MATERNAL HYPOTHYROXINEMIA AND FETAL BRAIN DEVELOPMENT: A BIOCHEMICAL, ENZYMIC, METABOLIC AND MOLECULAR BIOLOGICAL INVESTIGATION

Thesis submitted for the Faculty of Science at the University of London for the degree of Doctor of Philosophy

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1995
To my parents
Acknowledgements

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Abstract

The developing brain is the most susceptible tissue to changes in the level of thyroid hormone (TH). Abnormal intrauterine TH environment may lead to catastrophic disturbances in brain development which are irreversible if thyroid status is not corrected. Inappropriate transference of maternal TH to the fetus was recently reported to affect many biochemical, neurotransmitter, and metabolic parameters in adult, juvenile, and fetal brains. Therefore, the effects of intrauterine TH environment on the expression of biochemical, enzymatic, metabolic, and molecular biological functions of the developing brain was investigated using fetuses from partially thyroidectomised (TX) rat model at different stages of gestation.

Maternal hypothyroxinemia adversely affected the fetal brain growth as manifested by a significant decrease in the DNA content, wet weight, and protein:DNA ratio at 15, 19, and 22 days of gestation (dg) respectively. The RNA concentration and RNAiDNA ratio were also found to be reduced at 19 dg. The protein phosphate and inorganic phosphate were both reduced in fetal brain from TX dams at 15 dg, then substantially increased at 19 dg. A further increase at 22 dg was also observed for the inorganic phosphate. The lipid phosphate concentration was increased in fetal brain from TX dams at 19 and 22 dg. In addition, maternal hypothyroxinemia was found to affect most of the aforementioned biochemical parameters in the fetal liver.

With respect to the placenta, maternal hypothyroxinemia was found to reduce the RNAiDNA ratio and placental weight at 19 and 22 dg respectively, and significantly increase the RNA concentration at 22 dg. the protein phosphate concentration was found to be affected too. This was manifested by the significant decrease at 15 dg, followed by a substantial increase at 19 dg.

Maternal hypothyroxinemia was without effect on the placental or fetal brain total lipid concentrations, while the cholesterol concentration was significantly reduced in fetal brain at 15 dg followed by an increase at 22 dg. Similar increase was also observed in the
placenta and fetal liver. In the fetal liver a significant increase in the total lipid concentration was observed at 22 dg.

Maternal hypothyroxinemia significantly increased the protein kinase C (PKC) activity in the fetal brain at 15 dg, which was then reduced at 19 dg. The cAMP-dependent protein kinase (PKA) activity, on the hand, was within normal values at all gestational ages studied. The effect of maternal hypothyroxinemia on Na\(^+\) K\(^+\)-ATPase and Ca\(^{2+}\) Mg\(^{2+}\)-ATPase was studied too. The activity of both enzymes were significantly reduced at 15 dg and 19 dg. This reduction was sustained at 22 dg for the Na\(^+\) K\(^+\)-ATPase activity, whereas for Ca\(^{2+}\) Mg\(^{2+}\)-ATPase activity a significant increase was observed. Measurement of the activity of the dephosphorylating enzymes; acid phosphatase and alkaline phosphatase, revealed no obvious effect of maternal hypothyroxinemia upon their activity.

The glucose uptake and metabolites of glucose utilisation: CO\(_2\), amino acids, protein, lipid, and protein lipids were all found to be affected at all gestational ages studied, except for CO\(_2\) where no changes were observed at 21 dg in the experimental fetal brain. Finally the mRNA expression of the glucose transporter (GLUT 1) was found to be significantly reduced in the fetal brain from TX dams at 16 dg, whereas no changes were observed at 19 and 21 dg.

It can be concluded, therefore, that maternal hypothyroxinemia causes a range of damaging effects on the fetal brain development which may be irreversible and, as no significant effects of maternal hypothyroxinemia were observed on the placental development, it is suggested that the placenta in the hypothyroxinemic condition is likely to function normally and the observed changes in the fetal brain development are related directly to maternal TH status.
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<td>4-methylumbelliferone</td>
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<td>2-DG</td>
<td>2-deoxy-D-glucose</td>
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<td>ATCC</td>
<td>American type culture collection</td>
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<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
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<tr>
<td>AChE</td>
<td>acetycholinesterase</td>
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<tr>
<td>BMR</td>
<td>basal metabolic rate</td>
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<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
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<td>cDNA</td>
<td>complementary DNA</td>
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<td>CNS</td>
<td>central nervous system</td>
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<td>dCTP</td>
<td>cytosine deoxyribose 5'-triphosphate</td>
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<td>D-I</td>
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<td>D-III</td>
<td>5-deiodinase (inner or tyrosyl ring)</td>
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<td>DABA</td>
<td>diaminobenzoic acid</td>
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<td>DEPC</td>
<td>diethylpyrocarbonate</td>
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<td>dg</td>
<td>days of gestation</td>
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<td>DIT</td>
<td>diiodotyrosine</td>
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<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<td>DTT</td>
<td>dithiotheritol</td>
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<td>EDTA</td>
<td>ethylene diamine terta acetic acid</td>
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<td>EGL</td>
<td>external granular layer</td>
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<td>GLUT</td>
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<td>dGTP</td>
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\[
\begin{array}{ll}
\text{I}^*/\text{I}^+ & \text{iodine free radical/iodinium ion} \\
\text{IQ} & \text{intellectual quotient} \\
\text{Kd} & \text{dissociation constant} \\
\text{KDa} & \text{kilodalton} \\
\text{KRP} & \text{Krebs ringer phosphate solution} \\
\text{LSC} & \text{liquid scintillation counter} \\
\text{MAG} & \text{myelin associated glycoprotein} \\
\text{MAP} & \text{microtubule associated protein} \\
\text{MIT} & \text{mono-iodothyronine} \\
\text{MMI} & \text{methimazole} \\
\text{MOPS} & \text{morpholinopropanesulphonic acid} \\
\text{mRNA} & \text{messenger RNA} \\
\text{N} & \text{normal; control; euthyroid} \\
\text{NADH} & \text{nicotinamide adenine dinucleotide (reduced form)} \\
\text{bp} & \text{base pair} \\
\text{PCA} & \text{perchloric acid} \\
\text{PCR} & \text{polymerase chain reaction} \\
\text{Pi} & \text{inorganic phosphate} \\
\text{PKA} & \text{cAMP-dependent protein kinase} \\
\text{PKC} & \text{protein kinase C} \\
\text{PTU} & \text{propylthiouracil} \\
\text{RNA} & \text{ribonucleic acid} \\
\text{rRNA} & \text{ribosomal RNA} \\
\text{rT3} & \text{reverse T3} \\
\text{SDS} & \text{sodium dodecyl sulphate} \\
\text{SSPE} & \text{sodium saline phosphate-EDTA} \\
\text{T2} & \text{3,3'-diiodothyronine} \\
\text{T3} & \text{3,3',5-triiodothyronine} \\
\text{T4} & \text{thyroxine}
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<td>thyroxine-binding globulin</td>
</tr>
<tr>
<td>TBPA</td>
<td>thyroxine-binding prealbumin</td>
</tr>
<tr>
<td>TBS</td>
<td>tris buffered saline</td>
</tr>
<tr>
<td>TCA</td>
<td>trichloroacetic acid</td>
</tr>
<tr>
<td>TETRAC</td>
<td>tetraiodo-acetic acid</td>
</tr>
<tr>
<td>TH</td>
<td>thyroid hormones</td>
</tr>
<tr>
<td>THNR</td>
<td>thyroid hormone nuclear receptors</td>
</tr>
<tr>
<td>TRIAC</td>
<td>triiodo-acetic acid</td>
</tr>
<tr>
<td>Tris</td>
<td>tris (hydroxymethyl)-methylamine</td>
</tr>
<tr>
<td>TSH</td>
<td>thyroid stimulating hormone</td>
</tr>
<tr>
<td>TX</td>
<td>thyroidectomised/hypothyroxinemic</td>
</tr>
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CHAPTER ONE : INTRODUCTION
1.1 Historical review

The first historical record of the thyroid dates back to the 2nd century, when the ancient Greek physician Galeneos considered it a mucus-producing organ with the purpose of lubricating the pharynx and larynx (cited in Ericson and Fredrikson, 1990). Vesalius in 1543 was the first to give a full description, but it was not until 1656 that the organ was named "the thyroid" (after the Greek word, *thyreos*, meaning oblong shield) by Wharton (cited in Werner, 1971), who believed that the function of the thyroid was solely cosmetic, especially for women giving the throat region more beautiful roundness (cited in Ericson and Freriksson, 1990). Others suggested the organ to be a lymphatic gland, or a receptacle for worms (cited in Werner, 1971). In the 19th century, the gland was considered to act as a vascular shunt (Harington, 1933), cushioning the brain against sudden increases in blood flow.

It was later observed that certain characteristic symptoms of diseases were always accompanied by changes in the size of the thyroid and, although endemic goitre with cretinism was described by Paracelsus in 1567 (cited in Granfield, 1962) and by other physicians living in the Alps and Central Europe, the relation between the disease, cretinism, and the thyroid gland was not recognised at that time (Dussault *et al.*, 1987). Fagge (1871) introduced the term sporadic cretinism to describe hypothyroidism outside endemic goitre regions, whereas the term "myxoedema" was introduced by Ord in 1878 to describe adult hypothyroidism (cited in Werner, 1971). In 1891, Murray reported that the symptoms of hypothyroidism could be relieved by twice weekly injection of an extract of sheep thyroid. This finding was correctly interpreted as indicating a major role for the thyroid gland in normal body function.

In 1895, an important step in the understanding of thyroid gland function was taken by Baumann who observed that this was the only organ in mammals that had the capacity to incorporate iodine into organic substances. This observation was supported by Oswald in 1899 (cited in Warner, 1971). In 1912, Gudernatsch showed that tadpole metamorphosis could be accelerated by feeding thyroid extract, an early indication of the
influence of thyroid hormone on maturation. Kendall in 1915 was able to isolate a crystallised form of L-thyroxine (tetraiodothyronine, T4) from alkaline hydrolysates of thyroid tissue, and the nature of the thyroid hormone was seemingly established by the chemical synthesis of T4 in 1927 by Harington and Barger. A further breakthrough in the understanding of the nature and structure of thyroid hormones was made by Gross and Pitt-Rivers in 1952 who isolated a compound with only three iodine atoms, triiodothyronine (T3), from the thyroid gland. Subsequently, this compound was isolated from plasma (Roche et al., 1952), and proved to be physiologically more potent and more rapid in onset of action than T4, and was clinically effective in the treatment of myxoedema. Four years later, the existence of biologically inactive rT3 reverse triiodothyronine (rT3) and diiodothyronine (T2) in thyroid tissue and plasma of rats was reported by the same group (Roche et al., 1956).

1.2 The thyroid gland

1.2.1 Development of the thyroid gland

The thyroid gland develops from an invagination in the floor of the primitive pharynx at the level of the first and second cartilaginous ring. This invagination, lined by cylindrical epithelial cells, can be distinguished in the human embryo between 16 to 17 days of gestation (dg) (Boyd, 1964) and in the rat at 10 dg (Romert and Gauguin, 1973). The invagination grows downward in front of the primitive pharynx and the developing hyoid bone. This down-growth being accomplished by proliferation of existing cells and recruitment of additional cells from the pharyngeal epithelium (Shain et al., 1972). The cells in the endodermal invagination which can be distinguished from the surrounding pharyngeal epithelium develops into a flask-like vesicle with a narrow neck, the ductus thyroglossus. The vesicle bifurcates and continues its down-growth to the final position on the front of the upper trachea. In humans, this process is completed by 45 to 50 dg and in rats by 13 to 14 dg (O'Riordan et al., 1985; McDougall, 1992).
During the down-growth and by the 7th week of human development, the connection of the thyroid to the pharynx is lost, and the cells of the thyroid are grouped into clusters. At about 11 weeks, a central lumen appears in each cluster, completely surrounded by a single layer of cells (Hifer, 1979; O'Riordan et al., 1985). In the human fetal thyroid, the production of T4 starts at 70-80 dg, but it does not respond to pituitary-secreted thyrotropin until the 22nd week of gestation (Fisher et al., 1977). Rat fetal thyroid, on the other hand, starts to secret thyroid hormones (TH) at 17.5-18 dg (Morreale de Escobar et al., 1989).

1.2.2 Gross structure
The thyroid gland is situated in the anterior neck. It consists of two pear-shaped lobes, which lie to the sides of the trachea, and the smaller isthmus joining the medial aspects of the lobes, which lies over the second and fourth tracheal rings (Fig.1). The lobes are about 5 cm long, 3 cm across at the widest part, and 2 cm thick at the broader lower pole. The inferior poles of the lobes extend to the sixth tracheal ring, and the superior poles to the thyroid cartilage. In the newborn human, the thyroid weighs 1.5 g, it increases progressively with age and size to approximately 20 g in adults (McDougall, 1992). The thyroid is amongst the most vascular organs, with four main arteries and a plexus of veins supplying it with blood and the euthyroid gland has a blood flow rate of about 5 ml/g/min. In Graves' hyperthyroidism this can increase 200-fold. There is also a rich lymphatic supply which follows the veins and communicates between the lobes within the thyroid. The recurrent laryngeal nerves run inferior to the thyroid, behind the lower third of the gland. They lie about 1 cm lateral to the trachea in close relation to the interior artery. The motor branch of the nerve innervates the laryngeal muscles and damage to that nerve causes ipsilateral paralysis of the vocal cord and a hoarse voice. The main nerve supply to the thyroid itself is sympathetic. It arises from the middle cervical ganglion and travels with the inferior thyroid artery. Some sympathetic vasoconstrictor fibres from the superior cervical ganglion reach the thyroid along with the superior thyroid artery. Some patients with an enlarged thyroid notice rapid fluctuations in the size of their glands and this is likely to be a
vascular phenomenon mediated by the autonomic nervous system. However, these nerves are not important in the formation and release of thyroid hormones (McDougall, 1992).

1.2.3 The secretory unit - the follicle

At the microscopic level, the characteristic feature of the thyroid is its organisation into follicles (Fig. 2a). The follicle is a spherical structure, consisting of a single layer of cuboidal epithelial cells (follicular wall) which encloses the follicular lumen (Gumbiner, 1987). The lumen is filled with a proteinaceous solution, termed "colloid". The diameter of follicles, even under normal physiological conditions, varies from 100 to 1000 μm (average 200-300 μm) (Laycock and Wise, 1983; McDougall, 1992).

The follicles contain enzymes for trapping iodide (peroxidase), iodinating tyrosine (iodinase), forming iodothyronine (coupling enzyme), recycling iodine (deiodinase) and for releasing thyroxine (T4) and 3,5,3'-triiodothyronine (T3) from the thyroglobulin (protease and peptidase) (Werner, 1971). In addition, each follicle is surrounded by a basement membrane, and lying between this membrane and the follicular cells are the parafollicular C-cells. These cells secrete calcitonin in response to an elevation in calcium, thus serving to regulate circulating calcium levels (O'Riordan et al., 1985; McDougall, 1992).

In situations where the gland is underactive (as in an iodine-deficient hypothyroid state), the follicles are distended with colloid and the follicular cells are thin and flattened with little cytoplasm visible under the light microscope (Fig. 2b). On the other hand, enhanced thyroid activity over a prolonged period is usually accompanied by a reduction in the colloid and the follicular cells, which become more columnar (Fig. 2c) (Laycock and Wise, 1983; O'Riordan et al., 1985).
Figure 1: Diagram showing the location of thyroid gland in the neck.

Adapted from McDougall (1992).
Figure 2: Histological diagram of mammalian thyroid follicle. (a) euthyroid follicle, (b) underactive follicles with flattened epithelial cells and increased colloid, (c) overactive follicles with columnar epithelial cells and reduced colloid.

Adapted from O'Riordan et al., (1985).
1.3 Thyroid hormones

Thyroid hormones have various general effects on metabolism and are particularly important for normal growth and development. There are two physiologically important thyroid hormones, T4 and T3.

The daily production of T3, the most active thyroid hormone, varies between 26 and 40 μg/70 kg body weight. Thyroxine is more abundant in the circulation and its production in healthy humans varies between 80 and 100 μg/70 kg body wt. The average T3 and T4 concentrations in the thyroid are approximately 0.02 and 0.3 μmol/g (15 and 200 μg/g) respectively, and plasma total concentrations are 1.4-3 nmol/l and 60-160 nmol/l respectively. The thyroid gland contributes some 20% of the extrathyroidal pool of T3; the remainder being produced by monodeiodination of the outer tyrosyl ring of T4 (Laycock and Wise, 1983).

1.3.1 Regulation of thyroid hormone function

The formation and secretion of thyroid hormones are largely controlled by thyroid stimulating hormone (TSH; thyrotrophin). TSH is produced in, and secreted from, basophilic cells (thyrotrophs) of the anterior pituitary. TSH is stimulated in turn by a tripeptide of the hypothalamus, thyrotrophin-releasing hormone (TRH).

In the human, plasma TSH levels have been shown to be elevated in hypothyroidism, decreased in Graves’ disease and promptly depressed by the injection of thyroid hormone. The normal set point of pituitary-thyroid function is the resting level of plasma thyroid hormone, for the maintenance of which specific levels of TSH are required. Secretion of TSH is inversely regulated by thyroid hormone so that deviation from the set point of control leads to appropriate graded changes in the rate of TSH secretion (O'Riordan et al., 1985).
1.3.2 Thyroid hormone synthesis

The synthesis of thyroid hormone requires iodine (Werner, 1971). This is normally ingested in the diet in the form of iodide and transported to the thyroid. The normal thyroidal intracellular iodide concentration is approximately 600 μg/g tissue, being 20-50 times greater than the plasma concentration (Laycock and Wise, 1983).

In the process of hormone synthesis, iodide is oxidised and then bound to tyrosyl residues in thyroglobulin; a protein which is synthesised in the thyroid follicular epithelium (Bjorkman and Ekholm, 1990). Thyroglobulin is an iodine-containing glycoprotein and comprises about 80% of soluble protein in the thyroid (Robbins and Rall, 1960). Most of the thyroglobulin content of the gland is stored in the follicular lumen, where it is the main constituent of the colloid (Laycock and Wise, 1983).

1.3.2.1 Iodine transport and oxidation

The thyroid has the ability to take up and concentrate iodide from the circulation. This process is called the iodide pump or the iodide trap. The basal plasma membrane of the follicle is responsible for concentrating the iodide, whereas the apical membrane may act as a permeability barrier between the cell and the lumen.

Iodide transport from the extrafollicular space into the follicle cells operates against an electrical gradient, and energy is required for transport (Bjorkman and Ekholm, 1990). After entering the cells, iodide moves with the electrical gradient from the cell to the follicle lumen. Na⁺ K⁺-ATPase seems to be of importance in the active transport across the basal plasma membrane (Gerard et al., 1985), and the inhibition of iodide accumulation in the thyroid by ouabain further indicates the involvement of Na⁺ K⁺-ATPase in iodide transport (Wolff and Maurey, 1961).

Once inside the cell, and before linking to tyrosyl residues present in thyroglobulin, the iodide (I⁻) is rapidly oxidised to a more reactive form. This process requires the presence of a peroxidase enzyme and H₂O₂. Thyroid peroxidase, is a membrane bound glycoprotein containing about 10% carbohydrate and, like other peroxidases, is a haemoprotein (Bjorkman and Ekholm, 1990). It is located mainly near the apical membrane.
of the follicle and oriented towards the follicular lumen (Laycock and Wise, 1983; Bjorkman and Ekholm, 1990). The oxidation of I\(^-\) to I\(_2\) may occur via one-electron transfer, where I\(^-\) is formed, or via two electron transfer, where I\(^+\) is formed. Both I\(^-\) and I\(^+\) can form I\(_2\) which subsequently iodinates the tyrosyl ring of the thyroglobulin.

\[
I^- + I^- \rightarrow I_2 \\
I^- + I^+ \rightarrow I_2
\]

Three other forms of oxidised iodine (iodine free radical I\(^-\), iodinium ion I\(^+\) and hypiodite IO\(^\cdot\)) may also participate in the iodination process, which indicates that there are at least four mechanisms for the iodination process. TSH stimulation increases peroxidase activity whereas suppression of TSH with T4 results in a decrease in the peroxidase activity.

### 1.3.2.2 Thyroglobulin synthesis

Thyroglobulin is a glycoprotein synthesised in the follicular cells. It is a large molecule of 660 KDa and contains approximately 10% (w/w) carbohydrate and 1% (w/w) iodine. Thyroglobulin is synthesised on the rough endoplasmic reticulum, then packaged in the Golgi apparatus. Golgi derived vesicles then fuse with the apical cell membrane and release their contents into the lumen of the thyroid follicle by exocytosis (O'Riordan et al., 1985).

### 1.3.2.3 Coupling

The next step in TH synthesis is the coupling process whereby T4 will be formed, and this reaction comprises three steps (Fig. 3):

a - oxidation of diiodotyrosyl residue to the activated form through the action of thyroid peroxidase. The oxidation of the diiodotyrosyl residue may be by one-electron oxidation (whereby both residues are oxidised to iodotyrosyl radicals) or by two-electron oxidation, implying removal of two electrons from one of the paired iodotyrosyl residue, the other residue remaining as anion.

b - coupling of activated iodotyrosyl residues within the same thyroglobulin molecule to form a quinol ether intermediate.
Figure 3: Scheme for oxidation and coupling of diiodotyrosyl residue to form thyroxine.

Adapted from Bjorkman and Ekholm (1990).
c - splitting of the quinol ether to form an iodothyronine residue.

1.3.2.4 Thyroid hormone secretion

The first step in TH secretion is endocytosis of the colloid from the follicular lumen. Hormone-containing thyroglobulin is sequestered into discrete intracellular compartments (endosomes) which are either colloid droplets formed by macropinocytosis, or micropinocytosis vesicles. In the next step, endosomes fuse with lysosomes; in these secondary lysosomes thyroglobulin and lysosomal enzymes are mixed, resulting in the hydrolysis of the thyroglobulin and the release of thyroid hormones. The fate of the hydrolysed products obtained (iodothyronines (T4, T3), mono- and di-iodothyronines, (MIT, DIT), and small peptide fragments) is incompletely known, but almost all the monoiodotyrosine (MIT) and diiodotyrosine (DIT) is deiodinated within the follicular cells and the released iodide is re-utilised in the follicle (Rosenberg and Gorwann, 1979). Little is known about the transport of T4 and T3 from the thyroid cell to the extra-cellular fluid, but it has long been thought to occur by passive diffusion (Bjorkman and Ekholm, 1990), although other investigators have suggested that the process is carrier-mediated (Oppenheimer and Schwartz, 1985).

1.3.3 Transport of thyroid hormone in the serum

Almost all TH in the circulation is reversibly bound to a carrier protein and only a small fraction (less than 1%) exists in the free form (McDougall, 1992). The three best known plasma carrier proteins are:

1-Thyroxine-binding globulin (TBG).
2-Thyroxine-binding prealbumin (TBPA).
3-Albumin.

Thyroxine-binding globulin is synthesised in the liver (Bartalena and Robbins, 1993), has a molecular weight of 56 kilodalton (KDa), and has a single binding site for which both T4 and T3 compete. In the case of T4, ≈75% is bound to TBG. The normal
range for TBG concentration in the plasma is 1.5-2 mg/dl (Krenning et al., 1978; Ekins, 1984).

The second carrier protein, TBPA, is also synthesised in the liver and has a molecular weight of 54 KDa. The name of this binding protein was changed recently to transthyretin (Bartalena and Robbins, 1993). It possesses two identical binding sites with a lower affinity for T4 and T3 than TBG. Consequently, it normally carries only ≈8% of circulating T4 (Ekins, 1984) and 25% of T3 (Robbins and Rall, 1960). The plasma concentration of TBPA is approximately 25 mg/dl.

Finally, the third plasma carrier protein is albumin, which is present at high concentration (3500 mg/dl). However, it carries only ≈16% of circulating T4 and about 30% of T3 and has only one iodothyronine binding (Ekins, 1984; McDougall, 1992).

1.4 Thyroid hormone metabolism

Following the discovery of biologically active T3 in 1952, it was proposed that most of circulatory T3 is generated by extrathyroidal deiodination of T4, which is considered to be the major metabolic pathway (Gross and Pitt-Rivers, 1952).

Monodeiodination accounts for up to 80% of total T4 disposal (Henneman, 1986). It is important for the production and the subsequent metabolism of tri-, di-, and monoiridothyronines, with about 80% of daily produced T3 and 94% of rT3 being generated in peripheral tissue by monodeiodination (Henneman, 1986).

Two major deiodination reactions have been identified. The first reaction is the phenolic ring (outer ring) deiodination reaction, and is referred to as 5'-deiodination. It is catalysed by two enzymes; type I 5'-deiodinase (5'D-I) and type II 5'-deiodinase (5'D-II). This reaction leads directly to the formation of T3 and is considered an activation pathway. The second reaction is the tyrosyl ring (inner ring) deiodination reaction, and is referred to as 5-deiodination. It is catalysed by only one enzyme, type III 5-deiodinase (5D). This reaction leads to the degradation of T3 and other iodothyronines having two iodine atoms in the tyrosyl ring, as well as catalysing the formation of rT3 from T4. Therefore 5D
deiodination is considered to be an inactivating pathway (Fig. 4) (Leonard, 1990). However, in addition to the outer ring deionation, type II 5'-deiodinase exhibits tyrosyl ring deiodination at pH 8.0, while outer ring deiodination is most active at pH 6.4 (Mol and Visser, 1985).

All three enzymes are membrane proteins associated with the microsomal fraction (Visser, 1990), but differ in their tissue distribution and catalytic specificity. The two enzymes 5'D-II and 5D are further distinguished from 5'D-I by their insensitivity to 6-n-propyl-2-thiouracil (PTU) inhibition and in general by their lower Michaelis constant (Km) values for substrate (Kaplan, 1984; Hesch and Kohrle, 1986; Visser, 1988; Leonard, 1990). The most common feature of all three enzymes is their need for a reduced thiol, such as mercaptoethanol, dithiothreitol (DTT) or glutathione, as a cofactor for their catalytic activity.

1.4.1 Type I deiodinase (5'D-I)

Type I deiodinase is an intrinsic membrane protein in all organs studied (Leonard and Rosenberg, 1978) and catalyses 5'-deiodination of both T4 and rT3, with rT3 being the preferred substrate. The Km of the enzyme for T4 and rT3 is much greater than the physiological tissue concentrations, therefore it has been suggested that 5'D-I does not play an important and regulatory role in TH metabolism, but is engaged in the catabolism of surplus hormone for iodide economy (Leonard and Visser, 1986; Leonard, 1990).

As mentioned earlier 5'D-I deiodinase is susceptible to PTU inhibition (Leonard and Rosenberg, 1978; 1980; Visser, 1979; Visser et al., 1979). 5'D-I is found in most body tissues including the thyroid, which appears to have the highest enzyme specific activity of all tissues (Green, 1978; Erickson et al., 1981; Laurberg and Boye, 1982). In rats, it is most abundant in the liver and the kidney and less so in the developing pituitary, eye and lung. In the brain, the enzyme activity appears to be present mostly in the glial cells, with little if any activity in neurons (Leonard and Larsen, 1985; Leonard and Visser, 1986; Leonard, 1992).
Figure 4: Monodeiodination of thyroid hormone

3, 5, 3', 5'-tetraiodothyronine (T4)

3, 5, 3'-triiodothyronine (T3)

3, 5'-triiodothyronine (rT3)

3, 5-diiodothyronine

3, 3'-diiodothyronine

3', 5'-diiodothyronine

3-monoiiodothyronine

3'-monoiiodothyronine

Thyronine
It has been proposed that reduced thiols such as mercaptoethanol, dithiothreitol (DTT) and glutathione act as cofactors (Leonard and Visser, 1986; Leonard, 1990). Since glutathione (GSH) is known to be the most abundant intracellular thiol (Leonard, 1990), it has been studied most extensively. It is thought that the ratio between reduced (GSH) and oxidised (GSSG) forms of glutathione is important in regulating 5'D-I activity (Sato and Robbins, 1981; Sato et al., 1983). Balsam et al (1976) have shown that NADPH may serve as the natural cofactor for GSSG reductase-mediated reduction of GSSG, thereby indirectly stimulate D-I activities. Thus GSSG reductase may serve as an important link between energy metabolism and cellular deiodination (Leonard and Visser, 1986).

As mentioned above, T4 and rT3 are the most commonly utilised substrates for 5'D-I (Leonard, 1990), with rT3 being by far the preferred substrate for hepatic deiodination (Leonard and Visser, 1986). With respect to rT3, 5'D-I does not display systematic preference for either inner ring or outer ring deiodination (Leonard and Visser, 1986). In general, type I outer ring deiodination is favoured by a slightly acidic pH of around 6.5, whereas inner ring deiodination is optimal around pH 8.0 (Mol and Visser, 1985). This is the reason why conversion of T4 to rT3 is readily observed at high pH; since otherwise its further degradation to 3,3'T2 is too rapid (Leonard and Visser, 1986). Therefore the intracellular pH may determine whether an inner ring or an outer ring iodine is removed by 5'D-I (Leonard and Visser, 1986).

Another factor which can affect inner or outer ring deiodination is sulphoconjugation. Sulphation of the 4-hydroxyl of both T4 and T3 promotes deiodination of the tyrosyl ring, whereas phenolic ring deiodination of 3,3'-T2 is increased by this kind of modification (Visser, 1990). Furthermore, Visser and co-workers have illustrated that liver 5'D-I has some "wobble" at the catalytic site, allowing both 5' and 5-deiodination to take place, whereas, in the absence of 4'-sulphate modification, the activity of 5'-deiodinase is at least 100 times higher than that of 5-deiodinase (Visser, 1990).
1.4.2 Type II deiodinase (5'D-II)

The first indication of T3 production from T4 in brain and pituitary was observed by Silva and Larson (1978), and also reported by Cheron (1979). It has been found that in the brain 5'D-II is mainly localised to the neurons (Leonard and Larsen, 1985).

Although PTU injection was found to inhibit more than 97% of liver and kidney 5'-deiodinase, it had very little effect on brain and pituitary. Subsequent examination revealed the presence of 5'D-II in these tissues (Kaplan and Yaskoski, 1980; Leonard and Visser, 1986). This enzyme has been identified in other mammalian tissue including brown adipose tissue (Leonard et al., 1983) and placenta (Kaplan and Shaw, 1984). Thyroxine appears to be the preferred substrate over rT3, with a Km of 0.5 to 2 nM for T4 and 1.5 to 10 nM for rT3 (Visser et al., 1982; Kaplan and Shaw, 1984). Reverse T3 is believed to be a competitive inhibitor of T3 production from T4.

It has been shown that brain and pituitary 5'D-II is sensitive to changes in circulating thyroid hormones; thyroidectomy leads to an increase in the enzyme activity, whereas enzyme levels falls by 80-90% within 2-4 h after injection of a saturating dose of T3 to thyroidectomised animals (Leonard et al., 1984; Leonard, 1990). These changes in the enzyme response to thyroid hormone were claimed to be due to an alteration in the number of catalytic units in the tissue (Leonard, 1990).

The conversion of T4 to T3 plays a fundamental role as the first step in the molecular mechanism of TH action; by serving as the source of bioactive TH for target tissues. Nowhere is this more important than in the brain where it has been reported that the intracerebral conversion of T4 to T3 contributes some 75-90% of the T3 bound to brain cell nuclei, with only 10-25% of the intracerebral T3 being obtained from the circulation. Furthermore, the time span for the equilibration of locally produced T3 with that bound to nuclear receptors is rather long (12-15 h) when compared with that observed in liver and kidney (30-90 min). This slow equilibration of T3 with its receptor is likely to be due to the rapid degradation of the imported T3 from the circulation rather than the relatively slow conversion of T4 to T3 in brain (Silva et al., 1982). This indicates the ability of the brain to
rapidly degrade T3 derived from the circulation, thereby removing a potent competitive substrate that could adversely affect T3 generation by 5'D-II (Leonard, 1992).

1.4.3 Type III deiodinase (5D)
This is most abundant in the skin (Leonard, 1990) and the central nervous system (Kaplan and Yaskoski, 1980; Roti 1981a,b, 1982a,b; Leonard, 1992). It catalyses the inner ring deiodination of T4 and T3, with some preference for T3 over T4. In the central nervous system, the activity of this enzyme is inversely regulated with respect to 5'D-II to ensure optimal regulation of intracellular T3 levels. In the brain and skin, 5D may also play an important role in the clearance of circulating T3 levels and production of plasma rT3 (Kaplan, 1984; Hesch and Khorle, 1986; Leonard, 1990). Moreover, it has been suggested that the placental enzyme may limit the passage of plasma T4 and T3 from the mother to the fetus (Roti et al., 1983). Like 5'D-II, 5D shows no sensitivity to PTU inhibition. In the brain 5D is predominantly present in the glial cells and is responsible for T3 disposal (T3 → 3,3' T2). Furthermore, in cell cultures from fetal and neonatal rat brain, the highest activity is found in astroglial cells and the lowest activity in oligodendrocytes. High activity of this enzyme has been detected in embryonic tissues and placenta suggesting that the enzyme has an important role in protection of the developing tissues during critical periods against active TH (Visser and Schoenmakers, 1992).

The effect of thyroid status on 5D activity has been investigated in different tissues. Brain 5D activity was reported to be increased in hyperthyroid rats and decreased in hypothyroid animals (Kaplan and Yaskoski, 1980; Huang et al., 1986). In chick embryo heart cells, 5D activity was stimulated by addition of T4 or T3 to the cultures. The activity of 5D in skin was also decreased by hypothyroidism, but little change was observed in hyperthyroid animals (Visser and Schoenmakers, 1992).

1.4.4 Ontogeny of 5' and 5-monodeiodinase activities in the central nervous system
Thyroid hormones play a crucial role and are necessary for normal growth and development of the vertebrate CNS (Valverde et al., 1993). Several studies have been
performed to investigate the role of 5' and 5-monodeiodinase in controlling TH homeostasis in early development.

In rats, it has been demonstrated that low levels of 5'D-II have been detected in fetal brain at 17 dg. This increases 4-folds between 19 and 21 dg (Ruiz de Ona et al., 1988). No activity was demonstrable in the cerebellum or the median eminence-hypothalamus before age 7 postnatal days, even with the stimulus of hypothyroidism (Kaplan and Yaskoski, 1981). In these three brain regions, the activity of 5'D-II was found to peak at about 28 days after birth, and then decline to lower adult levels (Kaplan, 1986). Simultaneously, the deactivating pathway (5D activity) attains maximum values in cerebral cortex, cerebellum, median eminence-hypothalamus and eyes (Kaplan and Yaskoski, 1981; McCann et al., 1984). After birth this activity was reported to decline progressively to adult levels over 1 to 3 weeks (Kaplan, 1986).

In chicken brain, total 5'-monodeiodinase has been detected at day 12 of embryogenesis (Valverde et al., 1993). A high activity of 5'D-II was detected as early as day 13 of embryogenesis, which seems to be coupled with the peak of neuroblast proliferation occurring at this stage of development (Borges et al., 1986). In contrast, only a low 5D activity was detected during this period. This supports the neurogenic importance of T3. However, a decrease in the 5'D-II activity at days 14-17 of embryogenesis, the period of greatest synaptogenesis, was reported (Valverde et al., 1993). Moreover, similar changes in cerebral deiodinating activities were reported during synaptogenesis in the rat (McCann et al., 1984). These findings strengthen the notion that synaptogenesis is accompanied by a mechanism which protects the brain from over exposure to T3 and suggests that local deiodination plays a central role in the processes of cerebral maturation.

A biphasic pattern of cerebral 5'D-II displayed a second increase in chicken which begins at day 18 of embryogenesis reaching the highest level on day 20 (Valverde et al., 1993). Such an increase in 5'D-II activity was also reported in sheep cerebral cortex during peripartum period (Sing-Yung et al., 1978).
1.4.5 Other metabolic pathways

Several other less important pathways of thyroid hormone metabolism have been reported, briefly they are:

1 – Ether bond cleavage: This represents perhaps the least important metabolic reaction of thyroid hormones in the human (Engler and Burger, 1984; Visser, 1990), although it may be more significant in rats. It is an antioxidant reaction that yields diiodotyrosine as one of the products and occurs especially when tissue peroxidase activity is stimulated.

2 – Side chain modification: This is an oxidative deamination and decarboxylation reaction, considered to be another minor metabolic pathway of iodothyronines where, in healthy humans, conversion to tetraiodothyroacetic acid (TETRAC) accounts for only 2% of total T4 turn over, whereas about 14% of T3 is transformed to triiodothyroacetic acid (TRIAC) (Engler and Burger, 1984).

3 – Conjugation: This is an accelerating reaction and includes glucuronidation and sulphation which, as mentioned above, enhances the deiodination process.

1.5 Thyroid hormone nuclear receptors (THNR)

Thyroid hormones are involved in complex arrays of developmental and physiological responses in many tissues of higher vertebrates (Wolff, 1964). The diversity of thyroid hormone responses, suggests that the hormone acts at multiple cellular levels, perhaps via different mechanisms (Schwartz et al., 1993). It is generally accepted, however, that the primary mechanism of TH action is mediated through a nuclear receptor, to modulate the expression of specific genes in target cells (Thompson et al., 1987). Nevertheless, both the cell membrane and the mitochondrion may comprise additional, less important, sites of action.

