BIOCHEMICAL EFFECTS AND MOLECULAR MECHANISMS OF THYROID HORMONE ACTION IN THE DEVELOPING RAT BRAIN

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

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ABSTRACT

Evidence has recently accumulated favouring the notion that maternal thyroid hormones (TH), specifically T4, traverse the placenta from mother to fetus during pregnancy and exert influences critical to normal fetal brain development. The aim of this project was to assess whether lack of T4 transfer resulted in any alterations to the biochemical systems of the fetal brain, and if so, to attempt to identify some of the molecular mechanisms responsible.

The progeny of hypothyroxinemic rat dams demonstrated organ-specific reductions in weight and protein content during the neonatal period, after which compensation took place. In the brains of the progeny, specific reductions were observed in the levels, localisation, and temporal expression of a number of Con A binding glycoproteins both in membranes and in the cytosol. The activities of certain lysosomal enzymes were also found to be reduced.

T3 stimulated the incorporation of [$^3$H]-fucose into glycoproteins in astrocytic cultures, where it became concentrated in the plasma membrane. Time course experiments suggested that regulation occurs at the transcriptional rather than translational level. It is therefore possible that TH is involved in the regulation of neurogenesis partly via controlling the expression of cell-surface glycoproteins.

A study of the ontogeny of TH binding to nuclear receptors in separated neuronal and glial nuclei from different regions of neonatal rat brain revealed high levels of binding in both neurons and glia which diminished with age. An early postnatal peak in receptor number was due to high levels in the glia. A significant neonatal increase was also noticed in the affinity of the receptor for hormone in glial nuclei. It is suggested that this may be evidence for the presence of at least two types of receptor, which are expressed in a temporally-controlled, and possibly cell specific manner, and may be involved in co-ordination of TH action on the developing brain.
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LIST OF ABBREVIATIONS

Abbreviations were the same as those recommended in (378), with the following additions:

4-MU 4-methylumbelliferone
B bound
B/F bound/free
BGT3 brain glucose transporter 3
cAMP cyclic AMP
CBG corticosteroid binding globulin
cDNA complementary DNA
CNS central nervous system
CNTF ciliary neurotrophic factor
Con A concanavalin A
CTBP cytosolic thyroid hormone binding protein
D diffusion/mixing rate constant
D-I (5-, or 5'-) deiodinase
D-II 5'- deiodinase (outer, or phenolic ring)
D-III 5-deiodinase (inner, or tyrosyl ring)
DABA 3,5-diaminobenzoic acid
DIMIT 3,5-dimethyl-3'-isopropyl-L-thyronine
DTT dithiothreitol
EEG electroencephalogram
EGL epigranular layer
FCS fetal calf serum
FE fractional efflux
fT3 free triiodothyronine
fT4 free thyroxine
GABA γ-aminobutyric acid
GFAP glial fibrillary acidic protein
GH growth hormone
GS glutamine synthetase
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<td>T4</td>
<td>thyroxine</td>
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<td>TBG</td>
<td>thyroxine binding globulin</td>
</tr>
<tr>
<td>TBS</td>
<td>tris buffered saline</td>
</tr>
<tr>
<td>TCA</td>
<td>trichloroacetic acid</td>
</tr>
<tr>
<td>TCA (cycle)</td>
<td>tricarboxylic acid cycle</td>
</tr>
<tr>
<td>TD</td>
<td>thyroidectomised dam</td>
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<tr>
<td>TETRAC</td>
<td>tetraiodo-acetic acid</td>
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<td>TH</td>
<td>thyroid hormone</td>
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<td>THNR</td>
<td>thyroid hormone nuclear receptor</td>
</tr>
<tr>
<td>TRH</td>
<td>thyrotropin releasing hormone</td>
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<tr>
<td>TRIAC</td>
<td>triiodo-acetic acid</td>
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<tr>
<td>Tris</td>
<td>tris(hydroxymethyl)-methylamine</td>
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<tr>
<td>TSH</td>
<td>thyroid stimulating hormone (thyrotropin)</td>
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<td>[fH]e</td>
<td>free hormone concentration (bolus at equilibrium)</td>
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<td>[fH]w</td>
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CHAPTER 1 : INTRODUCTION
1.1 Historical Perspectives

Recent years have seen dramatic advances in our understanding of the molecular mechanisms through which thyroid hormones (TH) operate. The importance of the thyroid gland for growth and brain function was, however, first recognised over a century ago by Fagge, who first linked sporadic cretinism to atrophy of the thyroid gland (1). Several comprehensive reviews of the subject have appeared in the literature (2-4).

