyroid hormones thyroxine (T4) and triiodothyronine (T3) back into the blood (Taurog, 1996). The thyroid hormones are carried throughout the circulatory system by specific proteins. In humans about 15% is bound to transthyretin (TTR), 10% by albumin and the majority 75% by thyroxine-binding globulin (TBG) (Schussler, 2000). TBG is the least abundant protein but most efficient T4 binder (Fink et al., 1986). The main effect of TBG is to increase the metabolic rate throughout the body so more energy per unit time is used. T4 is more prevalent but weaker whereas T3 is scarcer but 4 times more potent. Therefore, when the body needs to increase its metabolic rate, it will convert T4 to T3 producing a larger effect.

3.3 - Negative Feedback and Changing Needs

Negative feedback maintains fairly constant levels of T4 in the circulating blood. However, if the needs of the organism change this system is unable to provide the necessary adjustments. One such change that negative feedback is unable to accommodate for is exposure to very cold environments, in which the rate of TH production must change. However, TRH

control of the thyrotropes can provide this adjustment (Porter, 1977), by releasing more TRH into the portal blood vessels, thyrotropes will respond by producing more TSH, leading to increased secretion of T4.

Small amounts of T4 are essential in maintaining the functions of the TRH secreting neurons of the hypothalamus. Reichlin and Mitnick (Reichlin and Mitnick, 1973) demonstrate intra hypothalamic administration of small doses of T4 augment TRH secretion in thyroid deficient animals. Larger doses result in a decrease in TSH secretion. In these studies it was not ruled out that T4 may have traveled to the pituitary and acted there.

3.4 - Hypothalamic-Pituitary-Thyroid Axis (HPT)

Optimum concentrations of hormones must be present in order that they function properly. It has been suggested that target glands have important roles in regulating the secretory activities of the pituitary gland (Dot-ton, 2000; Hadley, 1996). Friedman and Colleagues (Friedman et al., 1979) found that in addition to promoting secretion of THs, TSH acts on the thyroid gland to stimulate protein and RNA synthesis.

Feedback loops in the hypothalamic pituitary axis/pathway are very complex because three integrating centers are involved: hypothalamus, anterior pituitary, and the endocrine target of the pituitary hormone. When hormone secretion increases or decreases this causes a change in the secretion of other hormones because of the feedback loop that links them. In pathways with multiple hormones the down- stream hormone usually feeds back to suppress the hormones that controlled its secretion. For example T4 and T3 synthesized by the thyroid gland feeds back to suppress the TRH secreting cells of the hypothalamus (Koller et al., 1987; Rondeel et al., 1989; Segersen et al., 1987b) and the thyrotropes of the adenohypophysis/anterior pituitary which

secrete TSH (Chopra, 1996; Rondeel et al., 1988; Scanlon and Toft, 1996), this relationship is called long-loop negative feedback (negative feedback from a peripheral endocrine gland hormone to the hypothalamus and the anterior pituitary) (Silverthorn, 2004). In short-loop negative feedback the pituitary hormone TSH feeds back to decrease hormone secretion by the hypothalamus. Animal studies by Bergland and Colleagues (Bergland and Page, 1978) support this belief as exogenous pituitary hormones injected into the hypothalamus caused an inhibition of TRH secretion. These feedback systems allow for hormones to stay within an appropriate range to elicit an appropriate response.

Thyrotropes of the adenohypophysis synthesize TSH and it travels via the bloodstream to the thyroid glands. Once T4 and T3 are produced by the thyroid follicular cells and released back into the blood, they travel back to the pituitary gland to exert negative feedback control over TSH secretion. If too much TSH is secreted, T4 levels rise transiently leading to elevated levels of T4 which then suppress TSH secretion. This causes a decline in T4 output. As T4 levels fall, inhibition is lifted and more TSH is secreted.

Chapter 4: Thyroid Hormone Receptors

And the Deiodinases

This chapter will provide an overview of the thyroid hormone receptors; their characteristics, structure, transport and the biological responses they induce. Thyroid hormones (TH) can affect brain maturation by interacting with nuclear receptors thereby regulating gene expression. In addition a brief discussion of the deiodinases will be included as they are the peroxidase enzymes involved in the activation or deactivation of the thyroid hormones. They are an important point of physiological control as 80% of brain T3 is produced through activation of these enzymes (Guandano-Ferraz et al., 1999;Peeters et al., 2005; St Germain et al., 2005).

4.1 - Thyroid Hormone Receptors

4.1.1 - Mitochondrial Receptors

Within the inner mitochondrial membranes of cell types in the liver, kidney, myocardium and skeletal muscle, high affinity receptors for T3 have been identified. Such cell types have been implicated in the calorigenic action of the hormones. However, structures that do not display changes in oxygen consumption; adult brain, spleen and testes have been found not express these receptors (Sterling et al., 1978). Data from hypothyroid rats reveal an acceleration of oxidative phosphorylation by mitochondrial vesicles due to physiologic concentrations of T3 (Sterling, 1979).

4.1.2 - Characteristics of the Nuclear Receptors

High-affinity, low capacity receptors within the nuclei mediate most of the thyroid hormone actions (Baxter et al., 1979; Oppenheimer et al., 1975). Tissues of the liver, kidney, pituitary gland, brain, heart and circulating leukocytes have similar protein properties with molecular weights of 50,000 - 60,000. In patients showing target organ insensitivity the numbers of protein molecules are sometimes reduced (Chait et al., 1982). Thyroid receptors exhibit a 10 fold greater affinity for T3 than for T4, and T3 is suggested to be the physiologically important regulator of thyroid receptor action (Muñoz and Bernal, 1997; Zoeller and Crofton, 2000). Eberhardt and Colleagues (Eberhardt et al., 1980) and Oppenheimer and Dillman (Oppenheimer and Dillman, 1978a) suggest that nuclear proteins are true hormone receptors, support for this concept comes from the fact that studies in rat show hormone sensitivity is directly related to the numbers of nuclear binding sites for T3, and the magnitude of the response varies with the numbers of T3 molecules bound (Martin, 1985; Valcana and Timiras, 1978).Nuclear receptors are (non- histone) proteins that are ligand modulated /hormone activated (non- histone) transcription factors and thereby act by modulating gene expression (Baxter et al., 1979; Eberhardt et al., 1980; Muñoz and Bernal, 1997). They are able to influence gene expression in the absence of the hormone, reflective in repression of the target genes which is relieved upon ligand binding, although ligand independent stimulation of transcription occurs (Muñoz and Bernal, 1997). In contrast to steroid hormone receptors, they bind DNA in the absence of hormone which usually leads to transcriptional repression. Hormone binding is associated with a conformational change in the receptor that causes it to function as a transcriptional activator (Tsai and O'Malley, 1994). Nuclear receptors are made by euthyroid, hyperthyroid and thyroid hormone-deprived subjects (Martin, 1985). The receptors/holoreceptors (a complete receptor whose subunits are capable of transducing intracellular signals) are composed of a core subunit

that directly binds the iodothyronines and a regulatory component, which modifies the binding affinities of the core subunits. Assembled in the cytoplasm, the core proteins act to concentrate T4 for translocation to the nuclei. It has been suggested that if the regulatory components are absent, the receptor binds T4 more tightly than T3 (Tsai and O'Malley, 1994). When no hormone is present, core proteins enter the nucleus and enter into the formation of holoenzymes that have much higher affinity for T3,as T4 is biologically active only when present in very high concentrations (Lazar, 1993; Oppenheimer and Dillman, 1978b; Zhang and Lazar, 2000). Separately translocated, the regulatory components are suggested in directing the protein hormone complexes to the appropriate DNA segments. Both magnesium and calcium maintain the concentrations of hormone free receptors with the nucleoplasm (Rodriguez-Pena and Bernal, 1982).

4.1.3 - Thyroid Hormones Transport to Nuclear Acceptor Sites

T4 and T3 rapidly enter cells, traverse the cytoplasmic compartment, and penetrate the nucleus. Translocation to the nucleus is not accelerated by the binding of THs to cytosolic proteins (Latham et al., 1978). Attachment to holoreceptors is suggested to be brief under physiologic conditions. Oppenheimer, (Oppenheimer, 1979) demonstrated that 50% of labelled T3 returned to the cytoplasm within 15 minutes. T3 may be recycled or extruded across the plasma membrane, while some cell types deiodinate/ inactivate T3 (Oppenheimer et al., 1994 ;Tsai and O'Malley, 1994; Yen and Chin, 1994).

4.1.4 - Occupancy of Nuclear Receptor and Biological Response

Most target cells in euthyroid subjects have 10-15% of T3 attached to the nuclear receptor sites (Martin, 1985). The total number of nuclear receptors and their occupancy can vary

widely. For instance, a kidney or liver cell nucleus contains 50% more nuclear receptors than a typical neuron nucleus (Oppenheimer and Dillman, 1978a). Palmero and Colleagues (Palmero et al., 1991) demonstrated in rat testis that hypothyroidism drastically lowers the percentage of receptor occupancy. Auto regulatory mechanisms are evident in certain cell types for defending their T3 levels (Leonard et al., 1981). When an excess of T4 is presented to the brain most of it is converted to rT3, and when plasma T4 is low, the conversion to T3 is accelerated. Unfortunately this mechanism is limited as either too much or too little T4 impairs functions. Latham and Colleagues (Latham et al., 1978) report that hypothyroidism has little effect on receptor numbers in the pituitary, although toxic doses of T3 slow receptor replenishment. It has also been evidenced that malnutrition of some forms slows T4 entry into the cytoplasm thus reducing nuclear occupancy (Okamura et al., 1981).

Thyroid hormones are essential for maintenance of lung heart and brain during late fetal and early postnatal life and of skeletal structure during juvenile stages and reproductive structure afterward (Dussault and Ruel, 1987; Hyyti et al., 2005; Longcope, 1991; Myant, 1971). The production of surfactant is in part regulated by T3 and the thyroid hormone numbers are highest when the fetus is preparing to breathe air and fall after birth (Baines et al., 2000; Ballard, 1982). Steady increases in receptor numbers have been reported in liver maturation (Malik and Hodgson, 2002).

T3 receptors are more numerous in fetal and neonatal brains than in the adult organ. Critical periods exist for the influence of thyroid hormones on the brain (Berbel et al., 1993; Dowling et al., 2001; Leonard et al., 1982; Madeira et al., 1991; Madeira et al., 1992; Nicholson and Altman, 1972a; Nicholson and Altman, 1972b) and the hormones must also at this time be

present in the correct concentrations (Leonard and Koehrle, 1996). Thyroid deficient human fetuses suffer impairment in brain maturation that is unresponsive to hormones administered later (Dussault and Ruel, 1987;Myant, 1971;Porterfield and Hendrich, 1993;Timiras and Nzekwe, 1989).

In an animal model, Ben-Baruch and Colleagues (Ben-Baruch et al., 1981) reported that, under physiological conditions, T3 participates in regulation of synaptogenesis and cerebellar neuron proliferation. In addition, influences on the set points for pituitary-thyroid feedback have also been described (Reichlin and Mitnick, 1973), (reviewed in Martin, 1985).

4.1.5 - Thyroid Hormone Receptor Structure and Expression

The thyroid hormone receptors are members of a nuclear superfamily of receptors, which includes: retinoic acid, retinoid X, vitamin D, steroids, prostoglandins and orphan receptors with no recognizable ligand (Evans, 1988; Muñoz and Bernal, 1997). Common to this superfamily of receptors are six similar domains (Ribeiro et al., 1995) two ligand-binding regions and a central region that constitutively binds to DNA (Malik and Hodgson, 2002). Triiodothyronine (T3) is bound with an affinity 10 times greater than thyroxine (T4) and either activates or represses gene expression upon ligand binding (Muñoz and Bernal, 1997). Mammalian thyroid receptors are encoded by two genes designated alpha and beta. In humans, TRα is located in chromosome 17 and TRβ is on chromosome 3 (Malik and Hodgson, 2002; Muñoz and Bernal, 1997). Alternative splicing of the primary transcript for each gene generates different alpha and beta receptor isoforms, TRα1, TRα2, TRβ1 and TRβ2 having been cloned in humans (Lazar, 1993;Muñoz and Bernal, 1997; Williams, 2000). The TRα1, TRβ1 and TRβ2 encode a ligand binding receptors while TRα2 is the only isoform that is unable to bind T3 because of its unique C terminus and functions to suppress expression of genes containing TREs (Apriletti et al., 1998).

Thyroid hormone receptors are comprised of three functional domains: (1) At the amino terminus a transactivation domain which interacts with other transcription factors to form complexes that either activate or repress transcription; (2) DNA binding domain which binds to sequences of promoter DNA called hormone response elements; and (3) At the carboxy-terminus a ligand-binding and dimerization domain.

Expression patterns of TR isoforms in a region and time specific fashion in the brain is complex (Muñoz and Bernal, 1997). The predominant TR isoform expressed in the brain is TRα1 although all receptor isoform mRNAs are expressed. In the central nervous system (CNS) from E14 to adulthood TRα1 is widely expressed (Bradley et al., 1989;Mellstrom et al., 1991) and from E19 to P0 TRα1 has been shown to be expressed in area CA1 of the hippocampus and the outer part of the cerebral cortex (Bernal 2009). In adult rats TRα 1 expression is pronounced in the cerebellum, hippocampus, cerebral cortex, olfactory bulb and striatum (Bernal 2009).

Expression patterns of receptor isoforms during development is different for TRα1 and TRβ1 with TRβ1 expression during the fetal period being restricted and low and increasing during the postnatal period through adulthood. At E15.5 TRβ1 mRNA is detectable in the upper tegmental neuroepithelium, and from approximately P7 TRβ1 is expressed in the cerebral cortex (Bernal 2009). In the mouse, overlapping distribution of both receptor subtypes has been evidenced with some differences in the hippocampus, hypothalamus and amygdala. In some cells of the cerebellum, preferential expression of one isoform has been noted, for example Purkinje cells express TRβ1 and granular cells express TRα1 (Bernal 2009).

In addition TRβ2 has been found to be widely distributed in the brain in layers ll through V1 of the cortex and Purkinje cells of the cerebellum (Lechan et al., 1993). In approximately the 10th week post conception the receptor protein is present at low levels in the human fetus (Bernal

and Pekonen, 1984). From the 10th week of gestation both the receptor and ligand T3 are present in the brain at enough concentrations to result in about 25% occupancy of the receptor (Ferreiro et al., 1988). Although predominant neuronal expression of T3 receptor mRNA has been suggested, astrocytes and oligodendrocytes have been evidenced to express T3 receptors in neural cell cultures as well (Luo et al., 1986; Yusta et al., 1988).

4.1.6 - Thyroid Hormone Receptor Interaction with DNA

By binding to specific DNA acceptor sites called T3 response elements (T3REs), the T3 receptors modulate transcription (Glass, 1994; Muñoz and Bernal, 1997). Mangelsdorf and Colleagues, (Mangelsdorf et al., 1995) report that the thyroid hormone receptors will bind TREs constitutively, independent of ligand occupancy. A TRE is comprised of two AGGTCA half sites in several different arrangements (direct repeats, inverted repeats or pallindromes) by four nucleotides. A requirement for efficient binding and function is for the two half site sequences to have a specific orientation (Muñoz and Bernal, 1997; Umesono et al., 1991).

In the central DNA- binding domain of the thyroid receptor, there are two sets of four cysteine residues, and each set chelates a zinc ion, forming loops known as zinc fingers. The two zinc containing modules mediate the specific recognition sites and spacing specificity for the receptor to bind genomic DNA (Ribeiro et al., 1998). Part of the first zinc finger interacts directly with nucleotides in the major groove of the TRE DNA, while residues in the second finger interact with nucleotides in the minor groove of the TRE (Apriletti et al., 1998). Thyroid hormone receptors can bind to a TREs as monomers a homodimers, but in the most prevalent model of T3 action the majority bind in the form of a heterodimer with the retinoid X receptor (RXR) another member of the nuclear receptor superfamily that binds 9-cis retinoic acid (Malik and Hodgson, 2002; Muñoz and Bernal, 1997). Heterodimer formation is thought to enhance

DNA binding affinity (Malik, 2002). Additionally, RXR is a receptor and a partner for other nuclear receptors, its participation in other activation pathways may modify the cellular response to T3 (Muñoz and Bernal, 1997). In fact, evidence for mutual interference between vitamin D3 and T3 signaling has been described (Yen et al., 1996) (reviewed in Muñoz and Bernal, 1997).

4.1.7 - Ligand- Free State of Receptor versus Ligand - Bound State of Receptor

Thyroid hormone receptors bind TRE DNA regardless of whether they are occupied by T3. Generally unliganded binding of the thyroid hormone receptor to DNA leads to repression of transcription, while in the presence of T3 the hormone receptor complex activates transcription (Brent, 1994; Muñoz and Bernal, 1997; Tsai and O'Malley, 1994; Zhang and Lazar, 2000).

In the absence of T3, the transactivation domain of the receptor in a heterodimer formation with RXR, are bound to co-repressor molecules (Kliewer et al., 1992; Malik and Hodgson, 2002). A portion of this co-repressor complex has histone deacetylase activity (HDA), which results in a closed or turned-off chromatin configuration on genomic DNA. The overall effect is to repress transcription of the target gene (Nagy et al., 1997). Alternatively, binding of T3 to its receptor promotes a conformational change in the receptor allowing it to release the co-repressor molecule and recruit and bind the co-activator protein. The co-activator complexes have histone transacetylase (HAT) activity (Ribeiro et al., 1998) and enzymatic acetylation of the nucleosome allows for an open chromatin configuration of the TRE on genomic DNA. Torchia and Colleagues, {Torchia, 1997) suggests that the open configuration of chromatin facilitates assembly of the transcription machinery and increases the rate of mRNA transcription of the thyroid-responsive genes.

4.2 - Deiodinases

Important points of physiological control are the deiodinases. In fact, 80% of brain T3 is produced through activation of these enzymes {Guandano-Ferraz, 1999; St Germain, 2005; Peeters, 2005}. In addition it has been reported that coordinated regulation among DII and DIII provide the homeostatic regulation of intracellular T3 concentrations, thus having a developmental role in the maturation and function of the brain (Asteria, 1998; Croteau et al., 1996; Guandano-Ferraz et al., 1997; Kohrle, 1999). Croteau and Colleagues (Croteau et al., 1996) posits a similar situation in humans.

For the nuclear receptor, T3 has ten time's greater affinity and efficacy than T4, although T4 is secreted at much higher levels. As such T4 should be considered a pro-hormone that requires deiodination to T3 in order to be biologically active (Hassi et al., 2001). The major determinant of cellular supply of thyroid hormone is considered to be circulating concentrations of free thyroid hormone, however, it has been suggested that other factors may play a role in modulating thyroid hormone the deiodinase which to converts T4 to T3 determine the availability of free T3 to bind to thyroid receptors (Chan et al., 2002). Each one of the deiodinases possesses specific qualities and the complex tissue-specific and developmental pattern of their expression indicates their ability to impact a variety of physiological systems (St Germain et al., 2005). Iodothyronines deiodinases are part of the selenodeiodinase enzyme system and are responsible for regulating the local delivery of T3 to the thyroid receptors and for thyroid hormone metabolism, they include: type 1=DI; type 2 = D2; and type 3 = D3 (Chan et al., 2002; Malik and Hodgson, 2002).

Type 1 deiodinase D1 accounts for approximately 30-40% of extrathyroidal production of T3 and is present predominantly in the thyroid tissue, kidney and liver and less abundant in the central nervous tissue (Berry et al., 1991;Malik and Hodgson, 2002; Mandel et al., 1992).

Type 1 (D1) catalytic activity is inhibited by 6 propyl-n-thiouracil (PTU) (Kohrle, 2000; Toyoda et al., 1997), which is the drug used to inhibit thyroid function in all of the experiments in this thesis. Deiodinase 1 converts T4 to T3 by catalysing the outer ring deiodination of T4. Type 1 deiodinase is a dual enzyme either activating or deactivating thyroid hormone (Gilbert and Zoeller, 2010).

Type 2 deiodinase is found in the skeletal muscle, pituitary and the central nervous system and contributes 60-70% of extrathyroidal production of T3 (Leonard et al., 2000). D2 is the active enzyme residing in tanocytes or the astrocytes that line the third ventricle (Cooper et al., 1983). It is reported to be present in human cerebral cortex, caudate nucleus and hippocampus, where these neurons co-express nuclear T3 receptors (Asteria, 1998; Guandano-Ferraz et al., 1997). D2 is relatively insensitive to PTU (Bianco et al., 2002). In rodents and humans the highest tissue activities have been reported in the anterior pituitary, brown fat and the CNS (Salvatore et al., 1996; Silva and Larsen, 1985). This deiodinase is thought to be critically important in maintaining thyroid hormone action in the CNS as the supply of active hormone T3 is dependent upon the cellular uptake of free thyroid hormones and intracellular deiodination of T4 by D2. Although inactivation of T4 and T2 are accomplished by both D1 and D2 systems, the major in activator is type 3 deiodinase , which catalyses the tyrosyl deiodination of T4 to rT3 and T3 to T2 (both inactive metabolites) (Malik and Hodgson, 2002). It also converts rt3 to rT2 (Bianco et al., 2002). Type 3 deiodinase is predominantly localized in neurons (Bernal, 2002a; Chan et al., 2002; Chan et al., 2005) and placenta (Kaplan and Yaskoski, 1981; Koopdonk-Kool et al., 1996).

Chapter 5: Background and Significance

Thyroid hormones are essential for brain maturation and function (Bernal, 2007). During development TH's are required to perform specific actions during critical time windows and deficiencies of even short duration can cause irreversible brain damage (Anderson et al., 2003 ; Bernal, 2005, 2007). This chapter will address the single most important cause of preventable brain damage after starvation, iodine deficiency (ID). Its metabolism, the physiologic consequences during pregnancy as an increase in iodine uptake is required. During a normal pregnancy a balance must be achieved between an increased need for thyroid hormone and the availability of iodine, and this balance requires a profound physiological adaptation of maternal thyroid function. In addition IDs role in both hypothyroidism and hypothyroxinemia will be reviewed as well as the consequences of TH dysregulation on brain development.

5.1 - Relative Importance of Thyroid Hormones to the Fetus

The prevailing idea during most of the second half of the 20th century was that embryological development took place in the absence of thyroid hormone, as it was believed that the placental "barrier" system drastically limited the transfer of hormone from mother to fetus. This limited transfer was thought to be necessary to protect fetal tissue from toxic concentrations of fT4 and T3 before it was developmentally required (Morreale de Escobar et al., 2004a). However during the last decade experiments in both human and animal models point to a role for the maternal transfer of thyroid hormone in brain development throughout fetal life (Burrow et al., 1994; Lavado-Autric et al., 2003; Man and Serunian, 1976; Morreale de Escobar et al., 2004a). Also receiving increasing acceptance is the idea that the developing brain is critically dependent on maternal T4 early in pregnancy, before fetal thyroid function onset at midgestation.

This is a period when the mother is the only source of thyroid hormone for the fetus (Calvo et al., 2002;Glinoer and Delange, 2000; Lavado-Autric et al., 2003). Evidence of a prenatal requirement of TH for normal brain development was provided by Goodman and Gilbert (Goodman and Gilbert, 2007) who suggested that thyroid hormone insufficiencies may contribute to the induction of some forms of cortical dysplasia.