Until recently, little was known regarding the nature of the T3 nuclear receptor, mainly due to unsuccessful attempts at purification by conventional methods (Puymirat, 1992). However, the nuclear receptors that have been examined so far are of similar size in all tissues (Nakai et al., 1988; McDougall, 1992). Characterisation of partially purified TH
receptor extracted from nuclei of rat and human thyroid hormone-responsive tissues and rat pituitary tumour cell lines have shown that it is a non-histone protein, tightly bound to the chromatin and of 55 KDa molecular mass. The protein binds T3 and T4 with Kd's of 0.2 to 1.0 nM and 2 to 10 nM, respectively (Oppenheimer et al., 1977; Silva and Larsen, 1986; Nikodem et al., 1990).

1.5.1 THNR structure

Because many oncogenes are thought to be derived from normally occurring non-oncogenic counterparts in the cell (proto-oncogenes), it was not surprising that a cellular analogue to v-erbA (an oncogene found in avian erythroblastosis virus) would be found. Subsequently, it was discovered that there are several genes with erbA-like sequences which encode for a number of hormone receptors. Amongst these genes are those encoding receptors for the steroid hormones, retinoic acid, vitamin D, and the TH. Their similar mode of action in modulating the expression of target genes lead to the identification of steroid/TH receptor gene superfamily (Evans, 1988). Analysis of this multigene family has shown that the amino acid sequence of the receptors follow the same general pattern and can be subdivided into 5 major functionally distinct regions, A/B, C, D, E and F (Fig. 5).

The A/B region is located at the amino terminus and is thought to be important in governing the specific interaction of the receptor with other proteins. The C domain is the most highly conserved amongst members of the family and contains two type II zinc (Zn) fingers which are responsible for DNA recognition and dimerization (Goldberg et al., 1989; Nikodem, 1990; Tsai and O'Mally, 1994). In a recent report by Schwartz et al., (1993) they indicated that a short amino acid segment in the base of the first zinc finger serves to group receptors into subfamilies. One group includes the THNR, estrogen, retinoic acid and vitamin D receptors, and the other group includes glucocorticoid, androgen, mineralocorticoid, and progesterone receptors. A short segment of the base of the second finger serves to differentiate among members of the subgroups.

The fourth "D" region is variable in both length and sequence, and may serve as a hinge interposed between the C and E regions. The E region is considered to be the ligand-
binding domain (Sap et al., 1986). The sequence of this region is unique to each receptor, as the region allows for specific ligand binding (Nikodem et al., 1990). Finally, located at the C-terminal is the F region, for which no specific function has been identified (Tsai and O'Mally, 1994).

Figure 5: Steroid/thyroid hormone receptor superfamily. The domains are labelled A-F. C domain is the DNA binding site and the E domain is hormone binding site.

Adapted from Lazar (1993).
Chromosome hybridisation studies have revealed that there are two separate but similar T3 receptor genes encoding human THNR proteins, one on chromosome 17, the other on chromosome 3, and they are referred to as α and β respectively (Weinberger et al., 1986). The α form of T3 receptor has 96% amino acid homology with the v-erbA protein and a molecular mass of 45 KDa. Alternate splicing of the α gene transcript results in the formation of three mRNAs α1, α2I and α2II. In the rat α1 codes for a nuclear protein of 410 amino acids with a molecular mass of 45 kDa and has high affinity for T3, while the two similarly sized α2I and α2II code for T3 non-binding proteins of 492 amino acids and 53 kDa molecular mass (Strait et al., 1990; Puymirat, 1992). The α2 and α1 isoforms are identical from amino acid 1 to 370. The α2 isoforms has carboxyl sequence of 120 (human) 122 (rat) amino acids that replaces the critical 40 amino acids ligand-binding segment of α1. This sequence can not bind to TH whereas the α1 isoform is capable of binding to TH (Lazar, 1993; Schwartz, et al., 1993).

The β form has 82% amino acid identity to the v-erbA protein. Two isoforms of the β gene product have been identified and these are referred to as β1 and β2, both of which bind T3. β1 encodes a protein of 461 amino acids with a molecular mass of 52 kDa and is expressed in all thyroid hormone responsive tissues like liver, kidney, and heart. β2 mRNA is localised in the anterior pituitary of the rat, where its expression is negatively regulated by thyroid hormone. It codes for a protein of 514 amino acids with a molecular mass of 62 kDa. β2 differs from β1 by an additional 53 amino acids at the amino terminus and it is unique in the A/B domain (Nikodem et al., 1990; Strait et al., 1990; Puymirat, 1992; Lazar, 1993). All of these THNR proteins possess a high degree of homology in the DNA-binding domain (Fig. 6, 7).
Figure 6: Schematic processing of α and β genes alternative splicing.

Adapted from Puymirat (1993).

Figure 7: Multiple THNR isoforms of rat-erbA derived amino acid sequence. The number of the amino acid sequence are indicated above each box. The number inside each box refer to the percent nucleotide identity to the THNR β2.

Adapted from Puymirat (1993).
1.5.2 Gene expression

In recent years, the mechanism by which T3 controls the synthesis of specific proteins has received considerable attention. There is now a large body of evidence which shows that T3 acts at the level of gene expression, mediated by the interaction of a receptor-TH complex with specific regulatory DNA sequences located in the vicinity of T3 target gene promoters. The effect of T3 on the level of target mRNA appears to be tissue-specific, correlating with the presence of high affinity nuclear binding sites for T3 in the TH responsive tissue (Nikodem et al., 1990). Thyroid hormone can modulate gene expression in either a positive (initiation or stimulation of transcription) or a negative (inhibition of transcription) manner depending upon the target gene (Nikodem et al., 1990). Evidence of a positive effect of TH on the level of gene expression is manifested by malic enzyme gene expression, where T3 was found to increase the rate of transcription in both liver and heart (Dozin et al., 1986; Nikodem et al., 1990). Positive regulation by TH affects, amongst others, acetyl coenzyme A carboxylase and glucose-6-phosphatase dehydrogenase (McDougall, 1992). On the other hand, negative regulation of gene expression by T3 has been studied less extensively; the gene for the β-subunit of thyroid stimulating hormone (TSH), α- glycoprotein subunit (Shupink et al., 1985), and β-myosin heavy chain (Khan et al., 1986; Nikodem et al., 1990) are all negatively regulated by T3.

1.5.3 Tissue distribution

The concentration of THNR varies widely from one tissue to another. Of the tissues studied, the anterior pituitary contains the highest density of receptor sites (6,000 per cell), while the testis showed the lowest (16 per cell). The adult brain contains about 2,000 binding sites, whereas in neonatal brain the number is about 2-fold higher (Gullo et al., 1987; De Nayer and Dozin, 1989). Analysis of the tissue distribution of THNR isoforms has shown that β2 mRNA is abundant in the anterior pituitary but undetectable in the liver, heart, cerebrum, and other organs of the adult rat. This isoform is also detectable in the hypothalamic, arcuate, paraventricular and ventromedial nuclei of adult rat as well as the developing hippocampus and striatum. Unlike β2, mRNAs encoding α1, α2 and β1
isoforms are expressed in virtually all tissues although they have characteristic distribution patterns. Thus, β1 is highly expressed in brain, liver and kidney (Schwartz et al., 1992; Lazar, 1993). The α1 isoform is in highest abundance in skeletal muscle and brown fat whereas the α2 isoform is extremely prominent in the brain (Lazar, 1993). It is worth mentioning that, recent studies have shown that this inactive form of THNR (α2) can block the effect of T3 mediated by α1 and β1 receptors (Koenig et al., 1989; Puymirat, 1992). All the aforementioned THNR isoforms (α1, α2 and β1) are regulated by T3. For example, levels of α1 and α2 mRNA decrease modestly in variety of rat tissues, with the notable exception of the brain, as a consequence of T3 administration. In contrast β1 mRNA is unresponsive to T3 except in the pituitary (Lazar 1993).

1.5.4 Ontogenesis of brain nuclear receptors
Triiodothyronine binding sites are present in nuclei from 7-week old human embryo (De Nayer and Dozin, 1989). In the human fetal brain low concentration of THNR have been detected at 10 weeks of gestation, and they increase ten-folds by 16 weeks of gestation, coincident with the period of neuroblast multiplication, indicating that TH may play a role in neuroblast proliferation and/or differentiation.(De Nayer and Dozin, 1989; Obregon et al., 1989). In rats, nuclear T3 binding sites are also present in the brain at 14 dg; well before the onset of fetal thyroid function, and the receptors attain a constant concentration of 50-80% of the adult level during the phase of neuroblast proliferation (Ferriero, 1988). After birth they rapidly increase in number and remain at high level from 2-3 postnatal weeks, then decrease to the adult value. During the postnatal period, the T3 binding capacity is about 1.7-2 fold higher than that of adult brain. The pattern of development of T3 binding sites in rat brain varies, depending on the brain area analysed (Coulombe et al., 1981). In the cerebrum, the peak value of T3 binding capacity occurs around birth while in the cerebellum, it occurs later; around the 14th postnatal day. Furthermore, the maximal number of T3 binding sites is 3-fold higher in the cerebrum than in the cerebellum (Puymirat, 1992).
The cellular localisation of T3 binding sites has been estimated in purified nuclei of neurones and glial cells during brain development (Hubank et al., 1990). The presence of T3 binding sites has been reported in both cell types. On postnatal day 5, the number of T3 binding sites is 2-fold higher in glial than neuronal nuclei. Between day 5 and 21 it decreases 3-fold in glial nuclei but remains stable in neuronal nuclei (Hubank et al., 1990).

In studies carried out on sheep it has been reported that THNR are present in fetal lamb tissue at 50 days of gestation (neuroblast proliferation period) and levels increase in subsequent days reaching a maximum in the brain and liver at 82 days of gestation (Ferreiro et al., 1987). Furthermore these authors have shown that the occupancy of the fetal lamb receptor at 100 day of gestation is high (60-70%) in the brain, whereas in the liver and lung it approximates only 10%.

The ontogeny of mRNA THNR isoforms is particularly interesting. In rat, chicken and amphibian, the α1 isoform is expressed at a higher level than β1 early in development, including the period before the thyroid gland is formed (Lazar 1993). In the recent work of Forrest et al (1991), wide expression of the α isoform in early embryonic stages (chicken) has been reported, whereas the β isoform was sharply induced in white matter and granule cells after the migratory phase at embryonic day 19 coinciding with a known hormone-sensitive period. Apart from expression in the earlier proliferating and migrating granule cells, the α form mRNA was also found in more mature granular and Purkinje cells layers after hatching, indicating a possible role of THNR α in both immature and mature neural cells. In rats, the α1 isoform is also predominant in the developing brain between 14th-15th fetal day (Puymirat, 1992), and at postnatal day 7, then it peaks in expression during the 3rd postnatal week in cerebral cortex, amygdala, hippocampus, and cerebellum (Mellstrom et al., 1991; Lazar, 1993). The fact that the α1 THNR mRNA is the predominant mRNA that codes for functional T3 receptors in the developing brain before birth, may suggest that the α1 receptors have a specific role during the first step of neuronal differentiation, including neurite outgrowth, synaptogenesis and the development of neurotransmitter activities (Puymirat, 1992).
The expression of β1 in the rat brain was notable after birth; during the first 15 days of neonatal life especially in the mitral cell layer of the olfactory bulb, caudate nucleus and hippocampal field, whereas in other regions it is expressed later during development (Mellstrom et al., 1991; Puymirat et al., 1991; Puymirat, 1992). This rapid increase coincides with the increase of serum and brain T3 levels (and with brain growth), suggesting that the β1 receptor may be involved in the postnatal effects of thyroid hormone on brain development, as in man, where mutations in THNR β are associated with neurological and growth abnormalities (generalised thyroid hormone resistance syndrome). Thus, the β1 receptor may mediate the effects of T3 on later stages of neuronal differentiation; including neurite outgrowth, synaptogenesis and the development of neurotransmitter activities (Forrest et al., 1990, 1991; Puymirat, 1992; Lazar 1993).

1.5.5 Extra-nuclear receptor

Although THNR are known to be the major site of TH action, there are many reports indicating the existence of several extra-nuclear binding sites (Segal and Ingbar, 1986). These sites are located in the cellular membrane, mitochondrion, and the cytosol. The mitochondrial binding sites are most probably localised in the mitochondrial membrane (Sterling et al., 1978). Several reports have suggested the presence of these sites in the inner membrane where oxidative phosphorylation takes place (Racker, 1970; Lehninger, 1975), while others have indicated its presence in the outer mitochondrial membrane (Jorgensen, 1976). In a study by Sterling (1986) it has been reported that adenine nucleotide transferase, an enzyme responsible for ATP and ADP transfer across the mitochondrial membrane (to and from the cytoplasm respectively), is a T3 binding site in the inner mitochondrial membrane. Binding of T3 by mitochondrial preparations is heat labile and pH dependent, with maximum values at pH 8.0; is independent of the cations Na⁺, K⁺, Ca²⁺ and Mg²⁺, and unaffected by EDTA and the reducing agent dithiothreitol (DTT). The T3-binding moiety appears to be a protein, since binding is completely abolished by treatment with protease. However, the number of these binding sites is uncertain; some reports indicate the existence of only one site whereas others indicate the
presence of two sites in the inner mitochondrial membrane (Segal and Ingbar, 1986). Thyroid hormone action directly on the mitochondrion is thought to increase oxidative phosphorylation reaction by increasing the rate of ATP synthesis and O2 consumption as well as protein synthesis (Segal and Ingbar, 1986).

Thyroid hormone binding sites in the plasma membrane have been identified in many tissues including human placenta, rat brain, liver, kidney and thymocytes; the GH3 pituitary cell line and rabbit adipocytes (Mendosa et al., 1977; Mashio et al., 1982; Alderson et al., 1985; Segal and Ingbar, 1986). These sites are thought to be of two types: low capacity, high affinity with a Kd of 2 nM, and high capacity, low affinity (Kd 260 nM) (Segal and Ingbar, 1980). In the rat brain, studies have indicated the existence of specific T3-binding sites in synaptosomes with high maximal binding capacity in cerebral cortex and hypothalamus (Mashio et al., 1982, 1983). Whereas in the rat thymocyte, it has been reported that T3 binding was decreased by 80% by trypsin and pronase, by 50 and 15% by phospholipase A and phospholipase C respectively; and by 25-40% by neuraminidase, suggesting that protein, phospholipid, and sialic acid are components of the binding site (Segal and Ingbar, 1986). In addition to stimulating the activity of acetylcholine esterase and Na+K+_ATPase, plasma membrane binding sites may influence the transport of glucose, amino acids and nucleosides (Goldfine et al., 1975; Mendosa et al., 1977; Segal and Ingbar, 1980; Roeder et al., 1985).

Cytosolic TH binding sites are reported to be present in rat brain, heart and kidney, and in human carcinoma cell line (Nishii et al., 1989; Obata et al., 1989). It is believed that more than one binding site may be present with different affinities to TH in different cell type (Lennon et al., 1983) and in rat brain these binding sites are present in smaller amount in glial cells than that in neurons (Francon et al., 1985). These binding sites are thought to be involved in the regulation and the availability of TH to organelles including the nucleus; by serving as a storage and supply function (Francon et al., 1985).
1.6 Biological Effects of Thyroid Hormone Action

Thyroid hormone produces profound and adverse effects on the physiology, metabolism and biochemistry of most tissues and cell types of mammalian organisms (Sokoloff, 1971). Normal thyroid function is necessary for the attainment of normal body weight, structure and stature, and for proper maturation of nervous and other body systems (Legrand, 1986).

Perhaps the earliest discovery concerning the function of the thyroid gland was in regard to its effect on oxygen consumption and heat production (Sokoloff, 1971). Thyroid hormone deficiency is associated with a decrease in oxygen consumption; complete thyroidectomy can lead eventually to as much as a 50% decrease in the basal metabolic rate (BMR), which can be reversed by the administration of thyroid hormones. Conversely, hyperthyroidism can lead to increases in oxygen consumption and the BMR. This may be related to changes in mitochondrial respiratory chain components; for example, cytochrome c, ubiquinone, and the microsomal NADH cytochrome c reductase system are increased in hyperthyroidism but decreased in hypothyroidism (Sokoloff, 1971).

Numerous studies have established that thyroid hormones stimulate protein synthesis (Michels et al., 1963; Tata, 1964; Wolff and Wolff, 1964; Sokoloff et al., 1968). In tissues such as liver, kidney, and heart (which also respond with increased metabolic rate), administration of thyroid hormones to normal animals was found to stimulate the incorporation of amino acids into protein. However, no effect was observed on the rate of protein synthesis in mature brain, testis or spleen; tissues which retain normal rates of \( O_2 \) utilisation in hyperthyroidism (Gorden and Hemming, 1944; Mirsky and Borth-Khan, 1936). Furthermore, in vivo studies have shown that administration of TH stimulated nuclear DNA polymerase activity and the synthesis of rRNA (Tata and Widnell, 1966). These effects result in an increased cellular content of functional ribosomes and therefore ultimately result in an increase in the rate of protein synthesis (Tata, 1964; Sokoloff, 1971).

During development, TH are known to affect hepatic enzymes, pituitary growth hormones, bone growth, brain and skin maturation (Sokoloff, 1971). In addition, the
production of cartilage is partially controlled by thyroid hormone, and this may explain the growth defects and bone malformation observed in cretins (Timiras, 1988). Furthermore, many reports indicated a correlation between thyroid function and sexual maturation, with alterations in the anterior pituitary. A reduction in the number of maturing ovarian follicles and atrophy of the ovaries and uterus has been reported in hypothyroid rats and rabbits (Parrot, 1960; Hagino, 1971).

The developing mammalian brain undergoes marked morphological reorganisation, indeed differentiation, which appears to be dependent on the action of the thyroid hormones, a distinctive feature which characterises the brain from other body organs (Sokoloff, 1971). Therefore the effect of TH on brain development is discussed in further detail below.

1.7 Thyroid Hormones and Brain Development

The developing brain is the most vulnerable tissue with respect to changes in the level of thyroid hormone (Dussault, 1989). Deficiency or excess of TH leads to disturbances in brain development which are irreversible if thyroid status is not promptly corrected (Dussault, 1989). The precise role of this crucial effect of TH in brain development is unknown. However, a variety of in vivo and in vitro experimental models have been used to evaluate the effect of dysthyroid states (hypothyroidism in particular) on the developing brain. Behavioural, anatomical and biochemical findings are discussed in detail below.

1.7.1 Behavioural effects of thyroid hormones

Early studies on cretinoid animals described them as placid and more easily handled than normal animals (Eayrs, 1960). Learning difficulties have also been reported; for example, cretinous rats run a simple T maze more slowly with a greater error score than their normal littermates (Eayrs and Lishman, 1955; Eayrs, 1960). Adult rats thyroidectomised at birth make significantly more errors than controls in learning a simple escape-avoidance task in response to a conditioned auditory stimulus, suggesting a diminished sensitivity to
environmental change (Hamburgh, 1969). Hamburgh and Vicari (1957) have also reported some abnormalities in the auditory responses of hypothyroid mice.

There is a close relationship between the behavioural performance of the hypothyroid rat and the age at which the thyroid is destroyed. The impairment has proved to be severe in animals in which the thyroid is destroyed on the day of birth and to a less extent when hypothyroidism is induced at 10 postnatal days. Similarly, thyroid hormone replacement therapy in animals made hypothyroid at birth is effective only when begun during the first 10 days of postnatal age (Hamburgh, 1969). In a more recent study, maternal thyroidectomy was found to cause significant malbehaviour and reduced activity in rat progenies (Attree et al., 1992).

Behavioural abnormalities have been observed in human as well, where low IQ, specific learning disabilities, attention deficits, speech and language disturbances and a range of neurological problems have been observed in hypothyroid children and cretins (Pharaoh et al., 1980). Congenitally hypothyroid children can achieve a normal IQ when the thyroid hormone treatment is initiated within the first few months after birth (Smith, 1981).

All these observations point to an important role of the thyroid hormones during the critical period of central nervous system development where changes in thyroid hormone level have a great effect on neural maturation. On the other hand, adult hypothyroidism causes a range of psychological disorders, behavioural morbidity and motor dysfunction, where general slowing of psychomotor function and changes in mood and memory correlate significantly with general hypothyroid state (Mennemeir et al., 1993). Adult hypothyroidism has also been shown to be associated with dementia, which can be alleviated by replacement therapy with TH (Haupt et al., 1991). The hypothyroid state in human has also been correlated with depressive illness, and successful treatment of manic depression with high doses of T4 has been reported (Sinha et al., 1994). In contrast hyperthyroidism correlates with certain personality disorders and behavioural dysfunctions, such as increased anxiety, nervousness, irritability, depressiveness and agoraphobia (Rockel et al., 1987; Weller, 1984).
1.7.2 **Morphological effects of thyroid hormone**

Thyroxine and T3 distribution in the brain is uneven, and depends on the distribution and the density of binding sites. This may explain the variation in the morphological or biochemical changes noticed in different brain regions (Nunez, 1984). The first anatomical indication of developmental abnormalities resulting from abnormal thyroid function, was the brain size and shape. In rats made hypothyroid at birth, a reduction in brain water content has been reported (Sokoloff, 1971), as well as a brain weight loss and alteration in shape (Eayrs, 1960). A similar observation has been reported for fetal sheep thyroidectomized at 60 days of gestation where a reduction in brain size was evident particularly with respect to the cerebral hemispheres (Hetzel and Querido, 1980). Legrand reported in 1967 that the maturation of the cerebellum is greatly affected by neonatal hypothyroidism. Furthermore, neonatal hypothyroidism was found to inflict reductions in the size of the cerebellum as well as deficit in basket cell number, a preponderance of undifferentiated neurones, a reduction in synaptic density, and an increase in glial cell number (Balazs et al., 1968; Hetzel and Querido 1980; Smith, 1981).

Postnatal hyperthyroidism, on the other hand, accelerates differentiation, particularly in the cerebellum, in such a way as to terminate cell replication prematurely and changes the relative proportion of different cell types with fewer neurones in general and a major deficit in basket cells (Smith, 1981).

During normal development in most brain regions, neuronal proliferation is terminated at birth, whereas glial cells continue to proliferate in the early postnatal period. In the cerebellum, cell proliferation begins after birth in the extragranular layer (EGL) and continue for two weeks. Cell proliferation lasts for a longer time in the hippocampus while it may continues to the adult stages in other zones (Nunez, 1984). Many studies have shown that normal cell proliferation activity can be immensely disturbed by changes in thyroid hormone levels. For example in the cerebellum, the EGL proliferation process is normally completed by day 20 after birth but hypothyroidism was reported to cause a delay in the cell proliferation in the EGL (Nunez, 1984), and severe damage to the maturity of Purkinje cells, but the granular cells were less affected (Legrand et al., 1976). After a
period of mitotic activity, cells from the EGL normally migrate towards the inner granular layer, differentiate in the process and establish contacts with afferent mossy fibres and Purkinje cells to yield the normal "wiring pattern". Neonatal hypothyroidism delays the disappearance of the inner granular layer and decreases the number and density of synaptic contacts with Purkinje cells and short parallel fibres, resulting in a permanent impairment of neuronal connectivity. The critical period for this stage of development appears to be the first 21 days after birth (Dussault and Ruel, 1987).

Perhaps the most dramatic effect of hypothyroidism is a hypoplastic neuropil in the cerebral cortex and the cerebellum. Thyroid hormone deficiency in the cerebral cortex reduces the rate of growth of perikarya and axons (Eayrs and Taylor 1951; Eayrs, 1955; Nunez, 1984). In addition, the length and branching of pyramidal neurones, the density of axonal terminals, and the number of dendritic spines are all decreased (Eayrs, 1955; Hamburgh, 1969). In the cerebellum, hypothyroidism inflicts a deficit in the number, density and branching of dendritic spines of the Purkinje cell (Nunez, 1984; Timiras, 1988), which prevents or impairs the establishment of contacts among neurones. Decreased interneuronal contact is also consequent upon reduced or delayed synaptogenesis and in general, to alterations in the synaptic membrane, and may consequently lead to neuronal death (Duassalt and Ruel, 1987). Indeed, Eayrs (1966) suggested that the drastic reduction in axo-dendritic interaction may be responsible for the behavioural deficits in the cretinoid rat.

Perhaps the most noticeable morphological signs of neural differentiation is the formation of the myelin sheath around the axon. It has been reported that in hypothyroid animals, myelinogenesis of the developing brain is retarded and the total amount of myelin in some tracts is reduced. This may be related to the reduction in enzyme activities responsible for myelin synthesis and lack of axons as a result of thyroid hormone deficiency (Hamburgh, 1969). Therefore the timing and duration of thyroid hormones deficiency plays a major role in determining the specific damage inflicted either on brain region or cell type.
Adult hypothyroidism was also found to affect the brain morphology. Reductions in the granule cells of the dentate gyrus (Madeira et al., 1991) and pyramidal cells of the hippocampal CA1 region (Madeira et al., 1992) were reported. In the cerebral cortex adult thyroidectomy was found to cause an increase in the number of synaptic terminals' vesicles and the formation of coated pits in the mossy fibre nerve terminals. Administration of T4 can reverse such effect (Paula-Barbosa et al., 1985). Furthermore, adult thyroidectomy was reported to cause reduction in the numbers of dendritic spines along the apical shaft of pyramidal cells of the visual and auditory cortices (Ruiz-Marcos et al., 1980, 1983, 1988).

1.7.3 Biochemical effects of thyroid hormone
During normal brain development, the content of protein, DNA and RNA all rise. These changes are delayed and reduced in magnitude by thyroid hormone deficiency at, or shortly following, birth (Geel and Timiras, 1967; Balazs et al., 1968). Protein synthesis is depressed in the brain of the hypothyroid rat (Geel and Timiras, 1967), and decreases in protein and RNA per unit DNA have also been reported in rats made hypothyroid after two weeks of postnatal life (Balazs et al., 1968). The DNA content in the brain of the 70 day thyroid deficient fetal sheep was also reduced, indicating a reduction in the number of cells, which is probably due to slowed neuroblast multiplication. Neonatal hypothyroidism also causes a significant reduction in the DNA content of the postnatal cerebellum in the rat but not the forebrain. This change is most evident in the EGL of cerebellum at 14 day postnatally, which is probably equivalent in terms of brain development to 120-150 day period in sheep (Balazs, 1977; Hetzel and Querido, 1980). However, adult-onset hypothyroidism in rats is found to have no effect on the content or concentration of nucleic acid or protein (Ahmed et al., 1993).

Alteration in thyroid state also caused a depression in glucose uptake and metabolism as well as a severe reduction in amino acid uptake and synthesis in the brain of hypothyroid neonatal rat, which may be the underlying cause for the depression in the normal rate of protein synthesis. (Geel et al., 1967; Balazs et al., 1968; Hamburgh, 1969; Hendrich et al., 1984; Hendrich and Porterfield, 1991).
During the differentiation period, the neurones undergo dramatic changes in cell shape. Neurites develop and then differentiate into axon or dendrites. For this neurite outgrowth microtubules are required (Nunez et al., 1992). These microtubules are composed of tubulin (representing about 70% of the neurite proteins) and a number of microtubule associated protein (MAPs) which have been shown to act as promoters of microtubule assembly (Fellous et al., 1979). A reduction in microtubule number and function has been noticed in developing hypothyroid rat axons and dendrites (Stein et al., 1991). Furthermore neonatal hypothyroidism has been reported to cause changes in MAP composition which in turn impair neurite outgrowth and decrease the rate of microtubule assembly (Fellous et al., 1979).

It is known that one of the most severe effects of neonatal hypothyroidism is a lower deposition of myelin in the CNS. Hypothyroid brains have decreased amounts of cholesterol, cerebrosides, sulfatides, glycolipids and gangliosides in the myelin sheaths (Sokoloff, 1971; Ford and Carmer, 1977). These effects appear to be a consequence of decreased activities of enzymes involved in myelin lipid synthesis, such as cerebroside sulfotransferase and galactosyl transferase. Furthermore, myelination is a highly regulated timely event in the rat, starting a few days after birth and depending upon a proper differentiation of oligodendrocytes. Hypothyroidism delays the pattern of myelin associated glycoprotein (MAG) accumulation in different brain regions, consequently myelination does not proceed simultaneously throughout the brain (Rodriguiz-Pena et al., 1993).

The thyroid state has been shown to influence several neuronal functions such as neurotransmitter metabolic enzymes' activities and the number of neurotransmitter receptors (Puymirat, 1992). Thyroid hormone deficiency has been associated with a fall in the number of catecholamine, muscarinic, ß-adrenergic, acetylcholine and GABA receptors in neonatal brain (Geel and Timiras, 1967; Balazs et al., 1968; Pestesky and Burkart, 1977; Patel et al., 1980; Smith et al., 1980; Valcana and Timiras, 1981). The activities of GABA transaminase, acetylcholine esterase, glutamate decarboxylase and monoamine oxidase are all reduced, while choline acetyltransferase activity is increased (Geel and Timiras, 1967;

Neonatal hypothyroidism affects many other metabolic enzymes in rats such as succinic dehydrogenase (associated with synaptosomal mitochondrial and nerve terminals along with glutamate decarboxylase) the activity of which is reduced, while the activity of lactate dehydrogenase, (associated with cell sap) and that of glutamate dehydrogenase, (associated with mitochondria of nerve cell perikarya) are less affected by thyroid hormone deprivation (Balazs et al., 1968; 1969; Hamburgh, 1969). This indicates that the mitochondria of the nerve terminals are probably more sensitive to T4 or lack of it than those of cell bodies, and this indicates a selective effect of thyroid hormone (Hamburgh, 1969). Furthermore, the activity of the phosphohydrolases responsible for the maintenance of the normal ionic gradient; Na⁺ K⁺-ATPase has been reported to be reduced by neonatal hypothyroidism (Smith, 1988). On the other hand, thyroid hormone deficiency shows no effect on Mg²⁺-ATPase activity (Timiras, 1988), an indication of a selective effect of thyroid hormone on different enzymes.

1.8 Maternal hypothyroxinemia

1.8.1 Maternal thyroid hormone transfer across the placenta

Transference of the TH across the placenta from the mother to the fetus was the centre of scientific investigation since early this century. The literature holds many conflicting evidences regarding the permeability of the placenta to the TH.

Before dealing with these reports, it is important to point out that most studies indicating poor placental permeability were performed late in gestation; after the onset of fetal thyroid function. In addition, it is possible that different animal models may have different placental types, they will therefore vary in the degree of permeability to TH (Austin and Short, 1984). Furthermore, considerable debate has centred on the amount of TH which can be considered biologically significant for fetal development (Fisher et al., 1976; Ekins et al., 1989; Morreale de Escobar et al., 1989). For example, some reports
indicated the presence of low levels of TH in the fetal serum which was used to support the idea of lack of transport and function (Soloman and Friesen, 1968; Fisher et al., 1976; Dussault and Coulombe, 1980). It could be argued that, a concentration which may be considered insignificant if found in maternal tissues such as liver or lung, may be sufficient to initiate a response in the fetus. In addition, many of these studies were performed before the discovery of modern immunoassay techniques.

Perhaps the first report concerning placental impermeability to TH was produced by Courrier and Aron in 1929, using pregnant bitches and guinea pigs as an animal model. Several other studies using radiolabelled TH have also failed to detect placental transfer of T4 in rabbit and sheep. (Hall and Myant, 1956; Comline et al., 1970; Dussault et al., 1971), while T3 was reported to be transported in sheep in minimal amount (Dussault et al.; 1972). Data obtained at late pregnancy failed to detect any T3 transfer but indicated minimal transfer of T4 in rats (Fisher and Klein, 1981) and human (Fisher et al., 1976; Roy and Kobayashi, 1962; Thomas and Nathanielsz, 1983).

In contrast, and at the same period of time, other investigators have reported substantial transfer of TH from the mother to the fetus. In some cases these reports indicated a significant effect of these hormones on the fetuses that lacked TH. For example, a study carried out by Peterson and Young (1952) indicated that T4 crosses the placenta in guinea pigs. They found that the administration of T4 to pregnant mothers was able to diminish the hyperplasia of the fetal thyroid induced by maternal PTU administration. A similar study showed that radiolabelled T4 and T3 were able to cross rat placenta (Knobil and Josinovich, 1958). In a more recent study Sweney and Shapiro (1975) were the first to report labelled T4 in the placenta, the amniotic fluid, and the palatal and hepatic regions of 13 and 14 day old rat embryos after injection of tracer into the mother. Thyroid hormone transport across the human placenta has also been reported in normal pregnancies (Grumhach and Werner 1956).

A frequently cited explanation for the apparent failure of TH to transfer across the placenta is the existence in maternal serum (and, at later stages, in fetal serum) of specific TH binding proteins (primarily TBG) (Osorio and Myant, 1960). For instance, the levels
of specific thyroid binding proteins rise in maternal serum during human pregnancy. It has been suggested that this increase may prevent passage of TH from the maternal to the fetal circulation, thus serving to isolate or to protect the fetus from the maternal endocrine system (Osorio and Myant, 1960).

In order to gain a better understanding of the physiological role of specific hormone binding proteins, Ekins has produced a physiochemical model which challenges the validity of this view (Ekins, 1985; Ekins et al., 1989). In brief, the model predicts that a rise in serum TBG will preferentially enhance delivery to those tissues with high hormone demand. The rise in TBG may therefore help in the delivery of TH to the feto-placental unit.

Attention to placental TH transport during early gestation has thus been extensively studied in the last two decades, adding more support to Ekins' hypothesis. Two approaches have been employed to determine TH transport across the placenta. i) direct measurement of TH in fetal tissue before the onset of fetal TH synthesis. ii) measurement of transplacental passage of radiolabelled TH injected to the mother during pregnancy.

Thyroxine and triiodothyronine were found to be available to the rat conceptus at a very early stage of pregnancy; 4 days after uterine implantation (Obregon et al., 1984). These investigators have also reported the presence of T4 and T3 in embryos, placentae, and amniotic fluid at later stages of pregnancy; at 19 dg. In agreement with this, a report by Sinha et al. (1983) indicated that, following administration to pregnant dams, T4 could be found in the 5 day old rat conceptus. The same group also reported that measurement of fetal [125I]T4 levels (uptake / μg protein, at 9-10 dg) after 1 and 4 hr injection to mother, were equal or higher than that in maternal ovary, heart, and brain. Whereas [125I]T3 levels found in these fetuses were less than that in all maternal target organs studied and they further indicated that most of it was derived from local deiodination of [125I]T4 (Wood et al., 1984).

In order to confirm the maternal origin of iodothyronines found in early embryonic samples in normal rats, the effect of maternal thyroidectomy has been studied (Morreale de Escobar et al., 1989). Both T3 and T4 were found to be present in embryonic samples
obtained well before the onset of fetal thyroid function in fetus from normal dams. In contrast in embryonic samples from totally thyroidectomised mothers, T4 and T3 concentrations were below the limits of detection in all tissues, (including placenta) at least up to 17 days of gestation (Morreale de Escobar et al., 1985). After this a marked increase in both T4 and T3 concentration was observed. This agrees with the reported timing of 18 days of gestation for the onset of fetal TH secretion in the rat (Nataf and Sfez, 1961; Geloso, 1967). Radiothyroidectomy, however, with 131I performed in rats before conception had led to significant reduction in maternal serum T4 as well as fetal tissues T4 and T3 (Porterfield and Hendrich, 1992). Furthermore in recent studies carried out by Calvo et al (1992) and Morreale de Escobar et al (1992), maternal TH was found in fetal tissues after using a goitrogen (MMI), given to dams at 10 and 14 dg (respectively) to block fetal thyroid function. The pregnant dams were subsequently infused with 125I T4 from day 11 to day 21 and from day 15 to day 21 of gestation respectively. Therefore, the TH found in the fetus before 18 dgs are obviously of maternal origin. Furthermore, rabbits were also used to study placental transfer of TH, and it has been found that administration of relatively small amount of T4 or T3 to the 25 and 26 days pregnant rabbit results in transfer across the placenta. (Devasker et al., 1986).

In human, Vulsma et al (1989) have demonstrated the presence of considerable concentrations of T4 (ranging from 35 to 70 nM) in cord blood of congenitally hypothyroid neonates. Since those children suffered from a total organification defect, the only possible source of the T4 was from the mother. These findings are analogous to those from the rat demonstrating that when the fetal thyroid is impaired, considerable transfer of maternal TH occurs and this may mitigate tissue TH deficiency.

Thyroid hormones are known to play an important role in brain maturation. Their absence during development leads to irreversible brain damage (Eayrs, 1960; Ford and Cramer, 1977; Morreale de Escobar et al., 1983). However the timing at which the developing brain is more sensitive to TH deficiency is not well known. In early pregnancy and following injection of 125I T4 to pregnant rat dams at 10 dg, the tissue distribution of accumulated T4 in the fetus was found to be relatively favourable to the brain (Ekins et al.,
1986). Recent study by Porterfield and Hendrich (1992) have also indicated the presence of T4 and T3 in fetal brain in early fetal life; before the onset of fetal thyroid function. Although maternal thyroidectomy was found to have little effect on brain levels of T4 and T3 in mid- and late-gestation (Ruiz de Ona et al., 1988), it was found to significantly reduce the brain T4 and T3 levels at 13 and 16 dg (Porterfield and Hendrich 1992).