The field of thyroid physiology became established with observations of the effects of goitre removal in humans. Goitre and cretinism were known to be linked in ancient times; that they were due to deficiencies of substances produced in the thyroid was established in the last two decades of the 19th century (2-4). Experimental thyroidectomy of animals produced the symptoms of cretinism in respect of stunted growth, lethargy, increase in weight and hair loss (2-4). It was also during this period that hypothyroidism was first treated successfully with thyroid extract (5). The demonstration that administration of this extract increased the consumption of oxygen by experimental animals provided the first indications of the metabolic role of TH. At the same time, an early clue to TH chemistry was provided with the identification of the presence of iodine in the gland (2).

In 1912, Gudematsch provided the first quantitative demonstration that thyroid substances could influence maturation, when he accelerated tadpole metamorphosis by feeding them thyroid extract, thereby also providing the first sensitive method for testing TH and their analogues (6). The biologically active iodine-containing compound thyroxine (T4) was isolated and crystalised by Kendal in 1914 (7), and was finally synthesised by Harrington and Barger in 1927 (8). A succession of studies of the biological potency of various artificially created T4 analogues was instigated, culminating in the discovery of 3,5,3'-triiodothyronine (T3) in the thyroid by Gross and Pitt-Rivers in 1952 (9), and subsequently in serum by Roche and co-workers (10). This compound was identified as the most biologically active naturally occurring TH derivative in a variety of animal tissues. Gradually, increasing numbers of biological processes were recognised to be, to some extent, under TH control, both during periods of growth and development, and at the immediate or initiating level in metabolism.
1.2 Clinical Considerations

A variety of disorders arise from failure of systems through which TH exert control over both developmental and metabolic processes. These result from a lack or excess of hormone (hypothyroidism and hyperthyroidism respectively), and vary according to the degree of imbalance, and the time at which it occurs.

Cretinism is at the extreme end of the scale of dysfunction, and is caused by deficiency of TH during early development. Three different types of cretinism have been identified, and are distinguished on the basis of their etiology. The first results from congenital hypothyroidism, in which the fetus is unable to produce its own TH (11). This may be due to genetic abnormalities, or result from \textit{in utero} exposure to goitrogens or antithyroid drugs (12). On occasions, the disorder may arise secondary to TSH (thyroid stimulating hormone or thyrotropin) deficiency, or tertiary to a failure of thyrotropin releasing hormone (TRH) release (13).

Endemic cretinism has been defined as the condition displaying irreversible somatic and mental abnormalities which result from iodine deficiency in defined geographical locations (14). The symptoms of the disorder vary between regions, and it can be divided into two classes: myxoedematous cretinism, in which the damage results from severe hypothyroidism in the fetus, neonate and child, due to extreme iodine deficiency (15); and neurological cretinism, the clinical manifestation of which differs significantly from that of the myxoedematous form of the disease, and includes deaf-mutism, strabismus and diplegia (16).

Neurological cretinism, with its characteristic severe and irreversible effects on brain function, has historically been the most difficult to explain. In mothers from iodine deficient regions, the serum concentrations of T4 (total and free) are frequently below the normal range, whereas the serum concentrations of T3 (total and free) are normal or even raised (17,18). In many of these cases, the offspring are totally euthyroid (19).

Up to a billion people inhabit iodine deficient areas, and in certain regions almost all the population suffers from a degree of goitre. The children of women with this condition are therefore at risk from endemic cretinism, and approximately 10% of children are born with the most severe forms of the disease (19). Additionally, a range of subclinical, but significant, neurological deficits occur in the
children of these women, including poor motor co-ordination and cognitive dysfunctions (20).