Evidence for placental transfer comes from Vulsma and Colleagues (Vulsma et al., 1989)***,*** who found that the fetal thyroid begins producing hormones at about 12 weeks gestation, but before this the maturing brain is critically dependent on circulating maternal T4 concentration which are known to cross the placenta in significant amounts during the first trimester. Furthermore although a barrier exists, very small amounts of T3 and T4 of maternal origin have been found in fetal compartments by 4 weeks after conception, a period prior to the onset of fetal thyroid function (FTF). Research by Pop and Colleagues (Pop et al., 1999) supports findings that an important determinant of early fetal brain development is maternal thyroid function, because the fetal thyroid is unable to produce any T4 before 12-14 weeks gestation and a first trimester surge of maternal FT4 orchestrated by the conceptus is proposed as a biologically important event. As the cerebral cortex of the fetus depends on T4 for the production of T3 and for nuclear receptor binding and biological effectiveness. As such, an at risk population for neurologically disabled children would be women unable to increase their production of T4 early in pregnancy (Morreale de Escobar et al., 2004a, 2007; Pop et al., 1999), when this situation exits both pre and postnatal developmental alterations can be shown(Morreale de Escobar et al., 2004a; Morreale de Escobar et al., 2000).

Additional evidence for the importance of maternal thyroid hormone prior to FTF shows that treatment, such as with goitrogens or thyroidectomy used to induce maternal

hypothyroidism, interferes with some neuronal proliferation that is usually completed by embryonic day 12 (Narayanan and Narayanan, 1985). Additional migratory problems were reported by Dowling and Colleagues (Dowling et al., 2000) who reported that cells proliferating at E14-E15 that normally reach layer VI of the cortex by E16-E17 did not. Evidence for the local generation of T3 from T4 in the human cortex and the almost negligible influence of systemic T3 on brain was provided by Morreale de Escobar and Colleagues (Morreale de Escobar et al., 2004b) who demonstrated in the cortex T4 concentrations increased with postmenstrual age (PMA) as expected from the increase in fetal serum. T3, despite its low and almost constant circulating levels, showed a parallel significant increase with PMA in the cortex between 13 and 20 weeks to levels comparable to those reported in adults (2.5pmol/g) by midgestation.

Thyroid hormones (TH) can affect brain maturation by interacting with nuclear receptors thereby regulating gene expression. Bernal (Bernal, 2007) suggests that an excess or deficiency of hormone can lead to irreversible psychiatric and neurological symptoms that are associated with permanent alteration in brain structure and function. This is due to the fact that the thyroid system of the neonate is less able to tolerate fluctuations in THs due to its limited reserve capacity (Glinoer, 1997a; Glinoer, 1997b). Therefore it has been established that maternal status and the development of the child are related (Haddow et al., 1999b). The fetal brain has been shown to express TH receptors (Kilby et al., 2000) giving it the capability to bind TH if present and iodinases necessary to regulate the levels of T3 in the brain (Chan et al., 2002). The presence of thyroid hormone receptors (TRs) early in the development of the human fetal brain supports the hypothesis that developmental events sensitive to thyroid hormones might already occur before mid-gestation. Bernal and Pekones (Bernal and Pekonen, 1984) evidenced thyroid hormones in samples of the cerebral cortex studied 9 weeks PMA with concentration increasing

at least 10fold by 18 weeks. In addition, thyroid hormone receptors were occupied 25-30% by T3 during the study period. This finding supports the occurrence of the biological effects of the hormone in the cerebral cortex during the first trimester of gestation in the humans (Ferreiro et al., 1988). In addition, animal studies demonstrate that by mid-gestation T3 has been evidenced to partially occupy its nuclear receptors (Forrest et al., 1990; Perez-Castillo et al., 1985).

In addition to THs interaction with nuclear receptors, it has been evidenced to act as a ligand for several receptor types within the plasma membrane of the cell. The interaction of receptor and hormone typically initiates a cascade of secondary effects within the interior of the cell, often involving phosphorylation or de-phosphorylation of various other cytoplasmic proteins. One group of cytoplasmic proteins are the kinases, which are known to regulate cell proliferation, apoptosis, differentiation, survival and plasticity. Signaling pathways include: phosphorylation of Akt by direct administration of T3 into the hippocampus (Sui et al., 2008); phosphorylation of the mitogen activated protein kinase MAPK or ERK 1and 2 pathways by integrin receptor bound T4 and G protein coupled receptor activation (Lin et al., 1999) leading to direct activation of the MAPK pathway or indirect activation of the Adt signaling path. As such, Gilbert and Zoeller, (Gilbert and Zoeller, 2010) suggest a number of important actions of TH are mediated by the thyroid hormone receptor membrane receptors and are referred to as non-genomic actions.

Key events which are necessary for normal development of the brain occur along a distinct ontogenic timeline and thyroid hormones are necessary for the normal pattern and timing of such events (Gilbert and Zoeller, 2010). Under different windows of insufficiency, distinct patterns of functional deficits result. Recent work by Auso and Colleagues (Auso et al., 2004) has pinpointed a critical developmental window for the effect of TH insufficiency on cortical

migration in young offspring of dams that were treated with the goitrogen methimazole. The treatment resulted in a transient and very mild maternal TH deficiency, rendering the dam's hypothyroxinemic between E14 and E16. In the offspring, successive radial waves of neuronal migration into the somatosensory cortex of neurons were altered for neurons formed between E 14 and E 16, but also for those neurons generated on days E 17 - E 19, after goitrogen treatment ceased. Cytoarchitectural abnormalities included neurons appearing in aberrant locations and in cortical and hippocampal layers inappropriate for their date of birth. This could only be prevented by the replacement of T4 but only if the replacement was given during the critical period of corticogenesis.

5.2 - Hypothyroidism versus Hypothyroxinemia

According to the World Health Organization, ID is the single most important cause of preventable brain damage after starvation. Hypothyroxinemia and hypothyroidism due to ID cause decreased motor and mental function, cerebral palsy and reproductive failure (Dunn and Delange, 2001; Glinoer and Delange, 2000), despite dietary iodine supplementation and newborn screening programs in the U.S. (Harris and Pass, 2007). While up to 0.5% of pregnant women in Western Europe and North America exhibit overt/clinical hypothyroidism, up to 2.5% of them (1 out of 40) may have subclinical hypothyroidism which is undetected before pregnancy. Furthermore, hypothyroxinemia, often undetected by current screening standards, is present in between 6 and 12% of women of child bearing age, and as many as 25% of pregnant women in the United States have inadequate iodine intake levels during pregnancy

Studies in children with congenital hypothyroidism reveal the complex relationship between low TH and cognitive development. Although newborn screenings for CH has

eliminated the mental retardation associated with the disease (Brosco et al., 2006), the cognitive deficits remain, the severity depending on the duration of exposure to TH insufficiency, the degree of CH at diagnosis and the therapeutic treatment used (Hepworth et al., 2006; Oerbeck et al., 2007; Selva et al., 2005).

Hypothyroidism results from a decrease in the concentration of the thyroid hormones T4 and T3 (the active hormone in target cells) while exhibiting an elevation in TSH which is secreted by the anterior pituitary gland. Without sufficient amounts of iodine, the thyroid gland cannot synthesize adequate amounts of T3 and T4 and the resulting low blood levels of T3 and T4 are unable to exert the usual negative feedback on the hypothalamus (which secretes TRH) and anterior pituitary (which secretes TSH) (Chopra, 1996; Rondeel et al., 1988; Scanlon and Toft, 1996). Consequently, the pituitary continues to secrete TSH, which can exert a trophic action on the thyroid gland causing hypertrophy of the follicular cells (goiter) (Martin, 1985). Hypothyroidism is associated with some of the following symptoms in the early stages: fatigue, depression, bipolar affective disorders, loss of cognitive function, muscle cramps, joint pain, goiter, weight gain and bradycardia (low heart rate: less than sixty beats per minute), later symptoms include; slow speech and a hoarse, breaking voice, low basal body temperature and in females abnormal menstrual cycles (Bernal, 2007; Ganguli et al., 1996; Laureno, 1996).

Low T4 has historically been associated with high TSH, because regulation of thyroid functions through the hypothalamic - pituitary -thyroid negative feedback system. However, often not accepted, is a condition of low free T4 or T4 compared with levels found at the same stage of pregnancy in normal women ingesting enough iodine while TSH is not increased above normal. Hypothyroxinemia is a condition used to indicate whether or not clinical or subclinical (with TSH above normal values) hypothyroidism is present (Glinoer and Delange, 2000

;Lavado-Autric et al., 2003; Morreale de Escobar et al., 2000). This is an important condition, because current testing standards for hypothyroidism focus on circulating TSH levels, and therefore the majority of cases of hypothyroxinemia go undetected. This can occur under conditions of low dietary iodine intake where auto regulatory mechanisms sensing the decrease in circulating iodide respond by favouring the synthesis and secretion of T3 over T4. As a result, circulating T4 decreases, T3 however, does not decrease and may in fact increase thereby preventing an increase in serum TSH and clinical detection of hypothyroidism (Missler et al., 1994; Vagenakis et al., 1973). One must keep in mind that iodine deficient women are hypothyroxinemic not clinically hypothyroid (Choufoer et al., 1965; Hetzel, 1994), because circulating T3 is normal or even slightly elevated and sufficient for euthyroidism of most organs and tissues. In these women TSH rarely increases above normal (Glinoer, 1997b; Silva and Silva, 1982).

While negative feed-back mechanisms and preferential synthesis of T3 over T4 are useful regulatory mechanisms in controlling thyroid function several other auto regulatory mechanisms that will respond to a decrease in circulating iodide are: (1) iodine uptake; (2) thyroid vascularity; and (3) hyperplasia and serum T3/T4 ratios (Lavado-Autric et al., 2003; Morreale de Escobar et al., 2004b) (reviewed in Morreale de Escobar et al., 2004a), these changes are independent from TSH and occur even when hypophysectomized animals on TSH substitution or hypophysectomized rats are fed a diet low in iodine. Arntzenius and Colleagues {Arntzenius1991) has confirmed in man the autonomy of such auto regulatory mechanism from TSH, as evidenced in the use of iodine stores in the synthesis and secretion of T3 over T4.

Studies on thyroid function in pregnant women with moderate iodine deficiency have revealed no increase in TSH even among those women with the lowest first trimester free T4

levels however, increases in serum Tg and T3/T4 ratios were evidenced from the beginning (Glinoer, 1995, 1997, 2004). Thyroid hormone receptor isoforms have been found in the brain of the rat at the time of neural tube closure and are thought to mediate the effects of T3 (which has been synthesized from T4 transferred from the mother). As such, a hypothyroxinemic rat dam will have a fetus that is T3 deficient even if maternal T3 is normal. This is due to the fact that during early development serum-derived T3 hardly contributes to cerebral T3 (Calvo et al., 1990)

The risk for iodine deficiency-induced hypothyroxinemia is particularly high during pregnancy when the iodine requirement for synthesis of sufficient T4 is doubled (Morreale de Escobar et al., 2007). The requirement is substantial because the developing fetus is dependent upon maternal thyroid hormones as the fetal gland is not functional until mid-gestation (Calvo et al., 2002;Glinoer and Delange, 2000; Lavado-Autric et al., 2003). During the first trimester there is a major change in T4-binding globulin (TBG), one of three transport proteins for THs in the blood, and the major TH transport protein in pregnancy (Robbins et al., 1978). Because thyroid mainly crosses the placental barrier in the form of TBG - T4, and serum-derived T3 hardly contributes to cerebral T3, a hypothyroxinemic mother will have a fetus that is T3 deficient even if maternal T3 is normal (Calvo et al., 1990). To the developing brain, hypothyroxinemia is as problematic as hypothyroidism because the severity of mental retardation and CNS damage of the progeny is related to the degree of maternal hypothyroxinemia and not to changes in TSH or T3 (Choufoer et al., 1965; Glinoer, 1997a; Pharoah et al., 1976).

5.3 - Iodine Deficiency

Iodine deficiency (ID) is the most frequent cause of decreased motor and mental function, cerebral palsy and reproductive failure worldwide (Dunn and Delange, 2001; Glinoer and Delange, 2000). Therefore, the view that iodine deficiency disorders do not present a problem in certain European regions as well as countries like the United States (US) is unrealistic (Glinoer, 2004b). Although dietary iodine supplementation programs are present and additional screening is mandated for CH in new-borns in New York State since 1978 and included in every new-born screening (NBS) program in the U.S. (Harris and Pass, 2007)still the insufficiency persists. After starvation, the single most important cause of preventable brain damage was declared by The World Health Organization to be ID. Hetzel, (Hetzel, 1983) suggests that the potential irreversibility of the brain damage as well as the severity is related not just to the degree of ID but to the period in life when the exposure occurred. It has been suggested by Morreale de Escobar,(Morreale de Escobar et al., 2004a)that the currently reported statistics for diagnosing clinically or subclinically hypothyroid women would be higher if the new upper limit of 2.5 mU TSH/l were used as opposed to the upper limit for 'normal' TSH of approximately 5mU/l. In addition an even greater number of women would be diagnosed if serum FT4 and TSH first trimester specific ranges were available (Spencer, 2003).

A survey conducted in the U.S. revealed a marked decrease in iodine intake occurring during the period of 1988 - 1994 with a median urinary iodine excretion rate of 145µg/l as compared to a survey carried out between 1971and 1974 with an excretion rate of 300µg/l (Hollowell et al., 1998). The survey also showed that as many as 15% of the women were of child bearing age. Excretion levels in the range of moderate ID which is below 50µg/l during pregnancy were evidenced in almost 7% of the women (Dunn, 1998).

ID is inaccurately associated with clinical manifestations of hypothyroidism. Because of normal or increased circulating T3 people in situations of severe iodine deficiency are clinically euthyroid, this is due to auto regulatory mechanisms that permit their adaptation to inadequate iodine supplies (Missler et al., 1994; Vagenakis et al., 1973). In addition specific tissue can be hypothyroid without the individual as a whole being hypothyroid, as in brain tissue, that is dependent on T4 for the production of T3 and is T3 deficient and selectively hypothyroid (Hetzel, 1994) (reviewed in Morreale de Escobar et al., 2004b).Dunn, (Dunn, 1992) refers to these people as "dull" but when the hypothyroxinemia and the iodine deficiency are corrected they "wake up”. Maternal hypothyroxinemia which is characterized by low T4 with normal levels of TSH is the most common maternal thyroid disease impacting the developing fetus occurring in industrialized Western societies. It is potentially damaging for neurodevelopment of the fetus throughout pregnancy but especially so before midgestation, as the mother is the sole source of T4 for the developing brain (Morreale de Escobar et al., 2004a). As many as 25% of pregnant women in the United States have inadequate iodine intake levels during pregnancy, in fact they are half of what they should be. In addition, in western European populations, where non-pregnant women and schoolchildren have an adequate iodine intake, higher frequencies of this pregnancy related iodine insufficiency are being reported (Glinoer, 2004a; Morreale de Escobar et al., 2004a). Pop and Colleagues (Pop et al., 2003) suggests neurodevelopmental deficits in one out of two offspring from women in the Netherlands, with first trimester FT4 below the 10th percentile. In addition Caron and Colleagues (Caron et al., 1997) reports on a cohort of pregnant women from a town in France with extremely low urinary iodine excretion levels (75% of this cohort having excretion levels below 100μg/l). These findings highlight the need for local as well as longitudinal assessment of iodine deprivation during pregnancy because

mild-to-moderate ID occurs in areas that are not immediately recognized as iodine deficient (Glinoer, 2004b), as evidenced in the above studies.

In addition, more recent findings show that even mild degrees of ID are potentially adverse for the neurodevelopmental outcome of the pregnancy (Glinoer and Delange, 2000;Klein, 2001), as evidenced in Vermiglio and Colleagues, (Vermiglio, 2004) which was a 10 year follow up study examining the offspring of women who were hypothyroxinemic during the first half of pregnancy revealed the presence of an attention deficit hyperactivity disorder profile in 70% of the progeny.

5.4 - Thyroidal Economy in Pregnancy

Normal growth and development of many tissues is dependent on normal thyroid status, {Miell, 1993}.During a normal pregnancy a balance must be achieved between an increased need for hormone and the availability of iodine. For the maternal thyroid to meet the demand imposed by the fetus there are two prerequisites: (1) non impaired thyroid tissue; (2) a two fold increase in the iodine supply for synthesis of sufficient T4. Quite frequently women are unable to meet the demand for increased T4 production imposed by the fetus. The incidence is approximately 100 fold greater or more than that of CH (Morreale de Escobar et al., 2004a). The information available suggests that it imposes a considerable burden on the maternal thyroid gland (Glinoer, 2004b).

This balance requires profound physiological adaption of maternal thyroid function. Such increased hormonal requirements are the result of 3 factors which concurrently exert stimulatory effects on the thyroidal machinery, resulting in an altered thyroid economy (Glinoer, 1997b) During the first trimester there is a major change in T4-binding globulin (TBG) one of the three

transport proteins for thyroid hormones in the blood, and the major thyroid hormone transport protein in pregnancy (Robbins et al., 1978). Glinoer, (Glinoer, 1997b) reports a marked and rapid increase in serum TBG levels as a result of estrogen stimulation in response to increased levels of human chorionic gonadotropin (hCG). Such a marked increase in TBG levels leads to an increase in the binding capacity of serum for the hormone, which results in a rise in TSH concentrations causing stimulation of the maternal thyroid gland. The second factor is elevated hCG levels which exert a thyrotropic action by transiently stimulating the thyroid gland resulting in elevations in free T4 levels and a decrease in TSH levels. Finally, later in gestation the peripheral metabolism of thyroid hormones is modified through deiodination (increased placental deiodination of T4) and transplacental passage (Morreale de Escobar et al., 2007).

As events in the regulation of thyroid function in pregnant women from iodine sufficient areas require physiological adaptation, women from iodine insufficient areas face even greater challenges, as the adaptation of the thyroidal economy is from physiological to pathological. The factors affecting thyroidal stimulation remain the same as in the pregnant state: (1) rise in TBG; (2) and (2) thyrotropic action of hCG. However in iodine sufficiency physiological adaptation includes no relative hypothyroidism and no goitrogenesis. While in iodine deficient situations the pathological alterations include relative hypothyroidism as well as a goitrogenic stimulus (in mother and child) both alterations creating a negative feedback loop leading to a rise in TSH. In addition these physiological alterations in iodine deficiency lead to definitive pathological changes (Glinoer et al., 1995). As thyroid hormones are known to be important for optimal development of the human central nervous system and as epidemiological evidence has indicated that subtle deficiencies in circulating maternal thyroid hormones up to midgestation are associated with adverse neurodevelopment (Haddow et al., 1999b; Pop et al., 1999). Morreale de

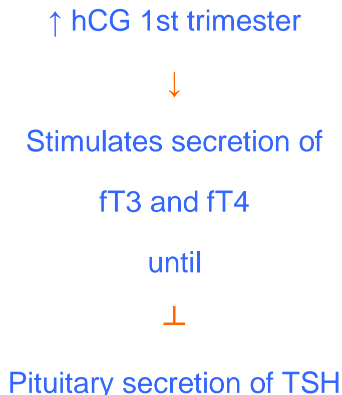
Escobar and Colleagues (Morreale de Escobar et al., 2007) posits that if all iodine disorders are to be avoided adequate iodine intake has to be ensured very early in pregnancy.

In conclusion, whether the intake of iodine is overtly or marginally restricted during pregnancy, the adequacy of the physiological adaptation of the thyroid machinery is not always achieved, leading to alterations in pathology that are in parallel with the degree of deprivation leading to increased thyroidal stimulation.

5.5 - Physiology of the Change Required by the Maternal Thyroid During Pregnancy

The conceptus, for its own benefit, imposes important changes on the function of the maternal thyroid with the onset of pregnancy. One such change is the increase in human gonadotropic chorionic hormone (hCG) in the maternal and fetal compartments which is required to maintain the pregnancy. Maternal circulating thyroid hormone concentrations are in large part determined by hCG. As the increased hCG during the first trimester causes increased secretion of fT4 and fT3 until hCG stimulation diminishes. In addition, this elevated concentration causes the secretion of the iodinated thyroid hormones to the point that TSH secreted by the pituitary is inhibited. This adaptation may be considered an example of the control the fetus exerts on the maternal endocrine system (reviewed in Morreale de Escobar et al., 2004a).

hCG (TSH like activity) → controls →T4 and T3



5.6 - The Physiologic Consequences of Iodine Insufficiency During Pregnancy

The increase in fT4 and fT3 resulting from the increased levels of hCG caused by the pregnant state present a need for an increase in iodine uptake (Glinoer et al., 1993; Guillaume et al., 1985). However, if the iodine is not sufficient, the maternal thyroid responds by auto regulation. This mechanism switches the synthesis and secretion of thyroid hormone toward the preferred use of the decreasing iodine supply in favour of T3 over T4 (Greer, 1968).Thus a decrease in maternal circulating T4 and fT4 occurs while the concentration of fT3 and T3 remains the same or may increase, inhibiting an increase in serum TSH as well as manifestations of hypothyroidism (Pharoah et al., 1973).As there is no increase in circulating TSH, the mother remains clinically euthyroid. However, there may be insufficient T4 available for the neurodevelopment of the fetus, as the fetal fluid levels of fT4 are defined by both the concentrations of maternal T4 or fT4 that have crossed the placental barrier and the concentration of T4-binding proteins. Determined ontogenically, the T4 binding capacity far exceeds the amounts of total T4 that reaches the fetal fluids, in addition it is independent of the maternal thyroid status. As such, the maternal circulating T4 and fT4 determines the available

fT4 for embryonic and fetal tissue and this would decrease in hypothyroxinemic women even if they were clinically euthyroid (Morreale de Escobar et al., 2004b).

*5.7 - **Metabolism of Iodine During Pregnancy***

Glinoer (Glinoer, 2004a) explains the main change in thyroid function during pregnancy is associated with the increased requirement of thyroid hormone beginning in the first trimester of gestation. Such production depends directly on the adequate availability of dietary iodine and the integrity of the glandular machinery, the thyroid. Iodine is converted to iodide and then absorbed from the gut. Each individual has an extra thyroidal pool of inorganic iodide (PII). The pool is composed of iodide of dietary origin that mixes with iodide resulting from the peripheral catabolism of thyroid hormones by deiodination. This pool maintains homeostasis and is in dynamic equilibrium with both the thyroid gland and the kidneys.

Iodide trapping is the mechanism by which the thyroid gland adapts to changes in the supply of iodine. Two situations that call for such an adaptation are when iodine intake is restricted as in Western Europe and secondly during pregnancy when there is an increased requirement for the synthesis of thyroid hormones. Augmentation of iodide trapping is basically the mechanism by which the thyroid gland adapts to changes in iodine supply and is a key to understanding thyroidal adaptation to iodine deficiency.