In addition to the direct measurements of TH in fetal tissues the presence of THNR in fetal tissues further indicates that the fetus has the potentials to utilise TH early in pregnancy. Thyroid hormone nuclear receptors were identified in whole rat embryos as early as 13 days in brain, and in liver, heart and lung from 16 days onwards; all before the onset of fetal thyroid function (Perez-Castillo et al., 1985). In addition, THNR gene c-erbA α, was found to be expressed in rat CNS by day 14; that is well before the onset of fetal thyroid function (Prati et al., 1992). With respect to humans, direct evidence of TH transfer is still lacking. However, nuclear receptors have been detected in the brain of a 10-week-old human fetus and in 7-week-old whole embryo (Bernal and Pekonen, 1984). Therefore the hormone and its receptor are present in fetal brain before the onset of fetal thyroid function as the secretion of T4 by fetal thyroid appears at 16-17 weeks of gestation, and that of T3 during the last trimester (Fisher and Kline, 1981). It is well known that the initial step of TH action is its binding to THNR and these findings indicate that TH is available to fetus at this stage of pregnancy. Therefore the presence of these THNR in fetal tissues, particularly in the brain early in pregnancy, suggests that TH may play a role in neurological development, and placental transport of maternal T4 is possible.

1.8.2 Maternal hypothyroxinemia and fetal development

Disorders resulting from iodine deficiency affect more than 800 million people worldwide; 400 million of them live in Asia alone and the rest are found mainly in Africa and Latin America (Ramalingaswami, and Kochupillai, 1986). Children born in the endemic areas are at risk from increased incidence of endemic cretinism; recent estimates suggest that the world has over 3 million overt cretins (Gaitan and Dunn, 1992). The most common form of endemic cretinism is the so-called 'neurological cretinism' which is characterised by
severe mental retardation, speech and hearing defects, together with abnormalities of gait and posture (Hetzel, 1987; Halpern et al., 1991). CT scanning in some patients showed extensive atrophy of the cerebrum and cerebellum and dilatation of the ventricles (Stanbury, 1986). A less common form of endemic cretinism is called myxoedema. This form is characterised by hypothyroidism, mental and intellectual deficiency, but not so severe as in neurological cretinism (Stanbury, 1986; Gaitan and Dunn, 1992).

Epidemiological studies have shown that endemic cretinism may be eliminated from the endemic areas when iodine is administered to women. However, to be effective, supplementation must be initiated prior to conception or early in pregnancy, implying that damage occurs in early pregnancy (Pharoah and Connolly, 1989; Stanbury and Hetzel, 1980; Pharoah et al., 1972).

The effectiveness of iodine supplement early in pregnancy, namely before the second trimester at which fetal thyroid is not in function, indicates that T4 rather than iodine is more important for the elimination of endemic cretinism. This goes with the frequently encountered low maternal serum T4 concentrations in areas of severe iodine deficiency while serum T3 remains within the normal levels (hypothyroxinemia) (Pharoah et al., 1976). This has been found to correlate with poor intellectual outcome, cognitive and motor function in offspring (Pharoah et al., 1981, Pharoah et al., 1984). Evidence has also been obtained suggesting a similar relationship between maternal T4 level in pregnancy and aspects of a child's cognitive capacity (Connolly and Pharoah, 1989). Therefore, the observed neurological deficits could be better explained by fetal deprivation of T4 early in pregnancy, and it can be suggested that maternal hypothyroxinemia and not iodine deficiency plays an important role in the broad spectrum of congenital abnormalities associated with severe iodine deficiency.

However, these studies were mainly performed in iodine deficient areas which necessarily implies that the fetuses and the children of the iodine deficient mothers are also subjected to iodine deficiency. Thus it can not be verified without hesitation which is the causative factor; deficiency of iodine or T4.
The investigations of Man and his group performed in iodine sufficient areas (North America) on the offspring of inadequately treated hypothyroxinemic women has shown decreased IQ relative to matched children from euthyroid or adequately treated women (Man et al., 1971; Man and Serunian, 1976). Furthermore those children showed poor vocabulary and poor speech which may suggest brain damage. Since adequate iodine was available for fetal thyroid function, this indicates that the lack of TH may be the cause of the abnormalities observed in those children. In addition, Escobar del Rey and co-workers (1986) reported that iodine-deficient diet resulted in severe reduction in T4 concentration in plasma, liver, and lung of pregnant rats and in all embryonic samples obtained from these dams at 11, 17 and 21 dg; before and after the onset of fetal thyroid function. This further supports the possibility that adverse effects of maternal iodine deficiency on the progeny may be related to maternal TH deficiency and not iodine deficiency per se, thereby resulting in deficient transfer of thyroid hormone across the placenta to the developing fetus, thus damaging fetal brain in early pregnancy as fetal CNS depends on thyroid hormones for its normal development.

In order to study in more detail the effect of maternal thyroid status on fetal development, a range of animal models have been used, including thyroidectomised rat, sheep and monkey. Investigations on laboratory rodents have shown that hypothyroidism results in a range of reproductive compromises, including prolonged gestation, small litter size, increased stillbirth, increased fetal resorption, and smaller fetuses (Folly, 1938; Krohn and White, 1950; Parrot et al., 1960; Porterfield et al., 1975). In severely thyroid deficient animals, there was a 69% reduction in fertility (Parrot et al., 1960). Reproductive compromise has also been observed in the human (Man et al., 1971).

In addition, maternal hypothyroxinemia has been reported to cause delayed skeletal maturation, reduced wool growth in sheep as well as retarded brain development and lung development (MacIntosh et al., 1983; Potter et al., 1986; Devasker et al., 1986). Maternal thyroidectomy in rats has also been shown to cause reduction in fetal body and brain weight early in gestation (Morreale de Escobar et al., 1985, 1987; Pickard et al., 1993). However, in some of these studies and in others, the onset of fetal thyroid secretion did not result in
complete and immediate catch-up of growth (Porterfield and Hendrich, 1991; Morreale de Escobar et al., 1985).

The influence of maternal thyroidectomy on early brain development in rats was examined. Fetal brain DNA content and total protein content were found to be reduced at 15 and 19 dg (Pickard et al., 1993). Other workers have reported that maternal thyroidectomy results in a reduction in both cell number and size, as well as RNA content in fetal brain at 22 days of gestation. However, these parameters were found to be normal by adulthood (Porterfield and Hendrich, 1982). Similar observations have been reported in sheep, where fetuses from TX dams display reduced cell number (DNA content) in the cerebellum and cerebral hemispheres with the cell size (protein : DNA ratio) also being reduced in the cerebral hemispheres between 71 and 125 dg (Potter et al., 1986). In rhesus monkey, maternal radiothyroidectomy was reported to cause reduction in RNA:DNA and protein:DNA ratio in both cerebella and cerebellum in 150 dg fetus, with an elevation in the water content and a reduction in the non chloride space, an index of brain volume, (Holt et al., 1975).

Carbohydrates are thought to represent the major fetal metabolic substrate in rats and humans (Hoar et al., 1957; Hugget, 1929). Consequently, alterations in fetal or placental carbohydrate metabolism can have deleterious effects on development. Thyroid hormones are known to alter the metabolism of carbohydrate, protein, and lipids in non pregnant adults and will therefore most likely affect the maternal system in a similar manner. During the last few days of gestation, the level of fetal liver glycogen rises, and it is known that liver glycogen storage is important for preventing severe neonatal hypoglycaemia which can result in mental impairment and even death (Jolin and Montes, 1974). Porterfield et al. (1975) have reported that maternal thyroidectomy on day one of pregnancy prevents the normal late gestational accumulation of glycogen in liver and skeletal muscle of the fetal rat, resulting in a significant decrease of serum glucose by day 22 of gestation. Since glucose appears to be the major metabolic substrate for the fetus (Jolin and Montes, 1974), these findings may represent a primary cause for the observed
reduction in fetal weight, and the increased numbers of fetal resorptions (Porterfield and Hendrich, 1975; 1976).

One of the most acute and lasting effects of maternal thyroidectomy on offspring relates to amino acid metabolism and protein synthesis. Hendrich et al. (1984) reported that the transfer of amino acids from hypothyroxinemic mothers to their fetuses is impaired and the tissue (brain and liver) uptake of amino acids is decreased in fetuses. The abnormal metabolism of amino acids may only be one contributing factor. Nevertheless, both serum protein and glycoprotein synthesis in brain, liver, and skeletal muscle are reduced significantly in fetal, neonate, and adult progeny of thyroidectomised rat dams (Hendrich et al., 1984; Porterfield and Hendrich, 1982; Pickard et al., 1991). A probable reason for the impaired protein synthesis and amino acid uptake in fetal brain is that the fetuses are hypoglycaemic, and hypoglycemia prevents normal uptake of amino acids (Schulman et al., 1975; Hendrich et al., 1984).

An electron microscopic study of brain from severe neurological cretins in China has revealed changes in the appearance of the myelin sheath (Ma et al., 1986), which serves to insulate the axon of neurones and thereby facilitate conduction, (Norton, 1981). The myelination process was found to be significantly reduced in fetal brain at 150 dg of radiothyroidectomised rhesus monkey (Holt et al., 1975). The influence of maternal thyroid state on myelination has been studied in more details in the rat. Although no change was found in brain cholesterol, a major constituent of myelin lipid, in adult progeny of thyroidectomized rats, the activities of the marker enzymes was altered (Hajzadeh et al., 1990). These include 2',3'-cyclic nucleotide, 3'-phosphohydrolase, and 5'-nucleotidase, which were significantly reduced in medulla and midbrain. The activity of oleate esterase, which may play a role in myelin degradation (Hirsch et al., 1977), was increased however in paleocortex (Hajzadeh et al., 1990). Furthermore the concentration of an important myelin-specific galactolipid, cerebroside sulphate, is reduced in the midbrain and paleocortex (Sinha et al., 1992). Together, these findings indicate deficiencies in the normal pattern of myelinogenesis, with possible impairment of neural function.
The attributes of a number of neurotransmitter metabolites have been studied in the CNS of hypothyroxinemic dams progeny. Sinha et al. (1992) have studied the activity of two acetylcholine metabolic enzymes in adult brain progeny. Choline acetyltransferase (ChAT), the synthetic enzyme, was found to be increased in activity in paleocortex, whereas the activity of acetylcholinesterase (AChE), the degradative enzyme, was found to be decreased in paleocortex, midbrain, and cerebral cortex of adult progeny.

Glycoproteins play important developmental roles in cell-cell recognition and synaptogenesis, and are particularly abundant in synaptic complexes. They also comprise a considerable number of important intracellular enzymes essential in developing cells. The notion that synaptogenesis may be compromised as a consequence of maternal hypothyroxinemia during fetal life is supported by the finding that calcineurin, a calmodulin-dependent phosphatase which regulates neurite elongation, was compromised in young progeny (Sinha et al., 1992). Neutral and alkaline components of this activity was significantly reduced in whole brain of one-day-old pups of thyroidectomized dam, whereas in two month old progeny, neutral and acidic activities are increased, at least in the cerebellum (Ruiz de Elvira et al., 1989).

In addition to the aforementioned, a significant elevation was reported in nuclear T3 binding capacities in the paleocortex and the cerebellum of adult progeny of thyroidectomized rats (Sinha et al., 1991), an indication of a permanent perturbation of thyroid hormone regulatory and effector system as a result of maternal hypothyroxinemia. Notably, it is unlikely that the above findings have resulted from placental compromise since the biochemistry of hypothyroxinemic placenta at term is apparently normal (Sinha et al., 1991), neither can it be attributed to iodine deficiency because in most of these studies the dams were fed an iodine-replete diet. Therefore the changes observed are most likely due to lack of maternal TH, resulting in altered fetal thyroid hormone environment during early neurogenesis.
1.9 Rationale

Previous studies were mainly centred on the importance and role of TH on mammalian CNS development during late fetal or early postnatal life. The role of maternal TH in the development of early fetal CNS has been poorly studied, reflecting the previously widely-held belief of the impermeability of the placenta for TH. However, as discussed earlier (section 1.7.1.), it is now clearer that maternal TH crosses the placenta during early pregnancy. Furthermore the fetal brain has access to the transferred T4, and THNR are expressed early in development (Porterfield and Hendrich 1993).

Although several studies have been concerned with the effect of the maternal thyroid state, most were concentrated on hypothyroidism (Hendrich et al., 1984; Morreale de Escobar et al., 1985, 1987, 1992; Calvo et al., 1992; Porterfield and Hendrich 1993) in which the animals were totally thyroidectomised either surgically or by treatment with $^{131}$I. Since this does not represents maternal hypothyroxinemia and does not reflect conditions found in neurological cretinism in man, a more appropriate model of partially thyroidectomised rats (parathyroid-spared) was used. In this study the rat was used because it possesses a haemochorial placenta, similar to human. In addition, it allows easy manipulation in the environmental and nutritional conditions.

The aim of this study was to look into the effect of maternal hypothyroxinemia on the fetal CNS development during pregnancy. Thus we have studied in utero biochemical parameters which might be dependent upon the availability of TH to the fetus at different stages of fetal brain development, namely before and after the onset of fetal thyroid function as well as near term; at 15, 19 and 22 dg respectively. These parameters include the cell growth indices; protein, DNA, RNA as well as the phosphate pool on which a wide range of biochemical and metabolic function depends. The activities of fetal brain phosphorylated proteins; phospholipid / Ca$^{2+}$-dependent protein kinase or protein kinase C (PKC) and cAMP-dependent protein kinase (PKA) and phosphohydrolase proteins; Na$^+$ K$^+$-ATPase, Ca$^{2+}$ Mg$^{2+}$-ATPase, acid phosphatase and alkaline phosphatase were also studied.
To examine whether maternal hypothyroxinemia has a specific effect on the growth of fetal CNS or has a general effect—probably best sensed by the liver, fetal liver biochemistry was investigated too. In addition, in order to determine whether the neurochemical effects, seen in developing brain as a result of maternal hypothyroxinemia, are due to placental dysfunction, several biochemical parameters in N and TX placentae at various stages of gestation were also examined.

It is well known that glucose—required for a wide range of biosynthetic reactions like nucleic acids, amino acids and fatty acid, as well as its well known role as primary energy substrate, is believed to be affected by TH. Since brain is dependent on adequate supply of glucose throughout life, especially so during development, the role of maternal hypothyroxinemia on glucose metabolism and the expression of glucose transporters gene (GLUT 1 and GLUT 3) were investigated in fetal brain at 16, 19 and 21 dg.
CHAPTER TWO : MATERIALS AND METHODS
2.1 Materials

General laboratory chemicals were purchased from Merck (Dagenham, Essex) and were of Analar grade whenever possible. All fine chemicals were obtained from Sigma Chemical Co. Ltd (Poole, Dorset), and radiochemicals from Amersham International (Amersham, Bucks). All other materials purchased from other sources are indicated in the text.

2.2 Animal model

The animals used for breeding were normal male and female, and partially thyroidectomized (parathyroid-spared) female Sprague-Dawley rats. The normal (N) rats were bred in the local animal facilities, while the thyroidectomized (TX) rats were obtained from Charles River (UK) Ltd. (Margate, Kent, U.K.). The animals were maintained under normal animal house conditions at a constant temperature of 22 °C on 14/10 h light/dark cycle and fed a standard, iodine-replete diet ad libitum (average iodine intake of 36 μg/day). The drinking water of the TX dams was supplemented with calcium lactate (0.1%, w/v). Before mating, blood samples were obtained from the tail vein of N and TX dams to assess circulating T4 and T3 levels. After mating, the dams were stunned and killed by cervical dislocation at 15, 16, 19, 21 and 22 days of gestation. Blood was taken by cardiac puncture, added to heparinized tubes, then centrifuged (2,500 g for 10 min). Plasma was stored at -20 °C for subsequent T4 and T3 measurement. The conceptuses were dissected out and the fetuses and placentae were separated. After determination of fetal body weight, brain and liver were dissected. Placental, fetal brain, and fetal liver weights were determined. Tissues were either used fresh (metabolic studies) or stored at -20 °C for subsequent analysis.
2.3 Tissue preparation

For metabolic studies, fresh fetal brain tissue was minced on ice using scalped blade. This was added to precooled incubation vials as described in section 2.12, while for other biochemical studies, including assay of enzyme activity, frozen tissues were thawed at 4 °C, chopped, then homogenised to yield a 10% (w/v) homogenate. Homogenisation was carried out on ice using an all-glass homogeniser (20 upward and downward strokes). The medium used for homogenisation was routinely 0.25 M sucrose, but in the case of the protein kinase assays, this was substituted with the appropriate 1X reaction buffer (section 2.9.1.1). For samples homogenised in sucrose, the homogenate was divided into 0.1 ml aliquots and stored at -20 °C for further analysis.

2.4 Determination of thyroid hormone

The levels of plasma thyroid hormone (total T4 and T3) in both N and TX dams were determined by radioimmunoassay using commercial kits obtained from North East Thames Region Immunoassay Unit (NETRIA; London, U.K.), following the standard protocols supplied. The sensitivities of these assays are 3.19 nmol/l for total T4 and 0.29 nmol/l for total T3.

2.5 Protein determination.

2.5.1 Reaction with the Folin Phenol Reagent

In principle, alkaline copper sulphate reacts quantitatively with compounds containing two or more peptide bonds to produce a violet colour; the intensity of which is proportional to the number of peptide bonds present. Maximum colour results after reduction of the diluted Folin Phenol reagent at pH 10 by the alkaline copper protein. This latter reaction is complete in 30 minutes (Lowry et al., 1951).
Solutions:

A) Solution (1): 2% (w/v) sodium carbonate in 0.1 M NaOH.
B) Solution (2): 0.5% (w/v) copper sulphate in 1% (w/v) sodium potassium tartrate.
C) Alkaline copper solution: mixture of 49 ml of solution A and 1 ml of solution B (freshly prepared).
D) Folin-Ciocalteau reagent: commercial stock solution, diluted with an equal volume of distilled water.
E) Standard solution of bovine serum albumin (BSA; 1.0 mg/ml water).

Method:

To variable amounts of BSA standard solution (10-200 µg) or aliquots of tissue homogenate (final volume of 1.2 ml) alkaline copper sulphate solution (3 ml) was added and the reactants were mixed. After standing for 15 min, diluted Folin reagent (0.3 ml) was added and the tubes were mixed rapidly. After 30 min, the absorbance was read at 500 nm against a reagent blank. All homogenate and standard samples were assayed in duplicate and a standard curve was drawn by plotting the optical density versus the amount of BSA (Fig. 8). Each point represents the mean ± SD of fifteen experiments.

2.5.2 Reaction with Bradford's Reagent

This method was usually used for samples with estimated protein concentrations of <10 µg/ml. The principle of this method is based on the absorbance shift from 465 to 595 nm which occurs when Coomassie Brilliant Blue G-250 binds to protein in an acidic medium. This is a rapid method and can be assayed within two min but it is stable for one hour. Although it is four times more sensitive than Lowry’s method, there is a slight non-linearity in the response pattern (Bradford 1976).
Figure 8: Protein standard curve: reaction with Folin Phenol Reagent.

Figure 9: Protein standard curve: reaction with Bradford's Reagent.
Solutions:

A) Coomassie Brilliant Blue G-250 reagent: a commercial reagent was used (Pierce Ltd, Luton, Beds).
B) Standard solution of BSA (25 µg/ml water).

Method:

Bradford solution (0.5 ml) was added to an equal volume of standard solution (1-5 µg) or tissue homogenate. The solution was mixed, left for 2 min, and the resultant blue colour was read against a reagent blank at 595 nm. A standard curve was drawn by plotting optical density versus amount of BSA concentration (Fig. 9). Each point represents the mean ± SD of seven experiments assayed in duplicate.

2.6 Nucleic acids determination

2.6.1 Nucleic acid extraction

Solutions:

A) 10% (w/v) Trichloroacetic acid (TCA)
B) 5% (w/v) TCA

Method:

Nucleic acid was extracted from tissue homogenate using the method of Schneider (1945), with modification. Aliquots of tissue homogenate were precipitated with 5 vol. 10% TCA. The mixture was chilled on ice for 15 min and then centrifuged (2,000 g for 15 min at 4 °C). The pellet was re-extracted with 10 ml 10% TCA, chilled and re-centrifuged. The pellet was resuspended in 5% TCA (1.5 ml) and placed in boiling water for 20 min. After cooling, the tubes were centrifuged (2,000 g for 15 min). The supernatant was collected, the extraction was repeated and the resulting supernatants were combined and stored at -20 °C for DNA and RNA determination.
2.6.2 DNA determination

2.6.2.1 Diphenylamine reaction

DNA was usually determined by the diphenylamine reaction. In this reaction, deoxyribose, released from DNA by acid hydrolysis, reacts with diphenylamine under acidic conditions to form a blue product with absorbance maximum at 595 nm. Because RNA is also present in the reaction mixture and could interfere at this wavelength, a reading was also taken at 650 nm which was subtracted from the reading at 610 nm. The difference is proportional to the quantity of DNA in the solution (Burton 1956; Schneider 1945).

Solutions:

A) 5% (w/v) TCA.
B) Calf thymus DNA (1 mg/ml) in 5% TCA.
C) Diphenylamine solution: 1% diphenylamine in 0.5 M sulphuric acid and 15 M acetic acid. Freshly prepared just before the assay.

Method:

To aliquots of nucleic acid extract standard solution (25-800 μg DNA) in a volume of 1.5 ml, an equal volume of diphenylamine solution was added. After mixing, tubes were placed in a boiling water bath for 20 min. Once cooled, the blue colour was read in the spectrophotometer against a reagent blank at 610 and 650 nm. A standard curve was drawn by plotting the difference in absorbance against the content of DNA concentration (Fig. 10). Each point represents the mean ± SD of five experiments assayed in duplicate. The assay was based on the methods described by Burton (1956) and Schneider (1945), as modified by Zamenhof et al. (1964).
2.6.2.2 3,5-Diaminobenzoic acid (DABA) reaction

In case of limited amount of samples or low DNA levels (< 20 µg/ml), a modification of the method of Setaro and Morley (1976) was used. In this procedure, deoxyribose reacts with 3,5-diaminobenzoic acid (DABA) under acidic conditions to yield a fluorescent product.

Solutions:
A) Standard solution: 1 mg/ml DNA in 5% (w/v) TCA.
B) DABA 30 mg/ml in water (1 M HCl was added dropwise to aid dissolution).

Method:
To standard (5-100 µg DNA) or diluted tissue extract, DABA (1 ml) was added (final volume 2 ml). The mixture was then placed in a water bath at 60 °C for 30 minutes, cooled and then read in a (Perkin-Elmer) fluorimeter at 420 nm excitation and 520 nm emission wavelength. A standard curve was drawn after reagent blank subtraction by plotting the fluorescence against DNA content (Fig. 11). Each point represents the mean ± SD of five experiments assayed in duplicate. The assay was based on the method described by Setaro and Morley (1976).

2.6.3 RNA determination

When heated in concentrated HCl, RNA pentose forms furfural. This is reacted with orcinol in the presence of ferric chloride to produce a green colour which absorbs at 660 nm (Schneider, 1945).

Solutions:
A) 5% (w/v) TCA.
B) RNA standard solution (1 mg/ml in 5% (w/v) TCA).
C) Orcinol solution: 1% (w/v) orcinol and 0.5% (w/v) ferric chloride in concentrated HCl.

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Figure 10: DNA Standard curve (Diphenylamine reaction).

Figure 11: DNA Standard curve (Fluorimetric method).
Method:
Orcinol solution (1.5 ml) was added to an equal volume of standard RNA solutions (15-150 μg) or nucleic acid extract. Tube contents were mixed and placed in a boiling water bath for 25 min. After cooling, the green colour was read in a spectrophotometer against a reagent blank at wavelength of 660 nm. A standard curve was drawn by plotting the absorbance against the amount of RNA (Fig. 12). Each point represents the mean ± SD of five experiments assayed in duplicate. The assay was based on the method described by Schneider (1945).

Figure 12: RNA standard curve.
2.7 Total lipid estimation

2.7.1 Lipid extraction

Solutions:
A) Chloroform:methanol (2:1 v/v).
B) Potassium chloride (KCl) 0.1 M.

Method:
One volume of tissue homogenate was mixed with 4 vol. of chloroform:methanol and kept at room temperature for 1 h. The mixture was centrifuged (1500 g for 20 min), the aqueous layer was removed and 1 ml of KCl was added to the organic layer, samples were left for 5 min then centrifuged (1500 g for 10 min). The organic layer was transferred to another tube. The lipid was extracted again from the initial aqueous layer and extracts were combined. This was then dried under a stream of oxygen-free nitrogen gas. The dried lipid was resuspended in known volume of chloroform:methanol (2:1) and stored at -20 °C.

2.7.2 Total lipid assay

The principle of this method, which is modified from Pande et al. (1963), involves the oxidation of lipids in acid-dichromate. The appearance of a light orange colour representing chrome alum (maximum absorbance 585 nm) is due to the reducing action of lipids on acid chromate and was read against acid-chromate as a blank.

Solutions:
A) Sulphuric acid (1.4 M).
B) Acid-dichromate reagent: (2% (w/v) Potassium dichromate in 5.4 M sulphuric acid. The potassium dichromate was powdered with mortar and pestle and the sulphuric acid was added slowly, ensuring that the temperature did not
exceed 100 °C. The reagent was stored in a dark and discarded if undissolved residue was present.

C) Palmitic acid standard solution (1 mg/ml absolute ethanol).

Method:
After complete removal of the solvent from the extracted lipid or the palmitic acid standard (75-300 μg) under a stream of oxygen-free nitrogen gas, acid-chromate reagent (3 ml) was added. Control tubes were also included which did not contain any lipid (reagent blank). The tubes were placed in a boiling water bath for 15 min and, after cooling, 4.5 ml water was added. After mixing, the solutions were recooled. The colour intensities were measured at 585 nm against the reagent blank. A standard curve was constructed by plotting the optical density versus the amount of lipid standard (Fig. 13). Each point represents the mean ± SD of five experiments assayed in duplicate.

2.7.3 Cholesterol determination
The method is based on the observation that ethanol provides complete cholesterol extraction and leaves behind interfering substances. The extracted cholesterol reacts with the ferric chloride-sulphuric acid reagent to produce a coloured product with peak absorbance at 560 nm (Fiske and Subbarow, 1925; Franey and Amador, 1968).

Solutions:

A) 0.1% (w/v) Ferric chloride in ethyl acetate.
B) Concentrated sulphuric acid.
C) Cholesterol standard solution (1 mg/ml in absolute ethanol).

Method:
The lipid extract was dried, then dissolved in 0.5 ml absolute ethanol. Ferric chloride solution (2 ml) was added to sample and standard (10-200 μg). Concentrated sulphuric acid (2 ml) was carefully added and after mixing, the tubes were allowed to cool for at least
10 min. A control blank of 0.5 ml ethanol was treated in the same manner. The samples were read against the control blank at 560 nm. A standard curve was constructed by plotting optical density against amount of cholesterol standard (Fig. 14). Each point represents the mean ± SD of five experiments assayed in duplicate.
Figure 13: Total Lipid standard curve.

Figure 14: Cholesterol standard curve.
2.8 Phosphate determination

2.8.1 Total inorganic phosphate

The principle of this method is that inorganic phosphate reacts with ammonium molybdate in an acidic medium to form phosphomolybdic acid. This product is extracted in butanol-benzene, then reduced with stannous chloride, to give a blue colour which is measured in the spectrophotometer at a wavelength of 730 nm (Martin and Doty, 1949).

Solutions:

A) Standard phosphate solution, Na₂HPO₄ 0.25 mg/ml.
B) Isobutanol:benzene (1:1(v/v)).
C) 2.5 M Sulphuric acid.
D) 10% (w/v) Ammonium molybdate.
E) Ethanol solution: 0.006% (w/v) stannous chloride in 0.3 M sulphuric acid and 87% (v/v) ethanol.
F) 10% (w/v) Stannous chloride in concentrated HCl.

Method:

To 0.2 ml aliquots of sample or standard (50-150 μg) in quadruplicate, 0.3 ml sulphuric acid, 2 ml butanol:benzene and 0.2 ml ammonium molybdate were added. Tubes were shaken vigorously and centrifuged (2000 g for 20 min). An aliquot (1 ml) of the organic phase was transferred to a clean tube and ethanol solution (2.5 ml) was added. Tubes were mixed and 10% stannous chloride solution (20 μl) was added. Tubes were then read in the spectrophotometer against the reagent blank at a wavelength of 730 nm. A standard curve was drawn by plotting absorbance against the amount of inorganic phosphate (Fig. 15). Each point represent the mean ± SD of ten experiments assayed in quadruplicate.
2.8.2 Lipid Phosphate

Method:
Total lipid was extracted from tissue homogenate (section 2.7.1). The dried lipid was digested with 0.5 ml perchloric acid (70%) at 80 °C for 45 min. Samples were cooled, 1 ml water was added and the liberated phosphate was extracted and measured as described above (section 2.8.1).

2.8.3 Protein-associated phosphate

Method:
Tissue homogenate protein was precipitated by the addition of 3 vol. TCA (30%). After standing on ice for 15 min, tubes were centrifuged (2500 g for 10 min). The pellet was digested in perchloric acid (0.5 ml) (section 2.8.2) and inorganic phosphate was measured as described above (section 2.8.1).
2.9 Enzyme assays

2.9.1 Protein Kinase activity
The most common method for assaying protein kinase activity is to measure the transfer of radioactive phosphate to a substrate protein or peptide. Although effective, the large quantities of $^{32}$P used makes such assays somewhat hazardous. An alternative non-radioactive method for cAMP-dependent protein kinase (PKA) and protein kinase C (PKC) is the Pep Tag procedure (White and Shultz, 1992). These assays utilise brightly coloured, fluorescent peptide substrates that are highly specific for the kinases in question. The amino acid sequence of the Pep Tag A1 peptide (PKA substrate) is L-R-R-A-S-L-G, whereas the amino acid sequence of the Pep Tag C1 peptide (PKC substrate) is P-L-S-R-T-L-S-V-A-A-K. The intense pink colour is imparted by the addition of a dye molecule to the Pep Tag peptide substrate. Phosphorylation by PKC or PKA of their respective substrates alters the charge of the peptide from a net charge of +1 to a net charge of -1. Phosphorylated and unphosphorylated substrates can be rapidly separated on an agarose gel at neutral pH. The phosphorylated species migrates towards the anode while the unphosphorylated substrate migrates toward the cathode.

2.9.1.1 Protein Kinase C (PKC) assay
A kit supplied by Promega (Maidason, U.S.A) was used.

Solutions:

A) Tris-HCl buffer, 50 mM; pH 8.0.
B) Glycerol 80% (v/v).
C) Reaction buffer (5X): 6.5 mM CaCl$_2$, 5 mM Dithiothreitol (DTT), 50 mM MgCl$_2$ and 5 mM ATP, in 0.1 M HEPES; pH 7.4.
D) PKC activator solution (5X): 1 mg/ml phosphatidyl serine
E) Pep Tag C1 Peptide, 0.4 mg/ml in water.
F) PKC control (2.5 μg/ml PKC, 20 mM Tris-HCl (pH 7.5), 2 mM EDTA, 2 mM EGTA, 1 mM DTT, 20 mM K₃PO₄ and 50% glycerol.

G) Gel solubilization solution.

F) Tissue samples were homogenised (10% w/v) in 1X reaction buffer (section 2.3). The protein was determined by Lowry’s method.

Method:
To microfuge tubes, 5 μl 5X reaction buffer, 5 μl Pep Tag C1 Peptide and 5 μl sonicated 5X activator solution were added. The tubes were incubated at 37 °C for 1 min, 10 μl tissue homogenate (85 μg) was added and the incubation continued for a further 30 min. The reaction was stopped by placing the tubes in a water bath at 95 °C for 10 min. The samples were then stored at -20 °C for electrophoresis. For positive controls, the tissue homogenate was replaced with purified rat PKC provided as supplied by the kit manufacturer. For negative controls, the activator solution was replaced with distilled water.

Gel electrophoresis
An agarose gel (0.8%, w/v) was prepared by dissolving 1.75 g agarose in 220 ml 50 mM Tris-HCl buffer, pH 8.0 by boiling. After cooling to 60 °C, the gel was poured. The electrophoresis buffer was 50 mM Tris-HCl; pH 8.0. Loading of samples was expedited by the prior addition of 1 μl glycerol. The gel was run at 95 V for 15 min. The coloured Pep Tag peptide bands were visualised on an ultraviolet transilluminator and photographed.

Spectrophotometer quantitation
Using a razor blade, the negatively charged phosphorylated bands were excised from the gel, keeping the total volume uniform. The volume was approximately 250 μl. Each excised band was placed into a graduated microfuge tube and heated at 95 °C until the gel slice melted. To each tube, 75 μl gel solubilization solution, 100 μl glacial acetic acid and 200 μl distilled water was added. While the gel was still liquefied, samples were then
vortexed and the absorbances were read at 570 nm against liquefied agarose containing no Pep Tag Peptide.

2.9.1.2 cAMP-dependent Protein Kinase (PKA) assay

A kit supplied by Promega (Maidason, U.S.A) was used.

Solutions:

A) Reaction buffer (5X): (100 mM Tris-HCl (pH 7.4)) 50 mM MgCl₂ and 5 mM ATP in 100 mM Tris-HCl (pH 7.4).
B) PKA activator solution (5X): 5 μM cAMP in water.
C) Pep Tag A1 Peptide, 0.4 mg/ml in water.
D) PKA control: 2.5 μg/ml bovine heart PKA catalytic subunit in 350 mM K₃PO₄ and 0.1 mM DTT.

Method:
The samples were prepared and assayed in the same way as for PKC (section 2.9.1.1) except the sample protein concentration was 150 μg and the incubation temperature was 28 °C.

2.9.2 Acid phosphatase (EC 3.13.2)

Acid phosphatase is one of some 40 enzymes known to be contained in lysosomes. Enzyme activity was determined by the use of 4-methylumbelliferyl-phosphate as a substrate. The product, 4-methylumbelliferone (4-MU), is highly fluorescent, especially at acidic pH.

Solutions:

A) Sodium citrate buffer (50 mM, pH 4.5).
B) 4-Methylumbelliferyl phosphate (0.012 mM in citrate buffer).
C) Glycine/NaOH buffer (50 mM, pH 10.4).
D) Tissue homogenate (10% w/v in 0.25 sucrose) (see section 2.3).
Method:
To 1 ml diluted homogenate (10-40 µg protein) an equal volume of substrate solution was added. The mixture was incubated at 37 °C in water bath for 15 minutes with shaking and the reaction was terminated by the addition of 3.0 ml of glycine/NaOH buffer. In the reagent blank, homogenate was added after stopping the reaction. The reaction mixture was read in a Perkin-Elmer fluorimeter at 360 nm excitation and 440 nm emission wave length. After blank subtraction the acid phosphatase activity was expressed as nmol 4-MU liberated/mg protein/h. The assay was based on the method described by Sinha and Rose (1972).

2.9.3 Alkaline phosphatase (EC 3.1.3.1)
The same procedure was followed as that for acid phosphatase, except that the incubation buffer was carbonate-bicarbonate (50 mM; pH 9.0) and the reaction contained 50-150 µg homogenate protein.

2.9.4 Na+ K+-ATPase activity (EC 3.6.1.3)
Plasma membranes of virtually all animal cells contain a Na+ K+-pump that actively pumps Na+ out of the cell and K+ into the cell against their concentration gradients. The Na+ and K+ gradients maintained by the Na+ K+-pump are responsible not only for the cell’s membrane potential but also for controlling cell volume and for driving the active transport of sugars and amino acids. The energy for this pump is derived from the hydrolysis of ATP to ADP with the release of inorganic phosphate. Like the Na+ K+ pump, the Ca2+ Mg2+ pump is a membrane ATPase that is phosphorylated and dephosphorylated during its pumping cycle. The activities of both enzymes were assayed by measuring the release of inorganic phosphate from ATP. In order to assess the activity of Ca2+ Mg2+-ATPase, the activity of Na+ K+-ATPase was blocked with the cardiac glycoside, ouabain. The method is based on the procedure of Baron and Khan (1985).
Solutions:

A) Buffer A: 100 mM NaCl, 20 mM KCl, 3 mM MgCl₂ and 1 mM EDTA in 100 mM Tris-HCl, pH 7.4.
B) Ouabain (3 mM in buffer A).
C) Tris-ATP (30 mM in buffer A).
D) TCA (30%, w/v).

Method:

Tissue homogenate was diluted in buffer A, or buffer A containing ouabain to give 0.5 mg protein/ml. Aliquots of sample (1 ml, in duplicate) were mixed with an equal volume of Tris-ATP and incubated for 15 min at 37 °C (or 4 °C for controls). Reactions were terminated by the addition of ice cold TCA (0.2 ml). The liberated inorganic phosphate was extracted and assayed as described earlier (section 2.8.1.). After subtraction of the 4 °C control, ATPase activity was expressed as μmol Pi liberated/mg protein/h.

2.9.4.1 Assay validation

Preliminary validation revealed the assay to be linear with 0.4-0.6 mg protein and incubation time of 30 min (Fig. 16 and 17).
Figure 16: Total ATPase activity (concentration course).