A range of progressively less serious disorders result mostly from iodine deficiency in later life, and are typically associated with some type of goitre. Other thyroid disorders include autoimmune disease, which can result either in hypothyroidism, or hyperthyroidism; and hyperthyroidism, resulting from a variety of causes (12).

In later life, most thyroid disorders can be adequately treated with hormone replacement therapy, as they cause metabolic dysfunctions which are swiftly remedied. Thyroid disorders in development are, however, far more serious. Treatment of neonatal children who are hypothyroid alleviates most of the developmental dysfunction which would otherwise result from lack of hormone, but permanent damage results if therapy is not introduced immediately (21). Indeed, there is considerable evidence to indicate that, especially in the case of neurological cretinism, therapy must begin in the first trimester of pregnancy, before the onset of fetal thyroid function, if irreversible damage is to be avoided (22). It is therefore clear that thyroid disorders in the pregnant woman are of crucial importance to fetal development, and must be treated appropriately. Considerable controversy exists as to the correct therapy under all circumstances. This stems from arguments regarding the time at which TH become important for development, and whether it is maternal thyroxine, or elemental iodine that is required from the mother in early gestation (23).

1.3 Ontogenesis of Thyroid Function

The thyroid originates as an outpouching of the ventrobuccal endoderm, first apparent in the human at day 17 of gestation. The gland develops from a bilobed pouch into a mass of tissue which descends to its final position in the anterior lower neck, where differentiation is completed (24,25).

The human thyroid gland first begins to secrete TH at approximately the 80th day of gestation. In the rat this occurs at gestational day 18-19 (term 22 days). In both species secretion follows the appearance of follicular spaces and the development of the capacity of the gland to concentrate iodine and form colloid. Hormone synthesis and secretion then increases to term in humans, and until about
postnatal day 24 in the rat (26).

Iodine is concentrated by incorporation into the protein thyroglobulin, which is secreted into the follicular lumen for storage. Hormone (mostly T4, but also T3) is manufactured from this store and secreted directly into the blood, where it is transported mostly bound to plasma proteins (99.9% for T4; 99% for T3), the main one being thyroxine binding globulin (TBG). In the human serum total T4 is first detected at about 10-12 weeks at a reported level of 26 nM and the concentration rises steadily until approximately 200 nM (27). The free hormone levels rise in parallel until the 24th week, after which the concentration of TBG plateaus out at about 6 mg/dl. From this point on, free hormone level rises at a slightly greater rate than that for the total hormone. Serum T3 levels are extremely low throughout gestation, whilst those of the inactive metabolic product of T4, rT3 (3,3',5'-triiodothyronine), are relatively high, implying that the majority of tissue T3 is likely to be produced by local deiodination of T4 during fetal life (28).

In the rat, total T4 is very low at birth, and total T3 is undetectable. Serum levels rise to a neonatal peak at around day 18 for total T4 (approximately 90 nM), and day 30 for total T3 (approximately 1.25 nM), before both level off towards adult levels (Figure 1)(29).

The maturation of the control systems which regulate TH secretion are of considerable importance in the ontogeny of thyroid function. TH function is monitored by the hypothalamus, which reacts to diminished serum concentrations of T4 by releasing TRH, which in turn stimulates the anterior pituitary to secrete TSH. TSH causes the stimulation of a large number of factors associated with the synthesis and release of TH, including acting as a growth stimulator. TSH is therefore the hormone responsible for the goitres of hypothyroid subjects, and measurement of this hormone is the usual method of assessment of thyroid function.

It appears that during early thyroid development, both the growth of the gland, and the secretion of thyroglobulin are independent of TSH. Pituitary TSH becomes detectable at about the same time that TH secretion commences in both the human and the rat, but remains low until approximately the 16th week (human) (24). After this time, concentrations in the pituitary rise sharply, followed by rising serum levels as secretion rates increase towards mid-gestation. Hypothalamic TRH levels rise in parallel with TSH secretion rates also increasing significantly at mid-gestation (30).
Figure 1: Ontogenesis of the serum levels of thyroid hormones and their controlling hormones during the neonatal period in the rat.