The normal adult uses 80µg of iodide to produce TH; the system is balanced with a 35% uptake of iodine by the thyroid assuming an adequate intake (150µg/d) of iodine in the non-pregnant condition. From the 80µg of iodide produced per day 15µg is depleted in the feces and the remaining 65µg is split between the thyroid compartment and urinary losses (Hays, 1993). As such, a metabolic balance is in equilibrium with 150µg in and the same amount out and 80µg

available for daily hormone production. Therefore with 150µg /d in non- pregnant adult the system is able to maintain plentiful intra thyroidal iodine stores of 15-20mg (Delange et al., 1988).

During pregnancy however, two changes take place altering the preconception steady state. When the iodine supply is restricted or severely deficient, pregnancy will trigger a cascade that leads to excessive glandular stimulation. There will be a 1.5 fold sustained increase in TH production requirements (80-120µg/d of hormonal iodide) and renal iodide clearance will increase by approximately 1.3 to 1.5 fold. This renal clearance has been referred to as an iodine leakage which lowers the circulating levels within the extra thyroidal pool of inorganic iodide and induces a compensatory increase in thyroidal clearance of iodide. Taken together these mechanisms increase thyroidal activity (Aboul-Khair et al., 1964; Liberman et al., 1998). ID is said to become significant when iodine intake falls below 100µg/d as the recommended dietary allowance of iodine for pregnant and lactating women is between 200-250µg/d. Therefore, when daily iodine intake is restricted 70µg/day the system is unbalanced despite the increase in glandular uptake. As iodide entry from uptake and recycling is not sufficient to keep up with the increased need for TH production. Therefore, in order for TH production to continue, iodine stored in the gland must be used, leading to an overall depletion of intra thyroidal iodine stores (Glinoer, 1997a; Glinoer, 2001).By about the end of the first trimester the already low intra thyroidal stores of iodide worsen and if the condition persists during the first half of gestation, its severity progresses throughout the pregnancy. This depletion of iodide, which is necessary for the synthesis of thyroid hormones, may lead to severe enough iodine deficiency to cause both fetal and maternal hypothyroxinemia (low T4 normal TSH) from early gestation onward. As thyroid hormones are crucial for brain development during fetal and early postnatal life (Glinoer

and Delange, 2000).Additional consequences of inadequate hormone availability during critical periods of brain development include: mental retardation irreversible brain damage and neurological abnormalities (Calvo et al., 2002; Morreale de Escobar et al., 2000).

5.8 - Thyroid Hormones and Brain Development

Recently it has been established that subtle decreases in maternal TH alter fetal brain development (Auso et al., 2004; Morreale de Escobar et al., 2004b). Children born to women with subclinical thyroid disturbances have been observed to have impairments in intellectual function and neuropsychological development (Morreale de Escobar et al., 2000).

Data from animal studies suggest that developmental disabilities associated with modest thyroid impairment may result from altered development of the cerebral cortex (Ruiz-Marcos, 1989). In particular, the development of radial glial cells, which are necessary for the formation of the neocortex, is altered by gestational hypothyroidism (Lavado-Autric et al., 2003; Martinez-Galan et al., 2004). Thus, lack of TH during development of the cerebral cortex may result in profound changes to cortical cytoarchitecture. Evidence for abnormal cortical neuron migration comes from Goodman & Gilbert (Goodman and Gilbert, 2007) who reported the development of a subcortical band heterotopia with consistent bilateral localization in the corpus callosum, following low dose PTU treatment during gestation in a rat model of hypothyroxinemia. Because the heterotopia is comprised of neurons immunopositive for inhibitory markers Gad-67, calretnin, and parvalbumin, there is evidence that tangential neuron migration, in addition to radial migration, may be altered by TH insufficiency suggesting more than just radial glia are altered. In addition cell migration and cytoarchitecture in the somatosensory cortex and

hippocampus of the progeny of dams fed a low iodine diet was found to be aberrant or inappropriate with respect to their birth dates (Lavado-Autric et al., 2003).

TH also affects genes involved in neuronal differentiation, cell migration and myelination (Asntisteban and Bernal, 2005; Konig and Neto, 2002). TH insufficiency has long been associated with altered myelination (Sharlin et al., 2008) possibly as the result of guiding embryonic stem cells toward an oligodendrocyte (myelin cell) fate. Support comes from the recent finding that TH insufficiency guides a glial-restricted precursor of oligodendrocytes and astrocytes, (GRP)/oligodendrocyte type II astrocyte (02A) precursor cells, away from an oligodendrocyte fate and toward an astrocyte fate (Sharlin et al., 2008). So it is tempting to consider that cortical dysplasia’s are the result not only of altered migration, but the altered differentiation of cells that were otherwise destined to become oligodendrocytes.

Several studies have demonstrated that the formation of cortex (Dowling et al., 2001; Leonard et al., 1982; Martinez-Galan et al., 2004), cerebellum (Legrand, 1979; Nicholson and Altman, 1972a; Nicholson and Altman, 1972b), and hippocampus (Madeira et al., 1991) are sensitive to TH levels during critical windows of development.

Therefore, TH plays a critical role in fetal life especially in brain development (Benvenuti et al., 2008).Studies have revealed that the expression of genes related to cell migration, myelination and neuronal differentiation are affected by TH (Asntisteban and Bernal, 2005; Konig and Neto, 2002). Additionally, the development of radial glial cells, which are necessary for the formation of the neocortex is altered by gestational hypothyroidism (Martinez-Galan et al., 2004). Autric and Colleagues (Lavado-Autric et al., 2003) suggest that early maternal hypothyroxinemia alters both fetal brain histogenesis and cytoarchitecture in the rat.

Supporting the importance of an adequate and early supply of maternal TH for neurodevelopment, Auso and Colleagues (Auso et al., 2004) altered TH availability during the early stages of development in the rat, which is equivalent to the end of the first and beginning of the second trimester in humans, a crucial period affecting neurogenesis irreversibly. Examined was the final location of cells that had been generated during early periods of neurogenesis in offspring of dams treated with MMI, a goitrogen, for the 3 days between E12-E15 (embryonic day). Treatment resulted in a transient and very mild maternal TH deficiency, rendering the dams' hypothyroxinemic between E14 and E16, a time point when the mother is the only source of TH for the developing fetus, and in the rat. Secretion of iodothyronines by the fetal thyroid starts at 17.5 to 18 days after conception. Embryonic days 14-16 are a time of very active neurogenesis with migration of radial neurons into the developing cerebral cortex and hippocampus. This short period of moderate maternal TH deficiency was sufficient to alter successive radial waves of neuronal migration into the somatosensory cortex of neurons formed between E 14 and E16 and also those neurons generated on days E 17 - E 19 when goitrogen treatment ceased. Thyroid hormone deficiency resulted in cytoarchitectural abnormalities with neurons appearing in aberrant locations and in cortical and hippocampal layers inappropriate for their date of birth. This could only be prevented by the timely infusion of T4. However, this was of no benefit if delayed beyond the critical period of corticogenesis. To extrapolate this study to man, this period would define that time in human gestation when the fetal cerebral cortex is especially sensitive to changes in the availability of maternal TH, within first half of pregnancy.

Chapter 6: Developmental Hypothyroidism Alters Neocortical Volume

Abstract

Although severe CH has long been associated with mental retardation and developmental deficits, it has only recently been established that even subtle decreases in maternal thyroid hormone (TH) alter fetal brain development and cause impairments in intellectual function. The present study was designed to determine whether an additional consequence of developmental TH insufficiency is an alteration in neocortical volume, as differentiation of cells in the brain during the period of corticogenesis appears to be affected by hypothyroidism (as reviewed in Sharlin et al., 2008). In addition, ubiquitin- dependent programmed cell death during cortical histogenesis appears to be regulated by TH (Pasquini et al., 2000), which suggests that developmental hypothyroidism may cause long-term changes in the number of cortical neurons. Together these findings indicate that developmental hypothyroidism alters neocortical development potentially leading to abnormalities in structural volume. Pregnant dams were exposed to propylthiouracil (PTU; 0, 3 or 10ppm) in their drinking water from gestational day 6 through lactation (PND31). Offspring from each dose group were perfusion-fixed on postnatal day (PND) 23 or PND86. Brains were sectioned coronally on a vibratome in a serial manner through the portion of the brain containing the dorsal hippocampus. Neocortical and whole brain volume estimates were made on every 5th section using a Cavalieri probe (Image J). A ratio of neocortex to whole brain volume was calculated to account for changes in brain volume induced by hormone insufficiency. In PND23 offspring a significant increase in the ratio of neocortical to whole brain volume was observed ($p = 0.019$). Dunnett t post-hoc test showed that the difference between 0 and 10ppm was significant ($p^* = 0.010$). The difference between 0 and 3ppm had a trend toward significance ($p = 0.056$). To determine whether this observation was due to an increase in the neocortex or a decrease in subcortical volume a morphometric analysis of cortical thickness was also performed. The analysis of cortical thickness substantiated that the increase in the cortex to whole brain volume ratio was a result of an increase in cortical thickness ($p^* = 0.017$) with post hoc test showing a significant difference between the 0 and 10ppm groups ($p^* = 0.015$). To determine if this effect was permanent, sections from PND86 euthyroid adult offspring were also examined. No significant differences were detected in neocortical or whole brain volume measurements, however, when the ratio of cortex to whole brain was measured a significant group difference was observed ($p^* = 0.026$). The Tukey - Kramer post-hoc analysis showed that the difference between 3 and 10ppm was significant ($p^* = 0.048$). Together, these results suggest that increasing levels of maternal thyroid insufficiency causes an increase in cortical volume that persists into adulthood and may account for functional deficits following developmental TH insufficiency. The observed developmental increase in neocortical volume is similar to what has been observed clinically in patients with autism.

6.1 - Introduction

A growing body of evidence suggests that the fetal brain requires adequate levels of thyroid hormone (TH) for normal development (Morreale de Escobar et al., 2000). Significant levels of thyroxine (T4) in the blood of premature infants has been reported by Vulsma and Colleagues (Vulsma et al., 1989) and Calvo et al., (Calvo et al., 2002) demonstrated that fetal tissue is exposed to significant levels of TH. In addition the fetal brain has been shown to express TH receptors (Kilby et al., 2000) giving it the capability to bind TH if present as well as the iodinases necessary to regulate the levels of triiodothyronine (T3) in the brain (Chan et al., 2002). Previous studies which examined the relationship between TH insufficiency and brain development focused on the effects of severe hormone deprivation during the early postnatal period (Bernal, 2002a, 2007). However, there is a growing body of evidence suggesting that subtle decreases in maternal TH during the prenatal period can alter fetal brain development (Glinoer and Delange, 2000; Klein et al., 2001) .

A number of clinical reports describe learning disabilities, neuropsychological deficits, and impaired psychomotor development accompanied by decreases in IQ in children whose mothers were hypothyroid during pregnancy (Haddow et al., 1999b; Man et al., 1991; Morreale de Escobar et al., 2004b; Pop et al., 2003; Pop et al., 1999; Zoeller, 2004). Animal studies support the clinical evidence that brain development is dependent on adequate levels of TH. The formation of the neocortex (Dowling et al., 2001; Leonard et al., 1982; Martinez-Galan et al.), cerebellum (Legrand, 1979; Legrand, 1984; Nicholson and Altman, 1972a; Nicholson and Altman, 1972b), hippocampus (Madeira et al., 1991) and auditory structures (Berbel et al., 1993) are sensitive to TH levels during critical windows of development. Developmental hypothyroidism has been shown to interfere with cellular migration and differentiation in the

hippocampus (Madeira et al., 1991; Madeira and Paula-Barbosa, 1993; Madeira et al., 1992) and auditory structures in the rat (D Forrest, 1996; Rueda, 2003; Rusch, 2001). There is also evidence of delayed or poor myelination in developmentally hypothyroid animals (Rodriguez-Peña et al., 1993; Walters and Morell, 1981). Alvarez-Dolado (Alvarez-Dolado and Bernal, 2000) demonstrated that developmental hypothyroidism affected the expression of L1, a gene that codes for an adhesion molecule involved in formation of the corpus callosum. The expression of reelin and dab1, proteins that regulate neuronal migration are also affected by thyroid hormone (Alvarez-Dolado and Nakajima, 1999). Further evidence of a prenatal requirement of TH for normal brain development was provided by Goodman and Gilbert (Goodman and Gilbert, 2007) who observed the presence of a bilateral heterotopia in corpus callosum of developmentally hypothyroid rats.

Differentiation of cells in the brain during the period of corticogenesis appears to be affected by hypothyroidism, with glial-restricted precursors being guided away from an oligodendrocyte fate to become astrocytes (Sharlin et al., 2008). Finally, ubiquitin-dependent programmed cell death during the period of cortical histogenesis appears to be regulated by TH (Pasquini et al., 2000), which suggests that developmental hypothyroidism may cause long-term changes in the number of cortical neurons. Together these findings indicate that developmental hypothyroidism alters neocortical development potentially leading to abnormalities in structural volume. However, no studies to date have specifically measured whether developmental hypothyroidism in the rat alters neocortical volume. Hasegawa (Hasegawa 2010) using magnetic resonance imaging (MRI) demonstrated that a ratio of specific structure versus whole brain volume measurements can be used to determine whether specific brain areas are selectively vulnerable to developmental hormone insufficiency. In the present study, stereological and

morphometric techniques were used to determine whether developmental hypothyroidism specifically alters neocortical volume relative to the volume of the whole brain. Using this approach neocortical volume was compared between control rats and juvenile as well as adult rats following developmental hypothyroidism.

6.2 - Materials and Methods

All experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by an institutional animal care and use committee. Measurements were performed on slide sets of brain tissue from animals used in the study reported by Goodman and Gilbert (Goodman and Gilbert, 2007). In some cases additional animals were generated to increase sample size. Timed pregnant Long-Evans rats were purchased from Charles River (Raleigh, NC) on gestational day (GD) 2 and housed individually. Animal rooms were maintained on a 12/12 light/dark schedule, and animals were permitted free access to food (rat chow; Purina, St. Louis, MO) and tap water.

6.2.1 - Developmental hormone insufficiency

Beginning on GD 6 and continuing through lactation on postnatal day (PND) 30, dams were rendered hypothyroid by addition of 3ppm or 10ppm of the thyroid hormone synthesis inhibitor propylthiouracil (PTU; Sigma, St. Louis, MO) to the drinking water. Control dams continued to receive normal tap water (0ppm PTU). The day of birth was designated PND 0 and litters were culled to 10 pups on PND3 - 4, retaining the maximal number of males per litter. On PND30, the offspring were weaned, transferred to plastic cages (two to four per cage) and were permitted free access to food and tap water. A subset of animals (one per litter) was sacrificed on PND23 and additional groups of animals were sacrificed on PND86.

6.2.2 - Brain sectioning and Nissl staining

Offspring from each dose group were sacrificed on PND23 (0ppm n= 7; 3ppm n = 8; 10ppm n = 9) and PND86 (0ppm n = 7; 3ppm n = 6; 10ppm n = 11) with an overdose of phenobarbital (100 mg/kg, ip) and perfusion-fixed through the aorta with 4% paraformaldehyde. Brains were removed and sectioned in the coronal plane on a vibratome (50 μm) in a serial manner from the beginning of the hippocampus until the dorsal and ventral hippocampus were connected (corresponding to roughly 5.8 mm caudal to bregma).Every 5th section was slide mounted, processed for Nissl staining and coverslipped.

6.2.3 - Cortical Measurements

Cortical and whole brain volume estimates were made on scanned images of slide mounted sections. Images were analysed using Image J software, and a point counting grid with a random starting point and a 1.4mm2 box superimposed on the image. Cortical volume measurements were made using points located between the dorsal surface of the corpus callosum and the pia mater for each section, and then totaled for all sections in an animal. Whole brain measurements used points located throughout all grey and white matter, including subcortical regions for each section, and then totaled for all sections in an animal. Volume (V) estimates for the neocortex and the whole brain were calculated using the Cavalieri method according to the formula: $V = \Sigma P * a(p) * t$, where $a(p)$ is the area between grid points, ΣP is the sum of the number of grid points intersecting the structure of interest and t is the estimated thickness between consecutive slide-mounted sections based on microscope z-axis measurement of the final section thickness after processing. For the linear measurement of cortical thickness, a total of 3 measurements per tissue section were taken for 5 anatomically matched Nissl-stained sections

(corresponding to plates 29 - 37 in (Paxinos, 1998) and the means recorded. Linear measurements were made from the dorsal surface of the corpus callosum to the pia matter using Image J Software. Volume and thickness measurements were compared across PTU treatment groups using a one way ANOVA with planned comparisons between the control group and each experimental group.

6.3 - Results

6.3.1 - Cortical and Whole Brain Volume Estimates PND23

Figure 6.1 shows a summary of neocortical volume, whole brain volume, and the ratio of neocortex to whole brain volume in PND23 rats developmentally exposed to PTU (0ppm, 3ppm or 10ppm) from GD6 through lactation PND30. One way ANOVA with treatment as independent factor did not indicate a significant treatment effect for the neocortical volume ($p = 0.096$; Figure 6.1A) or whole brain volume ($p = 0.085$; Figure 6.1B) when volume was analysed separately. However, the ANOVA indicated a significant treatment effect for the ratio of neocortical to whole brain volume ($p^* = 0.019$; Figure 6.1C). Dunnett t post-hoc test showed that the difference between 0 and 10ppm was significant ($p^* = 0.010$). The difference between 0 and 3ppm had a trend toward significance ($p = 0.056$).*6.3.2.*

6.3.2- Cortical Linear Measurements PND23

To demonstrate that the difference in the neocortical to whole brain volume ratio between groups of developmentally hypothyroid animals was due to an increase in the size of the cortex and not to a decrease in subcortical volume, linear measurements from the same stained sections of cortex from PND23 animals were examined. Neocortical thickness was significantly increased in PTU exposed rats ($p = 0.017$) with multiple comparisons with bonferroni correction showing a

significant difference between the 0 and 10ppm groups (p* = 0.015), but not between 0 and 3ppm groups (p = 0.450; Figure 6.1D).This substantiated that the increase in neocortical to whole brain volume ratio was a result of an increase in the size of the neocortex and not due to a decrease in subcortical structures.

6.3.3 - Cortical and Whole Brain Volume Estimates PND86

To determine if the increase in cortical volume evidenced in PND23 animals was evident in adult animals, slide-mounted Nissl-stained sections from PND86 animals developmentally exposed to PTU (0ppm, 3ppm or 10ppm) from GD6 through lactation PND30 were examined. Figure 6.2 shows that at this time point, no significant differences were detected in cortical volume (p = 0.812; Figure 6.2A) or whole brain (p = 0.258; Figure 6.2B) volume measurements. However the one way ANOVA with treatment as an independent factor revealed, a significant treatment effect for the ratio of neocortical to whole brain volume (p*= 0.026; Figure 6.2C). Dunnett t test showed that the difference between 3 and 10ppm animals was significant (p* = 0.048), while the difference between 0ppm vs 3ppm and 0ppm vs 10ppm was not (p = 0.955; p = 0.074 respectively).

6.3.4 - Cortical Linear Measurements PND86

A morphometric analysis of neocortical thickness of the brains from adult offspring that were developmentally exposed to PTU (0ppm, 3ppm or 10ppm) from GD6 through PND30 was also performed. For this comparison there was no significant difference between the controls and the treated groups (p=0.33, Figure 6.2D).

6.4 – Conclusion / Discussion

In general, increasing levels of maternal TH insufficiency caused an increase in the ratio of neocortical to whole brain volume and these effects persisted into adulthood and euthyroid status. The morphometric analysis indicated that the alteration in neocortex to whole brain volume at PND23 was not due to a disproportionate decrease in subcortical structures even though the increase in the ratio of neocortical to whole brain volume persisted. The observed increase in cortical volume may underlie some of the behavioural deficits associated with developmental hypothyroidism (Haddow et al., 1999b; Sui et al., 2005).

It is interesting to consider what may account for the disproportionately large cortex following developmental hypothyroidism. It is unlikely that neuronal hypertrophy or enhanced dendritic arborization account for this finding (Ruiz-Marcos et al., 1979).. A more likely explanation is an alteration in the composition and organization of the cortical cells. TH deficiency has been reported to alter the developmental fate of progenitor cells away from an oligodendrocyte fate (Johe, 1996; Murray and Dubois-Daleq, 1997; Raff et al., 1983; Sharlin et al., 2008).Support for altered organization comes from Auso et al., (Auso et al., 2004) and Lavado-Autric and Colleagues(Lavado-Autric et al., 2003) who observed alterations in neuronal migration and Goodman & Gilbert(Goodman and Gilbert, 2007), who observed the presence of a bilateral heterotopia, composed of neurons and glial cells, in the corpus callosum of rats treated with PTU during gestation. Together, these findings indicate that TH insufficiency alters cell differentiation and organization, which may have a significant impact on the number of neurons and glial cells in the cortex. Chapter 7 investigates whether an increase in the number of neurons contributes to this change in neocortical volume.

Normal brain development occurs along a distinct timeline that requires adequate levels of TH at specific time points to proceed (Gilbert and Zoeller, 2010). For example, Auso and Colleagues (Auso et al., 2004) pinpointed a critical developmental window when adequate levels of TH are necessary for proper neuronal migration during corticogenesis in offspring of hypothyroxinemic dams. Our findings obtained from animals on PND23 and PND86 may be the result of a delay of or interference in normal developmental processes resulting in a larger cortex. Therefore the actions of TH during development are varied, as they are required to carry out particular actions during explicit or precise time windows. Deficiencies can lead to permanent alterations in brain development, the consequences dependent on the timing of the beginning and duration of TH insufficiency (Anderson, 2003; Berbel et al., 2007; Bernal, 2005; Koibuchi N, 2006 Epub 2006 May 12; Morreale de Escobar et al., 2004b). In conclusion, this study provides further evidence that neocortical development is particularly dependent on adequate amounts of maternal TH and that behavioral deficits present in offspring of hypothyroid mothers are likely due to abnormal neocortical development.