Figure 17: Total ATPase activity (time course).
2.10 SDS-Polyacrylamide gel electrophoresis of proteins

Since proteins carry a net charge at any pH other than their isoelectric point, they will migrate if placed in an electric field, and their rate of migration will depend upon their charge density (ratio of charge to mass). In the mid-1960's a modified version of this method-known as SDS polyacrylamide-gel-electrophoresis (or SDS-PAGE) was developed. This system was designed to dissociate all proteins into their individual polypeptide subunits. The most common dissociating agent used is the ionic detergent, sodium dodecyl sulphate (SDS). In this method, the protein mixture is usually denatured by heating at 100 °C in the presence of excess SDS and thiol regent (to cleave disulphide bonds). Under these conditions, most polypeptides bind SDS in a constant weight ratio (1.4g of SDS per gram of polypeptide). The intrinsic charges of the polypeptide are insignificant compared to the negative charges provided by the bound detergent, so that the SDS-polypeptide complexes have essentially identical charge densities and migrate in polypeptide gels of the correct porosity strictly according to polypeptide size. Therefore the molecular mass of the sample polypeptides can be determined by reference the mobility of polypeptides of known molecular mass treated under the same electrophoric conditions (Weber and Osborn, 1969).

Solutions:

A) 0.2 M Sodium Phosphate buffer stock solution; pH 7.1 containing 0.2% (w/v) sodium dodecyl sulphate (SDS).

B) Electrode buffer:Phosphate buffer stock solution diluted 1:1 with double distilled water.

C-i) Sample buffer I (for protein concentrations ≤ 2 mg/ml) : 5% (v/v) phosphate buffer stock solution, 1% (v/v) 2-mercaptoethanol and 1% (w/v) SDS.

C-ii) Sample buffer II (for protein concentrations 2-5 mg/ml) : contains 5% (v/v) buffer stock solution, 2% (v/v) 2-mercaptoethanol and 4% (w/v) SDS.
D) Acrylamide : bisacrylamide (30:0.8% w/v), (Protogel; National Diagnostic, Somerville, U.S.A).

E) Ammonium persulphate solution : 1.5% (w/v) ammonium persulphate prepared in water immediately before use.

F) Bromophenol blue solution : 0.25% (w/v) in appropriate sample buffer.

G) 7.5 % acrylamide gel : 35 ml phosphate buffer stock, 17.5 ml protogel, 3.4 ml ammonium persulphate solution and 75 µl TEMED. The volume was made up to 70 ml with water.

H) Detergent solution : 0.1% (v/v) Triton X-100.

I) Fixing solution : 11.4% (w/v) TCA and 3.4% (w/v) sulphosalicylic acid in 30% (v/v) methanol.

J) Staining solution (1) : contains 0.25% (w/v) Coomassie brilliant blue R-250 and 9.2% (v/v) glacial acetic acid in 45.4% (v/v) methanol. The mixture was filtered through Whatman no. 1 paper to remove insoluble dye.

Staining solution (2) : Silver staining kit (purchased from Sigma).

K) Destaining solution : 10% (v/v) acetic acid in 30% (v/v) ethanol.

L) Preserving solution : 10% glycerol (v/v).

M) Rainbow protein marker : contains a mixture of seven individually coloured purified proteins (1.4 mg/ml each). These proteins are: myosin (200 KD), phosphorylase b (97.4 KD), BSA (69 KD), ovalbumin (46 KD), carbonic anhydrase (30 KD), trypsin inhibitor (21 KD) and lysozyme (14.3 KD).

Method :

Gel preparation:

Horizontal gel electrophoresis was carried out using Multiphor system. The gel loading cassette (125 x 260 x 1 mm) was mounted according to the manufacture's instructions, using 10µl slot former. The gel solution was prepared by degassing the mixture of water, buffer and Protogel for 30 min with periodic stirring. Then the persulphate and TEMED solutions were added, mixed and immediately poured into the gel mould (avoiding air
bubbles) using a 50 ml syringe with a wide bore needle. After 1 h, the cassette was placed at 4 °C for 15 min. The gel was stored in a humidified chamber at room temperature for at least 12 h, and used within 4 days.

Sample preparation
Brain homogenate (2 mg protein/ml) from fetuses at 15, 19 and 22 days of gestation were prepared by diluting 13 μl sample with 10 μl sample buffer I (for silver staining) or 5 mg/ml sample was diluted 1:1 with sample buffer II (for Coomassie brilliant blue staining). The samples were then incubated at 100 °C for 3 min, chilled, then centrifuged (1 min in a microcentrifuge). To 250 μl supernatant, 10 μl of 2-mercaptoethanol and 10 μl of bromophenol blue were added. The protein marker was prepared by mixing 10 μl with 10 μl sample buffer. After boiling 2.5 μl 2-mercaptoethanol and 2.5 μl bromophenol blue were added.

Electrophoresis
The gel was placed on the cooling plate of the electrophoresis tank with the sample slots at the cathode. The electrophoresis tank was filled with electrode buffer, the wicks (Watman no. 1 paper) were put in place and the gel was pre-electrophoresed at a constant current of 150 mA for 30 min. Then 9 μl sample/standard mix were applied to slots and electrophoresis was started immediately at 20 mA for the first 10 min, then 200 mA for 4-5 h. The gel was cooled during electrophoresis by passing water at 8 °C through the cooling plate. At the end of the run, the distance migrated by the tracking dye was measured and the gel was fixed in fixing solution for 1 h at room temperature.

Gel staining
A—Coomassie brilliant blue staining
The gel was washed with distilled water and stained with staining solution for 1 h at room temperature with shaking. The gel was transferred to the destaining solution, which was changed after 30 min, then 60 min, and then every 90 min (with shaking) until the process

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was satisfactorily completed. The gel was soaked in preserving solution for at least 30 min and dried overnight between cellulose gel drying kit (Promega, Maidason, U.S.A).

B–Silver staining

This technique is approximately 100 times more sensitive than Coomassie brilliant blue staining technique and allows the detection of small quantities (10-50 ng) of most proteins. The staining procedure was performed according to the protocols supplied with the kit (Sigma).

2.11 Metabolic studies

2.11.1 Glucose metabolism

The brain is dependent on an adequate supply of glucose throughout life. As well as its importance as a primary respiratory substrate in the CNS, glucose is required for a wide range of biosynthetic reaction; the carbon skeleton is incorporated into amino acids, protein, nucleic acids and fatty acids. Therefore, during the critical period of CNS development any factor which may regulate glucose metabolism may be expected to exert profound effects on the development and the homeostasis of the CNS. In this study the effect of maternal hypothyroxinaemia on glucose metabolism in fetal brain at 16, 19 and 21 day gestation was investigated.

Solutions:

A) Krebs-Ringer Phosphate (KRP) Buffer: 118 mM NaCl, 4.74 mM KCl, 1.185 mM MgSO₄, 1.185 mM KH₂PO₄, 1 mM CaCl₂ in sodium phosphate buffer (16.15 mM pH 7.4).


C) Incubation solution: 10 mM (17.5 μCi) D-[U-¹⁴C]glucose in KRP buffer.

D) Washing solution: 10 mM glucose in KRP buffer.

E) Hyamine hydroxide (methylbenzethonium hydroxide).
Method:
Freshly dissected whole fetal brain was minced and transferred into precooled (4 °C) glass scintillation vials (0.5 brain/vial for 16 dgs and 0.25 brain/vial for 19 & 21 dgs) containing 2 ml incubation solution. An Eppendorf tube containing a filter paper wick and 0.2 ml hyamine hydroxide was added. The vials were gassed with oxygen for 20 s then tightly capped. For each sample, 6 vials were incubated at 37 °C for 1 h and 2 vials were kept on ice as controls. To terminate the reaction the vials were quickly cooled in an ice bath for 5 min. The Eppendorf tubes were removed, the vial contents were transferred to precooled tubes and immediately centrifuged (2000 g for 1 min at 4 °C). The supernatant was stored at -20 °C and the pellet was washed twice with 2 ml washing solution (at 4 °C). To the washed tissue pellet 1 ml water was added and samples were homogenised in a precooled (4 °C) hand-held homogeniser. Sample processing to yield various fractions is described in detail below.

2.11.1.1 Carbon Dioxide fraction
To determine radiolabelling of the carbon dioxide fraction, the filter paper wick and hyamine hydroxide were transferred from the Eppendorf tube to scintillation vials. The Eppendorf tubes were washed with 0.2 ml methanol and 0.2 ml water. Scintillant (4 ml) and dilute acetic acid (0.1 ml) were added to the vials for liquid scintillation counting.
The efficiency of counting was determined using a known amount of \([U^{14}C]\) glucose (0.004 μCi; 10 μl) prepared in an identical manner as sample (filter paper wick, 0.2 ml NCS, 0.2 ml methanol, 0.19 ml water, 4 ml scintillant and 0.1 ml dilute acetic acid) in duplicate.

2.11.1.2 Total uptake

To determine the total uptake of radioactivity, 25 μl homogenate was solubilized in 0.2 ml NCS, 4 ml scintillant was added followed by 0.1 ml dilute acetic acid. The efficiency of counting was determined using a known amount of \([U^{14}C]\) glucose (0.004 μCi; 10 μl) prepared in an identical manner as the sample (90 μl water, 0.2 ml NCS, 4 ml scintillant and 0.1 ml dilute acetic acid) in duplicate.

2.12.1.3 Total lipid

For the determination of \(^{14}C\) incorporation into total lipid, tissue homogenate (0.1 ml) was diluted to 1 ml with water and extracted with 2 ml chloroform:methanol. The mixture was centrifuged (2000 g for 10 min) and the organic layer was transferred into a scintillation vial. The aqueous phase was re-extracted and the combined organic layers were dried under a stream of air. Scintillation fluid (4 ml) was then added. The efficiency of counting was determined using a known amount (0.004 μCi; 10μl) of \([U^{14}C]\) glucose in 4 ml scintillant in duplicate.

2.11.1.4 Protein and protein-associated lipid

Protein was precipitated by adding an equal volume of 1 M PCA to 0.55 ml tissue homogenate. The mixture was incubated for 15 min on ice then centrifuged (2000 g for 15 min at 4 °C). The supernatant was retained and the pellet was washed with 1 ml 0.2 M PCA. The supernatants were combined, neutralised with KOH, then stored at -20 °C for ion exchange chromatography (see below). Lipid was extracted (twice) from the insoluble pellet using chloroform:methanol (3:1; sample processing of this fraction was the same as described for total lipid). Traces of solvent were removed from the extracted pellet using an
air stream, 50 μl double distilled water was added, followed by 0.2 ml hyamine hydroxide. 
After heating at 60 °C to dissolve the pellet, the sample was transferred to scintillation vials; 
the tubes were being washed twice with 2 ml scintillant. Dilute acetic acid (0.1 ml) was 
then added. The efficiency of counting was determined using a known amount of [U-
\(^{14}\)C]glucose (0.004 μCi; 10 μl) prepared in an identical manner to sample (40 μl water, 0.2 
ml NCS, 4 ml scintillant and 0.1 ml dilute acetic acid) in duplicate.

2.11.1.5 Ion exchange chromatography of PCA-soluble material
Cation exchange resin (Dowex-50W-X8, H\(^+\) form; 100 mesh; Sigma) was prepared by 
washing with: a) 5 vol. 1 M HCl, b) double distilled water until neutral, c) 5 vol. 1 M 
ammonia, d) double distilled water until neutral, e) 5 vol 1 M HCl and f) double distilled 
water until neutral. The washed cation exchange resin (2 ml) was poured into columns (~15 
cm long and 1 cm diameter). Neutralised PCA-soluble material was applied to the column, 
followed by a water (10 ml) wash (aqueous fraction; contains glucose and tricarboxylic 
acid cycle intermediates). Amino acids were eluted with 24 ml of 1 M ammonia (amino acid 
fraction). Aliquots (1 ml) of each fraction were prepared for liquid scintillation counter 
(LSC) by the addition of 4 ml scintillation fluid. The efficiency of counting was determined 
for each fraction by adding a known amount of radioactivity (0.004 μCi; 10 μl incubation 
mix) to scintillation vials followed by 0.99 ml water or 1M ammonia (as appropriate) and 4 
ml scintillant.

2.11.2 Glucose analogue uptake
The non metabolised D-glucose analogue, 2-deoxy-D-glucose (2DG), was used to 
investigate glucose uptake. This analogue is transported by the membrane facilitative 
transporters and is rapidly phosphorylated by hexokinase. The product of this reactions, 
2DG 6-phosphate, however, does not serve as a substrate for phosphohexoisomerase and 
can not be metabolised further. Phosphorylation by introducing a negative charge, 
therefore, initially transport the analouge within the cell.
Solutions:

A) Krebs-Ringer phosphate buffer, pH 7.4 (see section 2.12.1 for composition).
B) Stock analogue solution: 10 mM 2-deoxy-D-glucose in KRP.
C) Radiotracer solution: 1 μCi/μl; 2-Deoxy-D-[2,6-3H]glucose.
D) Substrate solution: solution B and C are mixed with KRP to give 500 μM (10 μCi/ml) radio tracer.
E) Stopping solution: 1 mM 2DG in KRP.
F) Washing solution: 0.5 mM 2DG in KRP.
G) NCS tissue solubiliser (Amersham International, Amersham, Bucks).
H) Scintillation fluid: Pico-fluor 40 (Canberra-Packard, Pangbourne, Berks).
I) 1.18 M Dilute acetic acid.

Method:

Freshly prepared brain tissue mince from fetuses at 16, 19, 21 days of gestation were added to 1.8 ml KRP in precooled (4 °C) 20 ml glass vials and gassed with oxygen for 20 seconds, then sealed with Subacaps. Vials were preincubated for 5 min at 37 °C (6 vials) or 4 °C (2 vials) for controls. After the preincubation period 0.2 ml substrate solution (at appropriate temperature) was injected through the rubber stopper and the samples were incubated for a further 10 min. The uptake was terminated by the addition of 2 ml stopping solution (precooled to 4 °C) and the vials were cooled on ice. To collect the tissue slices, the mixture was centrifuged (2000 g for 1 min at 4 °C). The pellet was washed twice with 3 ml washing solution by resuspension/centrifugation, then homogenised in 1 ml water. To determine uptake, the sample (0.25 ml) was transferred to scintillation vials, and 4 ml scintillant followed by dilute acetic acid (0.1 ml) and NCS (0.2 ml). The efficiency of counting was determined using a known amount of 2-Deoxy-D-[2,6-3H]glucose (0.01 μCi; 10 μl) prepared in an identical manner as sample (0.2 ml NCS, 0.24 ml water, 4 ml scintillant and 0.1 ml dilute acetic acid) in duplicate. The protein content was assayed using the Bradford procedure (section 2.5.2).
2.12 Molecular biological studies

2.12.1 RNA isolation

Fetal brain tissues at 16, 19 and 21 dg were dissected and stored at -70 °C and total RNA was isolated according to the method of Chomczynski and Sacchi (1987).

Solutions:

A) DEPC-treated water: 0.1% (v/v) in water, left overnight in the fume cupboard, then autoclaved. All solutions used were prepared in DEPC water.

B) Denaturing solution (Solution D): 4 M guanidium thiocyanate (Sigma), 25 mM sodium citrate; pH 7, 0.5% sarcosyl (N-lauroylsarcosine; Sigma), stored at room temperature.

C) Working solution: 0.72% (v/v) 2-mercaptoethanol in solution D.

D) 2 M Sodium acetate; pH 4.0, autoclaved.

E) Phenol (Sigma): saturated with DEPC-treated water, stored at 4 °C.

F) Chloroform:isoamyl alcohol (49:1, v/v)

G) Isopropanol (propan-2-ol; Merck)

H) Ethanol (75% v/v).

I) SDS (0.5% w/v) (sodium salt; Sigma), autoclaved.

Sterile equipment used throughout.

Method:

The samples were homogenised in solution D (10% homogenate) in a sterile hand-held homogeniser. Aliquots (3.6 ml) of homogenate were transferred to 15 ml Falcon tubes, then 0.36 ml sodium acetate, 3.6 ml of water-saturated phenol and 0.72 ml of chloroform:isoamyl alcohol were added. The mixture was shaken well between each addition. The contents were transferred to baked Corex tubes and kept on ice. After 15 min the tubes were centrifuged at 10,000 g for 20 min (at 4 °C).
The aqueous phase was carefully transferred (avoiding contamination with the DNA interface) to a fresh tube and an equal volume of isopropanol (-20 °C) was added, and, after vigorous shaking, was placed at -20 °C for ≥ 1 h. The mixture was then centrifuged (as above), the supernatant was decanted and the pellet was dissolved in 0.3 ml solution D. Nucleic acid was precipitated in a sterile Eppendorf tube by addition of an equal volume of ice cold isopropanol. After precipitation at -20 °C for ≥ 1 h, the samples were centrifuged in a microfuge (top speed for 10 min at 4 °C). The pellet was washed with 1 ml 75% ethanol (-20 °C) followed by centrifugation (twice, as above). The supernatant was removed and the pellet was left to air dry for 15 min. The RNA was then dissolved in 0.5 % SDS (0.1 μl/mg wet weight extracted) at 65 °C (dri-block) for 15 min with periodic mixing, then stored at -70 °C.

2.12.2 Spectrophotometric standardisation

The sample was diluted with DEPC-water (1 in 500 and 1 in 1000 dilutions), vortexed. Blanks were 0.5% SDS treated in an identical manner to the sample, then the absorbance was read at 260 and 280 nm. The ratio (1.7-2) was taken as an indication of protein contamination.

2.12.3 Sample preparation for Northern transfer

Solutions:

A) 0.5 M EDTA; pH 8, autoclaved.

B) MOPS-acetate-EDTA buffer (5X MAE); pH 7-7.5: To 0.1 M MOPS, 40 mM sodium acetate and 5 mM EDTA were added.

C) Sample mix (516 μl total volume): To 66 μl 5X MAE buffer, 116 μl formaldehyde, 333 μl formamide and 1 μl ethidium bromide (EtBr; 10 mg/ml solution; Sigma) were added.

D) Gel loading solution (0.2 % bromophenol blue, 1 mM EDTA in 50 % glycerol):
To 0.05 g bromophenol blue (Sigma), 50 µl of 0.5 M EDTA, 12.5 ml glycerol (Sigma) and 25 ml DEPC-water were added and stored at 4 °C.

E) SDS (0.5%): autoclaved.

F) Formaldehyde (37%; Sigma).

G) Formamide (Sigma).

F) Agarose (sigma)

method:

A) Gel Preparation

A mixture of 2.67 g agarose, 133.3 ml DEPC-water, 44.4 ml 5X MAE were boiled. When hand hot, 40 ml formaldehyde was added, mixed then poured into the gel tray. The gel tank was filled 2.21 gel buffer (1X MAE Buffer in DEPC-water) and, before adding the sample the gel was pre-electrophoresed for 10 min at 100 V.

B) Sample preparation and electrophoresis

To 48 µl sample mix (with no EtBr), 12 µl sample (diluted to give the required µg in 11 µl) was added. The mixture was heated at 55 °C for 15 min., then placed on ice. Then 6 µl gel loading solution was added. After mixing, the gel was loaded with 60.5 µl into each well. Another set of samples were prepared (but with EtBr, to ensure that equal amounts of RNA extract was applied in each lane) in the same manner. The gel was run at 100 V for 2-3h at room temperature. The two ribosomal RNAs, 28s and 18s exhibited a ratio of approximately 2:1 (Fig. 18).

2.12.4 Northern transfer

Solutions:

A) Blotting buffer: Saline-sodium phosphate-EDTA buffer (SSPE; 20X): 0.2 M phosphate buffer, pH 7.4, containing 2.98 M NaCl and 0.02 M EDTA, autoclaved.
B) Hybond-N nylon membrane (Amersham).
C) Whatman 3MM paper (46 x 57 cm).
D) Staining solution: EtBr (0.5 µg/ml) in 0.1 M ammonium acetate.

Method:
Over a glass dish filled with 1L 20X SSPE, a glass plate (platform) was placed. The glass plates were then covered with a triple layer of wick (Whatman paper) and left to saturate with 20X SSPE. The gel (with no EtBr) was placed on the wick and surrounded with cling film to avoid short circuiting of the blotting buffer. An exact size of nylon membrane (wet with 20X SSPE) was carefully placed on the gel, avoiding air bubbles, then covered with 3 wet (20X SSPE) sheets of Whatman paper, cut to the same size as the membrane. A stack of hand towels was placed on the top, followed by a glass plate and finally a 0.5 kg weight. The transfer was allowed to proceed overnight. RNA was fixed to the membrane by baking in the oven at 80 °C for 2 h. The membranes were stored in Whatman paper under vacum (dessicator) at room temp until used.

2.12.5 RNA prehybridisation/hybridisation

Solutions:
A) Saline-sodium phosphate-EDTA buffer (SSPE, 20X concentrate).
B) 2X SSPE.
C) Dextran sulphate (Sigma) (28.57% w/v in 17.14X SSPE), stored at -20 °C.
D) Denhardt's reagent, 50X concentrate (1 % w/v BSA, Ficoll and polyvinylpyrrolidone): reconstituted lyophilizate (Sigma) with DEPC-water and stored at -20 °C.
E) Formamide (Sigma). To deionise, 200 ml was added to 10 g AG501-X8(D) mixed bed resin (Sigma) and stirred for 1 h. This was filtered through Whatman no. 1 paper to remove the resin and stored at 4 °C in the dark.
F) DNA (salmon testis DNA; 10 mg/ml; sonicated, denatured, phenol-chloroform extracted) (Sigma), stored at -20 °C.

G) SDS (10%); autoclaved.

H) Prehybridisation solution (freshly prepared; 10 ml): 6X SSPE, 2X Denhardt's reagent, 5% Formamide, 20 μg/ml DNA (boiled for 5 min and chilled on ice before addition) and 1.58 ml DEPC-water.

I) Hybridisation solution (freshly prepared; 5 ml): 10% (Dextran/SSPE), 2X Denhardt's reagent, 50% formamide, 20 μg/ml DNA (boiled for 5 min and chilled on ice)

J) Washing solutions:
   i) 2X SSPE-0.1% SDS
   ii) 1X SSPE-0.1% SDS
   iii) 0.1X SSPE-0.1% SDS

Method:

Prehybridisation was carried out in prehybridisation solution overnight at 42 °C. After which, prewarmed hybridisation solution (5 ml) containing denatured labelled probe (see section 2.12.6) were added and hybridisation was carried out at 42 °C overnight. Both prehybridisation and hybridisation were performed in a hybridisation oven (Hybaid; Teddington, Middlesex).

After the hybridisation process, the membrane was washed twice with 2X SSPE-0.1% SDS at room temperature for 10 min. This process was repeated twice at 55 °C (20 min pre wash). Subsequently, the membrane was washed with 1X SSPE-0.1% SDS, twice, then with 0.1X SSPE-0.1% SDS, twice, at 55 °C for 20 min. The final wash was carried out at 60 °C with 0.1X SSPE-0.1% SDS for 30 min. All washing solutions were prewarmed and the washing processes were performed with gentle shaking. Radioactivities on the membrane and in each washing solution were monitored with hand-held minimonitor. When washing was complete, the filter was wrapped in clingfilm, then placed in an autoradiography cassette with an intensifying screen (Hyperscreen,
Amersham) and exposed to preflashed X-ray film (Hyperfilm-MP, Amersham) at -70 °C for seven days.

2.12.6 Probe labelling: Megaprime DNA labelling system

Solutions:
A) Megaprime DNA labelling kit (Amersham); for use with radiolabelled dCTP
B) [α-32P]dCTP (3000 Ci/mmol; ICN 39011X or Amersham AA 0005).
C) TE buffer, pH 8 (1 mM EDTA in 10 mM Tris-HCl)

Method:
The probes (GLUT 1 and/or GLUT 3) concentration were adjusted to 5 ng/μl in TE buffer. To 25 ng (5 μl) probe, 5 μl primer solution was added, followed by heating to 95-100 °C for 5 min (boiling water bath) then incubation at 65 °C for 5 min. The mixture was allowed to cool to room temperature then briefly centrifuged (microfuge). To this mixture, 10 μl labelling buffer (dATP, dGTP and dTTP in Tris/HCl pH 7.5, 2-mercaptoethanol and MgCl₂), 2 μl enzyme (polymerase 1 Klenow), 23 μl water and 5 μl radiolabel were added, mixed then incubated at 37 °C for 30 min.

The labelled probe was immediately purified (section 2.12.7) to remove unincorporated 32P-nucleotide. To determine the specific activity of the probe, precipitation by TCA (section 2.12.8) was performed. After purification the fractions containing radiolabelled DNA were combined and denatured by heating to 95-100 °C for 5 min (boiling water bath) followed by rapid chilling on ice. The denatured probe was then added to hybridisation solution. Specific activity was usually greater 1.5 x 10⁹ dpm/μg.
2.12.7 Column purification of probe

Solutions:

A) Pharmacia NICK Column (17-0855-01)

B) TE buffer, pH 7.5

C) Scintillation fluid: Ecoscint A (National Diagnostics).

Method:

After removing the excess fluid, the column was rinsed once, then equilibrated with 3 ml TE buffer. Then equilibration buffer was allowed to enter the column after which the sample with TE buffer were applied (total volume 200 μl). After collecting the elute (200 μl, tube 1), a further 200 μl TE buffer was applied and the elute was collected (200 μl, tube 2). A total of 8 applications of TE buffer (150 μl each) were then eluted and collected (150 μl, tubes 3-10). This was followed by further 3 elutions with TE buffer (1 ml each), and collecting (1 ml, tubes 11-13). Radioactivity was counted in Scintillation counter.

2.12.8 Determination of incorporation by TCA precipitation

Solution:

A) 10% (w/v) TCA at 4 °C.

B) Glass fibre A discs (Sigma, 2.5 cm diameter).

Method:

In a Bijou bottle, the following were added, 2 μl reaction mix, 203 μl water and 50 μl carrier DNA, mixed well, then 5 μl were spotted on a glass fibre disc in a plastic scintillation vial and counted after addition of 10 ml scintillant (scintillation counter). To the remaining mix, 2 ml TCA was added and kept on ice for 15 min. The precipitated DNA was collected by vacuum filtration onto a glass fibre disc. The disc was washed 6 times.
with ice-cold TCA (1 ml), allowed to dry (10 min), then prepared for liquid scintillation counting.

2.12.9 Prehybridisation/hybridisation: oligonucleotide probe

The solutions were prepared in the same way as in section (2.12.5). The prehybridisation (5 ml) and hybridisation (10 ml) mixes consisted of 6X SSPE, 5X Denhart's reagents, and 50 μg/ml DNA. To the prewarmed hybridisation solution, 1 pmol/ml probe (see 3' end labelling in section 2.12.9.1) was added. The processes of prehybridisation and hybridisation were carried out in the same way as earlier (section 2.12.5) except the hybridisation temperature was 68 °C.

Washing solutions:

A) 6X SSPE

B) 6X SSPE-0.1% SDS

Method:

The membrane was washed twice with 6X SSPE at room temperature for 10 min, then twice with 6X SSPE-0.1% SDS at 68 °C for 10 min. Finally the membrane was washed once with 6X SSPE-0.1% SDS at 78 °C for 2 min. After warping membrane in a cling film it was autoradiographed (at room temperature, without screen using unflashed film) for four days.

2.12.9.1 Probe labelling: 3'-end labelling

Solutions:

A) 3'-End labelling kit (Amersham).

B) [α-32P]ATP (3000 Ci/mmol; Amersham)
Method:

In a sterile microfuge tube the following were added, 5 µl probe (5 pmol/µl), 0.5 µl Caryodyllate buffer, 7.5 µl [γ-32P]ATP (25.5 pmol), 2.5 µl terminal transferase and 7.5 µl DEPC-water. The mixture was incubated at 37 °C for 2 h. The labelled probe was purified same as earlier (2.12.7) except for all 15 fractions, 200 µl were collected.

2.12.10 Membrane stripping

The hybridised membrane was placed in a boiling solution (100 ml) of SDS (0.1% w/v in DEPC-water), and allowed to cool to room temperature whilst shaking. The membrane was autoradiographed for seven days to ensure that all the radioactivity had been removed.

2.12.11 Polymerase chain reaction (PCR)

Solutions:

A) Sterile distilled water.

B) GeneAmp PCR Core Reagents (Perkin-Elmer). Contains:

- AmpliTaq DNA Polymerase (5U/µl); dATP, dCTP, dGTP and dTTP (all 10 mM);
- 10X PCR Buffer II (500 mM KCl/100 mM Tris-HCl, pH 8.3); MgCl₂ (4 mM).

C) Primers: diluted with water to give 100 µM.

D) Plasmid template; GLUT 1 and/or GLUT 3 (ATCC, U.S.A).

E) AmpliWax PCR Gem 100 (Perkin-Elmer).

Method:

Human GLUT 1 and GLUT 3 cDNA were used. The former comprised the complete coding sequence plus upstream/downstream coding sequences (2.47 kb insert) in the vector PGEM3. The latter comprised the complete coding sequence plus upstream/downstream coding sequences (2.6 kb insert) in PBluescript SK-+. Plasmids were linearised before PCR. For GLUT 1 sequence, the freeze dried plasmid DNA (200 ng) was rehydrated in 17 µl water, to which 10X REact 4 bufer (20 mM Tris-HCl; pH 7.4, 5 mM MgCl₂ and 50 mM
KCl), and 1μl Smal (8-12 units; GIBCOBROL) were added. The mixture was incubated at 30 °C overnight. For GLUT 3, freeze dried plasmid (2 μg) was rehydrated in 20 μl water. To 1 μl of this a further 16 μl water followed by 2 μl of 10X REact 10 bufer (100mM Tris-HCl; pH 7.6, 10 mM MgCl₂, 150 mM NaCl) and 1 μl Sal I (10 units; GIBCOBROL). This was incubated at 37 °C overnight. The primers used for amplifying GLUT 1 were 3'CGTCGACACAGACCGT5' and 5'CCTTCGATGAGATCTG3'. The first primer will anneal to nucleotide from 1056 to 1070, and the second primer anneals to nucleotide from 1586 to 1600, resulting in the production of 545 base pair product. The primers used for amplifying GLUT 3 were 5'CAGAACCCTATGTAG3' and 3'TGTGCCCGGAAACTT5'. The first primer will anneal to nucleotide from 949 to 963, and the second anneals from 1633 to 1647, resulting in the production of 699 base pair. The PCR reaction was carried out in a programmable thermal cycler (Hybaid, model TR1). In a sterile tube the following were mixed; PCR buffer II (1.25X), Tris/KCl (12.5 mM/62.5 mM), MgCl₂ (4 mM), dATP (200 μM), dCTP (200 μM), dGTP (200 μM), dTTP (200 μM), primer 1 (1μM) and primer 2 (1 μM). One AmpliWax PCR gem was added and tubes were incubated at 80 °C for 5 min (to melt the wax), then cooled down to room temp (wax solidifies). A prepared mixture of (water (51 μl), 10X PCR buffer II (10 μl), template (10 μl; digested plasmid [100 pg Glut 1, equivalent to 10 pg target or 80 pg GLUT 3, equivalent to 10 pg target]), ampliTaq DNA Polymerase (0.5 μl; 5 U/μl;) was added at the top of the wax. The reaction mix was then placed in hybaid thermal reactor. The PCR reaction cycles were as follows:

1) 1 cycle: 4 min at 94 °C
2) 25 cycles:
   1 min at 94 °C
   1 min at 41 °C (GLUT 1) or 39 °C (GLUT 3)
   2 min at 72 °C
3) 1 cycle: 8 min at 72 °C
4) 1 cycle: 1 min at 30 °C
2.12.11.1 Analysis of PCR product: Agarose gel electrophoresis

Solutions:

A) MetaPhor agarose (FMC 50181; FlowGen).
B) Tris-borate-EDTA (TBE) buffer pH 8.3; (5X: 10 mM EDTA in 0.445 M Tris borate; sterile).
C) Ethidium bromide (10 mg/ml).
D) Gel-loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF and 15% Ficoll [Type 400; Pharmacia] in water).
E) DNA markers; 100 bp ladder (range 100-1500 bp; Gibco).

Method:
The agarose gel (1% w/v) was dissolved in 0.5 X TBE buffer (50 ml), boiled and after cooling (65 °C), 2.5 μl EtBr was added. Before pouring into the gel tray, gel loading buffer (2 μl) was added to PCR product (10 μl sample, ca. 100 ng DNA), or size marker (diluted 1 : 2), then 10 μl were applied to wells. The gel was run at 80-100 V for 1 h, then visualised under uv light and photographed (Fig. 19a,b).

2.12.11.2 Purification of PCR product

Solution:

A) TE buffer pH 7.5; (10 mM Tris-HCl, containing 1 mM EDTA pH 8).

Method:
The PCR product was purified using microcon-30 microconcentrators (Amicon). PCR product was diluted with TE buffer (0.5 ml), applied to microconcentrators then centrifuged (microfuge) for 12 min. The filtrate was discarded and this process was repeated three times. Then the sample reservoir was placed upside down in a clean vial.
(with cap removed) and centrifuged for 20 sec. The sample reservoir was then washed out with 20 µl TE and centrifuged for 20 sec. The wash was then combined.

2.12.11.3 Quantification of purified PCR product

solution:

A) TE buffer pH 7.5
B) TE buffer pH 7.5, containing 2 µg/ml ethidium bromide (TE/EtBr):
C) DNA standard (salmon testes DNA) solutions: 0 - 20 µg/ml in TE buffer;
standardised by spectrophotometry (A260 = 1 for a 50 µg/ml solution of DNA).

Method:
The purified PCR product concentration was determined by spectrophotometry at an absorbance of 260 nm. Then aliquots of the sample were diluted with TE to give 5 and 10 µg/ml solutions. Aliquots of TE/EtBr and sample or standard were mixed and checked on the top of uv transilluminator to confirm spectrophotometer readings (Fig. 19c).

2.13 Statistical analysis

All results were analysed for statistical significance using Two-way ANOVA test and post-hoc test. A p value of < 0.05 was taken as the minimum level of significance.
Figure 18: Gel electrophoresis of total RNA isolated from fetal brain from N and TX dams. lane 1) 16 dg N, lane 2) 16 dg TX, lane 3) 19 dg N, lane 4) 19 dg TX, lane 5) 22 dg N, lane 6) 22 dg TX.
Figure 19: Amplification of GLUT 1 and GLUT 3 cDNA by PCR.

a) Gel electrophoresis; lane 1) DNA size marker (100 bp), lane 2) GLUT 1 (20 μg/ml).

b) Gel electrophoresis; lane 1) DNA size marker (100 bp), lane 2) GLUT 3 (20 μg/ml).

c) Estimation of GLUT 1 concentration by visualisation on uv transilluminator, lane 1) 20 μg/ml, lane 2) 17.5 μg/ml, lane 3) 15 μg/ml, lane 4) 12.5 μg/ml, lane 5) 10 μg/ml, lane 6) 7.5 μg/ml, lane 7) 5 μg/ml, lane 8) 2.5 μg/ml, lane 9) 1 μg/ml, lane 10) 0 μg/ml.
CHAPTER THREE: RESULTS
3.1 Introduction

A variety of clinical and biochemical investigations support a role for maternal TH in fetal brain development (section 1.8.2). Central to these studies has been the development of progenies from hypothyroxinemic rat dam (TX) models. Preliminary characterisation of this model has revealed that maternal hypothyroxinemia is associated with a range of behavioural disturbances in adult progeny analogous to that suffered by offspring of hypothyroxinemic women in both iodine-deficient and iodine-sufficient environments. Although the biochemical disturbances underlying this CNS dysfunction have been investigated in some detail, studies have largely centred on juvenile and adult progeny of TX rat dams, with little attention being paid to the fetal brain. The purpose of this project was to concentrate on the biochemistry of fetal brain, particularly those stages of development both preceding and following the onset of fetal TH synthesis.

Proteins generally constitute somewhat more than half of the total dry mass of the cell, and their synthesis is central to cell maintenance, growth, and development. Protein synthesis is usually considered to start with the copying of DNA to messenger RNA (mRNA) (Alberts et al., 1983). In addition, all biological membranes, including plasma and internal membranes of eukaryotic cells, have a common overall structure; they are assemblies of protein and lipid molecules. While the specific functions of biological membranes are carried out largely by proteins, the basic structure is determined by the lipid bilayer. The nature of the lipid bilayer is of crucial importance for protein function, especially membrane enzymes such as Na+ K+-ATPase and Ca2+ Mg2+-ATPase, and others which depend on the presence of certain lipids for their normal function, for example, protein kinase C. The physical state of the bilayer (fluidity) is of importance and can be greatly affected by the kind of lipid and/or the chain length, or the extent of saturation of the fatty acids. Another determinant factor of membrane fluidity is cholesterol, which is present in a relatively large amount. In addition to its regulatory effect, cholesterol is thought to enhance the mechanical stability of the bilayer. Although changes in the content of brain protein, DNA, RNA, and lipids have been observed in adult progeny of hypothyroxinemic
dams, very little is known regarding the influence of maternal thyroid state on these parameters in the fetal brain. The possible involvement of insufficient placenta in the impairment of the fetal brain development was investigated too. In addition, to determine whether the effect of maternal hypothyroxinemia is specific to the CNS only or has a general effect the fetal liver growth was studied as well. However, because of difficulties in obtaining sufficient amount of liver from 15 dg fetus, fetal liver of only 19 and 22 dg were studied.