Adapted and redrawn from Fisher et al. (27).
This suggests that from independent beginnings, the hypothalamic-pituitary-thyroid axis starts to mature at around mid-gestation (24 weeks). Serum total and free T4 rise during this period, and continue to increase after a levelling off of both TSH and TRH levels, suggesting that the serum total T4 concentration suppresses the release of TSH and reflecting the maturation of the neuro-endocrine control system. In the human fetus, TSH falls from approximately 15 μU/ml at week 30 (a value which would indicate hypothyroidism in an adult), to 7 μU/ml just prior to term (24). These patterns are also apparent in the ontogenesis of the control of rat thyroid function, except that the events occur predominantly postnatally, indicated by the fall in serum TSH occurring between postnatal days 14 and 40. The main events of the ontogenesis of thyroid function in the rat are illustrated in Figure 1.

It is important to remember that all the values quoted here are serum concentrations. The levels of TH available to individual tissues, and the responsiveness of the latter are governed by a number of factors in addition to serum concentrations, including transport into the tissue, metabolism of the hormone, and receptor binding of the hormone, all of which may be controlled in specific manners. Thus if a tissue is capable of concentrating TH, and maximising its metabolism and ability to respond, it may be sensitive to relatively low serum levels of TH. In order to understand the relevance of these possibilities, it is essential to first consider some important aspects of the chemistry of TH, especially in relation to their biological activity.

1.4 Chemistry of the Thyroid Hormones

The most important hormone secreted by the thyroid is thyroxine. This iodothyronine is generally regarded as a pro-hormone, as it possesses only one tenth of the biological activity of T3. In tissues reported to be virtually impermeable to T3 (notably the brain), T4 may be the major source of T3 (31).

A few years after the discovery of T3, the metabolic products of deiodination, rT3 and T2 (diiodothyronine) were isolated in the thyroid and in plasma from the rat (32). These compounds, though similar to T4 and T3, were biologically inactive, and measurement of their concentrations in serum was only possible after the establishment of accurate radioimmunoassays in the 1970's.
In order to understand the relative potencies of the different TH analogues, it is necessary to examine the structure of the TH in relation to their activities. It is accepted that TH exert their actions predominantly through binding to protein receptor molecules (33). The most widely studied of these is the nuclear receptor, but other binding sites certainly exist at the level of the membrane, cytosol, and mitochondrion (33). The activity of receptors will be discussed in more detail after the chemical nature of the hormones themselves has been examined.

1.4.1 Structure of the iodothyronines

The iodothyronines (Figure 2) consist of two iodinated phenyl rings joined together by an ether linkage. The positions of the iodine atoms at 3 and 5 on the inner ring and the hydrogen atoms at 2' and 6' on the outer ring, results in a minimum energy configuration of the molecule in which the rings are perpendicular to one another, with rotation possible around the ether linkage. Where only one position on the outer ring is iodinated, as in the case of T3, two possible conformations exist: proximal, when the iodine of the outer ring is orientated towards the inner ring; and distal, when it is directed away. It is the latter form which is biologically active (34).

The hormones interact with the active sites within the receptor producing allosteric changes which render it active by causing an increase in the affinity of nuclear receptor binding to DNA. A large number of studies have examined artificially produced analogues of TH to identify the groups within the molecule which are necessary for its function, and have demonstrated three main structural requirements (see (32) for review). Firstly, a central lipophilic core is needed which is composed of two perpendicular phenyl rings linked at an angle of 120°. The ether link is therefore important in maintaining the stereochemical conformation of the molecule. The second requirement is the presence of non-polar groups at the 3 and 5 positions adjacent to the ether link, and at the 3' site. These need not necessarily be iodine atoms, as analogues which are alkylated at these positions are also biologically active (e.g. 3,5-dimethyl-3'-isopropyl-L-thyronine (DIMIT) and isopropyl T2 (3,5-diiodo-3'-isopropyl-L-thyronine). Any substitution of a polar group in these positions significantly reduces activity, as does any loss of a non-polar group on the inner ring, explaining the inactivity of rT3.
Figure 2: Structure of the thyroid hormones.