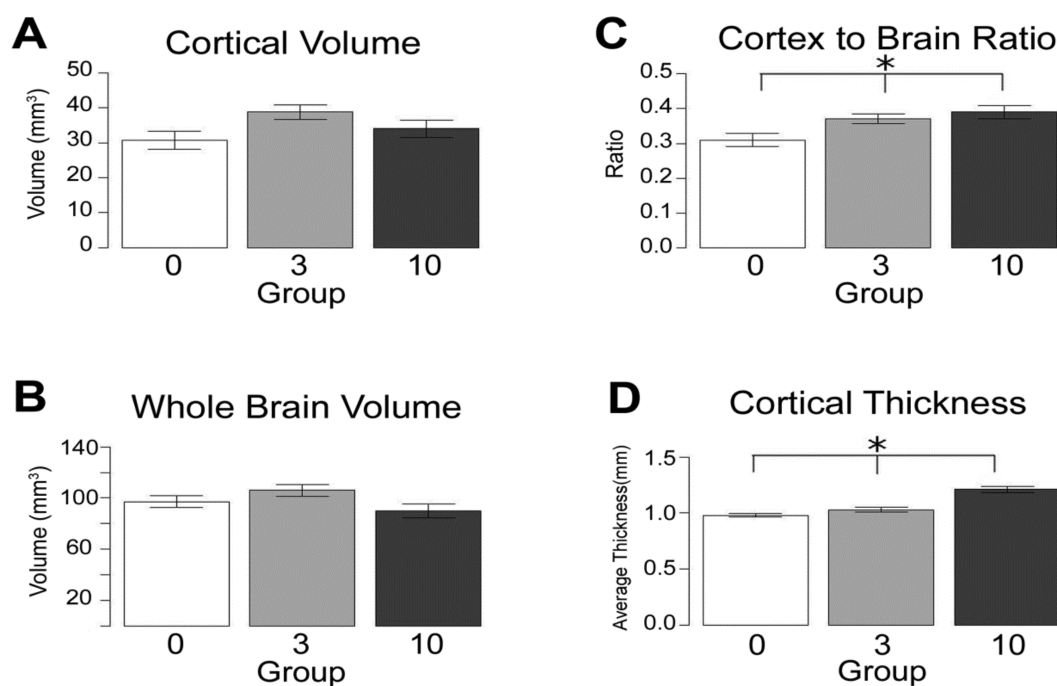


Figure 6.1: Changes in neocortical volume and thickness in PND 23 animals exposed to PTU (0, 3, 10ppm) from GD6 to PND30. No significant difference detected in A. neocortical volume (p = 0.096) or B. whole brain volume (p = 0.085). However a main effect of treatment was indicated for the ratio of neocortical to whole brain volume (p* = 0.019) with a significant difference between 0 and 10 PPM groups (p* = 0.010). **D.** Linear measurement of cortical thickness. The group difference for cortical thickness was significant overall (p* =0.017), with a significant difference between 0 and 10 PPM groups (p =0.015). Data was analyzed using a one way ANOVA followed by a Dunnett t test. Data are shown as Mean ± SEM and Differences with p values < 0.05 were considered significant. N = 0ppm: n= 7; 3ppm: n = 8; 10ppm: n = 9. N = 1 dam per treatment, 0ppm: n = 1, n = 2; 3ppm: n = 1, n = 3; 10ppm: n = 1, n = 3 pups per litter.

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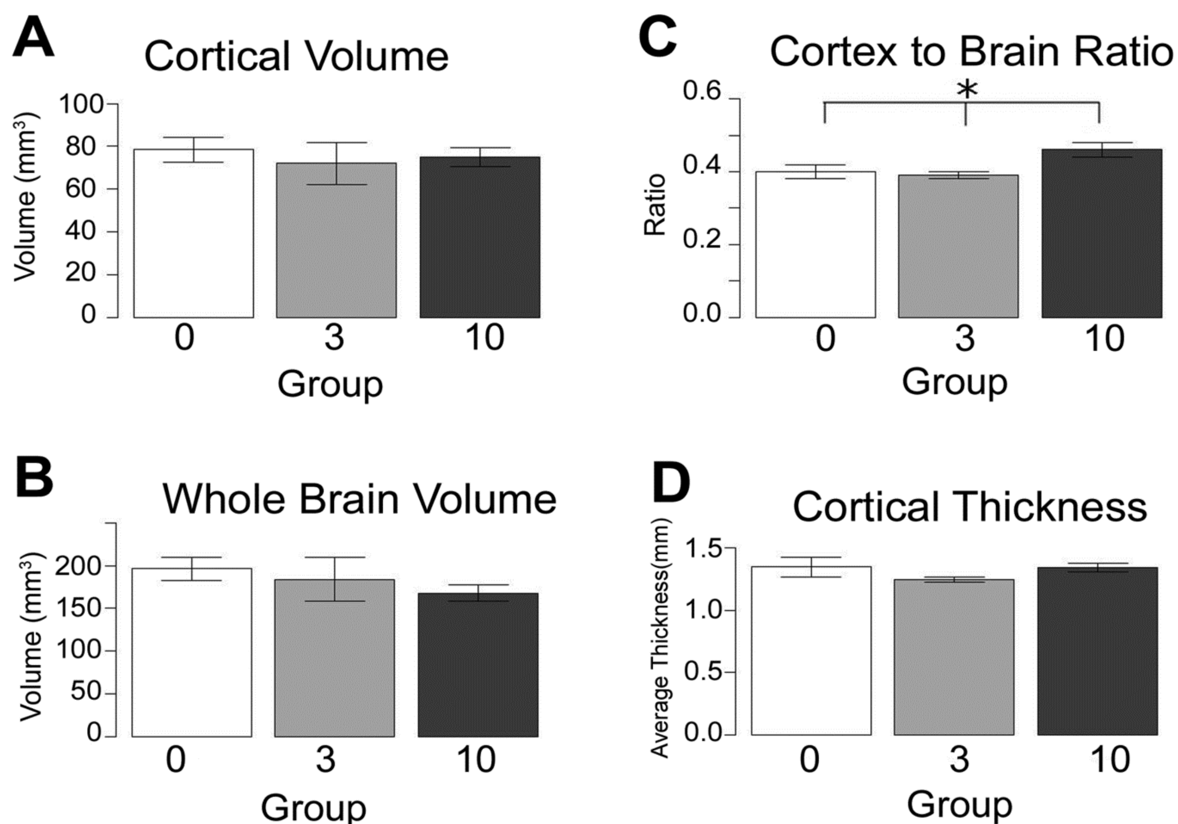


Figure 6.2: Changes in neocortical volume and thickness in PND 86 euthyroid animals exposed to PTU (0, 3, 10ppm) from GD6 through lactation PND30. No significant difference detected in A. neocortical volume (p = 0.812) or B. whole brain volume (p = 0.258) C**.** Ratio of neocortical to whole brain volume. The significant group difference for the ratio, present at PND23 was still present at PND 86 (p* =0.026). **D.** Linear measurement of cortical thickness. A comparison of cortical thickness in PND 86 animals developmentally exposed to PTU revealed no significant difference (p= 0.33). Data was analysed using a one way ANOVA followed by Dunnett t test. Data are shown as Mean ± SEM with p values < 0.05 considered significant. N = 0ppm n = 7; 3ppm n = 6; 10ppm n = 11. N = 1 dam per treatment, 0ppm: n = 1, n = 2; 3ppm: n = 1, n = 1; 10ppm: n = 1, n = 2 pups per litter.

Chapter 7: Developmental Hypothyroidism Alters Cellular Composition: A Stereological Analysis

Abstract

TH plays a critical role in fetal life especially in brain development (Benvenuti et al., 2008) as the formation of the neocortex has been shown to be sensitive to TH levels during critical windows of development (Dowling et al., 2001; Leonard et al., 1982; Martinez-Galan et al., 2004). Deficiencies during these periods can lead to irreversible impairment, cytoarchitectural and morphological abnormalities, disorganization and maldevelopment. Data from animal studies suggest that developmental disabilities associated with modest thyroid impairment may result from altered development of the cerebral cortex (Ruiz-Marcos, 1989). Thus, a lack of TH during development of the cerebral cortex may result in profound changes to cortical cytoarchitecture. Recently we have shown that the offspring of developmentally hypothyroid rats treated with PTU (0ppm, 3ppm or 10ppm); show a significant increase in cortex to brain ratio at post-natal day PND23 between groups of 0ppm and 10ppm PTU. To determine if this effect was permanent, sections from PND86 euthyroid adult offspring were also examined and the significant group difference for the ratio was still present. Thus the present study sought to determine if the increase in cortical volume previously reported can be accounted for by a change in the cellular composition of the cortex.Offspring from each dose group were perfusion-fixed on postnatal day PND23. Brains were sectioned coronally on a vibratome in a stereological manner through the dorsal hippocampus and transferred to 4 consecutive bins of cryoprotectant with every 5th section mounted onto treated slides. Bin 2 was chosen for the analysis stained for neurons with Antibeta III Tubulin and the composition of cortex was examined by estimating the amount of neuronal immunofluorescence in cortex. A stereological sampling technique was used to estimate (1) the percentage of the cortex occupied by neuronal immunofluorescence and (2) the concentration of neuronal immunofluorescence in the cortex. The analysis revealed a significant overall effect of treatment on the percentage of the cortex occupied by neuronal immunofluorescence (mm^3) ($p^* = 0.0001$) between the groups. When the concentration of neuronal immunofluorescence (mm^3) in the cortex was analysed by means of an ANOVA a significant overall effect was evidenced ($p^* = 0.0001$), with the 3ppm ($p^* = 2.07E-07$) and 10ppm ($p^* = 0.0012$) animals having a greater concentration of neuronal immunofluorescence in cortex compared to the controls, however not when compared to one another. Together these findings may provide insight into the causes of functional deficits following developmental thyroid insufficiency.

7.1- Introduction

Normal neonatal development in both rodents and humans is dependent on thyroid hormones (Calikoglu, 1999). In both human and animal models, early fetal brain development is regulated by the maternal thyroid (Morreale de Escobar et al., 1997;Pickard et al., 1997;Porterfield and Hendrich, 1991;Sinha et al., 1997). Both clinical observations and studies in the rat suggest that thyroid hormones effects on brain development occur before birth, as it has been reported that thyroid hormone of maternal origin crosses the placenta and reaches the fetus (Contempre et al., 1993; Morreale de Escobar et al., 1988). In addition, thyroid hormone receptors (TRs) are expressed in both human and rat brains before the onset of fetal thyroid function and occupancy is in the range known to elicit physiological effects (Bernal and Pekonen, 1984; Falcone et al., 1994; Ferreiro et al., 1988; Morreale de Escobar et al., 1988). Finally, Cao and Colleagues (Cao et al., 1994) report that neurological cretinism in regions of endemic goiter can be prevented by iodine therapy, however, only if initiated before the beginning of the third trimester. Therefore, from early embryonic stages onward thyroid hormones are essential for brain maturation (Bernal and Nunez, 1995; Morreale de Escobar et al., 2000).

Thyroid hormones play a crucial role in growth, differentiation and the physiological functioning of many organs, including the central nervous system (Oppenheimer and Schwartz, 1997; Porterfield and Hendrich, 1993). Neuronal growth, synaptogenesis and neuronal cytoarchitecture are strictly regulated by TH and any deficiencies during these periods can lead to irreversible impairment, cytoarchitectural and morphological abnormalities, disorganization and maldevelopment. These abnormalities include: altered neuronal proliferation and cellular migration (Goodman and Gilbert, 2007) delayed or poor myelination (Rodriguez-Peña et al.,

1993; Walters and Morell, 1981), cortical hypoplasia (reduced number of cortical neurons) (Kumar et al., 2006); and reduced dendritic spines or dysplasia's which are abnormalities in the numbers or shapes of dendrites (Bernal, 2005; Ruiz-Marcos et al., 1979), underscoring the requirement of TH for normal brain development (Lima et al., 1997; Oppenheimer and Schwartz, 1997). Several studies suggest that developmental disabilities ranging from mild dyslexia's to severe mental retardation can be attributed to alterations in cortical morphology resulting from abnormal cortical development (Crome, 1960; Galaburda et al., 1985; Humphreys et al., 1990).

Huttenlocker, (Huttenlocker, 1991) suggests that changes in synaptic and dendritic organization of the cortex may in fact underlie compromised brain function. This is supported by Kaufmann and Moser, (Kaufmann and Moser, 2000) who found that dendritic anomalies are the most consistent anatomical correlates of mental retardation (MR). As such, MR resulting from TH deficiency is posited to be a disorder in which subtle changes in neural circuitry are associated with devastating functional consequences (Thompson and Potter, 2000) the severity of the consequences related to the timing and duration of the insult (Calvo et al., 2002; Morreale de Escobar et al., 2000) which may explain both the behavioural irregularities and physiological abnormalities observed in both animal models and humans during thyroid dysfunction (Ahmed et al., 2008).

Recent findings suggest that even subtle decreases in maternal TH are adverse for the neurodevelopmental outcome of the pregnancy (Glinoer and Delange, 2000; Klein et al., 2001), and it is now well established that the mammalian brain is a target organ of TH during development (Ahmed et al., 2008). Clinical studies suggest that children born to women with subclinical thyroid disturbances have been observed to have impairments in intellectual development and neuropsychological function (Morreale de Escobar et al., 2000). Data from

animal studies propose that developmental disabilities associated with modest thyroid impairments may result from the altered development of the cerebral cortex (Ruiz - Marcos, 1989). Radial glial cells, which are necessary for the formation of the neocortex, have been found to have altered development following gestational hypothyroidism (Lavado-Autric et al., 2003; Martinez-Galan et al., 2004). In addition, it has been demonstrated that the formation of the cortex (Dowling et al., 2001; Leonard et al., 1982; Martinez-Galan et al., 2004) and cerebellum (Koibuchi and Chin, 1999; Legrand, 1979; Nicholson and Altman, 1972a; Nicholson and Altman, 1972b) are sensitive to TH levels during critical windows of development.

Thus a lack of TH during development of the cerebral cortex may result in profound changes to cortical cytoarchitecture, with specific alterations to the composition and organization of the cortical cells. For example, there is existing evidence that glial- restricted precursors are being guided away from an oligodendrocyte fate to become astrocytes (reviewed in Sharlin et al., 2008). Additional evidence for alterations in organization comes from Auso and Colleagues, (Auso et al., 2004) and from a study in which low dose PTU treatment in the rat during gestation resulted in the presence of a subcortical band heterotopia composed of neurons and glia with consistent bilateral localization in the corpus callosum (Goodman and Gilbert, 2007). In addition ubiquitin-dependent programmed cell death during the period of cortical histogenesis appears to be regulated by TH (Pasquini et al., 2000). Taken together these findings suggest that developmental hypothyroidism may cause long-term changes in the number of cortical neurons.

Recently we have shown that the offspring of developmentally hypothyroid rats treated with PTU (0ppm, 3ppm or 10ppm); show a significant increase in cortex to brain ratio at post-natal day PND23 between groups of 0ppm and 10ppm PTU. This increase persisted on PND86 when the rats returned to euthyroid status. The present study was designed to determine if this

increase in cortical volume can be accounted for by a change in the cellular composition of cortex. This was examined by estimating the amount of neuronal immunofluorescence in cortex.

7.2 - Methods and Materials

Three timed pregnant Long-Evans rats were purchased from Charles River (Raleigh, NC) on gestational day (GD) 2 and housed individually. Animal rooms were maintained on a 12/12 light-dark schedule and animals were permitted free access to food (rat chow; Purina, St. Louis, Mo) and tap water. All experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory animals

7.2.1 - Developmental hormone insufficiency

Beginning on GD 6 and continuing through lactation on postnatal day PND30, dams were rendered hypothyroid by addition of 3ppm or 10ppm of the thyroid hormone synthesis inhibitor propylthiouracil (PTU; Sigma, St. Louis, MO) to the drinking water. Control dams continued to receive normal tap water (0ppm PTU). The day of birth was designated PND 0 and litters were culled to 10 pups on PND3 - 4. On PND30, the offspring were weaned, transferred to plastic cages (two to four per cage) and were permitted free access to food and tap water.

7.2.2 - Brain sectioning

Offspring from each dose group were anesthetized with urethane (2.5 mg/kg, ip) and perfusion-fixed through the aorta with 4% paraformaldehyde on PND23 (0ppm n= 2; 3ppm n = 4; 10ppm n = 5). After brain extraction the brain was further fixed in paraformaldehyde overnight at 4 degrees C, and subsequently removed and sectioned in the coronal plane on a vibratome (50 µm). Sections were transferred to 4 consecutive bins of cryoprotectant with every 5th section being mounted on a treated slide.

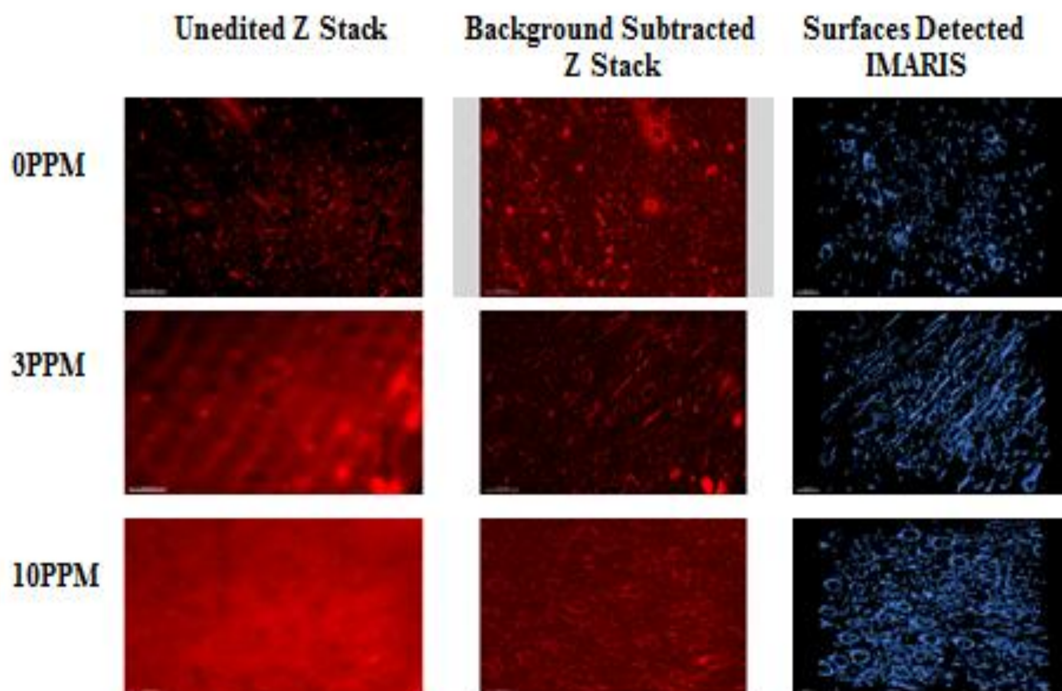
7.2.3 - Immunohistochemistry

Sections were removed from cryoprotectant (30% sucrose, with ethylene glycol and polyvinylpyrrolidone in PBS) and washed in PBS buffer on a shaker table twice for 5 minutes each time. Sections were then boiled in citric acid buffer for 20 minutes at a temperature of 80 degrees C. After boiling sections were allowed to cool under the hood and then washed in PBS buffer twice for 5 minutes each. Tissue was then placed in a blocking solution for 4 hours that contained .1% saponin in PBS, and 10% fetal calf serum in order to bind non-specific antigens. Sections were then incubated in a solution of primary antibody and blocking buffer 24 - 72 hours at 4 degree C. Anti-β-Tubulin III (1:500 dilution, raised in rabbit; Sigma-Aldrich, St.Louis, MO, USA) used to identify neurons. Sections were warmed to room temperature for 30 minutes on a shaker table. Followed by three 10 minute washes in PBS buffer with 0.1% saponin. The sections were then incubated in flurochrome conjugated secondary antibody Cy3 (donkey anti- rabbit, dilution 1:500; Jackson) diluted in blocking buffer for 4-6 hours at room temperature. Next the tissues were washed in 0.1% saponin in PBS buffer three times for 10 minutes each. Followed by three rinses for 10 minutes each in PBS buffer. Stained sections were then mounted on glass slides with Vecta shield and coverslipped.

7.2.4 - Quantification of neuron number

Immunostaining of cortex revealed an assortment of immunofluorescent neuronal components including partial and complete cell bodies and their projections as well as isolated neurites of varying lengths, it was not possible to measure the number of neurons directly. Instead, these immunofluorescent components were used as an indirect measure of neuronal content. Each brain was coronally sectioned in a stereological manner and transferred to 4

consecutive bins of cryoprotectant. Bin 2 tissue was stained with Antibeta III Tubulin to identify neuronal components and 10 tissue sections were selected for analysis. Using Stereoinvestigator (version 9.0, MBF Bioscience, Williston, VT) coupled to Nikon 90i microscope. A contour of each section of the cortex was traced at low power, and 10 randomly placed counting frame sites were established for each section. At each randomly placed counting frame site, the tissue was optically sectioned and the set of images was saved as a "z-stack". Thus a total of 100 Z stacks were collected for each animal And background subtracted using Image J software. IMARIS 3D image analysis software (version 7.2.1Bitplane, Inc. South Winsor Ct.) was used to determine (1) the number of the immunofluorescent objects and (2) their estimated volumes. From these data we calculated the (1) total number of neuronal components in the stack and (2) the percent of the stack volume occupied by the immunofluorescent objects.



Antibeta III Tubulin

Figure 7.1 Methods: This diagram displays three crucial steps from our methods. The first image from each of the three rows (0PPM, 3PPM and 10PPM dosage rats respectively) illustrates an example of a maximum intensity projection (MIP) of an unedited Z stack. The second image of each of the rows illustrates a MIP after the background was subtracted. The final image from each of the three rows shows the immunofluorescent components detected after our IMARIS procedure

7.2 – Results

The one way ANOVA with treatment as the independent factor indicated a significant treatment effect on the percentage of the cortex occupied by immunofluorescent neuronal components in PND23 rats developmentally exposed to PTU (0ppm, 3ppm or 10ppm) from GD6 through lactation (PND30) (p* = 0.0001 ; see Figure 8.2). Multiple comparisons test (Tukey HSD) showed a significant dose dependent increase in the percentage of the cortex occupied by immunofluorescent neuronal components (mm3) with the 10ppm group having the highest percentage of immunofluorescent neuronal components (p* = 0.0269).

Figure 8.3 shows the concentration of neuronal immunofluorescence in the cortex in PND23 rats developmentally exposed to PTU (0ppm, 3ppm or 10ppm) from GD6 through lactation (PND30). One way ANOVA with treatment as the independent factor indicated a significant treatment effect for the concentration of neuronal immunofluorescence in the cortex (p* = 0.0001). Multiple comparisons with bonferroni correction showed that the controls were significantly different from both the 3ppm (p* = 2.07E-07) and 10ppm (p* = 0.0012) animals exposed to PTU.

7.3 - Conclusion / Discussion

The precisely timed stages of cortical development can be divided into neurogenesis, neuronal migration and neuronal differentiation. In both rodents and humans there are discrete time windows spanning late prenatal and early postnatal periods when these events occur (Berger-Sweeney and Hohmann, 1997). In the rat, most cortical neurons are formed between embryonic days E14 – E 20, (Bayer et al., 1993; Uylings et al., 1991), 2-4 days after neuronal generation, migration occurs (Bayer et al., 1993); and differentiation which includes synaptogenesis, dendrite formation, cell maturation and the development of short and long

connections occurs during the first 3-4 weeks postnatal when synapses in the neocortex are formed and many cortical connections are refined (Greenough and Chang; Sur and Cowey, 1995; Wolff et al., 1995). Insults at particular developmental stages can lead to cytoarchitectural abnormalities including cortical ectopias or abnormal locations of neurons due to manipulations that interrupt cortical cell migration (Auso et al., 2004; Berger-Sweeney and Hohmann, 1997; Goodman and Gilbert, 2007); cortical hypoplasia, a reduction in cortical cell number due to manipulations that disrupt cortical neurogenisis (Berger-Sweeney and Hohmann, 1997; Kumar et al., 2006); and manipulations that disrupt cortical differentiation signals resulting in dysplasia's which are abnormalities in shapes or numbers of dendrites (Ruiz-Marcos et al., 1979). Each manipulation is capable of leading to alterations in connectivity patterns among neurons in the mature cortex (Berger-Sweeney and Hohmann, 1997).