3.2 Plasma T4 and T3 levels in dams

Circulating T4 and T3 levels were measured in normal and thyroidectomised dams at 15, 19 and 22 dg. The T4 level was found to be affected by the progression of pregnancy (Fig. 20a,b). In addition an expected treatment effect on the T4 levels is observed; when compared with N dams, thyroidectomy resulted in a large reduction (approximately 70%) in T4 levels at all stages of gestation.

In contrast, circulating T3 concentration was found not to be affected by age (Fig. 21a,b), while a significant treatment effect, though not expected, is observed. When compared with N dams, the plasma T3 level is also reduced, albeit to a lesser extent (approximately 50%) than T4 levels, by maternal hypothyroxinemia at all gestational stages studied. Similar observations were reported by Pickard et al (1993) using the same animal model (partially thyroidectomised rats).

The aforementioned findings indicate that the animals used in this study were hypothyroxinemic, as 30% of T4 and 50% of T3 were still present in the circulation, and their thyroid state is somehow similar to that found in endemic cretinism.
Figure 20: Plasma T4 concentration in N and TX dams.

a) Ontogenic profile

Values are mean ± SEM (n ≥ 5).

b) Statistical analysis: two-way ANOVA

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**Figure 21**: Plasma T3 concentration in N and TX dams.

a) **Ontogenic profile**

![Graph showing Plasma T3 concentration](image)

Values are mean ± SEM (n ≥ 5).

b) **Statistical analysis: two-way ANOVA**

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3.3 Reproductive performance of dams

The number of fetuses obtained from normal rats averaged approximately 12 per dam. This has significantly affected by treatment (Fig. 22a,b), in which litter size number is reduced to about only 6 to 7 in TX dams at all gestational periods studied. Similar reduction in the number of fetuses was also reported for hypothyroid and hypothyroxinemic rats (Morreale de Escobar et al., 1985; Porterfield and Hendrich, 1991; Ruiz de Ona et al., 1991; Pickard et al., 1993).

3.4 Effect of maternal hypothyroxinaemia on growth

3.4.1 Somatic growth

The fetal body weight, when examined by the two-way ANOVA test, was found to be significantly affected by age, but no treatment effect was observed (Fig. 23a,b).

3.4.2 Brain growth

As expected, fetal brain weight of the control group was significantly increased by age. A similar ontogenic pattern was observed for the experimental group, but the increase is slightly delayed with respect to controls. A significant treatment-related effect is also observed (Fig. 24a,b).

The fetal brain DNA content (cell number index) was found to be significantly affected by gestational age, but no treatment related effect was observed (Fig. 25a,b). Consistent with the recent study of Pickard et al., (1993) using the same animal model, maternal thyroidectomy was found to cause a significant reduction (53%; \( p < 0.05 \)) in cell number at 15 dg, after which no significant effect was observed (Fig. 25c). No ontogenic changes were observed for the fetal brain DNA concentration (cell density index), protein:DNA ratio and protein concentration over the study period. Maternal hypothyroxinemia, on the other hand, resulted in a significant increase (24%; \( p < 0.05 \)) in cell density at 22 dg (Table 1). This increase in late gestation was also observed by Pickard...
et al (1993) in fetuses from hypothyroxinemic dams. In addition, maternal hypothyroxinemia was associated with significant reduction (34%; \( p < 0.05 \)) in cell size at this stage of pregnancy, whereas protein concentration remained within normal values (Table 1). Thus, maternal hypothyroxinemia results in a slight delay in fetal brain growth. Before the onset of fetal TH synthesis, brain cell number has decreased and, although this was subsequently normalised, an increase in brain cell density along with decreased cell size has appeared near term.

An age but no treatment related effects were observed for the RNA concentration with highest values at 15 dg, which was then declined (Fig. 26a,b). A similar effect was also observed for the RNA:DNA ratio (Fig. 27a,b). Maternal hypothyroxinemia resulted in isolated reduction in both the RNA concentration (45%; \( p < 0.05 \)) and the RNA:DNA ratio (55%; \( p < 0.05 \)) at 19 dg.

The significant reduction in the RNA concentration (representing reduction in the ribosomal number) in the fetal brain from TX dam at 19 dg relative to the control did not match any reduction in protein concentration. This may indicate a reduction in the half life of RNA molecule concomitant with an increase in the turnover, which may explain the constant values for the protein concentration obtained from the experimental group relative to the control.

3.4.2.1 SDS-Polyacrylamide gel electrophoresis

The expression of certain proteins in brain and other tissues is controlled by TH (section 1.7.3). To investigate the susceptibilities of different fetal CNS proteins to maternal hypothyroxinaemia at early stages of development, SDS-PAGE was performed. Fetal brain from TX dams exhibited broadly similar pattern of proteins to controls at all gestational stages studied (Fig. 28a,b). This indicates that maternal hypothyroxinemia does not affect the expression of major fetal brain proteins. However using this technique, we were unable to detect any possible changes in the minor proteins which may have an adverse effect on the fetal CNS development.
Figure 22: The number of viable fetuses from N and TX dams.

a) Ontogenic pattern

Values are means ± SEM (n ≥ 5).

b) Statistical analysis: two-way ANOVA

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Figure 23: Fetal body wet weight from N and TX dams.

a) Ontogenic pattern

![Graph showing fetal body wet weight](chart)

Values are means ± SEM (n ≥ 5).

b) Statistical analysis: two-way ANOVA

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Figure 24: Fetal brain wet weight from N and TX dams.

a) Ontogenic pattern

![Bar graph showing fetal brain wet weight from N and TX dams.](image)

Values are means ± SEM (n ≥ 5).

b) Statistical analysis: two-way ANOVA

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120
Figure 25: DNA content in fetal brain from N and TX dams.

a) Ontogenic pattern

Values are means ± SEM (n ≥ 5).

b) Statistical analysis; two-way ANOVA

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c) *Post-hoc* analysis (Fisher PLSD test)

* p < 0.05, 15N vs 15TX
Table 1: Protein concentration, DNA concentration and protein:DNA ratio in fetal brain from N and TX rat dams.

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<tr>
<th>Age (days gestation)</th>
<th>Dam</th>
<th>Protein concentration (mg/g)</th>
<th>DNA concentration (mg/g)</th>
<th>Protein:DNA ratio (mg/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>N</td>
<td>55.38 ± 4.3</td>
<td>3.88 ± 0.8</td>
<td>15.58 ± 1.6</td>
</tr>
<tr>
<td></td>
<td>TX</td>
<td>52.31 ± 8.8</td>
<td>3.20 ± 0.3</td>
<td>17.64 ± 3.6</td>
</tr>
<tr>
<td>19</td>
<td>N</td>
<td>48.26 ± 3.8</td>
<td>3.47 ± 0.4</td>
<td>15.11 ± 2.8</td>
</tr>
<tr>
<td></td>
<td>TX</td>
<td>48.93 ± 2.7</td>
<td>4.17 ± 0.5</td>
<td>12.32 ± 1.3</td>
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<tr>
<td>22</td>
<td>N</td>
<td>49.11 ± 1.8</td>
<td>2.28 ± 0.1</td>
<td>22.37 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>TX</td>
<td>47.28 ± 1.9</td>
<td>3.66 ± 0.3*</td>
<td>14.84 ± 0.8*</td>
</tr>
</tbody>
</table>

Values are the mean of ± SEM (n ≥ 5), *p < 0.05. Fisher PLSD test. No age or treatment-related effects were observed by the two-way ANOVA.
Figure 26: RNA concentration in fetal brain from N and TX dams.

a) Ontogenic pattern

Values are means ± SEM (n ≥ 5).

b) Statistical analysis: two-way ANOVA

<table>
<thead>
<tr>
<th>Source</th>
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<th>Sum of Squares</th>
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<th>F-test</th>
<th>P value</th>
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</thead>
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<td>.0001</td>
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<tr>
<td>Treatment (B)</td>
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<td>9.12</td>
<td>1.28</td>
<td>.2689</td>
</tr>
<tr>
<td>A+B</td>
<td>2</td>
<td>30.26</td>
<td>15.13</td>
<td>2.13</td>
<td>.1441</td>
</tr>
</tbody>
</table>

c) Post-hoc analysis (Fisher PLSD test)

* p < 0.05, 19N vs 19TX
Figure 27: RNA:DNA ratio in fetal brain from N and TX dams.

a) Ontogenic pattern

Values are means ± SEM (n ≥ 5).

b) Statistical analysis: two-way ANOVA

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>Sum of Squares</th>
<th>Mean Square</th>
<th>F-test</th>
<th>P value</th>
</tr>
</thead>
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<td>16.65</td>
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</tr>
<tr>
<td>Treatment (B)</td>
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<td>1.77</td>
<td>1.66</td>
<td>.2095</td>
</tr>
<tr>
<td>A+B</td>
<td>2</td>
<td>6.56</td>
<td>3.28</td>
<td>3.07</td>
<td>.0647</td>
</tr>
</tbody>
</table>

c) Post-hoc analysis (Fisher PLSD test)

* p < 0.05, 19TX vs 19N
Figure 28: SDS-PAGE analysis of total protein of fetal brain from N and TX dams using (a) Coomasie brilliant blue staining, (b) Silver staining. Lane M) Rainbow™ protein marker, lane 1) 16 dg N, lane 2) 16 dg TX, lane 3) 19 dg N, lane 4) 19 dg TX, lane 5) 22 dg N lane 6) 22 dg TX.
3.4.3 Placental growth

The placental wet weight was found to be significantly affected by gestational age, with no treatment-related effect observed (Fig. 29a,b). Furthermore, marginally significant age-related effects were also observed for the DNA content (Fig. 30a,b) and the protein:DNA ratio (Fig. 31a,b). Maternal hypothyroxinemia on the other hand, caused a significant but isolated reduction (36%; \( p < 0.05 \)) in the protein:DNA ratio at 19 dg compared with the control (Fig. 31c). No such effects were observed for either the protein or the DNA concentrations (Table 2).

In summary, except for the protein:DNA ratio, maternal hypothyroxinemia was found to have no significant effect on placental wet weight, DNA content, protein, and DNA concentration at any gestational age studied.

The placental RNA concentration and the RNA:DNA ratio showed no age or treatment-related effects. While, the post-hoc test indicated that maternal hypothyroxinemia significantly increased (46%; \( p < 0.05 \)) the RNA concentration in the placentae of the TX dams relative to the control at 22 dg. Another isolated maternal hypothyroxinemia effect was observed for the RNA:DNA ratio, manifested by the significant decrease (59%; \( p < 0.05 \)) at 19 dg compared to the normal (Table 3).

Therefore, the main effects of maternal hypothyroxinemia on placental growth were a reduction in protein:DNA and RNA:DNA ratios at 19 dg, as well as a significant increase in the RNA concentration at 22 dg. This may indicate a reduction in the cell size at 19 dg, which was compensated for near term. No other changes were observed.
Figure 29: Placenta wet weight from N and TX dams.

a) Ontogenic pattern

Values are means ± SEM (n ≥ 5).

b) Statistical analysis: two-way ANOVA

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>Sum of Squares</th>
<th>Mean Square</th>
<th>F-test</th>
<th>P value</th>
</tr>
</thead>
<tbody>
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<td>102.3</td>
<td>.0001</td>
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<td>Treatment (B)</td>
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<td>.01</td>
<td>4.9</td>
<td>.355</td>
</tr>
<tr>
<td>A+B</td>
<td>2</td>
<td>.01</td>
<td>.003</td>
<td>1.1</td>
<td>.3341</td>
</tr>
</tbody>
</table>
Figure 30: DNA content in placenta from N and TX dams.

a) **Ontogenic pattern**

![Graph showing DNA content in placenta from N and TX dams.](image)

Values are means ± SEM (n ≥ 5).

b) **Statistical analysis: two-way ANOVA**

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>Sum of Squares</th>
<th>Mean Square</th>
<th>F-test</th>
<th>P value</th>
</tr>
</thead>
<tbody>
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<td>.06</td>
<td>3.8</td>
<td>.037</td>
</tr>
<tr>
<td>Treatment (B)</td>
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<td>.02</td>
<td>1.38</td>
<td>.2513</td>
</tr>
<tr>
<td>A+B</td>
<td>2</td>
<td>.02</td>
<td>.01</td>
<td>.78</td>
<td>.4696</td>
</tr>
</tbody>
</table>

128
Figure 31: Protein:DNA ratio in placenta from N and TX dams.

a) Ontogenic profile

Values are mean ± SEM (n ≥ 5).

b) Statistical analysis: two-way ANOVA

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>Sum of Squares</th>
<th>Mean Square</th>
<th>F-test</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
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<td>2483.26</td>
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<td>.046</td>
</tr>
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<td>Treatment (B)</td>
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<td>1043.88</td>
<td>2.95</td>
<td>.0986</td>
</tr>
<tr>
<td>A+B</td>
<td>2</td>
<td>1078.35</td>
<td>539.18</td>
<td>1.52</td>
<td>.238</td>
</tr>
</tbody>
</table>

c) Post-hoc analysis (Fisher PLSD test)

* p < 0.05, 19TX vs 19N
Table 2: Protein concentration, DNA concentration and protein:DNA ratio in placenta from N and TX rat dams.

<table>
<thead>
<tr>
<th>Age (days gestation)</th>
<th>Dam</th>
<th>Protein concentration (mg/g)</th>
<th>DNA concentration (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>N</td>
<td>35.12 ± 5.71</td>
<td>0.77 ± 0.12</td>
</tr>
<tr>
<td></td>
<td>TX</td>
<td>39.22 ± 3.20</td>
<td>1.06 ± 0.18</td>
</tr>
<tr>
<td>19</td>
<td>N</td>
<td>34.36 ± 1.17</td>
<td>0.53 ± 0.14</td>
</tr>
<tr>
<td></td>
<td>TX</td>
<td>30.72 ± 1.02</td>
<td>0.87 ± 0.26</td>
</tr>
<tr>
<td>22</td>
<td>N</td>
<td>36.14 ± 1.10</td>
<td>0.58 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>TX</td>
<td>39.92 ± 3.53</td>
<td>0.67 ± 0.08</td>
</tr>
</tbody>
</table>

Values are the mean of ± SEM (n ≥ 5). No age or treatment-related effects were observed by the two-way ANOVA.
Table 3: RNA concentration and RNA:DNA ratio in placenta from N and TX rat dams.

<table>
<thead>
<tr>
<th>Age (days gestation)</th>
<th>Dam</th>
<th>RNA concentration (mg/g)</th>
<th>RNA:DNA ratio (mg/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>N</td>
<td>4.18 ± 0.53</td>
<td>5.84 ± 0.84</td>
</tr>
<tr>
<td></td>
<td>TX</td>
<td>5.29 ± 0.92</td>
<td>4.99 ± 0.21</td>
</tr>
<tr>
<td>19</td>
<td>N</td>
<td>5.55 ± 1.13</td>
<td>11.96 ± 3.38</td>
</tr>
<tr>
<td></td>
<td>TX</td>
<td>2.95 ± 0.08</td>
<td>4.83 ± 1.22*</td>
</tr>
<tr>
<td>22</td>
<td>N</td>
<td>3.58 ± 0.19</td>
<td>6.18 ± 0.23</td>
</tr>
<tr>
<td></td>
<td>TX</td>
<td>6.63 ± 1.60*</td>
<td>10.68 ± 3.28</td>
</tr>
</tbody>
</table>

Values are the mean of ± SEM (n ≥ 5), *p < 0.05, Fisher PLSD test. No age or treatment-related effects were observed by the two-way ANOVA.
3.4.4 Liver growth

Fetal liver weight showed significant age and treatment-related effects (Fig. 32a,b). The fetal liver DNA content expressed opposite ontogenic patterns for the N and TX groups, with no consistent treatment effect (Fig 33a,b). These differences in the ontogenic pattern may explain the observed combined age and treatment-related effects. When compared with the control, the DNA content in the experimental group showed a significant increase (46%; \( p < 0.01 \)) at 19 dg, which was subsequently decreased (35%; \( p < 0.05 \)) at 22 dg (Fig 33c). Measurement of DNA concentration indicated significant age, treatment, and combined age and treatment-related effects (Fig. 34a,b). In N animals, the liver DNA concentration was unchanged between 19 and 22 dg. This, however, was not the case for the TX group in which the significant decrease (57%) observed between 19 and 22 dg. Maternal thyroidectomy, on the other hand, was found to significantly elevate (48%) the DNA concentration at 19 dg relative to the control liver. Protein concentration on the other hand, was found to be affected by treatment only (Fig. 35a,b).

With respect to the liver protein:DNA ratio, age and combined age and treatment-related effects were observed. The combined effect is sharper than the age effect alone, which could be explained by the differences in the ontogenic pattern for the N and TX groups (Fig. 36a,b). In addition, the TX group when compared with the control showed a significant decrease (43%; \( p < 0.05 \)) at 19 dg, followed by a significant increase (58%; \( p < 0.01 \)) at 22 dg. Measurement of the fetal liver RNA concentration, indicated that it was unaffected by either development or treatment (Table 4). However, this was not the case for the RNA:DNA ratio where age-related as well as combined age and treatment-related effects were observed (Fig. 37a,b). With respect to the latter it is more significant than the age effect alone. The findings were similar to those observed for protein:DNA ratio. In the N groups the values were similar at 19 and 22 dg, but in the experimental group, the values were depressed (63%; \( p < 0.05 \)) relative to the controls at 19 dg, then increased to reach their 22 dg level which was higher (68%; \( p < 0.05 \)) than the controls.
It can therefore be concluded that maternal hypothyroxinemia affected fetal liver growth in both gestational ages studied. This was manifested by the observed changes in all biochemical parameters investigated except the RNA concentration.
Figure 32: Fetal liver wet weight from N and TX dams.

a) Ontogenic pattern

Values are means ± SEM (n ≥ 5).

b) Statistical analysis: two-way ANOVA

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>Sum of Squares</th>
<th>Mean Square</th>
<th>F-test</th>
<th>P value</th>
</tr>
</thead>
<tbody>
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<td>Treatment (B)</td>
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<td>.01</td>
<td>.01</td>
<td>6.73</td>
<td>.0195</td>
</tr>
<tr>
<td>A+B</td>
<td>1</td>
<td>.01</td>
<td>.01</td>
<td>9.52</td>
<td>.0071</td>
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</tbody>
</table>
Figure 33: DNA content in fetal liver from N and TX dams.

a) Ontogenic pattern

![Graph showing DNA content in fetal liver from N and TX dams.](image)

Values are means ± SEM (n ≥ 5).

b) Statistical analysis: two-way ANOVA

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>Sum of Squares</th>
<th>Mean Square</th>
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<td>.11</td>
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<td>.2176</td>
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<tr>
<td>A+B</td>
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<td>4.68</td>
<td>4.68</td>
<td>69.95</td>
<td>.0001</td>
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</tbody>
</table>

c) Post-hoc analysis (Fisher PLSD test)

** p < 0.01, 19TX vs 19N
* p < 0.05, 22N vs 22TX
Figure 34: DNA concentration in fetal liver from N and TX dams.

a) Ontogenic pattern

Values are means ± SEM (n ≥ 5).

b) Statistical analysis: two-way ANOVA

<table>
<thead>
<tr>
<th>Source</th>
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<td>.0001</td>
</tr>
<tr>
<td>A+B</td>
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<td>59.83</td>
<td>59.83</td>
<td>35.07</td>
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</tr>
</tbody>
</table>
Figure 35: Protein concentration in fetal liver from N and TX dams.

a) Ontogenic pattern

![Graph showing protein concentration over gestation days.]

Values are means ± SEM (n ≥ 5).

b) Statistical analysis: two-way ANOVA

<table>
<thead>
<tr>
<th>Source</th>
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<td>Treatment (B)</td>
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<td>537.89</td>
<td>8.36</td>
<td>.0106</td>
</tr>
<tr>
<td>A+B</td>
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<td>1.5</td>
<td>1.5</td>
<td>.02</td>
<td>.8805</td>
</tr>
</tbody>
</table>
Figure 36: Protein:DNA ratio in fetal liver from N and TX dams.

a) Ontogenic pattern

Values are means ± SEM (n ≥ 5).

b) Statistical analysis: two-way ANOVA

<table>
<thead>
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<th>Source</th>
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<th>Mean Square</th>
<th>F-test</th>
<th>P value</th>
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<td>62.68</td>
<td>10.48</td>
<td>.0052</td>
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<tr>
<td>Treatment (B)</td>
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<td>.69</td>
<td>.69</td>
<td>.11</td>
<td>.739</td>
</tr>
<tr>
<td>A+B</td>
<td>1</td>
<td>104.56</td>
<td>104.56</td>
<td>17.47</td>
<td>.0007</td>
</tr>
</tbody>
</table>

c) Post-hoc analysis (Fisher PLSD test)

* p < 0.01, 19TX vs 19N and 22N vs 22TX
Table 4: RNA concentration in fetal liver from N TX rat dams.

<table>
<thead>
<tr>
<th>Age (days gestation)</th>
<th>Dam</th>
<th>RNA concentration (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>19</td>
<td>N</td>
<td>15.26 ± 4.06</td>
</tr>
<tr>
<td></td>
<td>TX</td>
<td>9.95 ± 0.78</td>
</tr>
<tr>
<td>22</td>
<td>N</td>
<td>12.21 ± 0.94</td>
</tr>
<tr>
<td></td>
<td>TX</td>
<td>15.88 ± 1.20</td>
</tr>
</tbody>
</table>

Value are mean ± SEM (n ≥ 5). No age or treatment-related effects were observed by the two-way ANOVA.
Figure 37: RNA:DNA ratio in fetal liver from N and TX dams.

a) Ontogenic pattern

![Graph showing RNA:DNA ratio in fetal liver](image)

Values are means ± SEM (n ≥ 5).

b) Statistical analysis: two-way ANOVA

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>Sum of Squares</th>
<th>Mean Square</th>
<th>F-test</th>
<th>P value</th>
</tr>
</thead>
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<td>5.82</td>
<td>7.98</td>
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<td>.03</td>
<td>.04</td>
<td>.8482</td>
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<td>A+B</td>
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<td>10.37</td>
<td>10.37</td>
<td>14.22</td>
<td>.0017</td>
</tr>
</tbody>
</table>

c) Post-hoc analysis (Fisher PLSD test)

* p < 0.05, 19TX vs 19N and 22N vs 22TX
3.5 Effect of maternal hypothyroxinemia on tissue lipids

3.5.1 Brain lipids

Lipids and proteins represent the main components of the cellular membrane, and play an important role in determining the activity of membrane proteins (Alberts et al., 1983); any disruption in lipid content or structure may have drastic effect on cellular function. Therefore, total lipid and cholesterol concentrations were measured in fetal brain to determine the effect of maternal hypothyroxinemia upon these parameters. Age and maternal thyroidectomy had no effect on the total lipid concentration (Fig. 38). The cholesterol concentration on the other hand was significantly affected by age, and by the combined age and treatment factors (Fig. 39a,b). The ontogenic pattern of changes in the control group between 15 and 19 dg is opposite that of the experimental group. However, between 19 and 22 dg both groups followed similar pattern. Furthermore, when compared with the control group maternal hypothyroxinemia had no effect on total lipid concentration. However, it was found to significantly reduce (66%; \( p < 0.01 \)) the fetal brain cholesterol concentration at 15 dg, followed by a significant increase (63%; \( p < 0.01 \)) at 22 dg (Fig. 39c). In short cholesterol concentration in the fetal brain although reduced at 15 dg, showed a tendency to normalisation towards the end of the pregnancy.

3.5.2 Placenta lipids

Total lipid and cholesterol concentrations were determined in placenta of normal and TX dams. Total lipid concentration was found to be stable over the study period in both N and TX dams, and maternal thyroidectomy had no significant effect (Fig. 40). The cholesterol concentration, on the other hand, showed significant age, treatment, and combined age and treatment-related effects (Fig. 41a,b). With respect to the age effect, a biphasic ontogenic pattern was observed for both N and TX dams. Maternal hypothyroxinemia, was found to elevate the placental cholesterol concentration (70%) only at 22 dg relative to the controls. This pattern is similar to that observed for fetal brain cholesterol concentration.

3.5.3 Liver lipids
3.5.3 Liver lipids

Unlike the cholesterol concentration, fetal liver total lipid concentration did not display any age-related effect. However, treatment and combined age and treatment-related effects were observed for both parameters (Fig. 42a,b) and (Fig. 43a,b) respectively. Maternal thyroidectomy, when compared with the controls, affected fetal liver total lipid concentration and cholesterol concentration in a similar way. While no significant changes were observed at 19 dg, total lipid concentration and cholesterol concentration in fetal liver from TX dams displayed significant increases (77% and 119%; respectively) at 22 dg.

From the total lipid and cholesterol results it can be suggested that the cholesterol was more susceptible to maternal hypothyroxinemia, and this has been affected in all tissues studied, mainly at 22 dg. At this age a significant increase in the cholesterol concentration was observed. This increase may be a compensation for the decline observed in the brain early in gestation, which may have existed in the liver as well. The total lipid was also increased at 22 dg, in the liver only.
Figure 38: Total lipid concentration in fetal brain from N and TX dams.

Values are means ± SEM (n ≥ 5).

The two-way ANOVA indicated no significant age or treatment-related effects.
Figure 39: Cholesterol concentration in fetal brain from N and TX dams.

a) Ontogenic pattern

Figure 39: Cholesterol concentration in fetal brain from N and TX dams.

Values are means ± SEM (n ≥ 5).

b) Statistical analysis: two-way ANOVA

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>Sum of Squares</th>
<th>Mean Square</th>
<th>F-test</th>
<th>P value</th>
</tr>
</thead>
<tbody>
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<td>3.03</td>
<td>1.51</td>
<td>4.34</td>
<td>0.0247</td>
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<tr>
<td>Treatment (B)</td>
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<td>.01</td>
<td>.01</td>
<td>.02</td>
<td>.8889</td>
</tr>
<tr>
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c) Post-hoc analysis (Fisher PLSD test)

* p < 0.01, 15N vs 15TX and 22N vs 22TX
Figure 40: Total lipid concentration in placenta of N and TX dams.

Values are mean ± SEM (n ≥ 5).

The two-way ANOVA indicated no significant age or treatment-related effects.
Figure 41: Cholesterol concentration in placenta of N and TX dams.

a) Ontogenic profile

![Ontogenic profile graph]

Values are mean ± SEM (n ≥ 5).

b) Statistical analysis: two-way ANOVA

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Figure 42: Total lipid concentration in fetal liver from N and TX dams.

a) Ontogenic pattern

Values are means ± SEM (n ≥ 5).

b) Statistical analysis: two-way ANOVA

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Figure 43: Cholesterol concentration in fetal liver from N and TX dams.

a) Ontogenic pattern

![Graph showing cholesterol concentration over gestational age.]

Values are means ± SEM (n ≥ 5).

b) Statistical analysis: two-way ANOVA

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3.6 Effect of maternal hypothyroxinemia on tissue phosphate and phosphate metabolic enzymes concentrations.

The presence of adequate free phosphate pool is essential for a wide range of biochemical and metabolic functions, specially during development where a high energy input is necessary to sustain the almost exponential increase in nucleic acid and protein synthesis, which characterise the explosive phases of neural development. Phospholipids are one of the major lipids present in cellular membrane and their presence with proteins is of importance in maintaining cellular structure and function.

Protein phosphorylation is widely recognised as one of the primary modes in regulation of cellular processes (Nakamura and DeGroot, 1983). Protein phosphorylation systems consist of three primary components; a protein kinase, a protein phosphatase and a substrate protein. Protein kinases catalyse the transfers of the terminal phosphate from ATP to serine, threonine, or tyrosine residues of the substrate protein, thus altering its functional properties.

Derangement in phosphate metabolism, or in protein phosphorylation system may therefore critically impinge upon normal fetal development, particularly the CNS. Therefore, the effect of maternal hypothyroxinemia on fetal brain phosphate pool; inorganic phosphate, protein phosphate and lipid phosphate, as well as protein phosphorylation activities were determined in fetal brains of TX dams at 15, 19, and 22 dg. Protein phosphate in the placenta and liver was measured as well.

3.6.1 Inorganic phosphate, protein phosphate and lipid phosphate levels
In fetal brain, neither age nor treatment alone showed any significant effect on the inorganic phosphate concentration. However, the combination of these two factors was found to have a significant effect. This effect appeared to be due to the ontogenetically divergent pattern in the two groups studied (Fig. 44a,b). Indeed the post-hoc test indicated that relative to the control, the inorganic phosphate in the TX group was significantly reduced (86%; p < 0.01) at 15 dg, but this was reversed by the significant increases (70%; p <
0.05) at 19 dg and (68%; \( p < 0.01 \)) at 22 dg (Fig. 44c). Measurement of protein phosphate concentration showed in addition to the age, combined age and treatment-related effects (Fig. 45a,b). The latter effect is largely due to the ontogenetically divergent pattern for the control and the experimental groups. Maternal hypothyroxinemia significantly reduced it (55%; \( p < 0.01 \)) at 15 dg. Subsequently this was significantly increased (39%; \( p < 0.01 \)) at 19 dg. Near term, however, protein phosphate concentration in TX group was within normal levels (Fig. 45c).

The lipid phosphate concentration showed both treatment and combined age and treatment-related effects (Fig. 46a,b). The treatment effect, was manifested by the increases in the lipid phosphate concentration (43% and 45%) at 19 and 22 dg receptively, relative to the control.

Therefore, it can be concluded that maternal hypothyroxinemia significantly affected fetal brain inorganic phosphate pool as well as phosphate metabolism at all gestational stages studied, while no changes were observed for the protein phosphate at 22 dg and for the lipid phosphate at 15 dg.

In the placenta the effects of age, treatment, and the combination of both factors were observed (Fig. 47a,b). The ontogenic pattern of the control group is opposite that of the experimental group and these differences were sharper at 15 and 19 dg. Placental protein phosphate concentration in the TX groups was found to be decreased (91%) at 15 dg relative to the controls. This was reversed by an increase of (82%) at 19 dg. Near term, however, placental protein phosphate concentration appeared to be normal. Measurement of protein phosphate in fetal liver at 19 and 22 dg indicated that it was influenced by age, treatment, and combined age and treatment-related effect (Fig. 48a,b). Compared with the control, maternal hypothyroxinemia substantially increased the fetal liver protein phosphate at 19 dg.
Figure 44: Phosphate pool (inorganic phosphate) concentration in fetal brain from N and TX dams.

a) **Ontogenic pattern**

Values are means ± SEM (n > 5).

b) **Statistical analysis: two-way ANOVA**

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c) **Post-hoc analysis (Fisher PLSD test)**

** * p < 0.01, 15N vs 15TX and 22N vs 22TX

* * p < 0.05, 19N vs 19TX
Figure 45: Protein phosphate concentration in fetal brain from N and TX dams.

a) Ontogenic pattern

![Graph showing protein phosphate concentration in fetal brain from N and TX dams.]

Values are means ± SEM (n ≥ 5).

b) Statistical analysis: two-way ANOVA

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c) Post-hoc analysis (Fisher PLSD test)

* p < 0.01, 15N vs 15TX and 19N vs 19TX
Figure 46: Lipid phosphate concentration in fetal brain from N and TX dams.

a) Ontogenic pattern

Values are means ± SEM (n ≥ 5).

b) Statistical analysis; two-way ANOVA

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<td>104.7</td>
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Figure 47: Protein phosphate concentration in placenta from N and TX dams.

a) Ontogenic pattern

Values are means ± SEM (n ≥ 5).

b) Statistical analysis: two-way ANOVA

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<td>6311314</td>
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Figure 48: Protein phosphate concentration in fetal liver from N and TX dams.

a) Ontogenic pattern

Values are means ± SEM (n ≥ 5).

b) Statistical analysis: two-way ANOVA

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<td>64.8</td>
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3.6.2 **Protein Kinase enzymes**

Amongst others the most widely studied serine/threonine-specific protein kinases are cAMP-dependent protein kinase (PKA) and phospholipid / Ca\(^{2+}\)-dependent protein kinase or protein kinase C (PKC). These enzymes are involved in the regulation of a large number of cellular processes, including cellular differentiation, gene expression, ion channel and receptor activation and neurotransmitter release. Both PKA and PKC are highly expressed in brain tissue (Nestler and Greengard, 1984) and play a role in several brain-specific functions, including neuronal differentiation, and neurite outgrowth (Abraham *et al.*, 1991). In view of the changes observed in fetal brain phosphate levels from TX dams the activity of these two enzymes was monitored in fetal brain to establish the effect of maternal hypothyroxinemia on fetal brain development.

The PKC activity was found to be significantly affected by age (Fig. 49a,b). Analysis of the data by *post-hoc* test revealed that maternal hypothyroxinemia relative to the control have a divergent effect, which is manifested by a significant elevation (44%; *p* < 0.01) in the PKC activity at 15 dg, followed by a significant decrease (24%; *p* < 0.05) at 19 dg, with activities being normal at 22 dg. (Fig. 49c) and (Fig. 50).

An age-related effect was also observed for PKA activity (Fig 51a,b). However, maternal hypothyroxinemia was without effect on the PKA activity at any gestational age studied (Fig. 52).

3.6.3 **Acid phosphatase and Alkaline phosphatase**

In addition to protein kinase activities, the activities of the dephosphorylation enzymes, acid phosphatase and alkaline phosphatase, were measured to help detecting the level of phosphate generation at steady state in control and experimental fetal brain homogenates.

The activities of acid phosphatase and alkaline phosphatase were both affected by age (Fig. 53a,b) and (Fig. 54a,b) respectively. Maternal hypothyroxinemia on the other hand was without effect on the activities of both enzymes at any gestational age studied.
Figure 49: PKC activity in fetal brain from N and TX dams.

a) Ontogenic pattern

Values are means ± SEM (n ≥ 5).

b) Statistical analysis: two-way ANOVA

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c) Post-hoc analysis (Fisher PLSD test)

** p < 0.01, 15TX vs 15N
* p < 0.05, 19TX vs 19N

Figure 50: Gel electrophoresis of PKC in fetal brain from N and TX dams. lane 1) 16 dg N, lane 2) 16 dg TX, lane 3) 19 dg N, lane 4) 19 dg TX, lane 5) 22 dg N, lane 6) 22 dg TX.

phosphorylated
non phosphorylated
Figure 51: PKA activity in fetal brain from N and TX dams.

a) Ontogenic pattern

![Bar graph showing PKA activity in fetal brain from N and TX dams over different ages.]

Values are means ± SEM (n ≥ 5).

b) Statistical analysis: two-way ANOVA

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Figure 52: Gel electrophoresis of PKA in fetal brain from N and TX dams. lane 1) 16 dg N, lane 2) 16 dg TX, lane 3) 22 dg N, lane 4) 22 dg TX, lane 5) 19 dg N, lane 6) 19 dg TX, lane 7) positive control.
Figure 53: Acid phosphatase activity in fetal brain from N and TX dams.

a) Ontogenic pattern

![Ontogenic pattern diagram](image)

Values are means ± SEM (n ≥ 5).

b) Statistical analysis: two-way ANOVA

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<td>37.65</td>
<td>1.04</td>
<td>.3688</td>
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</table>
Figure 54: Alkaline phosphatase activity in fetal brain from N and TX dams.

a) Ontogenic pattern

![Bar chart showing alkaline phosphatase activity in fetal brain from N and TX dams.](image)

Values are means ± SEM (n ≥ 5).

b) Statistical analysis: two-way ANOVA

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3.6.4 Effect of Maternal Hypothyroxinemia on $\text{Na}^+\text{K}^+\text{-ATPase}$ and $\text{Ca}^{2+}\text{Mg}^{2+}\text{-ATPase}$ Activity

The ouabain-sensitive $\text{Na}^+\text{K}^+\text{-ATPase}$ is a key plasma membrane enzyme controlling ion fluxes across cell membranes and plays a particularly important role in the neural cell membrane (Atterwill et al., 1985).

The ouabain-insensitive $\text{Ca}^{2+}\text{Mg}^{2+}\text{-ATPase}$ is also a plasma membrane enzyme and its activity is associated with the activity of calcium pump. This enzyme is regulated by the calcium binding cytoplasmic protein, calmodulin, as well as the lipid composition of the membrane bilayer. The activities of both enzymes were assayed in tissue homogenate of fetal brain from normal and TX dam at 15, 19 and 22 dg to assess the effect of maternal hypothyroxinaemia upon their activities.