**THYROXINE (T4)**

**3,5,3'-TRIOIODOTHYRONINE (T3)**

Outer (phenolic) ring    Inner (tyrosyl) ring
Finally, there is a requirement for polar groups at both ends of the molecule. The 4’ hydroxyl group is thought to be involved in hydrogen bonding to the receptor, as substitution with a non-polar group, such as caused by methylation, results in a loss of activity. The negative carboxylate ion of the alanine side chain is also important for activity, though the amino group is not. Binding is intensified if acetic acid is substituted for alanine in this position, as with 3,3',5-triiodo-L-thyroacetic acid (Triac). This was confirmed by the substitution of large groups onto the amino position, with no subsequent loss of activity implying that the amino group is situated outside the active site of the receptor (35).

### 1.4.2 Metabolism of the Iodothyronines

The most important types of iodothyronine metabolism involve deiodination, which is responsible both for increasing hormone activity, by converting T4 to T3, and degrading all iodinated thyronines. A considerable amount of work has been concentrated in this area in an attempt to clarify the mechanisms involved and understand their regulation (36).

Two distinct types of deiodination are immediately apparent: 5-deiodination, where the iodine is removed from the inner (tyrosine) ring; and 5'-deiodination, where the outer (phenolic) ring iodine is displaced (37). In fact, studies have demonstrated that at least three different mechanisms are involved, probably reflecting the operation of three separate isozymes (38). These are classed according to their substrate specificity and their susceptibility to inhibitory substances. On this basis, the processes are designated as follows: type I deiodination (D-I), where the iodine substrate can be situated on either the inner or outer ring, and the catalysis is inhibited by polythiouracil (PTU); type II (outer or phenolic ring) deiodination (D-II), where the target is specifically 5'-linked iodine; and type III (inner or tyrosyl ring) deiodination (D-III), where the substrate is exclusively 5-linked iodine. Neither type II nor type III deiodination is PTU-sensitive (Figure 3)(36,38).

The roles played by these enzyme systems in the determination of TH levels available to tissues, or even cell types cannot be ignored. This is particularly the case where alterations in the activities of the different systems may occur during development, thereby influencing the amount, or type of hormone available to
Figure 3: Monodeiodination of the iodothyronines.

Adapted and redrawn from Engler and Burger (36).
specific tissues at critical periods. It is, however, important to remember that
deiodinase enzyme activity cannot be taken in isolation to indicate a particular state of
TH responsiveness of a tissue.

Before explaining the mechanisms, localisations, and interpreting the
physiological relevance of each of these enzyme reactions, it must be pointed out that
much of the data so far produced regarding the operation, and in particular the
regulation of these processes are conflicting. This is, in part, a result of the range of
methodologies employed to study the reactions, but also of species and tissue
differences. Although it is therefore difficult to generalise, it is encouraging that
where human and rat work has been similarly performed, results have indicated
comparable systems at work (39).

The three isozymes share certain common features, including a requirement for
free sulphydryl groups as co-factors (albeit in different concentrations)(40). In vitro
experiments have typically employed dithiothreitol (DTT) at various concentrations to
fulfil this function. In vivo, it is thought that glutathione (GSH) may be the
operational co-factor (41). This has been studied most extensively in the non-specific
D-I, where it has been demonstrated that it is the proportion of glutathione in the
reduced form compared to the oxidised form that is crucial for enzyme activity. It has
been suggested that NADPH plays a role in the determination of this GSH(reduced):
GSSG(oxidised) ratio, and as such provides a link between energy metabolism and
deiodinase activity (38). This would imply the involvement of D-I in regulating the
levels of T4 to T3 or rT3 conversion in a manner directly linked to the energy
requirement.