It is well established that the mammalian brain is a target organ of TH during development (Ahmed et al., 2008), as the formation of the neocortex has been shown to be sensitive to TH levels during critical windows of development (Dowling et al., 2001; Leonard et al., 1982; Martinez-Galan et al., 2004). Impairments due to a lack of TH have been associated with permanent alterations in brain structure and function (Morreale de Escobar, 2004; Auso, 2004} and are suggested to result from compromised development of the cerebral cortex (Balazs et al., 1977; Ruiz-Marcos, 1989) with specific alterations to the composition and organization of cortical cells (reviewed in Sharlin et al., 2008). Several studies suggest that developmental disabilities ranging from mild dyslexia's to severe mental retardation can be attributed to alterations in cortical morphology which are the result of abnormal cortical development (Crome, 1960; Galaburda et al., 1985; Humphreys et al., 1990).As such the present study was designed to determine if the increase in cortical volume previously outlined in chapter 6 can be

accounted for by a change in the cellular composition of the cortex. A stereological sampling technique was used to estimate(1) the percentage of the cortex occupied by neuronal immunofluorescence (mm^3) (2) the concentration of neuronal immunofluorescence in the cortex (mm^3) of rats developmentally exposed to graded level of PTU (0ppm, 3ppm and 10ppm) to determine if anatomical alterations in cortex would be present. We found a dose dependent increase in the percentage of neuronal immunofluorescence in the cortex with treated groups being significantly different from controls and from one another, with the 10ppm animals exhibiting the greatest percentage of immunofluorescent neuronal components in cortex. These differences may account for the degree of severity of deficits exhibited in developmental hypothyroidism. When the overall analysis for concentration of neuronal immunofluorescent components in cortex was examined there was a significant effect of treatment, with treated groups having an increased concentration of neuronal immunofluorescent components in the cortex as compared to the controls. These findings suggest that treatment is causing a change in the composition of the cortex and may indicate either an increase in size of the neurons as has been reported previously in the rat cerebellum or an increase in the number of neurons (mm^3) in cortex. As an increase in the percent of the stack volume occupied by the immunofluorescent components implies an increase in size or the amount of space occupied by neuronal components (mm^3) and an increase in the total number of components in the stack (concentration of neuronal immunofluorescent components) suggests an increase in the number of neurons of cortex.

Our findings differ from those of Schwartz (Schwartz, 1983) and DeLong (DeLong, 1996) (reviewed in Thompson and Potter, 2000) who observed smaller and more closely aggregated cells in cortex partially due to a reduction in the development of axonal and dendritic

processes. However, our findings may indicate that the actual somas of the cells have increased in volume irrespective of dendritic and axonal reduction accounting for the increase in the percentage of the cortex occupied by neuronal immunofluorescence and the concentration of neuronal immunofluorescence in our animals. In addition several studies have examined alterations in morphology of specific cell types like the pyramidal cells of both the cerebral cortex and visual cortex where a decrease in dendritic spine number and altered distribution of dendritic spines was reported (Morreale de Escobar et al., 1983; Schwartz, 1983).However due to the different cell types and layered structure of cortex, the sampling of different areas and different layers of cortex and the differential techniques used, results across studies may vary. Finally Kumar and Colleagues (Kumar et al., 2006) report that compared with euthyroid animals hypothyroid rats had enhanced apoptosis of primarily neurons in the primary somatosensory cortex (S1) with the majority of apoptotic cells found in superficial cortical layers I-III, with minimal apoptosis occurring in the deeper layers. The proportional increase in apoptotic ells was observed throughout the developmental period (birth – PND 24) in the hypothyroid cortex; however, the highest increase occurred at PND8. As apoptosis results in a reduction in the number of neurons in cortex and our data suggests a neuronal increase one can speculate that the difference may be accounted for by the area Kumar and Colleagues (Kumar et al., 2006) examined and perhaps by the fact that our animals were sacrificed and processed at PND 23 after the highest increase of apoptosis reported by Kumar (Kumar et al., 2006) at PND8. Finally, our results could indicate a disruption in cellular differentiation with cells being pushed toward a neuronal fate as TH deficiency has been reported to alter the developmental fate of progenitor cells away from an oligodendrocyte fate (Johe, 1996; Murray and Dubois-Daleq, 1997; Raff et al., 1983; Sharlin et al., 2008).

When comparing our results to previous findings one must consider that our method included random sampling across all of cortex and was not area specific. Finally the current author must acknowledge that the results of this study may also have been influenced by the fact that more than one rat pup per litter was used in each of the experimental groups.

In conclusion, during development, TH has selective and permanent effects on processes that take place during fetal development. The present findings suggest that such processes that may have been affected during this study include cortical neurogenesis and differentiation, leading to possible alterations in connectivity patterns of the mature cortex which are the wiring for sensory integration and behavioural outputs (Berger-Sweeney and Hohmann, 1997) and may underlie some of the deficits seen in developmental hypothyroidism.

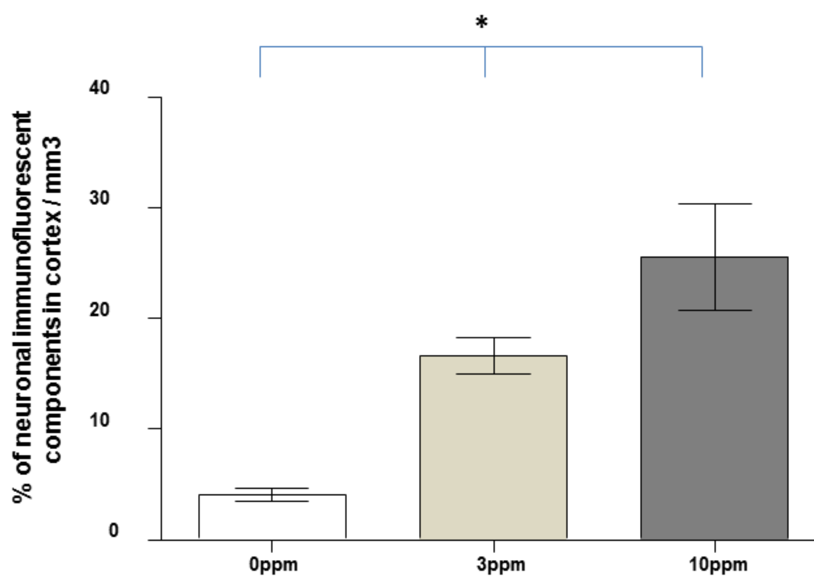


Figure 7.2: Percentage of cortex occupied by immunofluorescent neuronal components / mm $^{3.}$ To calculate percentage of immunofluorescent neuronal components per stack the volume of immunofluroescent neuronal components was divided by the stack volume for each stack in an animal for 11 animals exposed to 0ppm, 3ppm or 10ppm PTU. A significant main effect of treatment was shown (p* = 0.0001) with significant differences between all of the groups. Data for the stereology tasks was analyzed using a single factor ANOVA followed by Bonferroni multiple comparisons post-hoc test. Data are shown as Mean ± SEM. Differences with p values < 0.05 were considered significant. (0ppm: n= 2; 3ppm: n= 4; 10ppm: n = 5). N = 1 dam per treatment; 0ppm: n = 2; 3ppm: n = 9: N = 2 dams per treatment; 10ppm: n = 2, n = 3.

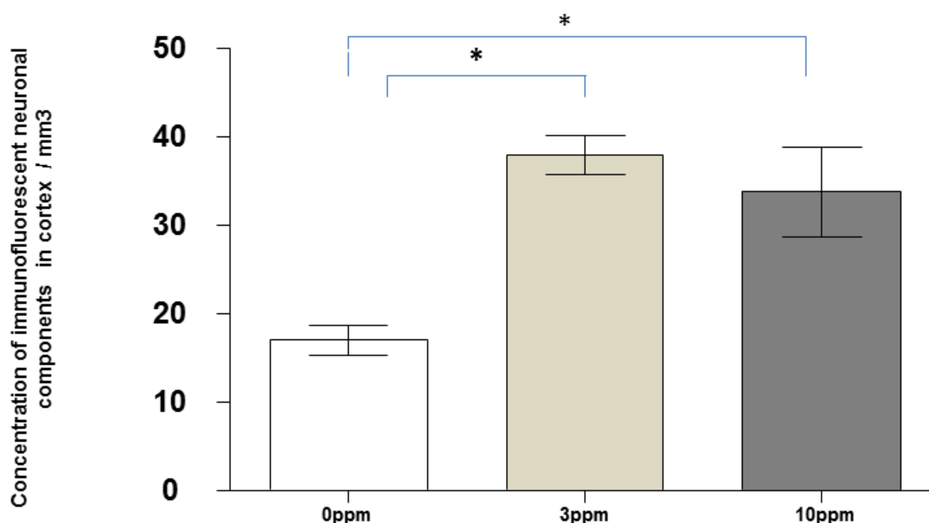


Figure 7.3: Concentration of immunofluorescent neuronal components in cortex / mm3. To calculate concentration of immunofluorescent neuronal components in cortex the number of immunofluorescent neuronal components was divided by the stack volume for each stack in an animal for 11 animals developmentally exposed to PTU (0ppm, 3ppm or 10ppm).. A significant treatment main effect was shown (p* = 0.001) with the controls being significantly different from either the 3ppm (p* = 2.07E-07) or 10ppm (p* = 0.0012).Data for the stereology tasks was analyzed using a single factor ANOVA followed by Bonferroni multiple comparisons post-hoc test. Data are shown as Mean ± SEM. Differences with p values < 0.05 were considered significant. (0ppm: n= 2; 3ppm: n= 4; 10ppm: n = 5). N = 1 dam per treatment; 0ppm: n = 2; 3ppm: n = 9: N = 2 dams per treatment; 10ppm: n = 2, n = 3.

Chapter 8: Developmental Hypothyroidism Effects on Social Behaviour in the Rat

Abstract

Developmental Hypothyroidism (DH) produces neuroanatomical changes in the rat including altered cytoarchitecture and neuronal migration and our recent finding of an increase in cortical volume. In the current study, offspring of dams in which a graded level of hormone insufficiency was produced by administration of propylthiouracil PTU (0, 3, 10ppm) in the drinking water (GD 6 through weaning) were evaluated to define the immediate and long-term consequences of milder forms of developmental hypothyroidism on certain aspects of behaviour. Maternal separation anxiety, social interaction, locomotion and perseveration behaviours were analyzed. Pups were removed from the mother on PND 6 and ultrasonic vocalizations in the range of 40 kHz were measured to test their separation anxiety. DH had a significant overall effect on PND6, (p* = 0.037) with a significant decrease in vocalizations in the rats exposed to 10ppm PTU when compared to the 3ppm group (p* = 0.025). In order to track activity patterns and social behaviours, weaned pups, implanted with radio frequency identification (RFID) transponders, were transferred to intermixed, RFID tracked, group housing configurations. Compared to controls, there was a dose dependent decrease in overall activity (PND 49-50) as the concentration of PTU increased (p*= 0.023). To determine the amount of time that animals spent interacting with cage mates, the degree of centrality (a social network analysis measure) was measured for each animal at selected time points each day over the course of 3 days (PND 47- 49).This analysis revealed no significant difference in the amount of time animals interacted with one another between the groups (p = 0.432). An additional test of sociability, the three chambered social approach task, was conducted. Groups (controls and 3ppm) were compared for time spent sniffing a novel animal or a toy, and time spent in the chamber with a novel animal. When time spent sniffing was analyzed there was no significant differences between the controls and 3ppm animals (p = 0.882; Novel Animal: p = 0.281; Toy). Again when time spent in the chamber of a novel animal was analyzed no significant differences were found between the controls and 3ppm groups (p = 0.537. Taken together, these results suggest that there is no difference in sociability between these groups. In order to access anxiety and locomotor activity, the open field test was employed. When time in the center zone was analysed the 3ppm group was found to spend significantly less time exploring the center than the controls (p* = 0.002) and when total distance travelled was analyzed there was no significant difference in locomotion between the groups (p = 0.090).In addition, animals were tested in the Barnes Maze to measure perseverative behaviour (repeated error hole visits), but no significant effect was observed. These results suggest that even modest DH, has consequences on the behavioural profile of these animals.

8.1 - Introduction

Normal development and maturation of the CNS is dependent upon thyroid hormones (Anderson et al., 2003; Bernal, 2002b). Hypothyroidism during human perinatal development results in profound alterations of mental capacities and neurological functions (Darbra et al., 2003a). Deficits in academic skills have been reported during childhood in humans with primary or secondary congenital hypothyroidism as well as a decrease in performance on neuropsychological tests in adulthood (Murphy and Nagy, 1976; Osterweil et al., 1992). Impairments have been associated with permanent alterations in brain structure and function (Morreale de Escobar, 2004; Auso, 2004) and are suggested to result from global reductions in brain size and compromised development of the cerebral cortex (Balazs et al., 1977; Ruiz-Marcos, 1989).In addition DH has been shown to interfere with cellular migration and differentiation in the hippocampus (Madeira et al., 1991; Madeira and Paula-Barbosa, 1993; Madeira et al., 1992) delay or disrupt myelination (Rodriguez-Peña et al., 1993; Walters and Morell, 1981) alter cell migration (Berbel, 2002;Goodman, 2007) and alter rates of neurogenesis (Sokoloff and Kennedy, 1973) (reviewed in Brosvic et al., 2002).

Studies examining the long-term behavioural effects of neonatal hypothyroidism in the rat report a general increase in spontaneous activity and a decrease in the ability to learn and habituate to maze tests (Davenport and Hennies, 1976; Schalock et al., 1977). In addition, studies on the effects of perinatal and postnatal TH insufficiency reveal an increase in exploratory and locomotor activities and a decrease in anxiety-like behaviours in adult rats (Darbra et al., 1995). Alterations in synaptic function and impairments in learning and memory have also been reported as a result of developmental TH insufficiency in young hypothyroid animals (Gilbert

and Sui, 2006; Sui et al., 2005). Long lasting behavioural impairments may be due to delaying restoration of thyroid hormone status (Glinoer and Delange, 2000; Morreale de Escobar et al., 2004b).

Until recently, experimental studies had been focused on severe hypothyroidism. However, it is now known that even subtle decreases in maternal thyroid hormone result in children with permanent intellectual impairment (Morreale de Escobar et al., 2000). Such impairments may be a result of altered synaptic function which Gilbert and Colleagues (Gilbert and Paczkowski, 2003; Gilbert et al., 2007) evaluated by conducting hippocampal electrophysiology in vitro and in vivo and found that paired pulse inhibition (representing the tone of local feedback circuits) was dose-dependently reduced following treatment with PTU. In fact, in the 10 ppm group, the highest PTU level tested, paired pulse inhibition was absent, suggesting local feedback inhibition is substantially weakened. In addition, PTU treatment increased CA1 population spike amplitude and long term potentiation in the CA1 dendritic region, consistent with enhanced excitability. Together, these data suggest there is reduced inhibitory input to principal neurons following developmental TH insufficiency.

In a previous study, outlined in Chapter 6, we found that the offspring of developmentally hypothyroid rats treated with varying levels of the thyroid synthesis inhibitor PTU (0ppm, 3ppm or 10ppm) showed an increase in cortical volume on (PND) 23. This increase resolved on PND 86 when the rats returned to euthyroid status. This study provided a means of modelling the effects of subtle thyroid insufficiency on early brain development while also offering a means to evaluate the dose- response relationship between altered serum hormones and neuroanatomical changes in the brain. We suspect that the cellular composition of cortex has been altered. Hence, the present study sought to determine if the alterations in morphology

would have both immediate and long-term consequences on behavior. We chose to accomplish this by using a battery of tests related to social behavior as Kooistra and Colleagues (Kooistra et al., 2001) report that children with a history of hypothyroidism during the perinatal period show a decrease in social behavior and an increased likelihood of introversion. In addition several nonsocial behaviors were examined; locomotion, working memory, repetitive behaviors and anxiety. Maternal separation anxiety related behaviour was tested at PND 6 by means of ultrasonic vocalizations (USVs), total errors, working memory errors, latency, and perseverative errors were tested at two time points PND 28 and PND 80 by means of the Barnes maze. General locomotor activity was assessed between PND 40-45 by open field testing. The three chambered social approach task was employed between PND 40-45 in order to test the sociability of the animals. Social interaction was evaluated from PND 31 until PND 86 by means of implanted radio frequency identification (RFID) transponders, which allowed the animals to be tracked without human intervention and total errors, working memory errors, latency, and perseverative errors were tested at two time points PND 28 and PND 80 by means of the Barnes maze.

8.2 - Methods and Materials

8.2.1 - Perinatal Hypothyroidism Model Developmental hormone insufficiency

A total of 96 Long Evans rat pups (0ppm: n = 25; 3ppm: n = 34; 10ppm: n = 37), from dams exposed to 0ppm, 3ppm or 10ppm propylthiouracil (PTU) (Sigma, St. Louis, MO) in their drinking water beginning on GD6 and continuing until postnatal day (PND) 31, were utilized for the following experiments. Control dams received tap water. Animals were housed in standard plastic cages unless otherwise noted. Litters were culled to 10 pups on (PND) 3 (day of birth = PND0). All animal treatments were in strict accordance with the National Institutes of Health

Guide for the Care and Use of Laboratory Animals. Animal rooms were maintained on a 12-h light, 12-h dark schedule, and permitted free access to food (rat chow; Purina, St. Louis, MO) and water.

8.2.2 - *Maternal Retrieval*

A total of 6 Long Evans rat mothers were removed temporarily from the home cage while PND4 pups were taken from their nest and placed in a diagonally opposite corner of the home cage (0ppm n =18; 3ppm n = 20; 10ppm n= 17). Mothers were returned and the lid was placed back on the home cage. Retrieval of pups was viewed from the side of the cage. The amount of time from the onset of the mothers return and retrieval of each pup was recorded methods previously described by Guariglia and Colleagues (Guariglia et al., 2011). (N = 2 dam per treatment; 0ppm: n =10, n = 8 pups per litter; 3ppm: n = 10, n = 9 pups per litter; 10ppm: n = 9, n = 8 pups per litter)

8.2.3 - Ultrasonic Vocalizations

A subset of animals were tested (0ppm: n=18; 3ppm: n= 20 10ppm: n=17) according to methods previously described by Guariglia and Colleagues (Guariglia et al., 2011). Testing occurred between the hours of 8 a.m. and 2 p.m. briefly individual animals were tested at PND6 by placing them in a 200ml beaker inside a Styrofoam box that housed a Ultrasonic frequency detector set to 40 kHz. The detector was then connected to a computer with Ultravox software that was able to detect and track the number of ultrasonic vocalizations (USVs) emitted by each pup. Three conditions were tested for each animal in all three conditions animals were maintained inside: condition 1 animal was placed in the 200ml beaker in the Sytrofoam box separated from its mother and littermates for a period of 15minutes and USVs were tracked;

condition 2 animal placed in a 200ml beaker that contained 2g of unrelated virile male home cage bedding the pup was placed on top of a coffee filter so as not to come into direct contact with the bedding for a total of 5minutes and USVs were recorded; condition 3 animal placed in 200ml beaker that was placed inside a tureen that contained 25ml of water maintained at 15 degrees C and then the beaker was placed inside the Styrofoam box the animal remained there for a total of 5 minutes and USVs were recorded. The criterion used to count the number of USVs was a minimum USV duration of 10ms with 5ms of silence between calls in order for the call to be considered independent. (N = 2 dam per treatment; 0ppm: n =10, n = 8 pups per litter; 3ppm: n = 10, n = 9 pups per litter; 10ppm: n = 9, n = 8 pups per litter)

8.2.4 - Barnes Maze Testing

A subset of animals were tested at PND28 and PND48 (0ppm: n = 2; 3ppm: n= 9; 10ppm: n= 8). The model is based on rodents' aversion to open spaces which motivates the test subject to seek shelter in the escape box. The maze tests the animal's memory for a position in space within the framework of visual spatial cues in an open environment (Coburn-Litvak et al., 2003). Fox and Colleagues (Fox et al., 1998) report that damage to the hippocampus leads to deficits in performance of this task. (N = 2 dam per treatment, 0ppm: n =1, n = 1; 3ppm: n = 4, n = 5; 10ppm: n = 4, n = 4 pups per litter)

Apparatus

The Barnes Maze consists of a white circular platform with twelve equidistant holes around the perimeter. The center of the maze is illuminated by a high wattage light bulb. Four clear plastic cups are randomly placed in the holes of the maze. Three of these cups serve as decoy escape holes while the fourth cup is intact and serves as an escape hole or goal box.

Testing occurred between the hours of 8 a.m. and 2 p.m. Testing began with all animals being habituated (3 trials) to the maze and returned to the home cage for 15 minutes between exposures. The first two days of testing (8 trials), the goal box was placed in the same location as during the habituation trials. Day three (trials 9-12) was used to test perseveration, the location of the hole from trials 1-8 was changed in order to test if the rat would adhere to the previously learned hole, or if they would be flexible and learn the new location of the escape hole (Guariglia et al., 2011). A period of no more than 24 hours between trials 4 and 5 as well as trials 8 and 9 was imposed.

Trial 1 (last habituation trial) the animal is placed in the center of the brightly lit maze and left to explore after ten minutes it is gently guided into the correct hole and left there for 2 minutes and then returned to its home cage. In the remaining trials the animal was placed in a random orientation into a start box in the center of the clean platform and maintained there for 15 seconds. After removal of the start box, a stop watch was begun to record total latency to escape. Errors (poking head or nose into incorrect holes) was recorded by location. Total number of errors, latency, perseveration, as well as working memory errors was tabulated. If the animal found the goal box it was left there for two minutes, if after two minutes it had not, it was removed from the platform and placed in the goal box for two minutes. During inter trial intervals animals were returned to their home cages which were maintained in the same location during the three days of testing.

8.2.5 - Open Field Testing

A subset of animals were tested between PND 40-45 (0ppm: n = 10; 3ppm: n = 19) (0ppm: N = 1 dam per treatment, n =10; 3ppm: N = 2 dam per treatment, n = 10, n = 9 pups per litter.

Methods as previously described by Gould and Colleagues (Gould et al., 2009). Rodents by nature like to seek and explore novel stimuli. The open field test (OFT) is a measure of general activity such as locomotor activity, exploratory behaviours and hyperactivity both in mice and rats, where quantity and quality of the activity can be measured. This paradigm is used to rule out an effect of a drug/ treatment (PTU) on motor ability which would be an unwanted confound. The open field (OF) is an enclosure, generally square, rectangular, or circular in shape with surrounding walls that prevent escape. The outcome of interest is movement.

The Open Field area consists of an empty bright square arena measuring 100 x 100cm surrounded by walls to prevent animal from escaping. A bright light bulb is illuminated over the arena. An individual rat is placed in the center of the arena by the tester and its behaviour is recorded by Cap Wiz (a computer software program) for 15 minutes. After 15 minutes the rat is removed from the box and placed back in its home cage. Anymaze software is used to analyze the pre- taped behaviour of the rat after completion of testing. The Open Field tests the conflict between the innate fear that rodents have of the central area of a brightly lit open field versus their desire to explore new environments.