The activity of fetal $\text{Na}^+\text{K}^+\text{-ATPase}$ was found to be influenced by age, treatment, and combined age and treatment-related effects (Fig. 55a,b). In deed, maternal hypothyroxinemia compared to the control, severely depressed the activity of this enzyme by (30%, 53%, 46%) at 15, 19, and 22 dg respectively. On the contrary, the activity of $\text{Ca}^{2+}\text{Mg}^{2+}\text{-ATPase}$ in fetal brain was only affected by age and combined age and treatment-related effects (Fig. 56a,b). The latter effect is related to the differences in the ontogenic pattern between the control and the TX groups. In early gestation, maternal hypothyroxinemia when compared with the controls, affected fetal brain $\text{Ca}^{2+}\text{Mg}^{2+}\text{-ATPase}$ activity in a similar way to that for $\text{Na}^+\text{K}^+\text{-ATPase}$; significant reduction (38% and 39%; $p < 0.01$) in the $\text{Ca}^{2+}\text{Mg}^{2+}\text{-ATPase}$ was observed at 15 and 19 dg respectively. At 22 dg, however, a statistically significant increase (73%; $p < 0.01$) in the $\text{Ca}^{2+}\text{Mg}^{2+}\text{-ATPase}$ activity was observed (Fig. 56c).
Figure 55: Na⁺K⁺-ATPase activity in fetal brain from N and TX dams.

a) Ontogenic pattern

b) Statistical analysis: two-way ANOVA

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<td>11416.35</td>
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Values are means ± SEM (n ≥ 5).
Figure 56: Ca\(^{2+}\)Mg\(^{2+}\)-ATPase activity in fetal brain from N and TX dams.

a) Ontogenic pattern

Values are means ± SEM (n ≥ 5).

b) Statistical analysis: two-way ANOVA

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c) Post-hoc analysis (Fisher PLSD test)

* p < 0.01, 15TX vs 15N, 19TX vs 19N and 22TX vs 22N
3.7 Effect of maternal hypothyroxinaemia on fetal brain glucose metabolism

The brain is dependent upon glucose throughout life. This sugar not only serves as the primary energy substrate for the CNS, but is also required for a wide range of biosynthetic reactions, including the synthesis of nucleic acids, fatty acids, amino acids, and certain transmitters. There is abundant evidence that carbohydrate represent the major fetal metabolic substrate in humans and laboratory rats (Porterfield et al., 1975). Therefore, any factor which regulates glucose metabolism may be expected to exert profound effect on the development and homeostasis of the CNS. Indeed early work of Porterfield and Hendrich (1975; 1976) has shown that maternal hypothyroidism reduced fetal serum glucose levels late in gestation; at 22 dg, and a recent study by Pickard et al (1990) showed that 2-deoxy-glucose uptake into neurones in culture is significantly enhanced by TH, and that brain cell slices and synaptosomes obtained from hypothyroid animals have reduced 2-deoxy-glucose uptake. The observed effects of inadequate thyroid hormone environment in utero upon the expression of biochemical functions of the CNS and behavioural output may well be due to an inadequate energy supply. It is therefore necessary to study the effect of TH upon glucose metabolism in the CNS during explosive growth period; when energy requirement is very high and restriction may lead to disastrous consequences. Thus, the effect of maternal hypothyroxinemia on in vitro glucose metabolism in the fetal brain was studied.

Because of the need for a relatively large amount of fetal brain tissue, fetal brain at 16 dg instead of 15 dg was used. Furthermore, in the previous experiments many rats were littered at 22 dg. Thus to be on the safe side fetuses were obtained at 21 dg rather than 22 dg.

3.7.1 Total intracellular $^{14}$C pool

Total intracellular radiolabel pool in control brain mince remained unchanged with gestational age. However, a treatment related effect was noted (Fig. 57a,b).
Figure 57: Total intracellular [U\textsuperscript{14}C]glucose pool in fetal brain mince from N and TX dams.

a) Ontogenic pattern

Values are means ± SEM (n ≥ 5).

b) Statistical analysis: two-way ANOVA

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<td>2043056</td>
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3.7.2 Carbon dioxide generation

The generation of CO$_2$ from glucose showed a biphasic pattern in both control and experimental samples (Fig 58a). Thus, the two-way ANOVA indicated significant age, treatment, and combined age and treatment-related effects (Fig. 58b). In addition, the experimental group when compared with the control, showed significant decrease (47% and 53%) in the CO$_2$ generation at 16 and 19 dg respectively. At 21 dg, however, CO$_2$ production was normalised in the TX brain mince.

3.7.3 Amino acid and protein synthesis

The labelling of amino acids from [U-$^{14}$C] glucose was found to be affected by age, and by the combined age and treatment factors (Fig. 59a,b). This combined effect of the age and treatment could be explained by the different ontogenic patterns for the control and the experimental groups. Thus, when examined by the post-hoc test, maternal hypothyroxinemia, relative to the control, was found to have significantly affected the incorporation of $^{14}$C into amino acids at all gestational ages studied. Although a large increase (86%; $p < 0.01$) in the amino acid labelling was observed at 16 dg, this was subsequently decreased (46% and 37%; $p < 0.01$) at 19 and 21 respectively (Fig. 59c).

Measurement of the incorporation of $^{14}$C into protein in brain mince indicated an age, treatment, and combined age and treatment related effects (Fig. 60a,b). The incorporation of $^{14}$C into protein remained fairly constant in the control group throughout the gestational ages studied, whereas maternal thyroidectomy was found to result in an increase (54%) at 16 dg followed by a significant reduction (44%) at 19 dg relative to the control. This pattern of effect of maternal thyroidectomy is similar to that observed earlier on for amino acid labelling, an indication of sever depression in brain protein synthesis in early fetal life.
Figure 58: The incorporation of \([U^{14}C]glucose\) into \(CO_2\) in fetal brain mince from N and TX dams.

a) Ontogenic pattern

![Ontogenic pattern graph](image)

Values are means ± SEM \((n \geq 5)\).

b) Statistical analysis: two-way ANOVA

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Figure 59: The incorporation of [U\(^{14}\)C]glucose into amino acids in fetal brain mince from N and TX dams.

a) Ontogenic pattern

![Graph showing incorporation of glucose into amino acids](image)

Values are means ± SEM (n ≥ 5).

b) Statistical analysis: two-way ANOVA

<table>
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<tr>
<th>Source</th>
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c) Post-hoc analysis (Fisher PLSD test)

* p < 0.01, 16N vs 16TX, 19N vs 19TX and 21N vs 21TX
Figure 60: The incorporation of [U$^{14}$C]glucose into protein in fetal brain mince from N and TX dams.

a) Ontogenic pattern

Values are means ± SEM (n ≥ 5).

b) Statistical analysis: two-way ANOVA

<table>
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3.7.4 **Total lipid and protein associated lipid production**

The incorporation of [U-\(^{14}\)C] glucose carbon skeleton into the total lipid and protein associated lipid was tested as well. The overall pattern of incorporation of the [U-\(^{14}\)C] glucose into total lipid in control and treated samples was similar to that of the amino acids (Fig. 61a). The two-way ANOVA revealed significant age and combined age and treatment-related effects (Fig. 61b). The control group showed a biphasic ontogenic pattern. This was not the case for the TX group which showed a gradual decrease in the radiolabel incorporation into the total lipid throughout gestational ages studied, which may explain the combined age and treatment-related effects. Thus, maternal hypothyroxinemia when compared with the control, appears to affect the radiolabel incorporation into the total lipid in fetal brain mince at all gestational ages studied. This is manifested by a significant elevation (62%; \(p < 0.01\)) at 16 dg, which was then reduced by (45%; \(p < 0.01\) and 46%; \(p < 0.05\)) at 19 and 21 dg respectively (Fig. 61c).

The incorporation of the [U-\(^{14}\)C] glucose carbon skeleton into protein associated lipids was found to be affected by age only (Fig. 62a,b), and maternal hypothyroxinemia was without significant effects at any gestational age studied.

3.7.5 **Glucose and tricarboxylic acid intermediate**

Finally the incorporation of [U-\(^{14}\)C] glucose into glucose / tricarboxylic acid intermediate pool in control group was found to be affected by age and by the combined age and treatment factors (Fig. 63a,b). Similar to the amino acids and the total lipid, the incorporation of [U-\(^{14}\)C] glucose into glucose / tricarboxylic acid intermediate pool in control group was found to follow a biphasic ontogenic pattern, whereas the TX group showed a gradual decrease throughout gestational period studied. With respect to maternal thyroidectomy relative to the control, it was found to significantly increase (60%; \(p < 0.01\)) the [U-\(^{14}\)C] glucose incorporation into glucose / tricarboxylic acid intermediate at 16 dg. This, however, was significantly reduced (48%; \(p < 0.01\)) at 19 dg with further (39%; \(p < 0.01\)) reduction near term (Fig. 63c).
3.7.6 **Glucose analogue (2-deoxy-glucose) uptake**

Unlike glucose, 2-deoxy-glucose is not metabolised beyond 2-deoxy-D-glucose-6-phosphate (2-DG-PO<sub>4</sub>), thus it can be used as a tool to determine the actual total glucose uptake *in vitro*. Therefore, measurement of 2-deoxy-D-[2,3-<sup>3</sup>H]glucose uptake in fetal brain mince showed age, treatment, as well as combined age and treatment-related effects (Fig. 64a,b). Maternal hypothyroxinemia effect was evident by the noted increase (37%) in the 2-deoxy-D-[2,3-<sup>3</sup>H]glucose uptake at 16 dg. This effect, however, was reversed and depressed by (39% and 31%) at 19 and 21 dg respectively.
Figure 61: The incorporation of [U^{14}C]glucose into total lipid in fetal brain mince from N and TX dams.

a) Ontogenic pattern

![Graph showing the incorporation of [U^{14}C]glucose into total lipid in fetal brain mince from N and TX dams.](image)

Values are means ± SEM (n ≥ 5).

b) Statistical analysis: two-way ANOVA

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c) Post-hoc analysis (Fisher PLSD test)

** p < 0.01, 16N vs 16TX, 19N vs 19TX
* p < 0.05, 21N vs 21TX
Figure 62: The incorporation of [U^{14}C]glucose into protein associated lipid in fetal brain mince from N and TX dams.

a) Ontogenic pattern

![Graph showing dpm/mg protein for different ages and treatments.]

Values are means ± SEM (n ≥ 5).

b) Statistical analysis: two-way ANOVA

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Figure 63: The incorporation of [U\(^{14}\)C]glucose into glucose/TCA intermediates in fetal brain mince from N and TX dams.

a) **Ontogenic pattern**

Values are means ± SEM (n ≥ 5).

b) **Statistical analysis: two-way ANOVA**

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c) **Post-hoc analysis (Fisher PLSD test)**

* p < 0.01, 16N vs 16TX, 19N vs 19TX and 21N vs 21TX
Figure 64: Uptake of 2-Deoxy-D-[2,6-^H]glucose into fetal brain mince from N and TX dams.

a) Ontogenic pattern

Values are means ± SEM (n ≥ 5).

b) Statistical analysis: two-way ANOVA

<table>
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3.8 Glucose transporters (GLUT1 and GLUT 3)

As mentioned before, the functional activity of brain is critically dependent on glucose as a major metabolic substrate for many metabolites. For glucose to be utilised in brain or any other tissue, it must be transported to the neurones and glial cells across the plasma membrane. This transport is mediated by membrane associated carrier proteins (glucose transporters; GLUT) which exist in different isoforms and have distinct tissue distribution (Pessin and Bell, 1992).

Two facilitative glucose transporters may be involved in the uptake and disposal of glucose in brain; GLUT 1 which is responsible for the transport of glucose across the blood brain barrier (Pardrige et al., 1990), and GLUT 3 which affects glucose uptake in neuronal cells (Burant et al., 1991). The above results regarding in vitro glucose analogue uptake (2-deoxy-glucose) in fetal brain from TX dams, indicated a significant increase at 16 dg followed by significant reduction at 19 and 21 dg. One explanation of these changes is that TH deficiency may have affect the gene expression of these transporters which are highly expressed in the brain. Unfortunately we were able to detect only faint signals (or in some times no signals) for GLUT 3 at all gestational age studied. This may be related to either experimental condition used and/or the expression of this isoform is not significant during pregnancy. The expression of GLUT 1, however, was detected at all gestational ages studied (Fig. 65). Surprisingly and in a sharp contrast to the results obtained for glucose uptake, GLUT1 expression at 16 dg was significantly reduced in fetal brain from TX dams relative to the control, whereas at 19 and 21 no changes were observed. To ensure that the same amount of RNA was used, the hybridised membrane was stripped then rehybridised with oligonucleotide probe using 3'-end labelling system. The autoradiography of the 18s RNA showed no differences between the signals for all samples (Fig. 66). Therefore, the observed increase in the glucose uptake at 16 dg, may be related to an increase in the GLUT 1 activity rather than an increase in the gene expression.
Figure 65: Northern blot analysis of GLUT 1 mRNA in fetal brain from N and TX dams. lane 1) 16 dg N, lane 2) 16 dg TX, lane 3) 19 dg N, lane 4) 19 dg TX, lane 5) 22 dg N, lane 6) 22 dg TX.
Figure 66: Expression of 18s RNA in fetal brain from N and TX dams. lane 1) 16 dg N, lane 2) 16 dg TX, lane 3) 19 dg N, lane 4) 19 dg TX, lane 5) 22 dg N, lane 6) 22 dg TX.
CHAPTER FOUR: DISCUSSION
4.1 Maternal TH state and fetal somatic growth

Maternal hypothyroxinemia, resulting from iodine deficiency, was reported to be associated with increased incidence of neurological disorders in the offspring (section 1.8.2). There is evidence suggesting that the neurological damage observed in these circumstances is sustained early in fetal life; before onset of fetal thyroid function (Pharoah et al., 1971). Studies using experimental animals showed that maternal hypothyroxinemia have multifaceted effects on fetal development, particularly fetal CNS. The presence of adequate levels of TH between 10 and 12 dg was reported to be important for normal neurogenesis in the fetal rat (Narayanan and Narayanan, 1985). In addition, the neurological changes observed in the brain of fetal sheep from thyroidectomised mothers (preconception), suggest impairment occurring during the phase of neuroblast multiplication in the cerebrum. This period occurs in the first half of gestation, a time when fetal thyroid is not functioning and the fetus is totally dependent upon maternal supply of TH (Ferreiro et al., 1987). In addition to its biochemical and enzymic effect on the fetal CNS (Devaskar et al., 1986; Ekins et al., 1989; Obregon et al., 1989; Porterfield and Hendrich 1992), maternal TH deficiency produces abnormalities in fetal carbohydrate and protein metabolism (Porterfield and Hendrich, 1976; Hendrich and Porterfield, 1980). Such fetuses were found to be unable to use amino acids properly, resulting in deficient protein synthesis in brain, liver, and skeletal muscle (Hendrich and Porterfield, 1977, 1980).

In our experiments, the primary requirement was to set up a model analogous to the hypothyroxinemic state of pregnant women that exist in iodine deficiency endemias, where most of the women have very low circulating T4, high TSH but near normal T3 levels (Hetzel et al., 1983; Pharoah et al., 1972). In order to avoid any extraneous influences on the maternal and fetal metabolism and to exclude additional factors other than maternal TH, antithyroid drugs (PTU and MMI) as well as iodine deficiency were avoided. Several other techniques employed by others were avoided as well, including $^{131}$I and total surgical thyroidectomy, which were used in most of Porterfield and Hendrich, and Morreale de Escobar and co-workers studies respectively. These two methods of thyroidectomy were
avoided because they depress the maternal levels of both T4 and T3 leading to a hypothyroid rather than hypothyroxinemic state. In addition, the use of $^{131}$I may result in its transference to the fetal system which in turn may damage the fetal thyroid. Consequently, partially thyroidectomised Sprague-Dawley rats were used as a hypothyroxinemic animal model, and the role of maternal TH state on the development of placenta and fetal brain and liver was studied before and after the onset of fetal thyroid function.

The measurement of maternal plasma T4 showed that the concentration of these two hormones decreased as pregnancy progressed. This pattern of reduction was also observed by Pickard et al. (1993) using the same animal model and by Morreale de Escobar, et al. (1988) in normal dams at 19 and 22 dg respectively. In thyroidectomised dams the plasma T4 and T3 levels showed that the animals are indeed hypothyroxinemic, since a significant reduction in the T4 levels was observed. Although maternal thyroidectomy caused significant reduction in the maternal T3 level, this reduction was, however, within the euthyroid level.

The results obtained in this study also showed that the TX rats have prolonged gestation and suffered considerable difficulties during parturition. In addition, our results confirm the reported adverse effects of maternal hypothyroxinemia and maternal hypothyroidism on the reproductive competence and the number of viable fetuses (Morreale de Escobar et al., 1985; Hadjzadeh et al., 1990; Hubank, 1990; Pickard et al., 1993; Porterfield and Hendrich, 1993). The precise role of TH in this process is not well known yet. This reduction in the litter size number may be attributed to increased resorptions. However, a reduction in the number of maturing ovarian follicles, and/or atrophy of ovaries cannot be ruled out (Parrot et al., 1960; Hagino, 1971). In addition, maternal thyroidectomy in rabbits was reported to result in an increase in the number of unruptured follicles, enlarged, polycystic ovaries, and a decrease in the number of ovulations (cited in Hendrich et al., 1976).

However, unlike the study of Pickard et al. (1993) who reported an isolated reduction in fetal body weight from TX dams at 15 dg, our results showed no such
changes at any gestational age studied. This remains so in adult progenies from hypothyroxinemic dams (Hadjzadeh et al., 1990). This may indicate that the placenta is functioning normally and that placental damage is minimal. However, in early postnatal period others reported significant reduction in the body weight of progenies from hypothyroxinemic rat dams, but this was shown to be reversed in adult progenies; their body weight exceeded that from control (Al Mazidi, 1989; Hadjzadeh et al., 1990; Hubank, 1990) suggesting a lag in development followed by an over compensation, therefore, this may indicate a long-term effects of in utero TH deficiency. Unlike maternal hypothyroxinemia, maternal hypothyroidism was found to cause a significant reduction in rat fetal and neonate body weight (Morreale de Escobar et al., 1985; Porterfield and Hendrich, 1982; Ruiz de Ona et al., 1991) and in fetal rabbits as well (Parrot et al., 1960).

4.2 Tissue growth

Thyroid hormones are known to be profoundly implicated in the development of fetal and neonatal brain, and inadequate supply of these hormones at crucial stages of development exerts irreversible, long term effects on neurological functions. Thus, the present study describes the ontogenic profile of various biochemical parameters in fetal brain from N and TX dams and attempts to delineate the direct/indirect effects of specific developmental and functional parameters.

Maternal hypothyroxinemia significantly affected the fetal brain weight. In a similar study, Pickard et al. (1993) reported a significant reduction in fetal brain weight at 15 dg, after which normalisation was sustained (Hadjzadeh et al., 1990; Hubank, 1990; Pickard et al., 1993). However, in case of maternal hypothyroidism significant reduction in fetal brain weight was reported in late gestation; at 20 and 21 dg (Morreale de Escobar et al., 1985).

The results obtained in this study and others, showed scattered changes in the cellular growth parameters throughout the pregnancy and in the neonatal periods, suggesting that TH effect may vary depending on the stage of development and the kind of
cellular growth. The DNA content, which has been defined as an index of cell number (Dobbing, 1981), was significantly reduced in fetal brain from TX dams at 15 dg in both this study and that of Pickard et al. (1993), albeit different methods of measurements were applied. This may indicate that maternal TH is crucial for neuronal proliferation, but this effect diminishes when the fetus is able to synthesise its own TH. However, this may not be true for the cellular maturation where a significant reduction in the protein:DNA was observed near term. The same argument applies for the RNA concentration and the RNA:DNA ratio. This kind of maternal TH action was reported in many other studies with monkeys, sheep, and rats. However, it is worth mentioning that in these studies different methods of thyroidectomy (total surgical thyroidectomy and the use of $^{131}$I) were used, resulting in severe hypothyroidism. Consequently, the findings may not be directly comparable. In studies performed by (Holt et al., 1975), the use of $^{131}$I in pregnant rhesus monkeys lead to complete ablation of the fetal thyroid gland. Therefore, the observed reduction in fetal brain growth parameters may be due to lack of both maternal and fetal TH. The same method of thyroidectomy was used for rats (Porterfield and Hendrich, 1982), in which a significant reduction in the DNA content was observed in fetal brain at 22 dg, and it also showed that T4 administration could block the reduction in DNA content. Similar observations were reported for fetuses from TX rat and sheep dams at 21 dg and between 84-125 dg respectively (Potter et al., 1981; Hetzel and Potter, 1983; Morreale de Escobar et al., 1985). However, These early reductions in brain cell number were found to be compensated for postnataley (Porterfield and Hendrich, 1982; Hubank, 1990) or at birth (Potter et al., 1981; Hetzel and Potter, 1983). Fetal cell number in rats represents predominantly neuronal cells (except the cerebellum), and cellular proliferation after birth is thought to be primarily glial (Zamenof and Marthens, 1971). Porterfield has pointed out that this normalisation in the cell number may be deceiving and may in fact be related to the increase in glial cells rather than a normalisation of the neuronal cells. However, a delayed action of maternal hypothyroxinemia was reported for 30 days old and adult rat progenies, in which significant reduction in the cell number was observed (Ekins et al., 1989; Al Mazidi 1989; Hubank, 1990). This late depletion in the cell number may possibly be
related to an increase in the rate of cellular death, whereas the observed reduction in the cell number early in gestation (at 15 dg) does not rule out the possible early loss of particular group of cells, which in turn may lead to further disruption in neural development at later stages (Jacobson, 1974).

Measurement of the protein:DNA ratio showed a significant reduction in the cellular maturation in fetal brain at 22 dg. Similar observation was reported by Pickard et al. (1993) using the same animal model. Likewise, depletion in the protein:DNA ratio was also reported in fetal brain from hypothyroid rat at 20 and 22 dg and in sheep between 84 and 125 dg (Potter et al., 1981; Porterfield and Hendrich, 1982; Morreale de Escobar et al., 1985). These changes were found to persist in early postnatal period and in adult progenies from hypothyroxinemic rat (Al Mazidi, 1989; Hubank, 1990). This reduction in the cell size index suggest a lag in the neurite maturation and in the cellular outgrowth, which may in turn cause retardation in the cellular connections and synaptogenesis. The possible retardation in neurite outgrowth may result in disruption of the myelination process. In fact such changes were reported for adult progenies from hypothyroxinemic rats (Hadjzadeh, 1991), where significant reduction in some of myelin proteins and myelin lipids of certain brain regions were reported.

Measurement of the fetal brain protein and DNA concentration were found to be not significantly affected by maternal thyroidectomy, except an isolated reduction in the DNA concentration observed at 22 dg. However, measuring these two parameters in neonates and adult rat progenies from TX dams showed significant alterations in protein and DNA concentration (Al Mazidi, 1989; Hubank 1990), indicating a possible delayed effect of maternal hypothyroxinemia. Reports about maternal hypothyroidism showed an early effect in which significant reduction in both parameters was observed in fetal brain at 21 dg, whilst the total protein concentration and the general pattern of major proteins were unaffected during the gestational period studied. This does not exclude the possibility of specific changes to minor proteins which were unable to be detected by the SDS-PAGE gel electrophoresis in this study. The same argument is applied for the RNA concentration. The RNA index represents mainly rRNA, thus any possible effect of maternal
hypothyroxinemia on the mRNA concentration can not be evaluated. Clearly, the results obtained from this study and others showed that the effect of maternal hypothyroxinemia on the cellular growth parameters in the fetal brain does not follow the same pattern. It can, therefore, be suggested that maternal TH effect is specific and different for each parameter as it has affected brain regions differently.

In normal rats, the maternal contribution of T4 to the fetal side of the placenta is important even after the onset of fetal thyroid function, as 74% of total TH in the fetus is of maternal origin (Morreale de Escobar et al., 1990). Maternal hypothyroidism was reported to considerably reduce maternal contribution of TH to only 10% of normal values (Morreale de Escobar et al., 1985). Besides its possible effect on fetal development, this reduction in maternal TH level may affect placental development as well. Therefore to investigate whether the aforementioned changes in fetal development have resulted directly from maternal TH deficiency or from abnormalities in placental development, a number of biochemical parameters were investigated in N and TX placentae.

In the present study, the wet weight of the placentae from TX dams increased in a manner similar to those from N dams in relation to age, with a marginally significant delay, which is similar to that observed for the fetal brain. However, using the same animal model no such changes were observed by Pickard et al. (1993). Thus, in a more severe TH condition; maternal hypothyroidism, occasional reductions in the placental weight was reported between 13 and 21 dg (Morreale de Escobar et al., 1985). The effect of TH on placental weight was further studied by Spencer and Robinson (1993) who reported that a daily injection of T4 (10μg) into pregnant rat significantly increased (by 20% ) the placental weight at 20 dg.

In agreement with the early study of Morreale de Escobar et al (1985), the protein concentration was found to be fairly constant in N placenta throughout the pregnancy, and maternal thyroidectomy was with no effect. This, however, contradicts the reported decline in protein concentration at 19 dg (Pickard et al., 1993), albeit being an isolated effect. In normal placentae, the DNA content and the protein:DNA ratio showed maximum values at 19 dg, indicating that the cellular proliferation / differentiation is complete at this stage of
pregnancy. A similar increase in the cell size index in normal rat placentae between 13 and 20 dg was reported by (Morreale de Escobar et al., 1985). Maternal hypothyroxinemia was without effect on either DNA content or DNA concentration, indicating normal cell number and density. This was not the case for the protein:DNA ratio, which was compromised at 19 dg, suggesting delayed cellular differentiation although normalisation was achieved at 22 dg. Early work of Pickard et al. (1993) tended to eliminate any effect of maternal hypothyroxinemia on any of these parameters. Data for maternal hypothyroidism differ from those obtained for maternal hypothyroxinemia, in which placentae from TX rats had lower DNA concentration and higher protein:DNA ratio than the control at 16 and 17 dg. Furthermore at 20 dg T4 supplementation to pregnant rats was found to have no effect on DNA, RNA, and protein concentration. No effects were also observed on protein:DNA ratio and RNA:DNA ratio (Spencer and Robenson, 1993). In this study however, maternal hypothyroxinemia was shown to lead to considerable increase in the RNA concentration at 22 dg, whereas the RNA:DNA ratio was significantly decreased at 19 dg. This increase may largely be due to the observed increase in the DNA content in placentae from TX dams, although this did not attain statistical significance Therefore, it can be concluded from our results that maternal hypothyroxinemia had no significant effect on cellular parameters, suggesting that the placenta in the hypothyroxinemic condition is likely to function normally, which is consistent with lack of effect on fetal body growth.

Reports regarding the effect of maternal TH state on fetal liver growth early in pregnancy are sparse and the results obtained in this study can not be satisfactorily verified. However, the available reports indicate the presence of maternal TH in fetal liver as early as 16 dg and the amount measured was higher than that obtained in the brain (Porterfield and Hendrich, 1991; Ruiz de Ona et al., 1991). This, however, was found to be significantly reduced with maternal thyroidectomy. In an earlier study, fetal liver TH concentration was found to be increased after the onset of fetal thyroid function, with maximum value at 20 dg for T4 and 22 dg for T3. Maternal thyroidectomy, on the other hand, reduces these levels between 17 dg and 22 dg except for days 19 and 21. These changes were either not observed or occurred to a lesser extent for liver T3; an indication of independent
deiodination system (Morreale de Escobar et al., 1985; Ruiz de Ona et al., 1991; Morreale de Escobar et al., 1993). Therefore, the presence of TH in fetal liver before onset of fetal thyroid function indicates that fetal liver growth may be influenced by maternal TH deficiency.

It is noteworthy to indicate that because fetal liver at 15 dg was not studied, we could not specify whether the alternations in fetal liver growth parameters at 19 and 22 dg, resulting from maternal thyroidectomy, were caused by a delayed effect of maternal T4 deficiency or have resulted from an early insult to the fetal growth. Data obtained in this study indicated that fetal liver is highly susceptible to maternal hypothyroxinemia, and that the first indication was the significant reduction in the fetal liver weight late in gestation. It would be relevant to mention that the same observation was reported for fetal liver from hypothyroid rat dams at 20 and 21 dg (Morreale de Escobar et al., 1985). This reduction in liver weight persisted even after birth; 35 days in rat progenies from hypothyroxinemic dams (Hubank, 1990). Results obtained for the fetal liver cell number from TX dams showed significant increase at 19 dg, which has diminished thereafter; near term. This increase in the cellular proliferation after the onset of fetal thyroid function, may suggest a compensation for an initial lag in cellular division resulting from diminish DNA synthesis. The protein:DNA ratio in the treated group showed exactly different pattern of changes. Therefore the observed reduction in fetal liver wet weight late in gestation may be due to reduction in cell number rather than reduced cell size. Additionally, although the fetal liver protein and DNA concentrations remained fairly constant in the control group, they were significantly affected by maternal hypothyroxinemia. In neonatal period, maternal hypothyroxinemia was found to have a lasting effect where liver weight, protein concentration, and protein:DNA ratio were all increased compared to the controls (Hubank, 1990), indicating an over compensation for the earlier decline. These changes in liver growth parameters may in turn have damaging effects on liver function. Such damages have been reported for 22 dg fetuses from TX rat dams, in which liver glycogen is depressed and glucose uptake and utilisation was greater than that in the control (Porterfield and Hendrich, 1976; Hendrich and Porterfield, 1977).
4.3 Total lipid and Cholesterol concentrations

The relation between TH and the lipid concentration was previously described by Lopez-Luna and Morales (1985) who indicated that TH plays an important role in regulating plasma triglyceride and cholesterol concentrations. Hypothyroidism in the human results in hypercholesterolemia, often accompanied by high plasma triglyceride concentration (Hazzard and Bierman, 1972; Lopez-Luna and Morales, 1985), whereas in the rat hypothyroidism induces hypercholesterolemia which is accompanied by a significant decrease in triglyceride concentration (Engelken and Eaton 1980; Dory and Roheim, 1981). Furthermore, the composition of the mitochondrial membrane was found to be influenced by TH (Paradies and Ruggiero, 1990), hyperthyroidism in rats increasing the negatively charged phospholipid in the mitochondrial membrane. Therefore, the role of maternal TH in regulating total lipid and cholesterol concentrations in fetal brain, liver, and placenta was investigated.

Although the total lipid concentration in fetal brain was found not to be affected by maternal hypothyroxinemia, this was not the case for the cholesterol concentration which was significantly increased at 22 dg. Similar changes for the cholesterol concentration were obtained for the placenta and fetal liver. However, maternal hypothyroxinemia had no lasting effect on brain cholesterol concentration as shown for the adult rat progeny (7 month) from TX dams (Hadjzadeh et al., 1990). The normalisation period for the cholesterol synthesis is not known. Therefore, it is difficult to speculate on the precise effects of these changes in the concentration of cholesterol on neural, hepatic, and placental development and function. This derangement in the cholesterol concentration, as a result of maternal hypothyroxinemia, may affect the normal developmental pattern of the cellular plasma membrane, which in turn may influence the functional activities of membrane proteins (Albert et al., 1983). It is known that the lipid composition of the plasma membrane is an important factor for regulating the physical state of the plasma membrane and the activity of certain membrane enzymes, such as Na⁺ K⁺-ATPase and Ca²⁺ Mg²⁺-ATPase and others. Therefore any changes in lipid composition and/or concentration may
significantly affect some membrane enzyme activities. In addition, it can be argued that these observed changes in the cholesterol concentration may have resulted from abnormalities in the activity of the enzymes responsible for cholesterol synthesis as a function of hypothyroxinemic state in utero or because most of the cholesterol in fetal tissue is produced locally from glucose metabolism. Therefore, any derangement in glucose metabolism may affect its synthesis. Since maternal factors would predominantly affect placental development, the same argument may apply for factors affecting placental cholesterol synthesis.

4.3 Effect of maternal hypothyroxinemia on phosphate content

The inorganic phosphate plays a crucial role in a variety of metabolic processes. During development, the availability of phosphorylated substrates, along with a high energy input, are necessary to sustain the exponential increase in nucleic acid and protein synthesis. Therefore any derangement in phosphate metabolism may restrict normal cellular development and function.

In this study, measurements of inorganic phosphate, protein phosphate, and lipid phosphate revealed that maternal hypothyroxinemia significantly disrupted phosphate homeostasis in fetal brain, fetal liver, and placenta. Generally, the protein phosphate concentration in placenta and fetal brain was significantly reduced at 15 dg but increased at 19 dg, this increase has been observed in fetal liver too. However, near term, maternal hypothyroxinemia had no significant effect on protein phosphate concentration in any of these tissues. Measurement of inorganic phosphate in fetal brain showed a similar pattern of changes at 15 and 19 dg, with a substantial increase at 22 dg as well. The changes observed in the placental protein phosphate may suggest that maternal thyroidectomy may have affected the phosphate environment in the maternal system which in turn resulted in the observed changes in the fetal brain and liver phosphate pool. However, this cannot be true when the fetal thyroid is functioning. At this stage of pregnancy, significant increases in the inorganic phosphate, protein phosphate, and lipid phosphate were observed in fetal
brain and liver. In addition, the placental protein phosphate showed a similar increase at 19
dg. Therefore, these increases after the onset of fetal thyroid function exclude the maternal
phosphate state as a causative factor, specially that maternal T3 is within normal levels, and
suggest that maternal T4, rather than maternal phosphate pool, is responsible for it. In
addition, this sudden contribution of fetal TH after complete starvation may have acted to
compensate for the early depletion. This effect of maternal TH on the phosphate pool could
be directly or indirectly influence the enzymes responsible for regulating the phosphate
homeostasis in fetal tissues, particularly the brain. Furthermore, the TH effects on brain
inorganic phosphate appear to have a lasting effect; a significant reduction has also been
observed in several brain regions in adult progeny from hypothyroxinemic rat dams (Al-
Mazidi, 1989). These deviations in the phosphate pool may affect the function and
metabolic efficiency of the affected tissues which in turn may have contributed to the
observed neurological and behavioural abnormalities (Attree et al., 1992; Porterfield and
Hendrich, 1993; Sinha et al., 1994).

4.4 Effect of maternal hypothyroxinemia on enzyme system

Extracellular signals, including a variety of neurotransmitters and hormones, produce
diverse responses in neurones. Mammalian brain may contain as many as 15,000 novel
brain-specific proteins (Browning et al., 1985). Such diversity would make it difficult to
identify protein molecules involved in specific neuronal function. However, it is known
that protein phosphorylation is one of the primary mechanisms utilised by eukaryotic cells
for postranslational regulation of protein synthesis and functions.

The phosphorylation state of cellular proteins is determined by several factors,
including activities of protein kinases and protein phosphatases. Two of many highly
expressed protein kinases in the brain are PKC and PKA (Abraham et al., 1991). Both
enzymes are involved in a large number of cellular processes including gene regulation, ion
channel and receptor activation, neurotransmitter release and cellular differentiation
(Browning et al., 1985; Abraham et al., 1991; Raymond et al., 1993).
Measurements of PKC and PKA activities in fetal brain from N dams showed a gradual increase throughout all gestational ages studied, indicating the importance of these two enzymes for brain development and function. Maternal hypothyroxinemia was without effect on PKA activity. However, it significantly affected the activity of PKC, which was manifested by a significant increase at 15 dg, followed by a significant decrease at 19 dg. Although this pattern of changes does not match that observed for protein phosphate, not all protein kinases were measured, neither was protein phosphatase activity. This, however, does not exclude the significance of the changes observed for PKC activity. Because of the role of PKC in the neuronal differentiation and synaptogenesis (Abraham et al., 1993), these observed changes in the PKC activity early in gestation may have inflicted an early damage to the normal pattern of the CNS development. Therefore, although the PKC activity was within normal levels near term, this does not rule out the possibility of a delayed damage resulting from abnormalities in PKC activity early in gestation. This notion is further supported by the study of Huang and Huang (1989), who reported that in the brain of Macaca Fuscicularis monkeys, the PKC I concentration was found to be high in neurones of the hippocampus, amygdala, cerebral cortex, and cerebellum which are important for learning and memory. During brain development, the PKC I was increased in parallel with the progress of synaptogenesis and was associated particularly with neuronal dendrites, which suggest that PKC I has an important role in memory functions. These finding may suggest the cause of neurological cretinism in which both intellectual abilities and motor competence have been compromised. Similar observations were reported for rat progenies from TX dams (Attree et al., 1992).

A more direct relation between TH and PKC and PKA activities was reported for adult hypothyroidism, where TH deficiency was found to elevate the PKC activity in the cerebral cortex, while PKA activity was increased in all brain regions studied (Ahmed et al., 1993).

The activities of two plasma membrane enzymes; Na⁺ K⁺-ATPase and Ca²⁺ Mg²⁺-ATPase were studied as well. Na⁺ K⁺-ATPase is a key plasma membrane enzyme controlling ion fluxes (Na⁺ out) and (K⁺ in) across the plasma membrane of neural cells.
(Atterwill et al., 1985). The activity of Na\(^+\)K\(^+-\)ATPase has been shown to increase with rat brain maturation, coincident with the establishment of synaptic connections (Lindholm, 1984). The enzyme exists as two subunits; a large (\(\alpha\)) subunit which is the ATP phosphorylation and ouabain binding site, while the other subunit (\(\beta\)) is a small glycosylated subunit whose exact function is still unknown. The activity of Na\(^+\)K\(^+-\)ATPase has been previously reported to be TH-dependent in neural and non neural tissues (Namura et al., 1990).