It is uncertain what, if anything, determines whether D-I exhibits 5-, or 5'-
deoiodinating activity. It has been noticed that in the rat, outer ring deiodination is
favoured at a pH of 6.4, whereas a pH of 8.0 leads to mostly inner ring activity, but
this is not universal (42). If it were the case, isolated microenvironments would be
necessary to maintain a pH different from that of bulk phase cytosol (pH 7.2 - 7.4). In
some instances, sulphation of the 4' hydroxyl group of the TH may direct activity.
5-deiodination (D-I) is reported to occur significantly faster on sulphated T3 and T4
residues than on normal TH, whilst no apparent 5' activity is observed (38).

It may be the case that D-I does not play a specific and sensitive regulatory role,
but is engaged in catabolism of surplus hormone for the iodide economy. Evidence
In favour of this view comes from the facts that rT3 is recognised over T4 as the preferred substrate for activity, and Michaelis constant of the enzyme are at a level far above physiological tissue values, with a Km for T4 of >1 μM (38). D-I activity is located preferentially in the kidney and liver of the rat, and less so in developing brain, pituitary, eye and lung. It is also very high in the thyroid itself, a position which suggests a metabolic, rather than developmental role for the enzyme (38).

D-II is responsible for local generation of T3 from T4 by 5' deiodination, and is particularly active in brain and pituitary, as well as placenta and brown adipose tissue. As with both other deiodinases, activity is membrane associated, and with a Km of 1 nM for T4 and 2-10 nM for rT3, the enzyme is highly responsive to plasma TH concentration changes (43). Activity at these physiological concentrations, and the short half life of the enzyme itself (30 minutes) suggests a regulatory role for the enzyme. After thyroidectomy, D-II activity rises sharply in brain and pituitary due to stabilisation of the enzyme, implying the existence of a rapid, extranuclear mechanism by which TH regulates D-II activity (44).

The enzyme responsible for hormone deactivation, 5'-D-III, has a similar pattern of distribution to D-II, being located in the central nervous system (CNS) of both human and rat, as well as the placenta, where cellular localisation in chorionic cells has been reported (45). This is the least studied of the deiodinases, and much variation exists in reported values for its Km, although about 40 nM for T4 in brain is considered a fair estimate (38). It seems likely, at least under given conditions, such as in the brain of the developing fetus, that D-II and D-III operate in a "push-pull" manner to homeostatically regulate the amount of T3 available to specific tissues, and the question of the regulation of deiodination is therefore an important one.

Under conditions of hypothyroidism, D-II activity in the rat brain is elevated almost immediately, acting to homeostatically maintain T3 levels by increased local generation from T4 (46). Similarly, D-III activity is reduced, though this takes a longer time (from 2 to 5 days after thyroidectomy in rats) (47). Deiodinases are thought to be of considerable importance during development, especially in the fetal brain and in the placenta, where their possible role in the control of TH availability to the developing conceptus will be discussed.

Data relating to regulation of deiodinases in the fetus is confusing, with a great deal of inconsistency. The most important common factor is that tissue differences
occur in the activities of the different deiodinases. Thus in the rat pituitary, D-II activity rises at a few days of postnatal age, increasing the conversion of T4 to T3 (48). D-II is certainly present in the fetal cerebrum at day 17 of gestation, and accordingly, it is possible that the activity of the enzyme in small subsets of brain cells may be sufficient to maintain the local concentration of T3 at levels needed to exert physiological actions (49). Interestingly, no D-II activity is recorded in the cerebellum at birth, but levels rise to a peak at day 28, coincident with the final stages of the maturation of the organ (50).

It is apparent that D-II:D-III linked homeostasis occurs in the fetal brain. D-III is already high in fetal cerebral cortex at gestational day 14, before the onset of fetal thyroid function, and peaks at days 17-19, after which it progressively declines until about the third postnatal week (51). As this would result in limiting the local supply of T3 in the developing brain at a time when the fetal thyroid is beginning to function, it raises the question of whether the fetal brain is being protected from the formation of T3, or whether the high activity of D-III is a reflection of a more biologically significant role for T4 at this developmental stage. This question still remains to be satisfactorily answered.