8.2.6 - Three Chambered Social Approach Task

A subset of animals was tested between PND 40 -45 (0ppm: n = 8; 3ppm: n= 9). The test used was an adaptation of the procedure previously described by Yang and Colleagues (Yang et al., 2011a) (N = 1 dam per treatment; 0ppm: n = 8 pups per litter; 3ppm: n = 9 pups per litter).

Apparatus

A rectangular three-chambered box with each box measuring 20cm (length) x 40.5cm (width) x 22 cm (height), the dividing walls are made of Plexiglas, with doorways measuring (10-cm width x 5-cm height) to allow access into each chamber. The center chamber is the start location. Weighted cups are placed on top of wire cups in order to prevent subject rats from climbing in phase III of the experiment and the empty wire cup is considered the novel object. The three chambered social approach task is widely employed as a standard test for sociability (Yang et al., 2011a). Sociability has been defined as the subject rat spending more time in the chamber with the target/novel rat than in either of the other two chambers one of which contains an inanimate novel object while the other is empty. A second measure is the time the test animal spends sniffing the target rat versus the time spent sniffing the novel object, this is said to be a measure of direct social interaction. A built in control measure of exploratory locomotion is offered by how many times the animal transitions across chambers (Crawley, 2007b, 2008; McFarlane et al., 2008; Moy et al., 2009; Moy et al., 2008b; Silverman et al., 2010a; Silverman et al., 2010b; Yang et al., 2009; Yang et al., 2011a).

Train target rat

Target rats were habituated in advance of the experiment by exposing them to an inverted wire cup and chamber. The target rats were placed in a clean wire pencil cup, and the cup was inverted into one side of the chamber. Training was repeated for two 15minute sessions. All target rats were age, sex and strain matched to the subject rat.

Acclimate subject rats

The acclimation and three phases of the assay took place on the same day.

All rats were transported to the behavioural testing area one hour prior to testing in their home cages. This was done in order that the animals recovered from the stress of transportation and acclimated. All subject rats were maintained in a quiet room nearby until all experiments for the day had been completed.

Phase I: The subject rat was habituated to the center chamber

The subject rat was placed in the center chamber of the test apparatus with both doors closed and allowed to remain there for 5minutes (recorded with a stop watch). Time spent grooming was recorded.

Phase II: The subject rat was habituated to all three chambers

Once the habituation period had elapsed the side doors of the testing apparatus were opened and the animal was allowed to habituate to all three chambers of the apparatus for 10minutes. All three chambers were empty with no wire cups. Time spent grooming was recorded.

Phase III: Test for sociability

At the conclusion of the second habituation period the subject rat was guided to the center chamber and both doors were closed. The empty wire cup (1) with the plastic cup containing weights was placed on the appropriate side of the chamber. The target rat was retrieved and placed into the target wire cup 2 and put in the opposite chamber from the empty cup, and a cup containing weights was placed on top of the wire cup. The doors on both sides

of the chamber were opened and time spent grooming, time spent sniffing and time in target rat chamber versus time spent in chamber with wire cup and no target rat were recorded. This sociability test period lasted for 10minutes.

8.2.7 - Social Interaction

At PND10 a subset of animals (0ppm: n = 5; 3ppm: n = 5; 10ppm: n = 12) were each implanted subcutaneously with a Trovan Unique radio frequency identification transponder (transponder size 11.5 x 2.2mm Microchip ID Lake Zurich, IL). The transponders are activated by the use of a handheld or stationary reader, identification and locations of individual animals can be recorded. (N = 2 dams per treatment, 0ppm: n = 2, n = 3; 3ppm: n = 2, n = 3; 10ppm: n = 6, n = 6 pups per litter)

At PND31 pups were transferred to one of four identical colonies each colony was maintained on one shelf of a four tiered metal rack (shelf one n = 6; shelf two n = 5; shelf three n = 6; shelf four n = 5). Each of the four colonies consisted of at least one animal per treatment group and each cage within the colony contained food, water and enrichment. Animals remained in their respective colonies until PND 86 when they were sacrificed. Figure 7.1 shows an overall schematic of how RFID is used to track movement of individual animals in the colony.

The goal of the experiment was to measure animal social behaviour in undisturbed colonies of groups of rats that had been developmentally exposed to graded levels of PTU (0ppm, 3ppm or 10ppm). The approach utilized was based on the premise that rats tagged with transponders that were socializing with one another would be located in the proximity of the same reader (McCloskey et al., 2011). As the data generated from this approach would be too large to analyze using traditional methods the following computational methods were employed: (1) Cluster analysis utilizing the K-means clustering technique produced a Hierarchical

clustering histogram of the four caging groups based on all of the RFID events for PND 49. The bars represent the proportion of time the animals spent in a given location (y axis) by each animal (1- 22, x axis) to each location where the animals lived (1-16 with each cage configuration having had 4 RFID readers, z axis; see Figure 7.2). (2) Adjacency matrix sampling, in which all time points were sampled during a 72 hour period, was employed in order to create a central graph based on multiple animal location adjacency matrices created throughout the day. In this analysis the strength of each dyad indicated by edge weight, the number of edges to other nodes or degree of centrality representing the animals involvement in the network and the eigen vector centrality or number of edges to other nodes weighted by edge weight can be ascertained (Butts, 2008; Freeman, 1979; Wasserman and Faust, 1994) (described in McCloskey et al., 2011) (see Figure7.3}. The degree of centrality or interaction between cage mates a quantitative social network analysis measure was computed for each animal at all-time points over the course of 3 days (PND 47- 49) (see Figure 7.10).

Components, description and methodology of RFID tracking system as previously described in McCloskey and Colleagues (McCloskey et al., 2011).

* Tag (transponder): stores identifying data to which it is attached (animals).

* Reader: placed at multiple places within the housing system gets data from tag and communicates with the backend computer

* Backend computer system consisting of RFID middleware, applications, hosts databases: processes information from the reader, and connects to the enterprise network, and to the Internet. The backend computer, which was connected to a series of Trovan LID 650 readers (Microchip ID, Lake Zurich, IL), updated a

text file each time an animal passed through one of the 4 antennae. The event in the text file included the animal ID (unique 10 digit alphanumeric code), time of entry, and reader number (1-10). Text files containing 24 hours of data entries were parsed using Matlab, by location (antenna) and a state matrix identifying the last known location for each animal was processed for each event.

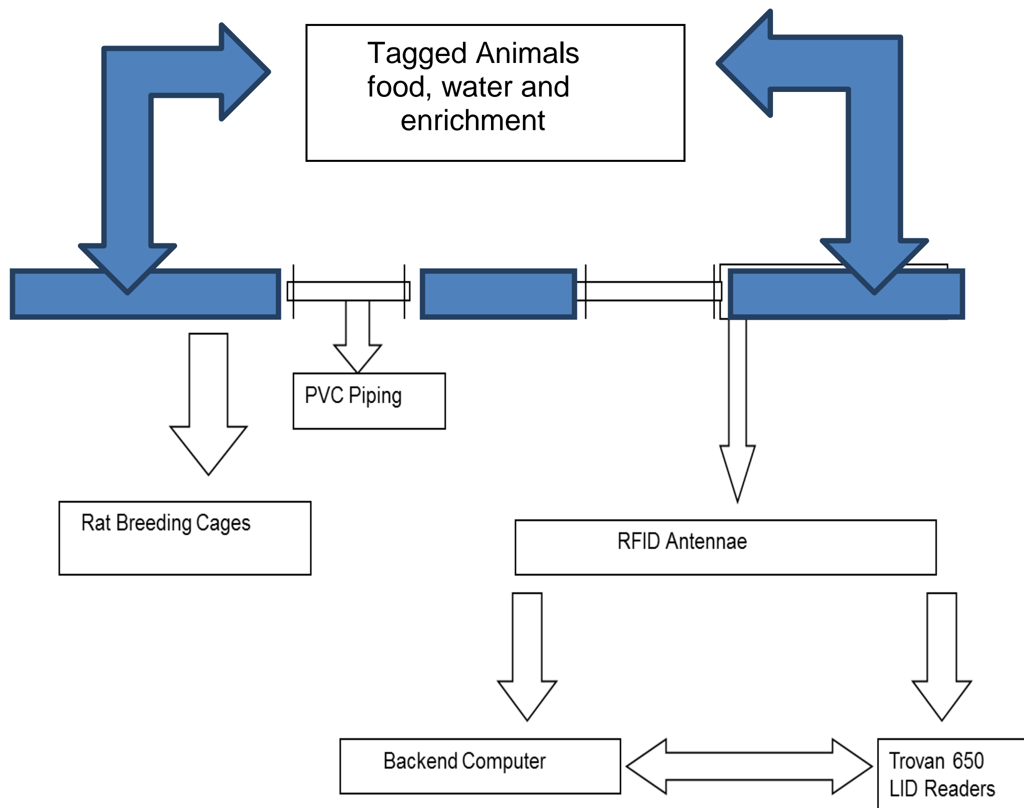


Figure 8.1: Schematic of how RFID is used to track movement of individual animals in the colony.

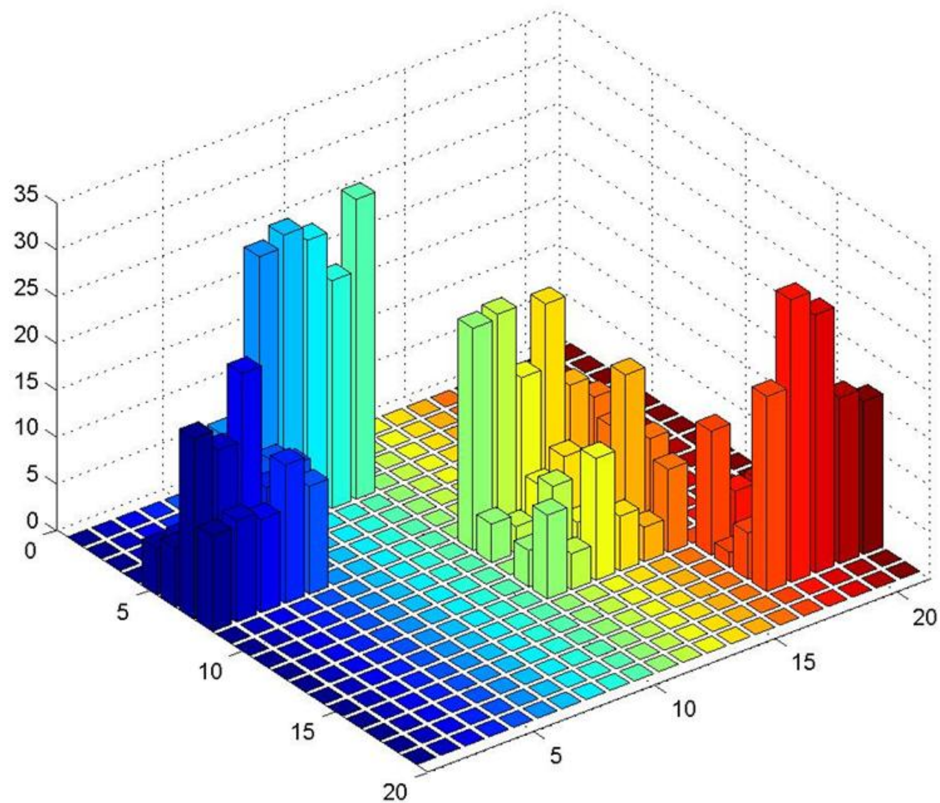


Figure 8.2: Place Preferences in the Housing Environment. Hierarchical clustering histogram of the four caging groups based on all of the RFID events for PND 49. The Y axis (bottom to top) represents the proportion of time animals spent in a given location. The X axis (left to right) represents the individual animals. The Z axis (back to front) shows the locations where the animals lived (each cage configuration had 4 RFID readers). There were no obvious differences in the location preferences for individual animals.

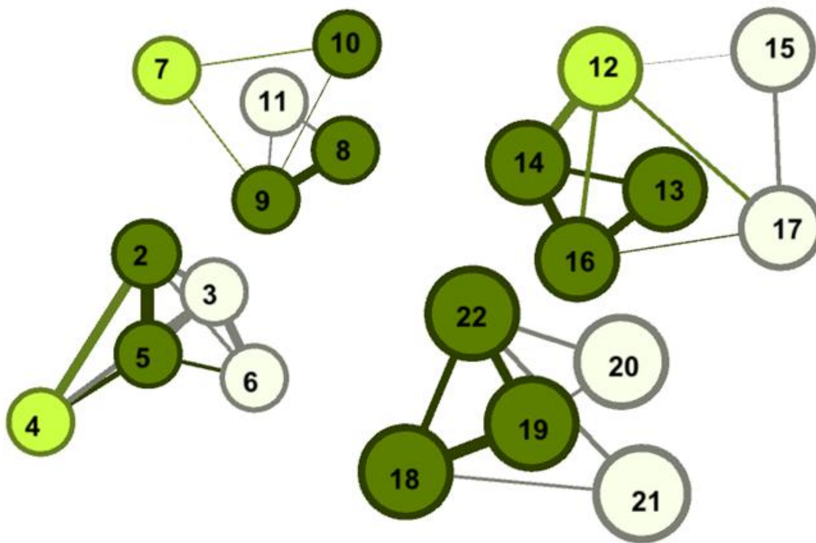


Figure 8.3: Network Graph 1 day of data. A network graph of the animals in their caging groups based on 1 day of RFID data. Node size represents the degree of centrality, which is an indicator of the number of other animals an animal shared a location with over the course of the day. Edge weight represents how much of the time each pair of animals spent together. White nodes: 0PPM, Light green nodes: 3PPM, Dark green: 10PPM.

8.3 - Results

8.3.1 - Maternal Retrieval

The one way ANOVA indicated no differences in maternal care, evaluated by latency to retrieve pups to their nest ($p = 0.25$; see Figure 8.4).

8.3.2 - Ultrasonic Vocalizations

The one way ANOVA with treatment as the independent factor indicated a significant main effect of treatment on the number of vocalizations emitted at PND6 ($p^* = 0.037$; see Figure 8.5A). Multiple comparisons with bonferroni correction revealed a significant difference in USVs between the 3ppm and 10ppm groups with the 10ppm group emitting significantly fewer vocalizations compared to the 3ppm group ($p^* = 0.025$). In addition, the one way ANOVA showed no significant differences in the mean duration of call length ($p = 0.219$; see Figure 8.5B).

The one way ANOVA revealed a significant treatment main effect on the number of USVs made by the groups in response to exposure to a stranger male bedding scent ($p^* = 0.0001$; see Figure 8.5C). A multiple comparisons with bonferroni correction showed the 3ppm animals differed significantly from both the 0ppm and 10ppm animals ($p^* = 0.001$; $p^* = 0.0001$ respectively) . When duration of USVs emitted in response to placement in stranger male bedding was analyzed using a one way ANOVA there was a significant treatment main effect in mean duration of call length between the groups ($p^* = 0.0006$; see Figure 8.5D). Multiple comparisons with a bonferroni correction showed a significant difference between the 3ppm and 10ppm animals ($p^* = 0.01$).

In separation experiments involving cold, the one way ANOVA indicated no significant treatment main effect in response to the cold at PND6 ($p = 0.131$ Figure 8.5E). However, the ANOVA showed a trend toward significance for the mean duration of call length ($p = 0.060$; see Figure 8.5F).

8.3.3 - Barnes Maze

PN28

Total number of errors, latency, working memory errors as well as perseveration was analyzed by means of an ANOVA at two different time points in rats developmentally exposed to PTU from GD6 through lactation (PND30). A 10ppm outlier (± 2SD from the mean) was removed from all analysis (0ppm n = 2; 3ppm n = 9; 10ppm n= 7). Time points were chosen in order to ascertain if possible effects in cognition at PN28 when the animals were hypothyroid were permanent at PND48 when the animals had returned to euthyroid status. No significant treatment main effects were evident at PND28 for total number of errors ($p = 0.299$; see Figure 8.6A), latency ($p = 0.349$; see Figure 8.6B), working memory errors ($p = 0.501$; see Figure 8.6C) or perseveration ($p = 0.420$; see Figure 8.6D) when analysed using a one way ANOVA.

PN48

Figure 7.7 shows a summary of total number of errors, latency, working memory errors as well as perseveration at PND48 when the animals had returned to euthyroid status. An outlier, greater than 2 standard deviations from the overall mean, in the 0ppm group for perseveration and an outlier in the 3ppm group for latency were replaced by the overall means for the groups in each analysis. The ANOVA showed no significant main effects for treatment in total errors ($p = 0.605$; see Figure 8.7A), working memory errors ($p = 0.289$; see Figure 8.7C) or perseveration ($p = 0.461$; see Figure 8.7D). However, latency or time to reach the goal box revealed a significant

treatment main effect (p* = 0.004 see Figure 8.7B), with multiple comparisons with bonferroni correction showed the controls being significantly different from both the 3ppm (p* = 0.002) and 10ppm (p* = 0.022) groups.

8.3.4 - Open Field Testing

Figure (7.8) illustrates open field activity for PND42 rats developmentally exposed to PTU from GD6 through lactation (PND30) and controls. Behavioural measures reported include time exploring the center area of the chamber, time exploring the outer area of the chamber and total distance travelled.

In order to measure anxiety like behaviour in the rodent, time spent in the center area is measured. If significantly less time is spent by the rodent exploring the unprotected center area that demonstrates anxiety-like behaviour in the rodent. Unpaired students t – test showed that 3ppm animals spent significantly less time exploring the central area of the chamber compared to the controls, (p* = 0.0023; see Figure 7. 8A). A two way ANOVA showed an effect of treatment (p* = 0.001; see Figure 7.8B) and a time effect (p* = 0.0190), reflecting an increase in activity in the center zone over time in the control group.

Rodents will typically spend a significantly greater amount of time exploring the periphery of the arena usually in contact with the walls. Unpaired students t-test revealed that the 3ppm animals spent a significantly greater amount of time exploring the periphery of the arena than did the controls (p* = 0.0009; see Figure 8.8C). In addition the two way ANOVA showed a significant effect of treatment (p* = 0.0001; see Figure 8.8D) with the 3ppm group spending the same amount of time in the outer zone for the duration of the test.

The most commonly used measure of overall exploratory /locomotor activity is total distance traveled. When the controls and 3ppm animals were compared for locomotor activity

over a 10 minute period an unpaired students t- test was employed and no significant difference between the groups was evident (p = 0.0900; see Figure 8.8E). When time course was employed in 2 minute bins over the course of 10 minutes a two way ANOVA was conducted and yielded a significant effect of treatment and time (p* = 0.034; p* =0.0001 respectively; see Figure 8.8F) .

8.3.5 - Three Chambered Social Approach Task

Grooming

Figure (7.9) shows a summary of grooming for phases I and II for PND 43 rats developmentally exposed to PTU from GD6 through lactation (PND30). Unpaired students t-test did not indicate significant differences in grooming (p = 0.0799; p= 0.4204; see Figure 7.9A Phase I and 7.9B Phase II respectively). Suggesting no difference in the level of interest the animals had in their environment.

Time spent sniffing

When the groups were compared for time spent sniffing both the novel animal and the toy unpaired students t-test showed there was no significant differences between the controls and 3ppm animals (p = 0.882; p = 0.281 respectively; see Figure 7.9C). However, both groups spent significantly more time investigating the novel animal when analyzed individually using an unpaired t test (0ppm: p* = 0.0003; 3ppm: p* = 0.0005; see Figure 7.9C).

Episodes of Sniffing

Unpaired students t-test showed no significant difference in the number of times the control verse 3ppm animals sniffed the novel animal or the toy (p = 0.9643 p = 0.1247; respectively; see Figure 7.9D). However, when the control and 3ppm subject rats were analyzed separately unpaired students t-test showed there was a highly significant increase in the number

of times the subject rats sniffed the novel animal compared to the toy (p* = 0.0003 p* = 0.0001; respectively; see Figure 7.9D).

Time in chambers

Unpaired students t-test revealed no significant difference in the amount of time the controls verse 3ppm groups spent in the chamber with the novel animal (p = 0.5375 ; see Figure 7.9E). However, the control group spent a significantly greater amount of time in the chamber with the toy (p* = 0.05). Controls showed no difference in the amount of time spent in either chamber (p = 0.2711 while, the 3ppm group spent a significantly greater amount of time in the chamber of the novel animal (p* = 0.0011; see Figure 7.9E).

8.3.6 - Social Interaction

A quantitative social network analysis was conducted to determine if the degree of centrality, a measure of social interaction, was influenced by developmental hypothyroidism. The one way ANOVA with treatment as the independent factor did not indicate a significant treatment effect on the amount of time animals spent with one another (p = 0.4321; see Figure 7.10). The one way ANOVA for both low and high activity periods showed no significant differences between the groups for interaction with cage mates (p = 0.311; Low Activity: p = 0.264; High Activity: p = 0.188; see Figure 7.10B and 7.10C respectively).

8.4 - Discussion

Developmental hypothyroidism is a disorder characterized by a reduction of the THs during a critical period of brain development, with the severity of the effects depending on the magnitude, the time of onset and the onset of treatment of the deficiency (Porterfield and Hendrich, 1993). Disruptions of TH can lead to morphological and functional alterations that

contribute to cognitive and neurological impairment related to behavioral changes (Pineda-Reynoso et al., 2010). The present study sought to determine if the alterations in morphology, including the change in relative cortical volume outlined in Chapter 6 and the change in the number of cortical neurons outlined in Chapter 7, led to behavioral manifestations. We chose to accomplish this by using a battery of tests related to social behavior as Kooistra and Colleagues (Kooistra et al., 2001) report that children with a history of hypothyroidism during the perinatal period show a decrease in social behavior and an increased likelihood of introversion. In addition, several nonsocial behaviors were examined; locomotion, working memory, repetitive behaviors as well as anxiety. However, one must acknowledge that none of the behavioral tests employed in this battery directly assay cortical functions.

The hypothyroid state of the PTU treated animals was corroborated by the presence of a subcortical band heterotopia with consistent bilateral localization in the corpus callosum (Goodman and Gilbert, 2007). Differences in eye opening were evidenced with the hypothyroid rats opening their eyes later than their euthyroid cohorts (unpublished observations). This retardation of the development of the CNS due to the thyroid hormone deficiency implied an alteration in neurodevelopment (Bernal, 2007). Pineda-Reynoso and Colleagues (Pineda-Reynoso et al., 2010) suggests that behavioural tests also reveal such alterations in the CNS. As the metabolic functioning of neurons during the life cycle of the rat is dependent on thyroid hormones and developmental hypothyroidism could modify long-term behavioural responses by causing structural changes at different brain levels (Bernal, 2007).