The measurement of Na\(^+\)K\(^+-\)ATPase activity in control fetal brain homogenate showed an age related increase. This was true for Ca\(^{2+}\)Mg\(^{2+}\)-ATPase only between 15 and 19 dg, after which a significant decrease was observed. Maternal hypothyroxinemia, however, significantly reduced Na\(^+\)K\(^+-\)ATPase activity at all gestational ages studied. This further supports the notion of the dependence of Na\(^+\)K\(^+-\)ATPase on TH (Namura et al., 1990). The Ca\(^{2+}\)Mg\(^{2+}\)-ATPase showed similar dependence on maternal TH deficits only at 15 and 19 dg, after which a significant increase in its activity was observed. These changes in both enzymes' activity suggest a direct action of maternal TH on Na\(^+\)K\(^+-\)ATPase and Ca\(^{2+}\)Mg\(^{2+}\)-ATPase activities, or indirect effect on the gene expression responsible for coding of these two enzymes. Additionally, any disturbances to membrane structure may be reflected in changes in enzyme activities as well. The disruption in the normal activity of Na\(^+\)K\(^+-\)ATPase may reflect a possible delay or derangement of the neuronal maturation and, together with the derangement of the Ca\(^{2+}\)Mg\(^{2+}\)-ATPase activity, may suggest ionic imbalance in the neuronal cells. Furthermore, the Na\(^+\)K\(^+-\)ATPase activity was also found to be reduced in adult progenies from hypothyroxinemic dam rats (Al Mazidi, 1989), whereas Ca\(^{2+}\)Mg\(^{2+}\)-ATPase showed no changes in its activity. This may indicate that maternal hypothyroxinemia has a lasting effect on Na\(^+\)K\(^+-\)ATPase which in turn may correlate with a compromise in synaptogenesis and synaptic functions. This suggestion is in agreement with the early study of Lindholm (1984) where the administration of T4 to neonatal rats (2 weeks old) from TX dams showed a significant increase in the activity of synaptic membrane Na\(^+\)K\(^+-\)ATPase, and in the activity of Na\(^+\)K\(^+-\)ATPase in general.
The effect of maternal hypothyroxinemia on the activities of the dephosphorylation enzymes; alkaline phosphate and acid phosphatase in fetal brain homogenate was studied in order to detect their role on the turn over of the inorganic phosphate. Maternal thyroidectomy was found to have no effect on the activity of both enzymes in fetal brain, similar to acid phosphatase when examined in neonates from hypothyroxinemic rat dams (Hubank, 1990). However, in adult progenies from hypothyroxinemic rat dams the activity of acid phosphatase was reduced in certain brain regions. This reduction was suggested to be consequent upon neither the observed reduction in cell number nor the reduction in the number of lysosomal particles containing this enzyme (Al Mazidi, 1989). Therefore these finding suggest that maternal hypothyroxinemia may have a delayed effect on the ontogenesis of acid phosphatase, and that more specific dephosphorylating enzymes may be involved in regulating the inorganic phosphate turn over in fetal brain and phosphate metabolic homeostasis.

4.5 Glucose metabolism

Glucose is thought to be the most important energy substrate in both developing and adult CNS, especially in the neuronal cells (Sinha et al., 1980; Farrell and Pardridge; 1991, Gerhart et al., 1992). Glucose metabolism and energy generation is most crucial during CNS development, since a host of energy dependent processes like cell division, protein synthesis, DNA and RNA synthesis, and cell migration proceed at explosive rates. Glucose metabolic status was therefore investigated in vitro.

Carbon dioxide production from glucose was found to be significantly reduced in the brains of 16 dg fetuses of TX dams. Since in hypothyroxinemic dams the developing brain prior to the onset of independent fetal thyroid function is essentially hypothyroid, the observed compromise in CO₂ generation is consistent with reported reduction in BMR in hypothyroid adult brain (Sokoloff, 1971). However, near term, CO₂ production is normalised and there are no differences between the normal and the experimental groups. This observation is in agreement with many other observations showing reduced oxygen
utilisation and CO₂ production, reduced BMR (by as much as 50%) and reduced thermogenesis (Sokoloff, 1971) due to total thyroidectomy, which can be reversed by thyroid hormone replacement. Increases in cytochrome C, ubiquinone and NADH cytochrome reductase in the hyperthyroid state also indirectly confirms our observations (Sokoloff, 1971). Reduced CO₂ production from glucose in the brain of 19 day old fetuses of hypothyroxinemic dams poses a problem. By that time the fetus is euthyroid due to local thyroid hormone production, although it can be argued that transition from hypothyroid to euthyroid state may have a latency period when the “fetal brain remains hypothyroid”, an assumption which remains to be proven.

In addition, reduction in CO₂ production at least in fetal brain from TX dams at 16 and 19 dg is in agreement with reports in the literature where hypothyroidism has been shown to reduce oxygen consumption in brain slices in vitro during development (Ghittoni & Gomez, 1964), whereas the treatment of young rats with T4 results in an increased rate of oxygen consumption in the brain (Hamburgh, 1969). Severe perturbation of brain glucose metabolism, namely a significant depression in glucose uptake as a result of neonatal hypothyroidism, has also been reported (Hendrich et al., 1984; Hamburgh, 1969; Geel et al., 1967; Balazs et al., 1968). Essentially these parameters are also being affected in maternal hypothyroxinemia because the fetal brain cannot get its full complement of T4 especially early in gestation.

The incorporation of glucose carbon into the lipid fraction (total lipid) is enhanced in the brain at 16 dg by as much as 62% relative to controls although significant reductions are evident at 19 and 21 dg. A similar pattern in the incorporation of glucose carbon into protein fraction is also observed at 16 and 19 dg followed by a normalisation by 21 dg. It would appear from our results that in early CNS development, glucose is being channelled into synthetic pathways at the expense of energy generation in fetuses from hypothyroxinemic dams. This happens in spite of a large radioactive carbohydrate pool in the cells at 16 dg. It is interesting that in spite of the relatively reduced glucose oxidation rate (i.e. reduced energy production) energy expensive synthetic processes such as protein, amino acid, and lipid synthesis from glucose are enhanced. This observation may indicate
the paramount requirement of glucose for developmental need during explosive tissue growth and differentiation. It is assumed that the energy need for development, may be met by alternative glucose metabolic pathways such as glycolysis and/or by more efficient operation of pentose phosphate pathway, suggesting glucose metabolic plasticity and compensatory mechanisms in the event of system failure as it were i.e. lack of oxidative hormone in this case, since development cannot be allowed to be compromised. Increases in the pentose phosphate shunts enzymes like glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase in developing rat brain has indeed been reported. This interpretation is consistent with the observation that intra uterine human brain can maintain a large degree of metabolic activity in extreme anaerobic conditions (Sinha et al., 1980). It is also evident that in spite of this metabolic plasticity the effects of the lack of intrauterine hormones on the developing CNS cannot be fully compensated for in terms of protein and nucleic acids acquisition as evidenced by the aforementioned deficit in protein, DNA and RNA concentration in the brain due to maternal hypothyroxinemia. Indeed, uptake studies with 2-deoxy-glucose in vitro also show an increased uptake in the experimental fetal brains (TX) at 16 dg and significant reduction in uptake at 19 and 21 dg. To explain this, there are at least three possibilities. First, since a large portion of glucose uptake, especially 2-deoxy glucose uptake, is phosphorylation-dependent it may be argued that the compromise in glucose and 2-deoxy glucose uptake observed in our study may be due to an decreased synthesis and/or activation of hexokinase enzyme, which is rate-limiting under these circumstances. As mentioned previously, brain-specific proteins are modulated as a function of thyroid hormone environment. On the other hand, it is also possible that the compromise is due to a disturbance of ATP homeostasis, which is at least partly dependent upon inorganic phosphate pool and other phosphate metabolic parameters which are compromises due to perturbed thyroid state in utero. The third possible explanation is that, in the absence of TH (at 16 dg) enhancement of glucose transporter proteins synthesis / activation occurred, followed by attenuation when thyroid hormone levels are normalised.

To examine this possibility, the expression of two facilitative glucose transporter isoforms; GLUT 1 and GLUT 3 were studied. Published studies indicated that the highest
levels of GLUT 1 are found in brain, placenta, and fetal tissues which then decline postnatally. GLUT 1 was the first glucose transporter to be cloned and its amino acids sequence is highly conserved. There is 98% homology between human and rat GLUT 1. This high degree of conservation implies that all domains of this 492-residue protein are functionally important. The other glucose transporter isoform is GLUT 3, present at variable levels in all adult human tissues and at highest levels in brain, kidney, and placenta. The ubiquitous distribution of GLUT 3 in human tissues suggests that it may be responsible, together with GLUT 1, for non insulin stimulated glucose transport. However, in the rat, monkey, rabbit, and mice the pattern of GLUT 3 expression is different from that observed in human, being found in high levels in brain whereas in other tissues it is very low or undetectable (Burant et al., 1991). The results obtained for mRNA expression of GLUT 3 and GLUT 1 indicated that GLUT 3 is undetectable in fetal brain. This may be related to experimental conditions used or that it is not significantly expressed in fetal brain. The GLUT 1 mRNA, on the other hand, was detectable as early as 16 dg which, however, was found to be significantly reduced as a result of maternal hypothyroxinemia. This contradicts the early observation of high glucose uptake at this age compared with the controls, which can be interpreted in several ways. First, it is possible that as a result of maternal hypothyroxinemia, this protein has a short half life whereas its activity was increased, which in turn leads to an increase in the glucose uptake but low mRNA expression. Another possible explanation for these changes is that GLUT 1 phosphorylation is controlled by PKC (Pessin et al., 1992), and early investigation of the effect of maternal hypothyroxinemia on PKC activity showed similar pattern of changes as for glucose uptake at 16 and 19 dg. Therefore it may be that the GLUT 1 activity rather than expression is responsible for the observed changes in glucose uptake.

It is likely that disturbed energy metabolism in the CNS in utero as a result of maternal hypothyroxinemia may be responsible, at least partially, for the observed neurological damage seen in cretins born to hypothyroxinemic mothers. However, the mechanisms of the observed damages and relationship between thyroid hormone availability and glucose metabolism cannot be explained and remain to be examined.
It is therefore clear that maternal hypothyroxinemia resulted in a range of biochemical deficits to fetal brain development. These damages have no consistent pattern, in which different parameters were affected to varying extent at different gestational ages. However, the cumulative effect of these derangements may inflect long-term irreversible damages resulting in the observed neurobehavioural compromise (Attree et al., 1992). Furthermore results obtained for the placenta showed limited damage to placental growth and normal body weight was obtained for fetuses from TX dams. This confirm the notion that maternal hypothyroxinemia rather than nutritional deficit is the reason for the observed damages in the fetal brain. In addition, the involvement of fetal iodine deficiency is disregarded since iodine has a free placental passage and the diet was iodine repleted.
4.6 Summary

In order to gain a better understanding about the role of maternal hypothyroxinemia on the fetal CNS development, an animal model of partially thyroidectomised rat was used. The aim was to obtain a hypothyroxinemic maternal state similar to that found in iodine deficient regions.

A range of cellular growth parameters were investigated in placenta and fetal brain, and liver. The results obtained for the placenta showed minimal changes to placental growth, as reflected by normal fetal body weight. On the other hand, maternal hypothyroxinemia has resulted in abnormal cellular proliferation and maturation in fetal brain. Furthermore, deviations in the cholesterol concentration and phosphate pool were observed. Maternal hypothyroxinemia was found to cause similar derangements of these parameters in the fetal liver albeit to different extent.

Measurement of PKC activity showed that it has been affected at 15 and 19 dg whereas normalisation was achieved at 22 dg. Due to its role in neurotransmitter release, cellular proliferation and gene regulation (Browning et al., 1985; Abraham et al., 1991; Raymond et al., 1993), it is possible that irreversible damage may have been inflected to the CNS which can be detected in behavioural studies (Attree et al., 1992), although the activity of this enzyme appears to be normal near term. The activity of Ca\(^{2+}\) Mg\(^{2+}\)-ATPase early in gestation was too affected, albeit in an opposite manner, showing a deficit at 15 dg followed by an increase at 19 dg. The Na\(^{+}\) K\(^{+}\)-ATPase activity was found to be depressed at all gestational ages studied indicating ionic imbalance and abnormal neuronal maturation.

The investigation of glucose metabolism in fetal brain in vitro revealed that total glucose uptake, CO\(_2\) production, and the incorporation of glucose carbon with the lipid and protein fractions were greatly affected by maternal hypothyroxinemia. The investigation of the gene expression of two facilitative glucose transporters (GLUT 1 and GLUT 3) indicated no expression of GLUT 3 at any gestational age studied, whereas the results obtained for GLUT 1 showed that it was significantly expressed as early as 16 dg, but depressed in fetal brain from TX dam at 16 dg. A number of factors may play part in these
disruptions in the glucose metabolism and glucose transporter gene expression, including disruption in activities or gene expression of enzymes involved in glucose metabolism and transport processes.

4.7 Future work

It is evident from this study that maternal hypothyroxinemia significantly affects normal developmental pattern of the fetal CNS. The results indicat that TH deficiency delayed neural maturation with possible varying effects on different cell types. It would therefore, be interesting if histological studies were performed and if cell type specific markers were investigated as well.

The results obtained from glucose metabolism study suggest possible relation between the disrupted glucose metabolic pathway in fetal CNS and the biochemical damages observed earlier. It is essential, therefore, to investigate the effect of maternal hypothyroxinemia on gene expression and transcriptional effects on mRNA for key enzyme systems involved in the CNS (like PKC), and in glucose metabolism in order to elucidate the molecular mechanisms involved.

Moreover, it would be worth investigating whether these observed abnormalities are reversible and at what stage of pregnancy if TH was administered to pregnant hypothyroxinemic dams.
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Transport of Thyroid Hormones to Target Tissues


Key-words: Thyroid hormone transport – free hormone hypothesis – placental control – maternal hypothyroxinemia – brain development.

Summary: Endemic iodine deficiency is associated with maternal hypothyroxinemia and a relatively high incidence of neurological disorders in the offspring. The previous assumption that the placenta is impermeable to maternal thyroid hormone, has resulted in the erroneous suggestion that iodine per se has an essential role in brain development. Furthermore, the observed factorial rise in thyroxine-binding globulin (TBG) in pregnancy has often been misinterpreted as preventing thyroid hormone loss to either the fetal compartment or excretory systems. However, physicochemical analysis of the role of specific binding proteins in hormone delivery, combined with epidemiological evidence and evolutionary considerations has led us to postulate that a) maternal thyroxine (T4) is transported to the fetus, and is of crucial importance in early fetal development, and b) TBG part of a control system specifically designed to maintain at an optimal level the T4 environment to which the developing fetus is exposed.

Placental transfer of maternal T4 in a variety of mammalian species (including humans) is now well established. Further experimental studies in rats have shown that perturbation of the intrauterine thyroid hormone environment during critical phases of brain development results in a spectrum of biochemical dysgenesis. For example, in fetal brains deriving from hypothyroid (Tx) rat dams, severe disruption of phosphate metabolism is observed and the ontogenesis of two enzyme activities associated with growth control, protein kinase C and ornithine decarboxylase, are compromised. Development of brain function is also impaired, as evidenced by the dysgenesis of certain neurotransmitter metabolic activities (choline acetyltransferase and DOPA decarboxylase). These findings may help explain the behavioural dysfunction observed in adult Tx dam progeny and are likely to be relevant to human subjects, since upwards of 1 billion people inhabit iodine deficient regions of the world.


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Transport von Schilddrüsenhormonen an ihre Zielgewebe


Eine physikochemische Analyse der Rolle der spezifischen Bindungsproteine bei der Hormonabgabe hat uns in Kombination mit epidemiologischen Daten und evolutionären Überlegungen zu folgenden Schlußfolgerungen geführt: a) das mütterliche Thyroxin (T₄) wird zum Fötus transportiert und ist von höchster Bedeutung in der frühen fötalen Entwicklung; b) das TBG stellt einen Teil eines Kontrollsystems dar, welches spezifisch dazu dient, die "T₄-Umwelt", der der Fötus ausgesetzt ist, auf einem optimalen Niveau zu halten.


Introduction

Thyroid hormones (THs) are known to be profoundly implicated in the development of the fetal and neonatal brain, an inadequate supply of these hormones at crucial stages of development exerting irreversible, long-term, deleterious effects on neurological function. Dietary iodine deficiency, resulting in maternal, fetal and neonatal hypothyroxinemia, is clearly of key importance in this context. However, although augmentation of the iodine supply -- particularly to pregnant women and young children -- represents an obvious means of preventing widespread neurological damage to the populations of iodine deficient regions, the administration of large, non-physiological, amounts of iodine (in the form, for example, of iodized oil) can itself depress TH synthesis, thereby exacerbating the very problems such action is designed to prevent. Meanwhile, correlations have also been claimed to exist between maternal hyperthyroxinemia and certain neurological disorders (e.g., schizophrenia [18]), suggesting that exposure of the fetal brain to elevated TH levels at critical periods of fetal development may likewise result in undesirable long term consequences. For this reason, amongst others, we have regarded as important the acquisition of a more detailed insight into the effects of maternal TH on neurological development throughout the entire span of fetal and neonatal life.

For a number of years, the consensus view amongst endocrinologists was that THs of maternal origin do not cross the placenta in significant amount (11). For this reason THs were not considered to be implicated in the early development of the fetus in general, or of the fetal CNS in particular (21). Studies in this area therefore centred almost entirely on the effects of fetal hypothyroxinemia per se. Nevertheless, for several reasons (some of which have been discussed in [5]), we doubted the validity of these ideas; we therefore initiated studies (ca. 1980) specifically intended to verify or disprove the experimental evidence and theoretical postulates on which such conclusions had been based.

The results of our preliminary studies (39) immediately gave further support to our suspicion that previous work on TH transport from mother to fetus was either irrelevant to the events occurring in early gestation (having been confined to animal studies late in pregnancy following the development of the fetal thyroid gland) or, in the case of the relatively few studies conducted during early pregnancy, had been misinterpreted.

Moreover, one of the principal explanations offered for the supposed absence of placental TH transport likewise appeared suspect, albeit superficially persuasive. This reflected the widespread notion that thyroxine-binding globulin (TBG) and the other serum TH binding proteins (thyroxine-binding prealbumin [TBPA] and albumin) serve to minimize hormone loss from the vascular compartment and, indeed, that this constitutes their principal physiological role (15). More particularly, the rise in TBG seen in pregnancy had been proposed as constituting a mechanism specifically designed to prevent passage of TH from maternal to fetal circulations (23).

Our disagreement with these ideas, coupled with both animal and clinical data suggesting that TH traverse the placenta early in pregnancy, and are implicated in the early development of the fetal neurological system, have led us to propose a comprehensive hypothetical model relating to the control of the TH environment to which the fetus is exposed. The basic components of this model are illustrated in Figure 1.

This model embodies several key ideas:

a) THs (particularly thyroxine [T₄]) of maternal origin are hypothesized to be essential to feto-placental well-being throughout gestation. Under "normal" conditions, maternal T₄ is postulated as being transported directly to the fetus early in pregnancy prior to the development of the fetal thyroid gland, where it is required for the proper development of the fetal neurological system; however, following the establishment of a fully autonomous fetal thyroid economy, T₄ supplied to the feto-placental unit is deiodinated within the placenta, thereby serving as an important source of the elemental iodine required by the fetus for the establishment of its own independent and stable TH supply.

b) A control system is envisaged as existing within the mother ensuring a sufficiency of the T₄ supply to the feto-placental unit. The main components of this
putative control system comprise TBG (whose elevated concentration during pregnancy is postulated as enhancing T4 release from the maternal thyroid and causing preferential transport of this hormone to the fetal-placental unit), and a pregnancy is postulated as enhancing T4 transport. 

These concepts challenge much that has previously been accepted in the thyroid field, including the supposed lack of TH transport across the placenta, the claimed unimportance of TH in early development of the fetal neurological system, and the postulated role of serum binding proteins in TH transport. It likewise resurrects an old hypothesis (which provoked considerable scepticism) relating to the existence and physiological significance of a "chorionic TSH". Nevertheless, despite its somewhat speculative nature, the model is compatible with a wide variety of clinical and experimental observations. Such evidence as can be marshalled against it can generally, on close examination, be shown to be suspect; indeed, we have not as yet encountered convincing experimental data showing the model to be invalid. Furthermore, it is teleologically attractive, providing the conceptual framework for the experimental studies reported in this presentation) evidence is increasingly accumulating of its fundamental validity. We therefore consider some of the more important observations and theoretical concepts relating to our proposals.

**Thyroid hormone-binding proteins and their role in hormone transport**

THs in human serum are largely bound by 3 proteins (34): albumin, TBPA, and TBG. Only a minute proportion of the total T4 present (ca. 0.02%) is free under in vitro equilibrium conditions. In the case of 3,5,3'-triiodothyronine (T3), the proportion is approximately ten-fold higher (ca. 0.2%). The concentration of TBG rises approximately two- to three-fold in human pregnancy; the concentrations of T4 and T3 likewise rise (albeit to a slightly lesser extent), the free concentrations of both hormones remaining approximately constant. These well known observations have formed the basis of the so-called free hormone hypothesis, i.e. the postulate that free thyroid and steroid hormone concentrations as determined in vitro govern hormone supply to target tissues. Though this hypothesis is widely accepted, the reasons for the existence of the specific TH binding proteins and for the characteristic changes in their concentrations which take place during gestation, remain unknown. For example, it has been variously suggested that serum binding proteins serve to solubilize hormone, to prevent glomerular loss, to reduce the effects on sensitive tissues of rapid fluctuations in serum hormone levels, and/or to ensure uniform distribution of hormone delivery to target organs. More recently, TBG has been claimed to reduce the effects of the free T4 concentration caused by variation in the serum level of free fatty acids. However, none of these suggestions explains the apparent absence of physiological effect in those individuals (predominantly males) in whom, for genetic reasons, TBG is totally absent.

Speculation on the physiological role of the specific thyroid (and steroid) hormone binding proteins has also occasionally centred on the possibility that they are in some way implicated in reproduction, as exemplified by the notion that the rise in TBG "protects" the fetus from maternal TH (23). Such speculation has stemmed in part from the marked changes observed in pregnancy in these proteins' maternal serum concentrations. Clearly the role of TBG and other binding proteins in TH transport merits consideration in the context of an examination of maternal, placental and fetal TH requirements throughout gestation. Coincidentally this issue is one which, because of its wider relevance to endocrinology and reproductive physiology, has attracted much recent interest and controversy. Discussion has particularly centred on the validity of the "free hormone hypothesis" of (thyroid) hormone transport and action.

**Hormone transport to target tissues: the free hormone hypothesis**

The "free hormone hypothesis" of hormone delivery is widely accepted amongst endocrinologists. It enshrines the concept that, in the case of hormones which exist in blood (largely) in protein-bound form, the serum free hormone concentration as determined under equilibrium conditions in vitro constitutes the key determinant of hormone action, the protein-bound concentration being physiologically irrelevant. A corollary is that only hormone in the free state is able to traverse target tissue capillary walls and penetrate target cells. These concepts reflect the observation that, in circumstances in which serum protein-bound hormone levels differ significantly from normal (e.g., during pregnancy,
Hormone transport to target tissues: "bound hormone" hypotheses

A number of investigators have sought explanations which challenge the free hormone hypothesis. For example, following observation of the differential effects of corticosteroids on the induction of hepatic and pancreatic aminotransferases in rats with altered serum corticosterone binding globulin (CBG) levels, Keller, Richardson, and Yates suggested that certain tissues are permeable to specific binding proteins (17), and thus accessible to protein-bound hormone per se. They therefore hypothesised that CBG "increases the specificity of the adrenocortical system by targeting corticosteroids according to features of microcirculation". Though Keller et al.'s hypothesis has subsequently been largely disregarded, similar ideas were more recently proposed by Siiteri et al. (37) who claimed to have observed intracellular localisation of CBG and sex-hormone binding globulin (SHBG), and have suggested that, following their structural modification, these proteins convey hormone directly into the nuclei of target cells. However, a major difficulty arising with all such concepts is that physiological manifestations of abnormality in binding protein levels would be expected to be readily apparent; moreover they offer no specific explanation of the characteristic changes of binding protein levels seen in pregnancy.

The ideas of Pardridge et al. in this area (24–27) have formed the basis of some 100 publications in major endocrine journals during the past 12 years and have commanded particular attention amongst endocrinologists and physiologists. Nevertheless, they have been the subject of considerable controversy. Pardridge's original postulate was that bound hormone was specifically directed to target tissues characterized by long capillary transit times (such as the liver), hormone delivery to tissues such as the brain (in which the blood transit time is relatively short) being essentially determined by the free hormone level. Based on this concept, Pardridge suggested a tissue-targeting role for TH and other hormone binding proteins, alterations in their concentrations during pregnancy resulting in increased delivery of T3 to the liver. Subsequently (in response to criticisms of its mathematical basis) Pardridge abandoned this hypothesis and advanced new ideas which — though retaining the central tissue-targeting role postulate embodied in his original ideas — envisaged entirely novel mechanisms governing hormone transport to individual tissues. These have been based on the proposition that the equilibrium constant governing protein binding of hormone is altered in the microvasculature of certain target organs, causing the intracapillary free hormone concentration to be elevated, and large amounts of (dissociated) bound hormone to be selectively transported to such organs. Pardridge has therefore continued to claim that the "function of plasma protein binding is the selective delivery of ligands to tissues in a way that varies from organ to organ" (25), implying — like Keller et al. — that variation in binding protein levels during pregnancy alters the distribution of hormone throughout the body. However, though the notion that binding proteins affect the delivery of hormone in this way is common to several recent challenges to the free hormone hypothesis (including our own), we have criticized Pardridge's proposals, believing them to derive from an oversimplified theoretical analysis of the kinetics of hormone efflux from target organ capillaries, causing crucial misinterpretations of experimental data (8). However, in part because of the interest Pardridge's views provoke, but more particularly because of the relevance of some of Pardridge's experimental observations — when correctly interpreted — to an understanding of the role of serum proteins in TH transport, this issue merits more detailed discussion here.

Pardridge's challenge of the validity of the free hormone hypothesis originally derived from a fundamental misunderstanding of the hypothesis itself (24). It should be noted that this does not propose that the amount of hormone delivered to an individual tissue is restricted to the amount initially present in the free state in the afferent blood supply. Illustrative of misunderstanding on this point, the observation that the "splanchnic extraction of testosterone or estradiol, or the brain extraction of progesterone, is on the order of 30–50%, which is 10-fold the percentage of free hormone in serum" (25) was originally viewed by Pardridge as contradicting the hypothesis. This misconception constituted the principal foundation of Pardridge's original proposal of an "apparent", in vivo, dissociation constant (Kd,app) deviating from the "absolute" constant (Kd) estimated in vitro (24), and whose value was supposedly given by Kd,app = (t), where (t) is the capillary transit time and (k) the capillary wall permeation constant (24, 25). The claimed manifestation of this effect was elevation of the "apparent" in vivo free hormone level in tissues (e.g., the liver) characterized by "long" capillary transit times (i.e. times comparable with the dissociation half-time of the protein-hormone complex), this phenomenon supposedly accounting for the high rates of unidirectional (radionlabelled) hormone efflux observed in Oldendorf-type experiments. In contrast, Pardridge and Landaw's revised hypothesis (27) proposed that the "major factor leading to the rapid transport in vivo of protein-bound ligands into tissues such as brain is an endothelial-induced decrease in the affinity of the plasma protein for the ligand" (27) arising, for example, from "a conformational change in the plasma protein" as it transits the target tissue.

The experimental basis for this revised proposal centred on the discrepancy between Pardridge and Landaw's observa-
tions on radiolabelled hormone uptake in brain and other tissues, and theoretical predictions based on a revised hormone efflux equation, described by these authors as the "modified Kety-Renkin-Crone" equation (26, 27), which takes the form:

\[
FE = l - e^{-\frac{k_{df}}{1 + K[P]} - \frac{k_{pt}}{k_{d}}} \tag{1}
\]

where \(FE\) = fractional efflux of radiolabelled hormone during a single pass through the target organ,

\(k_{df}\) = dissociation rate constant of bound hormone complex.

FE = 1 - e^{-\frac{k_{pt}}{k_{d}}} \tag{2}

where \(k_{d}\) = dissociation rate constant of bound hormone complex.

Clearly the simplified form of Eqn. 2 relied on by Pardridge reflects the assumption that \(k_{d} = 0\) (i.e., the protein hormone complex dissociates infinitely rapidly), implying that \(k_{p}/k_{d} = 0\). This assumption is clearly questionable. Moreover, inclusion of this term in the efflux equation is sufficient to explain all Pardridge's experimental observations (see, for example, Fig. 2).

Fig. 2. Unidirectional extraction of \(^{125}\)I\(\Gamma\)3 by rat brain plotted versus arterial albumin concentration (data reproduced from Figure 5 [27]). Note the typically good fit of Equation 2 to the experimental data using the dissociation constant determined in vitro. When the term \(k_{p}/k_{d}\) is omitted from the efflux equation, a 10-fold higher dissociation constant is required to obtain a good fit to the observations (27).

Inclusion of other similar terms in Eqn. 2 reflecting the presence in the hormone boluses used in Pardridge's experiments of albumin and other contaminating proteins also explain the data (see Eqn. 3).

\[
FE = l - e^{-\frac{k_{df}}{1 + K[P]} - \frac{k_{pt}}{k_{d}} + \sum K_i [P_i]} \tag{3}
\]

where \(\Sigma K_i [P_i]\) is the sum of the affinity constant/concentration products relating to any contaminating proteins present in the bolus.

In short, the discrepancies between experimental results and those predicted from Eqn. 1 provide no justification for the postulation of hitherto unsuspected intracapillary hormone release mechanisms of the kind proposed by Pardridge. Either of the more appropriate and exact equations (Eqns. 2 and 3) exactly fit the data.

Kinetic model of hormone transport

Pardridge's experimental results nevertheless provide some support for a proposition that we have frequently advanced, that circumstances may arise in a particular target organ in which dissociation of bound hormone exerts rate limiting effects on hormone delivery (6, 8). In such circumstances, variation in the bound hormone level will affect hormone delivery to the organ concerned, as is implicit in Eqn. 2, and exemplified by the data shown below. This proposition underlies our own challenge to the free hormone hypothesis, and our suggested explanation for the recent evolution and existence of serum binding proteins.

Though Eqn. 2 is capable of explaining Pardridge and Landaw's data, more complete theoretical analysis of the kinetics of hormone efflux from target tissue capillaries suggests that additional parameters affect the intracapillary free hormone concentration (or, more particularly, the concentration at the capillary wall, which governs the rate of hormone efflux into adjacent tissue).

Target tissue uptake of hormone can be shown to be a complex function of a number of factors (including the extravascular free hormone concentration, the capillary wall permeation rate constant, the rate of intracapillary free hormone diffusion and mixing, the intracapillary bound hormone level, and the dissociation rate of the bound hormone complex) some of which are "tissue specific". This proposition is illustrated in Figure 3, which shows (a and b) the high probability of recombination of free hormone molecules with binding proteins (following their release) as they migrate towards the capillary wall as compared with free molecules released in close proximity to the wall, and (c) the resulting variation in free hormone concentration across the capillary. In short, in consequence of hormone uptake in adjacent tissue, a free hormone concentration "contour" is established across the capillary diameter, the free hormone concentration at the capillary wall (which is the key determinant of hormone uptake by the organ) being depressed vis-à-vis the concentration at the capillary axis. Thus the major source of hormone exiting the capillary is the layer of blood immediately adjacent to the capillary wall, the free hormone concentration within this layer being depressed in consequence of target organ uptake. Furthermore, as shown in Figure 4, the higher the binding protein concentration (and concomitantly the bound hormone concentration) the higher the free hormone concentration at the capillary wall, notwithstanding maintenance at an unchanged level of the free hormone concentration as measured at
short distance before becoming rebound, whereas those released in close proximity to the wall are more likely to exit the capillary axis. The density of free hormone across the capillary [Ffj], varies with distance r from the capillary axis.

Such subtle effects are potentially of particular importance in the case of hormone transport from maternal to fetal circulations. The feto-placental unit is a relatively exceptional organ in that the fetus is not itself perfused by maternal blood, and the latter’s relationship to its (putative) maternal hormone supply is anatomically unique. Maternal blood within the placenta exists in the form of lacunae in the intervillus space, throughout which the villi of the chorion frondosum are distributed. Significant transport of T4 from maternal to fetal circulations would be anticipated to give rise to significant free hormone “depletion zones” within the maternal blood surrounding each individual villous. The anatomical structure within the placenta is thus exactly one in which a rise in T4 would be expected to enhance maternal T4 transfer to the fetal circulation.

Support for the belief that the feto-placental unit may be of special significance in this context is provided by the hitherto unexplained observation that a pregnancy-induced CBG rise is seen only in species characterised by haemochorial and haemoendothelial placentae (36). The nature of barriers to hormone transport between maternal and fetal circulations may underlie this unexpected correlation, differences in the permeability of these barriers providing a possible explanation both for species differences in the spectrum of hormone binding proteins present in the blood, and for the alterations in the levels of these proteins arising in pregnancy.

Our experimental observations on T4 placental transport in the rat nevertheless indicate that fetal T4 uptake is relatively high in the first 9 to 10 days of fetal life, but thereafter falls to low levels (39). Placental T4 accumulation remains high throughout pregnancy, the accumulated hormone being degraded to iodine (and reverse T3). Such degradation clearly provides a rich placental source of iodine for the developing fetal thyroid – a phenomenon likely to be of special importance when dietary iodine (and hence the iodide level in maternal blood) is low. Though the existence of TBG in the rat has only recently been demonstrated, and the changes in its concentration throughout life appear to be somewhat different from those seen in humans, such differences may reflect species differences in placental permeability to T4, gestation time, etc., as indicated above. T4 in rat plasma is nonetheless largely protein bound, and our observations are commensurate with the proposition that high levels of protein bound T4 in mammalian blood sustain a T4 supply to the feto-placental unit, and that human TBG may be of special importance in the particular circumstances characterising human gestation.

The presence in blood of a specific protein capable of enhancing T4 availability to the feto-placental unit might be expected to confer considerable evolutionary advantage, particularly in conditions of iodine scarcity, and in species such as man characterised by a long gestational period. It should be borne in mind in this context that, in conditions of iodine deficiency, serum total and free T4 values are frequently considerably lower than those seen in the iodine-replete populations of the US and UK, albeit serum T3 levels do not significantly differ.

A rise in serum TBG during gestation is potentially damaging to the mother if the maternal thyroid is incapable of fully responding to the stimulus to increased T4 secretion, causing further lowering of the serum fT4 level and hence a reduction in T4 supply to maternal tissues. If, on the other hand, the rise in T4 succeeds in causing an enhancement in the maternal total T4 level in serum, T4 delivery to the fetus may be maintained notwithstanding a reduction in T4 delivery to other maternal tissues. The finding that lack of maternal T4 in early fetal life is associated with irreversible neurological damage (30), and the recent observation that T4 alone is available to enter the fetal brain (20), are likewise entirely commensurate with the idea that T4 is of special importance in regard to the differentiation of the fetal CNS.

In summary, the reasons for the high protein-bound levels of TH in mammalian blood, and the raised levels of TBG seen in human pregnancy continue to be matters of speculation and controversy. We have attempted to show that detailed analysis of the physicochemical effects of binding proteins on hormone transport kinetics suggests that TBG is capable of selectively transporting T4 to the feto-placental unit, and that this would constitute a biological advantage in conditions of iodine deficiency. If maintenance of an adequate supply of T4 to the fetus
were indeed TBG's basic physiological raison d'être, total absence of TBG in males would clearly be of little consequence. Even in the extremely rare cases of total TBG deficiency in females, the high levels of T4 seen in the generally iodine-replete populations of Europe and the US might obviate or obscure any adverse consequences on the offspring that absence of TBG might otherwise cause.

Maternal hypothyroxinemia and fetal brain development

Detailed physicochemical analysis of the role of TBG in hormone delivery to the fetoplacental unit, coupled with epidemiological evidence indicating an association between maternal hypothyroxinemia and cognitive/motor dysfunction in offspring in both iodine-deficient and iodine-replete environments (19, 29), has resulted in a 3-part hypothesis (5, 9, 10), as summarised below:

1) The physiological role of the specific hormone binding protein is to modulate hormone supply to the fetoplacental unit in pregnancy.

2) A high requirement for T4 exists in the fetus prior to the development of an autonomous fetal pituitary/thyroid axis.

3) Deprivation of T4 early in fetal life results in irreversible, long term effects on the brain and neurological system.

It is now well established that adequate quantities of maternal T4 traverse the placental barrier and are available to the early fetus in the rat (9, 10, 22, 33, 43), the rabbit (4) and the human (at least when the fetal thyroid is inactive) (42). Consequently, current interest is focusing on the third part of the hypothesis. The results of our most recent studies are presented below.

We have developed a rat dam model which conforms to the thyroid state (hypothyroxinemia) of pregnant women in the iodine deficiency endemias. Briefly, Sprague-Dawley rat dams are partially thyroidecotomised (parathyroid spared; Tx) and maintained under standard conditions with free access to drinking water (supplemented with 0.1% [w/v] calcium lactate) and food (iodine-replete small animal laboratory diet). When circulating T4 values have fallen to ≤ 15 nM, the dams are mated with normal males. Age-matched, euthyroid (C) dams are also maintained and mated in a similar manner. The pregnancies are either terminated at specific gestational ages, or allowed to proceed to term, in which case the size of matched C and Tx dam litters are standardized.