Several other pathways of TH metabolism exist, which although are not directly regulatory, play an important role in determining TH tissue levels. These are catabolic, and include: conjugation, where as mentioned before, sulphate or glucuronide groups are substituted for the 4'hydroxyl group, thereby accelerating deiodination; conversion to acetic acid analogues preceding deiodination; and oxidative degradation, the major form of which is ether link cleavage, resulting in the destruction of the hydrophobic basis of the molecule (36).

In summary, the metabolism, particularly by deiodination, of TH is of considerable importance in determining tissue specific levels of TH both during development, and for the purposes of energy metabolism at the adult stage.

1.5 Molecular Mechanisms of Thyroid Hormone Action

1.5.1 The Thyroid Hormone Nuclear Receptor (THNR)

TH are responsible for a wide range of biochemical, morphological and
functional effects in metabolism and development, and for the regulation of other hormones and growth factors, in addition to feedback regulation of their own production. In recent years, some of the molecular mechanisms through which these functions operate have been identified. An understanding of these operations at the molecular level is necessary in order to explain how TH control of such diverse processes is targeted, both spatially and temporally, and what precise molecular interactions, instigated by TH, are responsible for the effects finally observed.

The nucleus was first proposed to be the site of action of TH in the mid 1960's by Tata and colleagues, whilst investigating RNA polymerase activity (52). These ideas were confirmed with the identification by Oppenheimer in 1972, of specific TH binding sites within the nucleus, which exhibited displacable binding of $^{125}$I\(\text{T}_3\) by both T3 and T4 (53). Early problems of distinguishing specific from non-specific binding (54) were gradually overcome, increasing the likelihood that the nuclear binding site was a receptor. Binding affinities of different T3 analogues generally reflected their biological activity (55), and saturating concentrations of hormone paralleled optimal concentrations for such activity (32). Binding sites were found only in hormone responsive tissues, their concentrations being found to correlate with the TH sensitivity of the cells (32,33). Finally, the nuclear sites demonstrated the typical low-capacity, high affinity ligand binding characteristic of receptors (32,33). Consequently considerable circumstantial evidence appeared to indicate that these sites were involved in the initiation of TH action.

TH nuclear receptors (THNR) were identified in most mammalian tissues, and in all vertebrate species containing significant concentrations of serum TH (56). Adult tissues containing high levels of THNR included liver, anterior pituitary, kidney and heart. Intermediate levels were found in the adult brain and lung, whilst levels in spleen and testis were either low or undetectable (57,58). The pattern of analogue binding was similar in all tissues. Assigning T3 an affinity of 1, preference of binding occurred in the following order: Triac (2-3)>Isopropyl T2 =T3 (1.0)>Tetrac =T4 (0.1)>rT3 (<0.001) (59). Separated areas of brain exhibited differential TH binding; binding was greatest in the cerebral cortex, falling away in a cranio-caudal direction to the cerebellum, which showed the fewest binding sites (60,61).

Receptors were shown to be chromatin-associated, and extractable using high
salt concentrations (e.g. 0.4 M KCl) (33,59). However, low numbers of THNR coupled with their instability, has rendered their isolation difficult (62). Salt extraction yields a mix of protein, of which the receptor contributes less than 0.01%. Different approaches have been employed to purify the THNR, without loss of stability. The best of these, involving a sequence of passages through different chromatographic columns, has yielded a purity of 5-10% (63). Receptors thus purified have a molecular weight of approximately 50,000, and a sedimentation coefficient of 3.5 S. Other characteristics of the THNR are described in Table 1.