Zippelius and Schleidt (Zippelius and Schleidt, 1956) report that the major role of USVs is thought to be communicative. Ultrasonic calls have been described in a variety of rodent infants (Sales and Pye, 1974) and in the rat were found to elicit and guide maternal search

(Smotherman et al., 1978). Therefore under a variety of experimental conditions we counted the number of USVs of PND6 rats developmentally exposed to graded levels of PTU (0ppm, 3ppm and 10ppm) to determine if the response to maternal separation would elicit differences in the number of USVs and to confirm if the differences were secondary to deficits in vocal ability caused by PTU treatment. The age for testing (PND6) was chosen because placing rat pups alone in a novel surrounding reliably elicits USVs from 3 to 18 days of age (Hofer et al., 1993b). We found that the 10ppm group evidenced a significant reduction in the number of USVs emitted in response to maternal separation as compared to the 3ppm group. However, the 3ppm treatment group tended to show a non-significant increase in the number of USVs made in comparison to the controls. Several studies suggest that distress perceived by pups to particular experimental situations is reflected by USVs (Moles et al., 2004). As such, the 3ppm treatment group can be considered to be more distressed by being separated from their littermates and mothers, while the response of the 10ppm group suggests that they did not find the situation distressing.

To determine if the reduction in USVs in the 10ppm group was due to abnormalities in vocal structures resulting from the PTU treatment, USVs in response to the cold were examined for a 5 minute period. The differences of this analysis were not significant and so these findings suggest that the developmental hypothyroidism due to PTU treatment had no effect on vocal ability and was not the reason for the findings in this group. Overall analysis found no significant differences between the groups suggesting similar levels of distress to the cold.

The groups were then exposed to the bedding of a foreign male in the event that PTU treatment caused thermoregulatory abnormalities that could have confounded the cold response test. There was a significant difference in the number of USVs emitted in these experiments,

with the 3ppm animals emitting significantly more USVs than either the controls or 10ppm animals. In addition there was a significant difference overall in duration of call length with 3ppm group emitting significantly longer calls than the 10ppm group. Taken together this suggests that the 3ppm group is more distressed by the encounter with the stranger male bedding scent than either the controls or 10ppm group.

The 10ppm group evidenced a significant reduction in the number of USVs in response to maternal separation, and based on the cold test the results were not due to structural abnormalities. It is imperative however, to determine that these results were due to developmental hypothyroidism due to exposure to graded levels of PTU (0ppm, 3ppm or 10ppm) treatment and not due to effects resulting from a decrease in maternal care caused by maternal exposure to PTU through gestation and until weaning. As such, a maternal retrieval test was run and mothers from all groups showed no significant differences in the time it took to retrieve their pups to the nest. This data suggests that maternal PTU treatment did not affect maternal care nor was maternal care the cause for the reduction in USVs found in the 10ppm group.

Experiments involving social manipulations seem to increase distress in the 3ppm treatment group, while they do not elicit the same response in the controls or 10ppm group. The results for both measures USVs emitted and duration of call for exposure to a male foreign bedding scent suggested that the 3ppm group was once again more distressed by the social manipulation than either the controls or 10ppm groups as they elicited more USVs with a longer duration of call. Finally the number of USVs emitted in response to the cold showed no difference between the groups suggesting similar levels of distress to this non- social measure. Taken together this suggests that the 10ppm treatment group may not be distressed by changes in

social situations or may have deficits in communication, while the 3ppm group exhibits what could be perceived as heightened distress to social situations.

The Barnes maze, a hippocampal dependent task (Fox et al., 1998) that tests for memory of a position in space within the reference framework of visual spatial cues in an open environment, was employed as a test in this behavioural battery (Coburn-Litvak et al., 2003). Developmentally hypothyroid animals were tested for total number of errors, latency to reach the goal box, working memory errors as well as perseveration at two time points PND28 and PND48. Testing at PND28 showed no effect of developmental hypothyroidism on these measures; however at PND48 latency or time to reach the goal box was significant overall with the controls taking longer to reach the goal box than either of the treated groups. This may suggest a decreased level of anxiety to the testing conditions in the controls as compared to the treated groups. Our findings differ from those of Davenport and Hennies, (Davenport and Hennies, 1976) and Schalock and Colleagues (Schalock et al., 1977) who reported a general increase in spontaneous activity and a decrease in the ability to learn and habituate to maze tests in hypothyroid rats. The increase in spontaneous activity in transiently hypothyroid rats has been suggested by Comer and Norton (Comer and Norton, 1985) to result of habituation in the controls to their test surroundings after a short time, while hypothyroid animals maintain the same level of spontaneous movement and exploration over time. In addition Sui and Gilbert (Gilbert and Sui, 2006), report an effect of 3ppm and 10ppm PTU on spatial learning in the Morris water maze at PND130-175. One must consider that these differing results may be attributable to the developmental timing of the deficiency, the differences in severity (van Wijk et al., 2008), or the choice of task or the age at testing.

In order to determine if developmental hypothyroidism caused any changes in locomotion in PND42 rats we exposed them to the open field testing which also provided an initial screen for anxiety related behavior in rodents (Ossenkopp et al., 1994; Prut and Belzung, 2003). The task is based on the natural tendency of rodents to explore and avoidance reaction to protect. Rodents that spend significantly less time in the center of the arena are considered to exhibit anxiogenic behavior or anxiety- like behavior.

The 3ppm treated animals were found to spend significantly less time in the center area of the arena and more time in the periphery of the arena than the controls, suggesting that moderate developmental hypothyroidism produces anxiety. In order to determine if the 3ppm animals were actually more anxious than the controls a segmental analysis was conducted on time in the center zone of the arena. The repeated measures ANOVA revealed a difference of only 10% between the groups in time spent in the center zone of the arena. This result might suggest that there are actually no differences in anxiety like behavior in the 3ppm verses control animals. However, one must acknowledge the possibility that the mildly hypothyroid group is more distressed by the tasks presented than the controls, which is consistent with previous findings in the USVs testing which revealed distress in this group in response to maternal and littermate separation and encounter with a stranger male bedding scent.

The most common measure of overall exploratory/locomotor activity is the total distance traveled. When the groups were compared for general locomotion no significant differences were detected. However a segmental analysis revealed an overall effect of treatment with the controls traveling slightly more over the testing period and an effect of time with the 3ppm treatment group traveling less in the later time bins.

In the present study locomotor activity was not affected in the developmentally hypothyroid rats which is inconsistent with locomotor activity studies conducted previously, where it was found to be increased when hypothyroidism was induced prenatally, perinatally or postnatally (Akaike et al., 1991; Darbra et al., 1995; Friedhoff et al., 2000; Negishi et al., 2005). In addition Negishi and Colleagues (Negishi et al., 2005) report that hypothyroidism induced perinatally caused hyperactivity in the female rat in the open field test. These discrepancies may be explained by difference in treatment period and method (PTU treatment or low iodine diet or thyroidectomy) as well as age at testing (Darbra et al., 2003b; Tamasy et al., 1986). In addition the experimental set-up could also explain the discrepancies between the present study and previous findings, since the period in which hypothyroidism is inducted is a major determinant for final cognitive performance. Additional studies by van Wijk and Colleagues (van Wijk et al., 2008) report very low levels of anxiety in their rats; this may in fact be consistent with our findings of distress as opposed to anxiety.

A standard assay for determining sociability in mice is the three chambered social approach task (Crawley, 2004). We sought to determine if there was a difference in sociability between developmentally hypothyroid animals and controls. Grooming during phase I and II of the assay was assessed and no differences between the controls and 3ppm animals were detected. This suggests that there was no difference in the level of interest the animals had in the novelty of their environment.

There was no significant difference between the controls and 3ppm animals in the amount of time they spent in the chamber with the novel animal. As sociability is defined as the subject animal spending more time in the chamber of the novel animal than in the chamber containing the novel object this finding suggests that there was no difference in the sociability of

these groups, as they both selected to be near a conspecific rather than be alone in a chamber with an object. However, it is interesting to note that the controls spent significantly more time in the chamber with the toy than did the 3ppm group. This finding may have been due to the 3ppm animals suffering from some level of distress which prevented them from seeking out more stimulation, the novel object and also interfered with their natural tendency to explore.

Treated animals spent a highly significant amount of time in the chamber of the novel animal and significantly less time in the chamber of the novel object. This finding may suggest that these animals have a high degree of sociability, as more than 75% of their time was spent in the chamber of the novel animal or, as mentioned earlier, they may have been apprehensive to explore.

There was no difference in the number of sniffing episodes between the groups with both groups sniffing the novel animal more. Additionally, there was no significant difference in the amount of time sniffing or investigating the novel animal or the toy between the groups with both groups spending a significantly greater amount of time investigating the novel animal. These data corroborate that there is no difference in direct social interaction between these groups.

Taken together both groups seemed to show a preference for social interaction. This suggests that developmental hypothyroidism does not lessen the effect of sociability in these rats. Which is consistent with a previously reported animal studies in which a test of sociability found that neonatal and perinatal hypothyroidism did not affect the number of social contacts or the time spent in social contact (Pineda-Reynoso et al., 2010).

The goal of the social interaction experiment was to quantify social behaviour by measuring activity and social behaviour of animals developmentally exposed to graded levels of PTU (0ppm, 3ppm or 10ppm). This design allowed for the animals behaviour to be captured in their own intact and undisturbed colony limiting stress and allowing for continued analysis over multiple time scales. Quantification was based on the premise that when animals spend more time with one another they are more social than those animals that spend more time alone.

All groups (0ppm, 3ppm and 10ppm PTU) spent about the same time interacting with cage mates. This data corroborates the findings for the social approach task in which no group differences in sociability were evidenced. Although not significant, the average number of interactions with cage mates was lowest for the 3ppm group yet these animals spent 75% of their time in the chamber with the novel animal verse the chamber with the toy in the social approach task suggestive of a high degree of sociability. A possible explanation may be that these animals were highly intrigued during testing by the novelty of one conspecific but when housed with many animals over an extended period of time they found it distressing and did not interact as frequently as the controls or 10ppm animals in the group housing situation.

In the USVs testing the 3ppm animals showed high levels of distress and may in fact have heightened anxiety to social situations. Open field testing also suggests that these animals may be more distressed than the controls as they spent less time in the center of the maze and more time in the periphery. Together the distress evidenced in these two behavioural assays may account for the 3ppm animals having the fewest average interactions with cage mates as compared to the controls or 10ppm animals.

In conclusion, there may be several reasons that could account for some of the discrepancies between the current findings reported for this battery of behavioral tests and those previously reported. The developmental timing of the deficiency, differences in the severity of the treatment (van Wijk et al., 2008), as well as treatment period and method employed (PTU treatment or low iodine diet or thyroidectomy) (Tamasy et al., 1986;Darbra et al., 2003). In addition, one must also consider that the choice of task or the age at testing may also be a factor in the disparity of results. Finally the current author must acknowledge that the results of this study may also have been influenced by the fact that more than one rat pup per litter was used in each of the experimental groups.

Figure Captions:

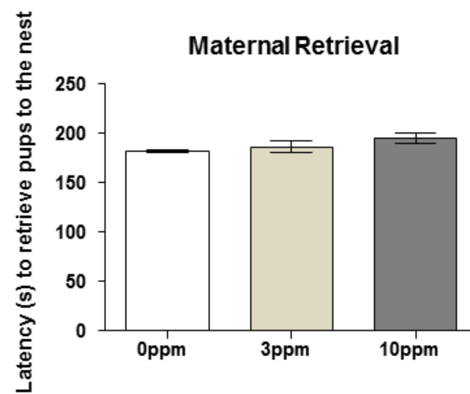
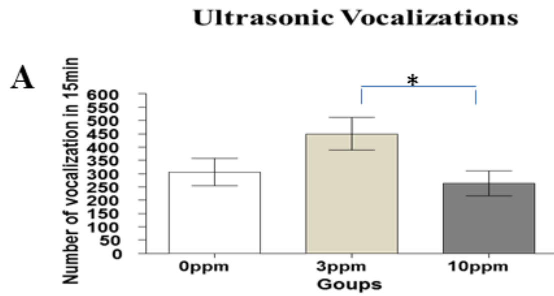


Figure 7.4: Maternal Retrieval. No significant differences were evidenced in latency to retrieve pups to the nest at PND4, ($p= 0.254$). Data for maternal retrieval tasks was analyzed using a one way ANOVA. Data are shown as Mean ± SEM and Differences with p values < 0.05 were considered significant. (0ppm: n = 2; 3ppm: n= 2; 10ppm: n = 2). N = 2 dam per treatment; 0ppm: n =10, n = 8 pups per litter; 3ppm: n = 10, n = 9 pups per litter; 10ppm: n = 9, n = 8 pups per litter.

A



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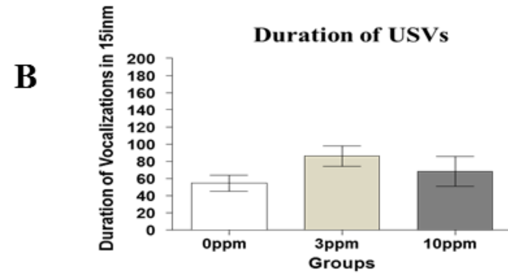

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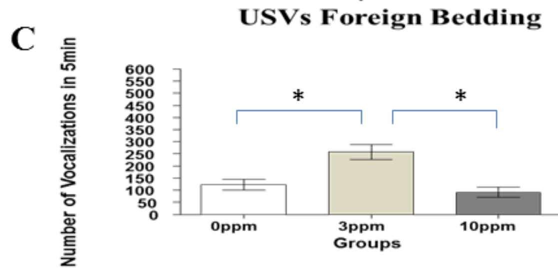
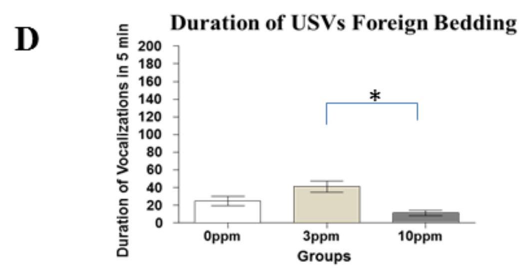
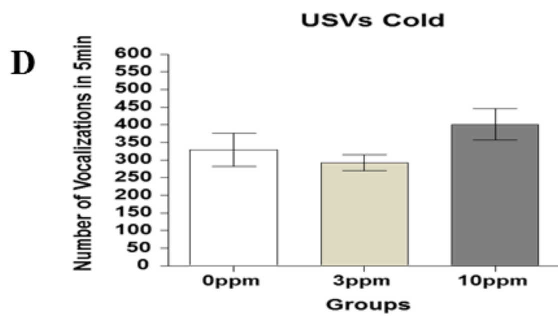


D



D



E

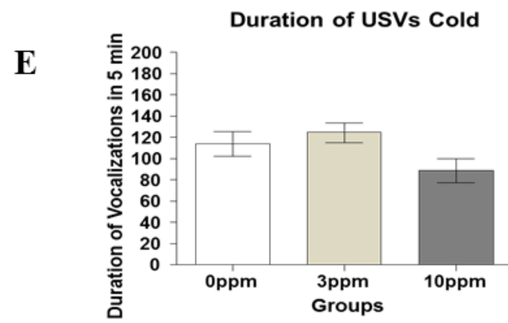


Figure 7.5: (A) USVs emitted in response to maternal separation. There was a significant overall effect of treatment on vocalizations at PND6**,** ($p^* = 0.037$) A significant reduction in the number of calls emitted in response to maternal separation was observed in the 10ppm group when compared to the 3ppm group ($p^* = 0.025$). (B) Duration of USVs emitted in response to maternal separation. No significant differences in mean duration of call length between any of the groups ($p = 0.219$) (C) USVs emitted in response to placement in stranger male bedding. There was a significant treatment effect on the number of USVs in response to exposure to a stranger male bedding scent ($p^* = 0.0001$). A significant difference was evidenced between the 3ppm animals and both the controls and 10ppms ($p^* = 0.001$; $p^* = 0.0001$ respectively) (D) Duration of USVs emitted in response to placement in stranger male bedding. A significant treatment main effect in mean duration of call length was shown ($p^* = 0.0006$). With a significant difference between the 3ppm and 10ppm animals ($p^* = 0.01$). (E) USVs emitted in response to cold**.** There was no main effect of treatment on the number of USVs emitted in response to cold ($p = 0.131$). (F)Duration of USVs emitted in response to cold**.** However, mean duration of call length had a trend toward significance ($p = 0.060$). Data for vocalization tasks was analyzed using a one way ANOVA followed by multiple comparisons with bonferroni correction Data are shown as Mean ± SEM and Differences with p values < 0.05 were considered significant. (0ppm: n=18; 3ppm: n= 20 10ppm: n=17). N = 2 dam per treatment; 0ppm: n =10, n = 8 pups per litter; 3ppm: n = 10, n = 9 pups per litter; 10ppm: n = 9, n = 8 pups per litter.

Barnes Maze PND28

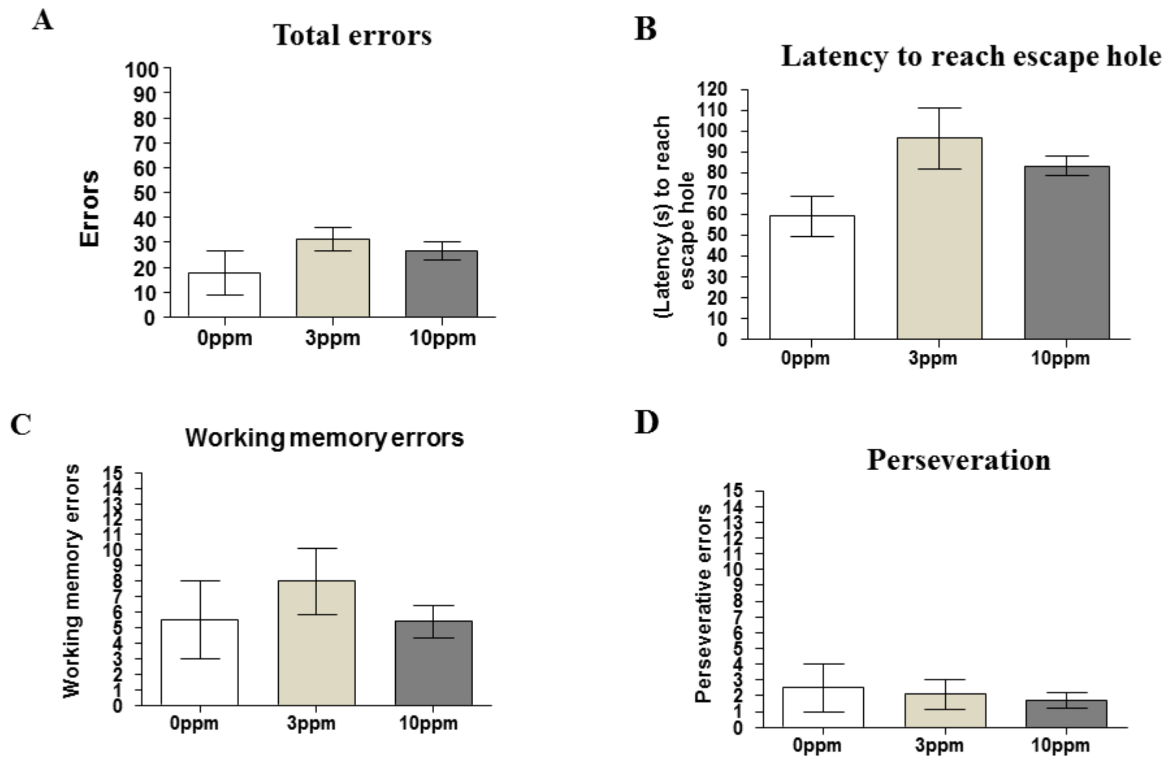


Figure 7.6: Barnes maze PND28. Animals were developmentally exposed to PTU (0, 3, 10ppm) through the mother's drinking water. **A.** Total errors, **B.** Latency to reach the escape hole **C.** Working memory errors, and **D.** Perseveration. No group differences were detected in the Barnes Maze at PND 28 (while the animals were still hypothyroid status). Total errors ($p = 0.299$), latency ($p = 0.349$), working memory errors ($p = 0.501$), and perseverative errors ($p = 0.899$). Data for Barnes maze tasks was analyzed using a one way ANOVA followed by multiple comparisons with bonferroni correction Data are shown as Mean ± SEM and Differences with p values < 0.05 were considered significant. (0ppm: n = 2; 3ppm: n= 9; 10ppm: n= 8). N = 2 dam per treatment, 0ppm: n =1, n = 1; 3ppm: n = 4, n = 5; 10ppm: n = 4, n = 4 pups per litter.

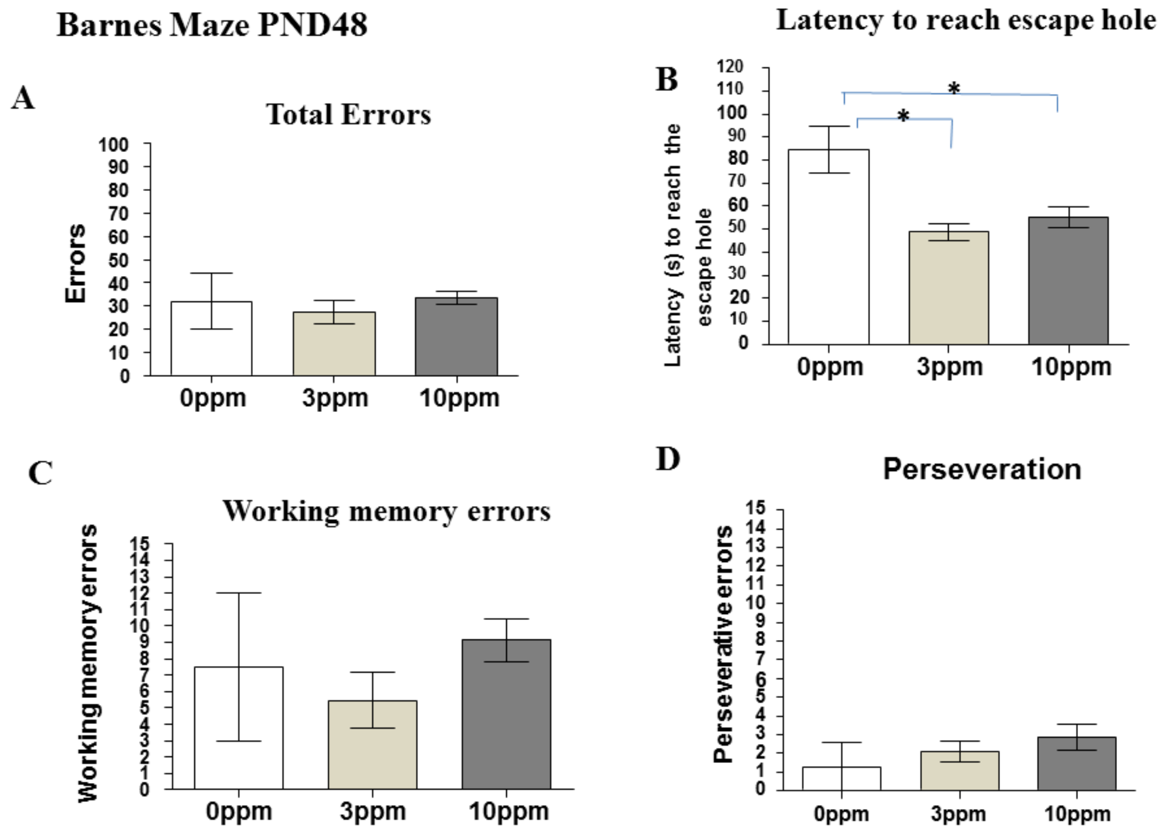


Figure 7.7: Barnes Maze PND48. Animals were developmentally exposed to PTU (0, 3, 10ppm) through the mother's drinking water. **A.** Total errors, **B.** Latency to reach the escape hole, **C.** Working memory errors, and **D.** Perseveration. When latency to reach the escape hole in the Barnes Maze was analyzed (when the animals returned to euthyroid status) a significant treatment main effect was shown (p* = 0.004) with the controls taking significantly longer than either the 3ppm (p* = 0.017) or 10ppm (p* = 0.022) groups to reach the escape hole. No group differences were detected for Total errors (p = 0.605), Working memory errors (p = 0.289), or Perseverative errors (p = 0.461). Data are shown as Mean ± SEM and Differences with p values < 0.05 were considered significant. (0ppm: n = 2; 3ppm: n= 9; 10ppm: n= 8). N = 2 dam per treatment, 0ppm: n =1, n = 1; 3ppm: n = 4, n = 5; 10ppm: n = 4, n = 4 pups per litter.