Phosphate and calcium metabolism

A wide range of biochemical and metabolic functions ultimately depend upon the availability of an adequate free phosphate pool (inorganic phosphate). A host of phosphorylated substrates, along with a high energy input, are necessary to sustain the almost exponential increase in nucleic acid and protein synthesis characteristic of explosive phases of neural development. Derangement in phosphate metabolism may therefore critically impinge upon the normal development of the fetal brain. Indeed, we have found the brain inorganic phosphate (P_i) concentration to be drastically reduced at 15 days gestation, but elevated at 19 and 22 days gestation in the Tx dam fetus (1). Similar changes are also observed for lipid phosphate and protein phosphate concentrations, at least at 15 and 19 days gestation (1). Of 2 protein kinase (PK) activities examined, PKC is elevated (by 23%; P < 0.05) at 15 days gestation, diminished (by 24%; P < 0.05) at 19 days gestation, but normal at term, whereas PKA is normal at all stages of gestation investigated. The intraterine TH environment also influences fetal brain phosphohydrodrolase activities in a selective manner: both Ca^{2+}-ATPase and Na^+, K^+ ATPase are adversely affected at 15, 15 and 22 days gestation, whereas acid and alkaline phosphatases are completely unaffected (1). The activity of brain Ca^{2+}-stimulated, calmodulin-dependant phosphatase (calcineurin) is also known to be severely compromised in newborn Tx dam progeny (38). These findings taken with those of others in endemic neurocrotic cretins, showing calcification of the basal ganglia and prominence of the Sylvian fissure due to mild asymmetry of the temporal lobes (13), strongly suggest an important influence of the intrauterine TH environment on phosphate and calcium metabolism in the fetal/neonatal CNS, possibly resulting in gross structural dysgenesis.

Neurotransmitter turnover

Various degrees of neurobehavioural and cognitive dysfunction, such as dippage, gait disorders, deaf-mutism, learning disability and severe mental retardation are primary features of neurologic and mixed type cretinism (31). A range of behavioural parameters (emotion, motor function, cognition and learning ability) are also compromised in adult pro

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**Fig. 4.** Calculated free T4 concentration contours across the capillary diameter for two different binding protein (albumin) concentrations, assuming the equilibrium concentration to be the same in each case. An increase in the binding protein concentration results in a higher free hormone concentration at the capillary wall, implying a greater hormone loss rate from the capillary.
ery of Tx dams (3, 14, 38). Such changes are strongly suggestive of disturbances in neurotransmitter function—indeed, an early study of adult progeny born to Tx dams has demonstrated brain region-specific compromise of monoamine oxidase, acetyl cholinesterase and choline acetyltransferase (ChAT) activities (9, 38). We have now investigated in detail the influence of maternal thyroid function on the early development of cholinergic and catecholaminergic metabolic systems. Preliminary results indicate that in Tx dam progeny, ChAT activity is reduced (by 24%; P < 0.02) at 19 days gestation but increased (by 30%; P < 0.05) at 21 days gestation. Although unaffected during the prenatal period, DOPA decarboxylase activity is transiently up-regulated in cerebellum (by 80%; P < 0.05) at 20 postnatal days and brainstem (by 44%; P < 0.02) at 30 postnatal days. The intrauterine TH environment therefore appears to exert both short term and long term influences on the development of neurotransmitter systems. Disruption of multiple neurotransmitter systems (epigenic influence) (12), may underlie the complex changes in behavioural function observed in both animal models (3, 14, 38) and neurological cretins (31).

Polyamine biosynthesis

Polyamine biosynthesis is closely linked with cellular proliferation, organ growth and development, including cellular specialisation of function. Omithine decarboxylase (ODC), the enzyme which catalyses the initial, rate-limiting step in polyamine biosynthesis, is known to be an excellent marker for growth, differentiation and maturity of a variety of systems (28). Rapid changes in activity occur in response to a wide range of stimuli, including growth factors and hormones. The brain enzyme is a sensitive marker of developmental anomalies brought about by a variety of environmental factors (40), including postnatal thyroid status (2, 16, 35, 41). We have therefore studied the fetal and postnatal ontogenesis of this enzyme in our animal model in order to investigate if the maternal thyroid status constitutes an additional important regulatory signal (32). Although the developmental profile of the enzyme is similar in both C and Tx dam progeny, brain ODC activity is reduced (by 22%; P < 0.05) at 15 days gestation but elevated (by 58%; P < 0.05) at 22 days gestation in the Tx dam fetus. Distinct, brain region-specific compromise is present in the postnatal period; activity being deficient in brainstem and cerebral cortex at 5 postnatal days, and brainstem, cerebral cortex and subcortex at 10 postnatal days (32). It is known that the ODC/polyamine system can influence the development of catecholaminergic nerve pathways and sensorimotor function (40). However, whether the observed changes in ODC ontogenesis are responsible for the associated effects on postnatal DDC ontogenesis (see above) and motor function (38) remains to be confirmed.

It is evident from our studies (3, 9, 10, 12, 32, 38, 43), and those of others (4, 14, 22, 33), that maternal T4 is available to the fetus during the critical period of neurogenesis and that an adequate intrauterine TH environment is necessary for normal brain development. Perturbation of TH homeostasis in utero results in irreversible neurobehavioural compromise. Restoration of the maternal TH environment during pregnancy by T4 replacement therapy may therefore correct/predict much of the neurological damage. Indeed, T4 therapy to pregnant women has recently been recommended by the FDA of the United States.

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References

Perturbation of Thyroid Hormone Homeostasis in the Adult and Brain Function


Summary: Although a critical role of thyroid hormones in mammalian brain development is well established and extensively documented, the adult CNS is often thought to be a thyroid hormone-insensitive organ. The presence in the adult brain of thyroid hormone, along with high levels of nuclear T3 receptors and the strict regulation of intracerebral T3 levels, coupled with overt psychomotor and cognitive dysfunctions in adult-onset dysthyroidism, casts doubt upon this assumption. We have therefore investigated the influence of thyroid hormones on the biochemistry, metabolism and molecular biology of adult rat brain regions and confluent neurons and astrocytes in culture. Our results and those in the literature show that brain nuclear T3 receptor and angiotensinogen mRNA levels and 5'D-II activity are dependent upon normal thyroid hormone concentrations. Several subfractions of cell signalling proteins (G protein α subunits) are compromised in hypo- and hyperthyroidism and the activities of protein kinases A and C are up-regulated in the hypothryoid state in a brain region-specific manner. The activities of acid phosphatase and aryl sulphatase A are compromised in the brain of hypothyroid rats, indicating a degree of lysosomal dysfunction, and several neurotransmitter metabolic enzymes and receptor systems are also affected. Metabolic experiments indicate that glutamate and acetate metabolism are compromised in the hypothryoid state, although glucose metabolism remains normal. Primary cultures of confluent neurons and astrocytes also strongly indicate a critical role for thyroid hormones in the control of amino acid uptake, protein synthesis, glycoprotein synthesis and 2-deoxyglucose uptake, in a cell-specific manner. In summary, our observations, taken together with those in the literature, demonstrate that thyroid hormones play a direct critical role in a range of biochemical and metabolic functions in the adult CNS. It is postulated that these deficits may be the underlying causes of the psychobehavioural, cognitive and motor disorders that accompany adult-onset dysthyroid states.

Einfluß von Störungen der Schilddrüsenhormon-Homöostase auf die Gehirnfunktion des Erwachsenen


Zusammenfassend zeigen unsere Beobachtungen in Zusammenhang mit den in der Literatur berichteten, daß die Schilddrüsenhormone eine direkte kritische Rolle in einer Reihe von biochemischen und metabolischen Funktionen des erwachsenen Zentralnervensystems spielen. Es wird daher die Forderung erhoben, daß diese durch Änderungen der Schilddrüsenhormonkonzentrationen ausgelösten Defizite der Zellfunktion die zugrundelie-
Introduction

Thyroid disorders are amongst the most prevalent pathological conditions in the world. Recent estimates have suggested that upwards of one billion people worldwide live under varying degrees of iodine deficiency and are at risk from the iodine deficiency disorders (IDD). Although mainly prevalent in developing countries, large pockets of iodine deficiency also exist in parts of Europe, including regions of Greece, Spain, Portugal, Italy, Germany and Ireland (25). A recent survey in Munich, Germany suggests that as many as 20% of women of college-going age have at least grade one goitre, and the incidence of cretinism in this area has been shown to be 1 in 4000 of the population. Similarly, in iodine-deficient areas of Sicily, the incidence of goitre may be as high as 44%, with a sizeable proportion of the children suffering some degree of cognitive dysfunction (14%), although overt neurological cretinism is rare (114). In the developing countries, the incidence of endemic iodine deficiency-related goitres is much higher; upwards of 60% of women of child bearing age may have palpable goitres and the incidence of overt neurological cretinism in offspring may approach 5 to 7% of total live births per annum. The diagnostic features of these cretins are diplegia, clonus, strabismus, deaf-mutism and severe mental retardation. Much larger number of children in these endemic areas also suffer from less overt disease states such as gait disorder, impaired motor coordination, loss of cognitive faculties, diminished IQ, partial deafness, speech defects and compromised school performance.

Apart from such childhood related disorders, adult-onset hypothyroidism is also common in the iodine deficiency endemic, although most women with goitre appear to be euthyroid and biochemically euthyroid. The primary features of this disease state, whether occurring in iodine-deficient or-replete conditions, are reduced basal metabolic rate (BMR) and body temperature, cerebellar ataxia, listlessness, lengthened reflex response time, lack of motor coordination, occasional psychobehavioural abnormality and, in extreme cases, overt psychotic behaviour similar to schizophrenic episodes (myxoedematous madness).

Hyperthyroidism, due to Graves' disease, nodular goitre, Hashimoto's thyroiditis or thyroid carcinoma however, is more common in the industrialised countries. The disease is manifested as a loss of weight, increased BMR, tremor of the extremities, hyperreflexia, sometimes ophthalmopathy, anxiety, confusion, psychotic state, and general behavioural disorders.

Taken together, the clinical features of adult-onset thyroid disorders strongly indicate disruption of central nervous system (CNS) function. Until recently, however, it was thought that the adult CNS was not a thyroid hormone-responsive tissue; the neurological dysfunction occurring secondary to effects on other organs such as liver and heart. This notion has nevertheless been challenged in recent years because of the presence of thyroxine (T4) and 3,5,3'-triiodothyronine (T3) in the brain, along with high levels of nuclear T3 receptors (particularly in neurons), coupled with the maintenance of intracerebral T3 levels within narrow limits. In this presentation, the available evidence indicating that the thyroid hormones play a critical role in the regulation of morphological, biochemical and behavioural function in the adult CNS is reviewed.

Thyroid hormone homeostasis

The adult rat brain contains significant amounts of both T4 and T3, with the molar ratio T3/T4 considerably greater than that in the circulation (31, 35, 51, 84, 113). Although bulk brain T3 and T4 levels of 2.0 to 2.5 and 3.5 to 4.0 nmol/kg wet weight, respectively, have been reported (31, 35, 51, 84), slight regional variations exist, especially in the case of T4 (113). Autoradiographic studies have demonstrated selective localization of [125I]T3 in discrete neural systems following intravenous administration to adult thyroidectomized rats (30, 32). Radiolabel is initially concentrated in nerve cell bodies in specific areas of grey matter and is subsequently transferred to related synapses by axonal transport; particular subregions of the cerebellum, hippocampus and thalamus becoming densely labelled (30).

Early work demonstrated that in cerebellum and cerebral cortex, the majority of intracellular T3 is derived from intracellular T4 (via local deiodination), rather than from circulating T3 (20, 21). A similar situation obtains in anterior pituitary, whereas plasma T3 is quantitatively more important in, for example, liver and kidney (105). Locally derived T3 has been determined to account for some 70 to 80% of nuclear T3 in cerebral cortex, whereas the value for cerebellum is somewhat lower at 50 to 60% (21). More extensive analysis of the CNS however, has revealed that in certain regions (hypothalamus, pons, medulla oblongata and especially spinal cord) circulatory T3 may be the major source of intracellular T3 (113).

The adult brain contains all 3 known types of iodothyronine deiodinase: type I 5'-deiodinase (5'D-I), type II 5'-deiodinase (5'D-II) and type III 5-deiodinase (5D-III) (66, 115). The 5'D-II activity is responsible for the local generation of T3 from T4 in vivo (107), whereas the main function of 5'D-I is thought to be the removal of 3,3',5'-triiodothyronine (rT3) produced as a consequence of 5D-III action (66). Studies of this latter enzyme have shown that T3 is the preferred substrate over T4 (KM's differ by an order of magnitude); its main role being the inactivation of T3 (59, 115).

The deiodinases are differentially distributed in the CNS (58) and, although primary cell culture studies indicate cell-specific localization as well, the data are somewhat contradictory. In serum-containing primary cultures, 5'D-I and 5D-III are predominantly localized in glial cells, and 5'D-II is neuronal (68). This latter observation is consistent with the presence of 5'D-II in neuroblastoma cells (99) and its preferential localization in the nerve terminal plasma membrane fraction of cerebral cortex (69). In serum-free medium however, significant 5'D-II activity has been observed in glial cells (11, 14, 15), whereas 5D-III may be present in both neurons (15) and glia (11, 19, 36). The discrepancies may in part be related to the composition of the culture medium and/or the differentiation state of the cells. In astroglia, for example, catecholamines, cyclic AMP, phorbol esters, fibroblast growth factors (FGF) and glucocorticoids all serve as inducers of 5'D-II (16, 17, 18, 65, 70), and many of these factors play a similar role in the regulation of 5D-III (19).
Thyroid hormone receptor systems

Early experiments employing saturation binding analysis revealed distinct cellular and regional distribution patterns of nuclear T3 receptors (TR) in the adult CNS. The neuronal nuclei have the highest concentration of TR, the oligodendroglial nuclei the lowest (44, 45, 95), with intermediate levels present in the astroglial nuclei. At the anatomical level, a caudal-rostral distribution pattern is apparent, with the cerebral cortex, the amygdala and the hippocampus containing the highest concentrations and the cerebellum the lowest (44, 95).

The nuclear TR are encoded by the proto-oncogenes c-erbAα and c-erbAβ (see [64] for a recent review). In the rat, the primary transcripts of both genes are alternatively spliced, yielding at least 5 different mRNAs, termed c-erbAα1, α2, α3, β1 and β2. However, only the TR-α1, -β1 and -β2 proteins exhibit T3 binding activity and can be considered true TR. Furthermore, transcription of a region of the noncoding strand of the c-erbAα gene locus results in the production of a third nonbinding protein, termed Rev-ErbAα (64).

The anatomical distribution of the various transcripts within the adult brain has been studied by several groups (9, 10, 13, 78). These studies have demonstrated widespread distribution of the c-erbAα1 transcript, with the c-erbAβ1 isomorph exhibiting a more restricted pattern. Nevertheless, considerable overlap exists between these two functional isoforms. In contrast the c-erbAβ2 transcript is largely, but not exclusively, localized to the pituitary. With respect to the transcripts encoding nonbinding variants, Rev-ErbAα is characterized by a unique and somewhat restricted distribution, whereas both c-erbAα2 and α3 exhibit almost identical patterns to that of c-erbAα1. Of the 3 c-erbAα-derivated transcripts, α2 is the most and α3 is the least abundant species. In view of the observation that the TR-α2 protein can inhibit T3-induced changes in gene expression mediated by the TR-α1 and -β1 proteins (63), at least in transient transfection studies, it has been suggested that the supposed nonresponsiveness of the adult brain may be related to the very high levels of the α2 transcript (13).

However, high levels of this transcript are also found in the developing brain, even during the critical period of thyroid hormone-dependency (10, 78).

Apart from nuclear TR, the adult brain contains both cytosolic (reviewed in [48]) and synaptosomal (74, 75) T3 binding sites. The latter are of particular interest since their binding capacity is higher in adult than developing brain (74). The synaptosomal binding sites, which are preferentially localized on the synaptic membrane, are differentially distributed throughout the brain; levels being highest in cerebral cortex and hypothalamus, but lowest in cerebellum (75). Their presence is consistent with the selective accumulation of thyroid hormone in synaptosomes following intravenous administration (29), and they may be related to the more recently described synaptosomal T3 transporters (61). Whether these sites play any role in thyroid hormone action in the brain, either at maturity or during development remains to be determined.

Hypothyroidism, as a consequence of thyroidectomy or propylthiouracil (PTU) treatment, results in a significant increase in nuclear T3 binding capacity (28, 47, 112) and a decrease in the affinity constant (28, 47). Induction of hypothyroidism by T3 administration has also been reported to result in a slight increase in receptor number, with no effect on the affinity constant (47). The up-regulation of the T3 receptor in hypothyroidism also bears a temporal relationship to the expression of the protooncogenes c-erbAα and c-erbAβ in brain regions. For example, preliminary experiments indicate slight increases in the levels of c-erbAα1 and β1 transcripts, at least in cerebellum and brainstem (but not cerebral cortex), during hypothyroidism (Fig. 1) (62). On the other hand, c-erbAα2 mRNA is unchanged in all brain regions (Fig. 1) (62), indicating perhaps that the thyroid hormones may play a role in the regulation of splicing of specific primary transcripts, including those encoding the TR. In contrast, other workers have reported that levels of brain c-erbAα1, α2 (80) and Rev-ErbAα (55) mRNAs are unaffected by thyroid status, although their data may indicate an increase in the latter transcript during hypothyroidism (55). Interestingly, the intranuclear thyroid hormone environment also appears to impinge upon the expres-
Adult-onset dysthyroid states and the brain

a) Morphology

Adult-onset hypothyroidism is without effect on biochemical indices of cell number, cell density and cell size (DNA content, DNA concentration and protein; DNA ratio in gross anatomical brain regions (1)). Nevertheless, the use of more sensitive morphometric techniques has revealed that cell number and other related parameters may be altered, at least in selected brain structures. Thus, reductions in the numbers of granule cells of the dentate gyrus (72) and pyramidal cells of the hippocampal CA1 region (73) have been reported. The number of pyramidal cells of the hippocampal CA3 region remains normal, but the volume of this cell layer is reduced, indicating an increased cell packing density possibly due to deficient elaboration of the neurite (73). The effects on cell number are essentially irreversible and may be brought about by increased cell death and, in the case of the granule cells, decreased proliferation (72, 73). Other workers however, have reported that T3 administration to adult rats is without effect on the morphology of the CA3 neurons, but results in a decrease in the apical dendritic spine density of the hippocampal CA1 pyramidal neurons (39). Further elucidation of the mechanisms and consequences of these hippocampal changes are likely to be central to our understanding of the cognitive dysfunction that is observed in hypo- and hyperthyroidism.

Thyroidectomy of adult rats has also been shown to result in morphological changes in the cerebellar, visual and auditory cortices. Both the numbers of synaptic vesicles and the formation of coated pits are increased in the mossy fibre nerve terminals of the cerebellar cortex (89). Administration of T4 can reverse such effects (89). Potential changes in synaptic function are also indicated by reductions in the numbers of dendritic spines along the apical shaft of pyramidal cells of the visual and auditory cortices (96, 97, 98). The changes in the visual cortex are reversible (98) and are observed as early as 5 days post-thyroidectomy, when brain thyroid hormone levels are severely depleted (96). The auditory cortex however, displays reduced sensitivity in that a considerably longer exposure time to hypothyroidism is required (97).

Despite these morphological changes, little is known regarding the influence of adult-onset dysthyroid states on cytoskeletal components. In vitro microtubule assembly is unaffected by the induction of a hypothyroid state in the adult rat, albeit significant effects occur during brain development (38). Although thyroid hormone influences the formation of filamentous actin stress fibres in confluent glial cells in culture, it has been reported to be without significant effect on beta-actin mRNA levels in these cells (37). Others however, have shown that thyroid hormone stimulates the accumulation of actin and tubulin proteins in primary brain cell cultures (23, 88).

b) General biochemistry

Adult-onset hypothyroidism is without effect on the content or concentration of nucleic acid or protein in gross anatomical brain regions (1). Furthermore, SDS-polyacrylamide gel electrophoresis of subcellular fractions indicates normal protein profiles (unpublished observations). Although the levels of quantitatively important proteins may be unaffected in the adult CNS, perturbation of thyroid hormone homeostasis affects specific functional proteins as described below.

The activity of the nuclear enzyme, RNA polymerase I, is reduced in the brain of adult hypothyroid animals; this effect being both rapid in onset (evident at 5 days post-thyroidectomy) and reversible by T3 replacement (26). The activities of the mitochondrial enzymes, succinate oxidase and succinate cytochrome c reductase, are also reduced in brain, but only after 60 days of hypothyroidism, whereas alpha-glycerolphosphate dehydrogenase remains completely unaffected (26). In contrast, changes in these 3 mitochondrial enzymes are apparent in the liver after 5 days (26).

Studies of lysosomal enzymes indicate that thyroid hormone action is both parameter-selective and region-specific in the adult brain. Thus aryl sulphatase A activity is reduced in cerebellum, and acid phosphatase activity is deficient in cerebellum and medulla of hypothyroid rats, whereas the activities of aryl sulphatase B and a range of glycospidases are normal (1). Furthermore, non-lysosomal alkaline phosphatase is deficient in sub-
cortex and midbrain (1), although other phosphohydrolases (Na+,K+- and Mg2+-ATPases) remain unaffected (unpublished observations). Despite the preferential localization of both aryl sulphatase A and acid phosphatase in neurons (109), general loss/compromise of this cell population is unlikely, since other lysosomal neuronal marker enzymes (β-D-glucosidase, N-acetyl-β-D-glucosaminidase and especially β-D-galactosidase) (109) are unchanged (1). The normality of N-acetyl-β-D-galactosaminidase activity (1) also argues against any gross effects on glia (110). Indeed, similar conclusions can be drawn from the normal expression of cell marker genes in the adult hypothyroid rat (54). Rather, since different lysosomal enzymes are packaged in different particles, it may be argued that only specific lysosomal subgroups are adversely affected (109).

Aryl sulphatase A, which plays an important role in the turnover of the myelin sheath, is known to be compromised in metachromatic leukodystrophy. Although the adult-onset form of this disorder results in impaired intellectual, emotional and motor function, the deficiency in the hypothyroid brain was by no means so complete (50 to 60% reduction) and restricted to only cerebellum (1). Nevertheless, other workers have reported region-specific changes in myelin-associated marker enzymes; 2',3'-cyclic nucleotide phosphodiesterase being reduced in whole forebrain and myelin-associated 5'-nucleotidase being increased in medulla (76), albeit proteolipid protein mRNA levels remain normal (54).

c) Metabolism

Substrate metabolism in the adult CNS as a function of perturbed thyroid hormone homeostasis has not been investigated in detail. Recently, 13C NMR spectroscopy has revealed that adult-onset hypothyroidism compromises in situ acetate metabolism (12). Radiotracer experiments in our laboratory however, indicate minimal disruption of glucose and acetate metabolism in tissue slices of various brain regions (unpublished observations and (2)). In vitro glutamate metabolism is compromised insofar as the incorporation of radiolabel into the CO2 fraction is reduced; the labelling of the protein, nucleic acid and lipid fractions remaining normal (2).

d) Neurotransmitter function

Chronic and acute hypo- and hyperthyroidism are associated with a spectrum of psychobehavioural disorders which are indicative of derangements in neurotransmitter metabolism/function. Indeed, it is generally accepted that hyperthyroidism is associated with an increase and hypothyroidism with a decrease in the catecholamine and indoleamine turnover rates in whole brain (reviewed in [92]), although contradictory evidence has been presented concerning the influence of thyroid status on regional levels of particular monoamines, as well as their synthetic rates (49, 92).

For example, hypothyroidism has been reported to increase the rate of dopamine synthesis in midbrain, brainstem and cerebrum, with hyperthyroidism producing a similar effect in midbrain. The rate of noradrenaline synthesis has been found by some workers to be independent of thyroid status, at least in brainstem, midbrain, striatum and cerebrum, whereas others have reported changes in discrete regions of hypothalamus (49, 92). Chronic T3 treatment however, produces an increase in the bulk phase serotonin synthesis rate, independent of changes in brain tryptophan accumulation (3).

At the enzymic level, hypothyroidism is associated with an increase in tyrosine hydroxylase activity in certain hypothalamic nuclei, but not in brainstem (92), whereas midbrain activity is either normal (92) or markedly increased (34). Hyperthyroidism however, is without effect on midbrain (34) or striatal (93) tyrosine hydroxylase. Tryptophan hydroxylase activity is also resistant to this condition, at least in midbrain (93). With respect to the degradative enzymes, a study of midbrain and cerebral cortex of hyperthyroid rats has revealed decreased catechol-O-methyltransferase activity but normal monoamine oxidase activity (93).

We have recently reported increased monoamine oxidase activity in the cerebellum of hypothyroid rats; all other brain regions remaining normal (1).

Pharmacological studies indicate that changes in central adrenergic and dopaminergic receptor sensitivities may accompany altered thyroid status in adults (3, 22, 34, 92). Saturation analysis supports this view: the numbers of cerebrocortical α1-, α2- and β-adrenoceptors are reduced in hypothyroidism (40, 41, 101), whereas only the β-adrenoceptors...
are influenced by T3 treatment (101). With respect to the dopaminergic system, the levels of D2 receptors are decreased in striatum as a result of hypothyroidism (22), although they are apparently unaffected during hyperthyroidism (3).

Less complete information is available on other transmitter systems. We have previously reported that in hypothyroidism, the activity of acetylcholinesterase is decreased in cerebellum, medulla and subcortex (1). However, information is lacking on the synthetic enzyme, choline acetyltransferase, and cholinergic receptor systems. Compromise of the cholinergic system is feasible in view of the observation that the number of dendritic spines on the cerebral pyramidal neurons are reduced, since these cells are normally connected by afferent cholinergic fibres from the basal nuclear system.

Opiate receptor binding is also known to be dependent upon thyroid status. The number of naloxone binding sites is increased in mice made hyperthyroid by T4 treatment (33). Conversely, Lofentanil (a morphine agonist) binding sites are decreased in both cerebellum and cerebral cortex of the hypothyroid rat (27).

With respect to the amino acid neurotransmitter systems, we have previously shown that GABA transaminase is reduced in all brain regions, whereas aspartate aminotransferase is selectively reduced in cerebellum and cerebral cortex (2). Hypothyroidism is without effect on the numbers of cortical GABAA binding sites, but these are decreased during hyperthyroidism (101), in association with an increased sensitivity to isoniazid- and picrotoxin-induced seizures (102). Parallel changes are not seen in benzodiazepine receptors (102), although imipramine binding sites are reduced in cerebral cortex and hypothalamus but increased in hippocampus as a consequence of hypothyroidism (101).

Thyroid status may also modify neuromodulator function. Hypothyroidism produces region-specific changes in the activities of adenosine metabolic enzymes; membrane-associated 5'-nucleotidase (responsible for extracellular adenosine production) being increased in cerebellum, cerebral cortex, striatum and hippocampus, whereas adenosine kinase (which plays a role in adenosine disposal after reuptake) is decreased in cerebellum, striatum, hippocampus and hypothalamus (76). It has been suggested that these changes may enhance adenosine-mediated presynaptic inhibition of excitatory neurotransmitter release (76). Further analysis of this neuromodulatory system has revealed that although the numbers of A1 adenosine receptors remain normal, hypothyroidism increases agonist-mediated inhibition of adenylyl cyclase activity (77). Indeed, subsequent work has shown that adult dysthyroid states can impinge upon the expression of receptor signalling mechanisms.

Modulation of the adult thyroid status influences the abundance of G-protein α subunits in synaptic compartments. During hypothyroidism, the expression of Gi1α, Gi2α and Goα are up-regulated in a region-specific manner which is characteristic for the particular subunit under study (86). Thus, Gi1α is affected only in cerebral cortex and striatum, whereas the level of Gi2α is additionally affected in medulla, hippocampus and hypothalamus and that of Goα is increased in all regions except hypothalamus (86). Induction of hyperthyroidism by T3 treatment for 3 days, down-regulates Gi1α and Gi2α in cerebral cortex but not medulla. In contrast, the expression of Goα is totally unaffected in all 3 regions (cerebellum, medulla, and cerebral cortex) by this short treatment schedule (85).

We have investigated the influence of hypothyroidism on the activities of protein kinases A and C (PKA and PKC, respectively) in various brain regions. The results show that PKC activity is up-regulated in cerebral cortex only, whereas PKA activity is elevated in all regions studied (Table 1).

Such changes, if with effect on the normal protein phosphorylation pattern, may impinge upon a multitude of targets, including neurotransmitter synthetic enzymes and receptors, ion channels, signalling pathways and cytoskeletal proteins (82).

e) Gene expression

It is well known that thyroid hormone action in the developing brain is mediated primarily at the level of the nucleus, through prior interaction with non-histone receptor proteins, with consequent changes in the transcription of target genes. Despite the accumulating morphological and biochemical evidence in favour of a role for the thyroid hormones in the adult brain, very few studies have considered their influence on gene expression. Apart from the influence of thyroid hormones on the expression of the c-erbA isoform mRNAs in adult CNS, as discussed above, we are aware of only 2 other studies.

Hypothyroidism, produced by PTU administration, has been shown to significantly reduce steady state angiotensinogen mRNA levels in diencephalon and brainstem of adult rats (52). Hyperthyroidism induced by T4 treatment over an identical time course is without effect on brain mRNA, despite an increase in liver levels (52). Nevertheless, addition of T3 to serum-free cultures of primary astrocytes increases angiotensinogen secretion, indicating that the effect of adult-onset hypothyroidism may be directly mediated (52).

Hypothyroidism has also been reported to decrease RC3 mRNA levels in the adult brain cortex and striatum, but is without effect on the expression of another neuronal gene, neuron-specific enolase (54). The effect is reversible: levels of RC3 mRNA can be normalised by T4 administration to hypothyroid animals for 5 days (54). Nevertheless, the

<table>
<thead>
<tr>
<th>Brain region</th>
<th>PKA Specific activity (OD units/mg protein)</th>
<th>PKC Specific activity (OD units/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>Tx</td>
</tr>
<tr>
<td>Cerebral cortex</td>
<td>0.638 ± 0.016</td>
<td>0.715 ± 0.013*</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>0.352 ± 0.011</td>
<td>0.629 ± 0.017*</td>
</tr>
<tr>
<td>Brain stem</td>
<td>0.492 ± 0.010</td>
<td>0.589 ± 0.009*</td>
</tr>
</tbody>
</table>

* P < 0.02; Tx versus N. Values are the means ± SEM of 5 animals.

Table 1. Specific activities of PKA and PKC in different brain regions from euthyroid (N) and hypothyroid (Tx) adult rats.
cellular function of the gene product remains to be determined.

Out of the very few specific genes that have been investigated, either in confluent cultured cells or adult animals, none appear to be completely under thyroid hormone control. Expression of such genes is not entirely obliterated by thyroid hormone deficiency but only modulated. It appears that expression of genes in the CNS are under the influence of multiple signals, thyroid hormones being only part of the overall control system. Nevertheless, attenuation or stimulation of gene expression in the CNS by thyroid hormones will place severe restrictions upon normal brain function.

f) Psychobehavioural function

This section includes short descriptions of psychobehavioural dysfunctions due to perturbed thyroid hormone homeostasis in humans, since this subject will be covered in greater detail elsewhere. A range of abnormal psychological disorders, behavioural morbidity and motor dysfunctions due to adult-onset hypothyroidism have long been known. A general slowing of psychomotor function is observed and symptoms resembling affective disorders, such as melancholia, psychotic behaviour and depression, may be present. These were originally identified by Sir William Gull (42), who coined the phrase "myxoedematous madness" to describe the pathology.

Such fundamental psychological parameters as memory and mood appear to correlate significantly with general hypothyroid state, and these changes may be irreversible (79). The administration of T4 to patients with subclinical hypothyroidism however, can improve memory skills and have beneficial effects upon other psychobehavioural symptoms (81). Hypothyroidism has also been shown to be associated with dementia which can be alleviated by replacement therapy with thyroid hormones (50) and loss of cognitive functions in elderly hypothyroid patients without dementia has also been reported (87). Rapid cycling bipolar affective disorders, Prader Willi syndrome and depressive illness have all been positively correlated with hypothyroid state in human subjects in recent years (5, 6, 46). Successful treatment of manic depression with high doses of T4 has also been reported (4, 111).

Disturbances of cognitive and motor function are also apparent in hyperthyroidism. However, short term thyroid hormone administration to normal adults has recently been reported to improve cognitive processing in a visual search paradigm (8). Patients often exhibit emotional lability and psychosis may develop, with delirium, stupor, coma and even convulsions in extreme cases. Psychopathological and neuropsychological symptoms have also been shown to be a common feature in subclinical hyperthyroidism, with abnormalities resembling a mainly depressive syndrome also present in remitted hyperthyroidism (7). Increased anxiety, nervousness, irritability, depressiveness and agoraphobia, have also been correlated with the subclinical hyperthyroid state (94, 117). Personality disorders and affective disorder-like syndromes are known to co-exist with hyperthyroidism in man.

It is postulated that thyroid hormones may induce a stimulatory state in the CNS in a region-specific manner, perhaps interacting through a variety of transmitter receptor systems as discussed in the previous section.

Conclusion

Experimental data from clinical investigation and animal studies clearly indicate the involvement of thyroid hormones in the maintenance of a variety of metabolic functions in the adult CNS. Any disturbance of thyroid hormone homeostasis will therefore impinge upon normal brain function. Of particular importance is the imbalance of transmitter systems of various kinds (cholinergic, catecholaminergic, glutamatergic and GABAAergic). Administration of psychotropic drugs also affects thyroid hormone homeostasis in the CNS, and administration of thyroid hormones appears to have some beneficial effects in the management of psychiatric states including affective disorders. However, a large number of studies investigating the direct relationship between thyroid hormone state in the CNS and transmitter functions have so far failed to find unambiguous answers. The exact role of thyroid hormones in adult brain function is therefore poorly understood at present, although the biochemistry of most brain regions appears to be compromised, at least to some extent.

These observations have important implications in the clinical management of hypo- and hyperthyroid conditions, in that the dose and duration of replacement therapy and/or administration of antithyroid drugs must be carefully controlled to avoid collateral damage to the CNS. Thyroid hormones have been used extensively (in combination with other medications) to control obesity in apparently normal subjects. The use of thyroid hormones for entirely social/cosmetic purposes should, if possible, be avoided and when unavoidable, regular monitoring must be instigated.

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In iodine deficiency endemias, the incidence of neurological cretinism has been correlated with maternal hypothyroxinemia. Although maternal thyroxine (T4) was thought to be unavailable and unnecessary for foetal brain development, recent studies in a variety of species indicate substantial transfer of T4 from mother to fetus in early pregnancy, and its subsequent localization in the foetal brain. Furthermore, work with a hypothyroxinemia rat dam model has indicated that the transferred maternal hormone is of critical importance for normal brain development in utero and CNS function in adult progeny. Nevertheless, more detailed investigations of the intrauterine mechanisms are required. We have therefore studied the influence of maternal hypothyroxinemia on foetal brain enzyme systems involved in transmembrane signal transduction, inositol phosphate-related cascades and calcium homeostasis.

Normal (N) and partially thyroidectomized (TX) rat dams were mated with normal males and the foetuses were obtained at various stages of pregnancy. Foetal brains were dissected out, homogenized in 0.32 M sucrose and assayed for protein kinase C, protein kinase A and Ca^{2+}-ATPase activities, using conventional procedures.

Protein kinase C specific activity was increased (by 23%; P < 0.05) at 15 days of gestation in TX dam progeny but decreased (by 24%; P < 0.05) at 19 days of gestation (soon after the onset of foetal thyroid hormone synthesis). However, activity was normalized near term (22 days of gestation). In contrast, protein kinase A specific activity was normal at all stages of gestation studied. Ca^{2+}-ATPase specific activity showed a different response; specific activities being decreased at 15 and 19 days of gestation (by 35-40%; P < 0.05) but increased (by 42%; P < 0.01) at 22 days of gestation.

In summary, maternal hypothyroxinemia may compromise the ontogenesis of selective signal transduction systems in the developing CNS. These changes may affect the expression of genes not under direct thyroid hormone control (catastrophic model).
P219 MATERNAL HYPOTHYROXINEMIA AND PHOSPHATE HOMEOSTASIS IN THE FOETAL BRAIN

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The intrauterine thyroid hormone environment prior to the establishment of independent foetal thyroid hormone synthesis has been postulated to be of crucial importance in brain development. Indeed, previous work has shown compromise in a range of biochemical, metabolic and behavioural functions in the CNS of progeny born to hypothyroxinemic rat dams. The aim of this study was to investigate the influence of maternal hypothyroxinemia on the homeostasis of phosphate metabolism in the foetal brain.

Normal (N) and partially thyroidectomized (parathyroid-spared; Tx) female Sprague-Dawley rats were mated with normal males and the resulting foetuses were removed at various stages of development. Total inorganic phosphate, lipid phosphate and protein phosphate was determined in brain homogenates, along with the specific activities of a range of phosphatases.

The concentration of inorganic phosphate was drastically reduced at 15 days of gestation (by 92%; $P < 0.005$) but increased at 19 and 22 days of gestation (by 220-240%; $P < 0.005$). Protein phosphate followed a qualitatively similar pattern, although no significant change was apparent near term, whereas lipid phosphate was decreased at both 15 and 22 days of gestation (by 73%; $P < 0.01$ and 91%; $P < 0.005$, respectively), but increased at 19 days of gestation (by 100%; $P < 0.005$). Na+K+-ATPase activity was deficient at all stages of gestation ($P < 0.01$), whereas Ca²⁺-ATPase, although reduced at 15 and 19 days of gestation (by 35-45%; $P < 0.01$), was increased near term (by 22%; $P < 0.02$). No change was apparent in either acid phosphatase or alkaline phosphatase specific activity at any developmental point.

In conclusion, maternal hypothyroxinemia is associated with severe perturbations of phosphate metabolism in the developing CNS which may restrict cellular proliferation and differentiation. Such changes may in part be responsible for the neural dysfunction observed in children born to hypothyroxinemic women.