Open Field

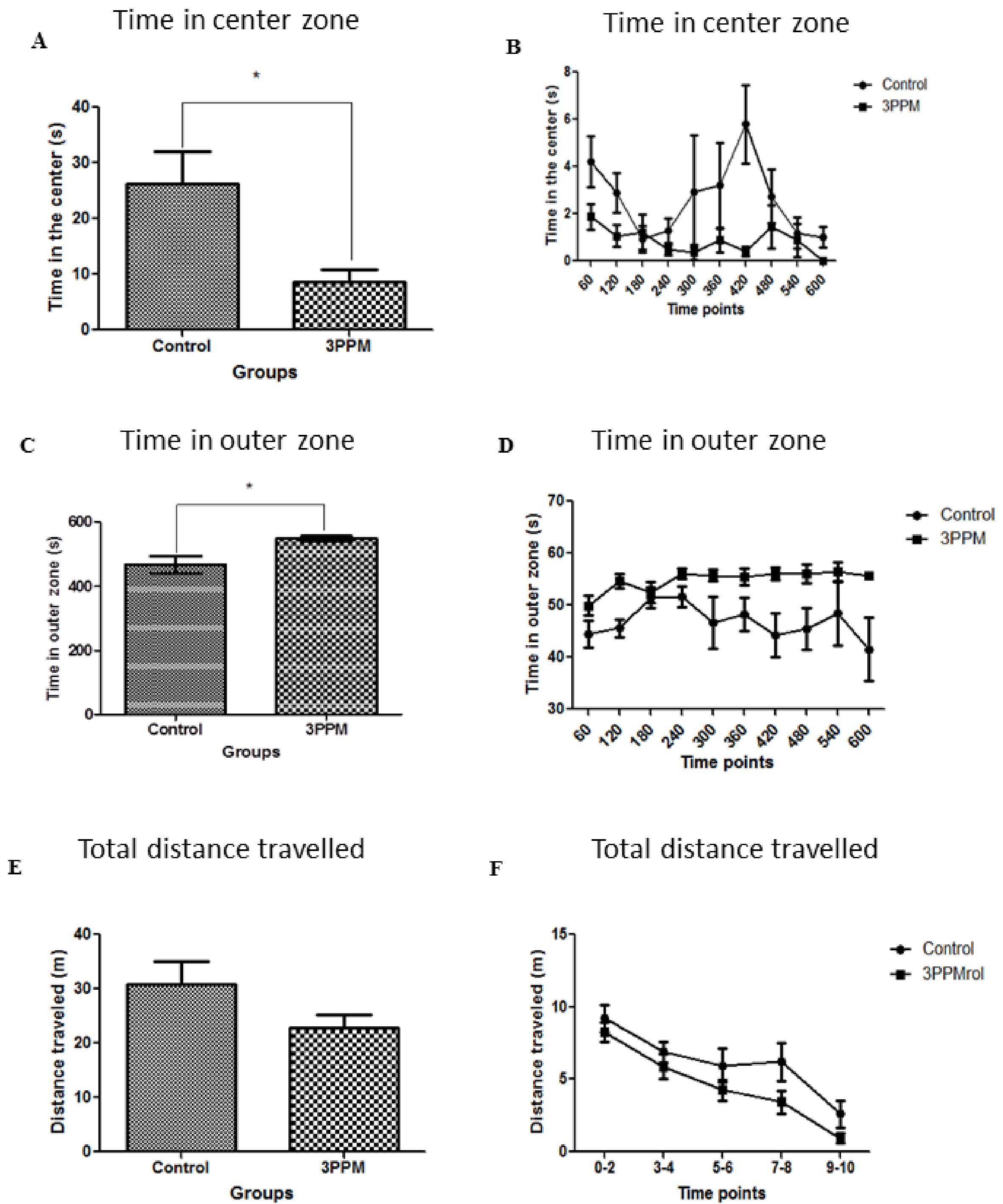
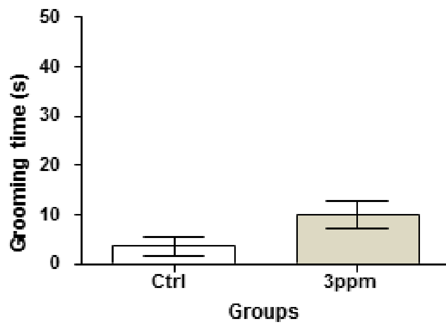


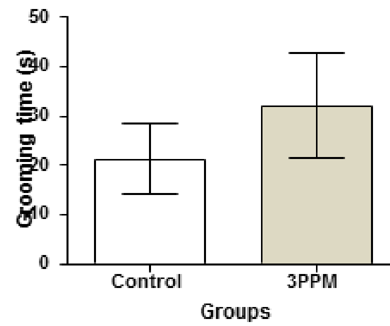
Figure 7.8: Open Field Test. (A) Time spent in the center zone. A significant treatment effect on the time spent in central area of the arena was shown with the controls spending more time in the central arena than the 3ppm animals ($p^* = 0.0170$). (B) The segmental analysis t revealed a highly significant difference in time spent in the center zone at 420 (s) with controls spending a greater amount of time in the center of the arena than the 3ppm group ($p^* = 0.001$). (C) Time spent in the outer zone. A significant treatment main effect was shown with the 3ppm animals spending more time in the periphery compared to controls ($p^* = 0.0140$). (D) Segmental analysis shows the time points in which the 3ppm and controls differed in time spent in the outer zone 420(s) ($p^* = 0.01$), 480(s) ($p^* = 0.05$) and 600(s) ($p^* = 0.001$). (E) Total distance travelled (m). No significant main effect of treatment was shown between the control and 3ppm groups for total distance travelled ($p = 0.0900$). (F) Segmental analysis shows total distance travelled (m) in 2 minute time bins across the 10 minute test period with no significant differences between the groups.. Data was analyzed using students t-test. For analysis involving multiple time points for the groups a two way ANOVA was used followed by Bonferroni multiple comparisons post-hoc test. Data are shown as Mean ± SEM. Differences with p values < 0.05 were considered significant. (0ppm: $n = 10$; 3ppm: $n = 19$). 0ppm: $N = 1$ dam per treatment, $n =10$; 3ppm: $N = 2$ dam per treatment, $n = 10$, $n = 9$ pups per litter.

Social Approach Test

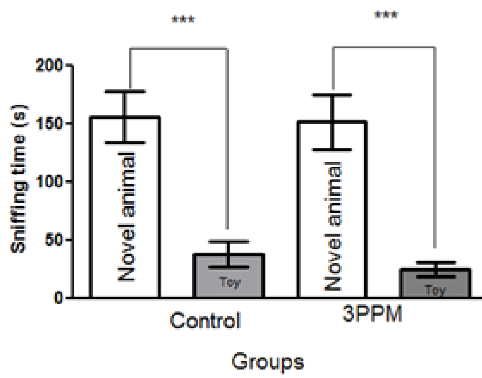
A Grooming (Phase I)



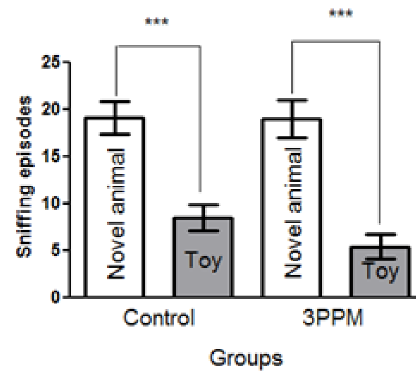
B Grooming (Phase II)



C Sniffing time



D Sniffing episodes



Time in the Chamber

E

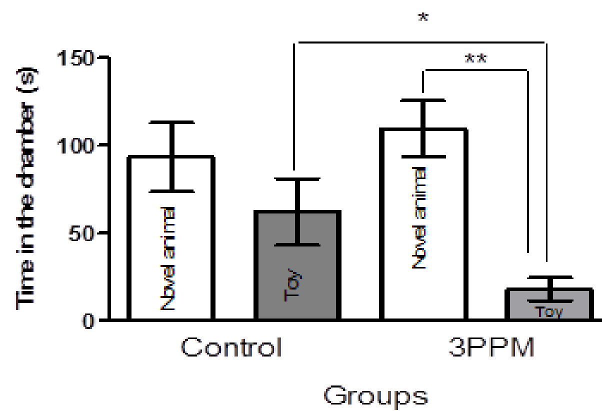


Figure 7.9: Social Approach. No significant difference in the time control verse 3ppm animals groomed in phase I (A) or phase II (B) of the experiment ($p = 0.079$; $p = 0.4204$). (C) Time spent sniffing no significant difference between the groups in the time investigating the novel animal or the toy ($p = 0.888$; $p= 0.281$; respectively), with both groups spending significantly more time sniffing the novel animal ($p^*= 0.0003$; $p^* = 0.0005$; respectively). (D) Sniffing episodes. Unpaired students t-test showed no significant difference between the groups for time sniffing ($p = 0.964$; $p = 0.124$; respectively), however, both groups spent more time sniffing the novel animal ($p^* = 0.0003$; $p^* = 0.0001$; respectively). (E) Time spent in the chamber. No significant difference in the amount of time the control verse 3ppm animals spent in the chamber of the novel animal ($p = 0.537$), however, the control animals spent a significantly greater amount of time in the chamber with the toy ($p^* = 0.058$). The 3ppm group spent a significantly greater amount of time in the chamber with the novel animal ($p^* = 0.0011$). Data was analyzed using unpaired t- test. Data are shown as Mean ± SEM Differences with p values < 0.05 were considered significant. (0ppm: $n = 8$; 3ppm: $n= 9$). N = 1 dam per treatment; 0ppm: $n = 8$ pups per litter; 3ppm: $n = 9$ pups per litter.

Degree of Centrality

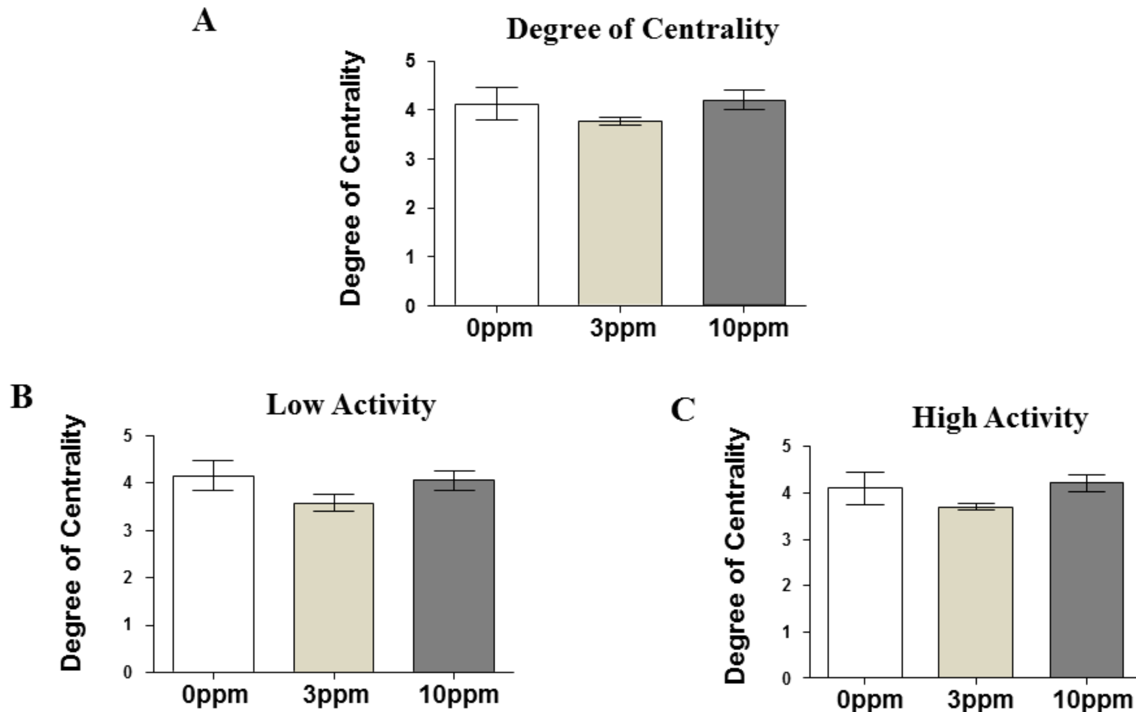


Figure 8.10: Social Interaction. (A)When the interaction between cage mates was measured for each animal over 72 hours, there was no significant main effect of treatment between the groups ($p = 0.4321$). (B) No significant treatment main effect was shown when low activity was analyzed between groups ($p = 0.311$). (C) High activity period revealed no significant treatment main effects ($p = 0.264$). Data for the degree of centrality was analyzed using a one way ANOVA. Data are shown as Mean ± SEM and Differences with p values < 0.05 were considered significant. (0ppm: n = 5; 3ppm: n = 5; 10ppm: n = 12) N = 2 dams per treatment, 0ppm: n = 2, n = 3; 3ppm: n = 2, n = 3; 10ppm: n = 6, n = 6 pups per litter.

Chapter 9 – Global Conclusion/Discussion

The central hypothesis of this work is that developmental thyroid insufficiency impacts the development of the rat cerebral cortex by altering cortical volume and the number of cortical neurons. In addition, as these neuroanatomical changes caused by milder forms of developmental hypothyroidism or hypothyroxinemia may have both immediate and long term consequences on certain aspects of behaviour, the investigation sought to determine if the alterations in morphology, including the change in relative cortical volume outlined in Chapter 6, and the change in the number of cortical neurons in the rat brain outlined in Chapter 7, led to behavioral manifestations. Hypothyroidism was induced by the administration of graded levels of the antithyroid agent propylthiouracil (PTU) to suppress thyroid hormone production.

The central hypothesis was confirmed by analyses of cortical volume in offspring of females treated with PTU during pregnancy. The number of neurons was estimated, using unbiased sampling techniques, to determine whether the cellular composition of cortex was altered following developmental TH insufficiency. To determine if these cortical alterations led to changes in behaviour, a battery of behavioural tests were performed which included maternal retrieval (PND 4), maternal separation anxiety (PND 6), Barnes maze (PND 48 and PND 86), social interaction social approach (PND 48-50), and open field (PND 46).

9.1 - Developmental Hypothyroidism Alters Neocortical Volume

Developmental TH insufficiency altered neocortical development. In general, increasing levels of maternal TH insufficiency caused an increase in the ratio of neocortical to whole brain volume and these effects persisted into adulthood and euthyroid status. The morphometric analysis indicated that the alteration in neocortex to whole brain volume at PND23 was not due

to a disproportionate decrease in subcortical structures, however this relationship resolved by PND86 even though the increase in the ratio of neocortical to whole brain volume persisted. The observed increase in cortical volume may underlie some of the behavioural deficits associated with developmental hypothyroidism (Haddow et al., 1999b; Sui et al., 2005).

TH deficiency has been reported to alter the developmental fate of progenitor cells away from an oligodendrocyte fate (Johe, 1996; Murray and Dubois-Daleq, 1997; Raff et al., 1983; Sharlin et al., 2008).Therefore, an alteration in the composition and organization of the cortical cells is a more likely explanation for our findings. Support for altered organization comes from Auso et al., (Auso et al., 2004) and Lavado-Autric and Colleagues(Lavado-Autric et al., 2003) who observed alterations in neuronal migration and Goodman & Gilbert(Goodman and Gilbert, 2007), who observed the presence of a bilateral heterotopia, composed of neurons and glial cells, in the corpus callosum of rats treated with PTU during gestation. Together, these findings indicate that TH insufficiency alters cell differentiation and organization, which may have a significant impact on the number of neurons and glial cells in the cortex.

9.2 - Developmental Hypothyroidism Alters Cellular Composition: A Stereological Analysis

Synaptic organization of the cerebral cortex has been suggested to be responsible for compromised brain function (Huttenlocher, 1991). Therefore we examined the percentage of neuronal volume (μ^3) in cortex occupied by Antibeta III Tubulin immuoreactivity and the concentration of neuronal particles(mm^3) in tissue of a rats developmentally exposed to graded level of PTU (0ppm, 3ppm and 10ppm) to determine if anatomical alterations in cortex would be present. We found a dose dependent increase in the percentage of neuronal volume (μ^3) with treated groups being significantly different from controls and from one another, with the 10ppm animals exhibiting the greatest percentage change in neuronal volume (μ^3). These differences

may account for the degree of severity of deficits exhibited in developmental hypothyroidism. When the overall analysis of concentration of neuronal particles (mm^3) in cortex was examined, there was a significant effect with treated groups having an increased concentration of neuronal particles (mm^3) in the cortex as compared to the controls.

These findings suggest that treatment causes a change in the composition of the cortex and may indicate either an increase in size of the neurons (μ^3) in cortex or an increase in the number of neurons (mm^3) in cortex. As an increase in the percentage of neuronal volume (μ^3) implies an increase in size or the amount of space occupied by neuronal particles and an increase in the concentration of neuronal particles (mm^3) suggests an increase in the number of neurons (mm^3) in cortex.

Our findings differ from those of Schwartz (Schwartz, 1983) and DeLong (DeLong, 1996) (Thompson and Potter, 2000) who observed smaller and more closely aggregated cells in cortex partially due to a reduction in the development of axonal and dendritic processes. However, our findings may indicate that the actual somas of the cells have increased in volume irrespective of dendritic and axonal reduction accounting for the increase in the percentage of neuronal volume (μ^3) and the concentration of neuronal particles (mm^3) in our animals. Our results are consistent with a disruption in cellular differentiation with cells being pushed away from an oligodendrocyte fate (Johe, 1996;Murray and Dubois-Daleq, 1997;Raff et al., 1983; Sharlin et al., 2008). When comparing our results to previous findings one must consider that our method included random sampling across all of cortex and was not area specific.

In conclusion, TH has selective and permanent effects on processes that take place during fetal development. Our findings suggest that processes that may have been affected include cortical neurogenesis and differentiation, leading to possible alterations in connectivity

patterns of the mature cortex which influence sensory integration and behavioural outputs (Berger-Sweeney and Hohmann, 1997) and may underlie some of the deficits seen in developmental hypothyroidism.

9.3 - Developmental Hypothyroidism Effects on Social Behaviour in the Rat

Until recently, experimental studies had been focused on severe hypothyroidism. However, it is now known that even subtle decreases in maternal thyroid hormone result in children with permanent intellectual impairment (Morreale de Escobar et al., 2000). The present study sought to determine if the alterations in morphology would have both immediate and long-term consequences on behavior. We chose to accomplish this by using a battery of tests related to social behavior as Kooistra and Colleagues (Kooistra et al., 2001) report that children with a history of hypothyroidism during the perinatal period show a decrease in social behavior and an increased likelihood of introversion. In addition, several nonsocial behaviors were examined; locomotion, working memory, repetitive behaviors as well as anxiety.

We found that the 10ppm group evidenced a significant reduction in the number of USVs emitted in response to maternal separation as compared to the 3ppm group. Although the 3ppm treatment group showed an increase in the number of USVs in comparison to the controls, it did not reach significance. Several studies suggest that distress perceived by pups to particular experimental situations is reflected by USVs (Moles et al., 2004). As such, the 3ppm treatment group can be considered to be more distressed by being separated from their littermates and mothers, while the response of the 10ppm group suggests that they did not find the situation distressing.

Experiments involving social manipulations seem to increase distress in the 3ppm treatment group, while they do not elicit the same response in the controls or 10ppm group. The results for both measures (USVs emitted and duration of call) for exposure to a male foreign bedding scent suggested that the 3ppm group was once again more distressed by the social manipulation than either the controls or 10ppm groups as they elicited more USVs with a longer duration of call. Finally the number of USVs emitted in response to the cold showed no difference between the groups suggesting similar levels of distress to this non- social measure. Taken together this suggests that the 10ppm treatment group may not be distressed by changes in social situations or may have deficits in communication, while the 3ppm group exhibits what could be perceived as heightened distress to social situations.

In the three chambered social approach task, the 3ppm treated animals were found to spend significantly less time in the center area of the arena and more time in the periphery of the arena than the controls, suggesting that moderate developmental hypothyroidism produces anxiety. In order to determine if the 3ppm animals were actually more anxious than the controls a segmental analysis was conducted on time in the center zone of the arena. The repeated measures ANOVA revealed a difference of only 10% between the groups in time spent in the center zone of the arena. This result might suggest that there are actually no differences in anxiety like behavior in the 3ppm verses control animals. However, it is possible that the mildly hypothyroid group was more distressed by the tasks presented than the controls, which is consistent with previous findings in the USVs testing which revealed distress in this group in response to maternal and littermate separation and encounter with a stranger male bedding scent.

There was no significant difference between the controls and 3ppm animals in the amount of time they spent in the chamber with the novel animal suggesting that there was no

difference in the sociability of these groups, as they both selected to be near a conspecific rather than be alone in a chamber with an object.

Taken together both groups seemed to show a preference for social interaction. This suggests that developmental hypothyroidism does not lessen sociability in these rats. This is consistent with a previously reported animal study in which a test of sociability found that neonatal and perinatal hypothyroidism did not affect the number of social contacts or the time spent in social contact (Pineda-Reynoso et al., 2010). Our data corroborate the findings for the social approach task in which no group differences in sociability were observed.

In summary, in USV testing the 3ppm animals showed high levels of distress and may in fact have heightened anxiety to social situations. Open field testing also suggests that these animals may be more distressed than the controls as they spent less time in the center of the maze and more time in the periphery. Together the distress evidenced in these two behavioural assays may account for the 3ppm animals having the fewest average interactions with cage mates as compared to the controls or 10ppm animals.

Taken together, the results presented here support the hypothesis that developmental hypothyroidism and hypothyroxinemia induced by chemical thyroid hormone suppression (PTU) cause alterations in the morphology of the cerebral cortex by altering cortical volume and changing the number of cortical neurons in the rat brain. Furthermore these alterations ultimately lead to changes in certain aspects of behaviour. These results have important clinical relevance because several studies suggest that developmental disabilities ranging from mild dyslexia to severe mental retardation can be attributed to alterations in cortical morphology resulting from abnormal cortical development (Crome, 1960; Galaburda et al., 1985; Humphreys et al., 1990).

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