Table 1 : Properties of the THNR

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular Weight</td>
<td>approx. 50,000</td>
</tr>
<tr>
<td>Sedimentation coefficient</td>
<td>3.8 S</td>
</tr>
<tr>
<td>Estimated half-life</td>
<td>4.5 h</td>
</tr>
<tr>
<td>Rate of synthesis</td>
<td>2,000 molecules/cell/h</td>
</tr>
<tr>
<td>Number per nucleus</td>
<td>approx. 15,000</td>
</tr>
<tr>
<td>Binding Capacity</td>
<td>1 pmol/mg DNA</td>
</tr>
<tr>
<td>Dissociation constant</td>
<td>0.5 nM</td>
</tr>
</tbody>
</table>

Based on determinations on rat hepatic, GH1 and GC cells (33,59). Some properties vary depending on cell type. Unless otherwise stated, throughout this thesis, dissociation constants always refer to T3.

Photoaffinity labelling of T3 allowed the production of an analogue which could be covalently bound to the THNR in intact cells or receptor preparations, by exposure to ultraviolet light (64). Examination of the SDS-gel electrophoresis profile of receptor preparations from the cultured pituitary cell-line GH1 labelled in this manner revealed the presence of distinct receptors with molecular weights of 47,000 and 57,000. These two forms apparently had similar synthetic rates, but different half-lives (6 hours and 2 hours respectively) (65). Several explanations for the presence of two receptors have been suggested (66). Firstly, the 57,000 Mr form may be a precursor of, or else the result of post-translational modifications of, the smaller form. Secondly, the two may be the products of two different genes, or else the
result of alternative splicing of the mRNA from the same gene. These latter possibilities are of particular interest in the light of the identification of the gene responsible for the production of the THNR, which is homologous to the avian erythroblastosis virus proto-oncogene, v-erb-A (67,68).

The v-erb-A gene product acts as a ligand independent transcription factor which is thought to be involved in the perturbation of the transcription of differentiation-linked genes during development (69). When operating in conjunction with the oncogene c-erb-B, complete transformation of host cells (fibroblasts and erythroblasts) ensues. In 1982, Vennstrom and Bishop, using v-erb-A as a probe, first identified a cellular homologue of the gene in the chicken (c-erb-A) (70). A human homologue was later located on chromosome 17 (71).

At the same time, studies of the glucocorticoid and steroid hormone receptors led to the cloning of their receptor cDNAs. Weinberger et al. noticed a considerable degree of homology between the amino acid sequences of the glucocorticoid receptor and regions of the v-erb-A protein product (67), and similar conclusions were reached regarding the steroid hormone receptors (33). Krust et al., examining the amino acid homology of the human and chicken oestrogen receptor, proposed that the receptor consisted of five or six domains, each with a specific function, such as DNA binding, or ligand binding (72). It became apparent that these regions were common to a number of receptors, including the THNR, with degrees of inter-receptor homology being higher in some regions than in others. The concept therefore arose that hydrophobic ring structures, such as the thyroid hormones are able to act as hormones via a superfamily of nuclear receptor proteins.

It has been proposed that this superfamily has evolved from an ancient precursor (73). The THNR is analogous in structure to certain early response genes (c-fos, c-egr) which play a role in growth control. Consequently, it has been suggested that the THNR has evolved from such a gene, acquiring in the process, ligand sensitivity.

1.5.2 Structure of the THNR

The generalised structure of the hydrophobic ring structure hormone receptors is illustrated in Figure 4.
**Figure 4**: Schematic representation of the structure of the c-erb-A, divided into regions.

<table>
<thead>
<tr>
<th>A/B</th>
<th>C</th>
<th>D</th>
<th>E/F</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>40</td>
<td>120</td>
<td>194</td>
</tr>
<tr>
<td>rc-erb-A α1</td>
<td>86</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>40</td>
<td>120</td>
<td>194</td>
</tr>
<tr>
<td>rc-erb-A α2</td>
<td>82</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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<thead>
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<th>A/B</th>
<th>C</th>
<th>D</th>
<th>E/F</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>94</td>
<td>174</td>
<td>248</td>
</tr>
<tr>
<td>rc-erb-A β1</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>1</td>
<td>147</td>
<td>227</td>
<td>301</td>
</tr>
<tr>
<td>rc-erb-A β2</td>
<td>DNA</td>
<td></td>
<td>HORMONE</td>
</tr>
</tbody>
</table>

Numbers outside boxes refer to the amino acid sequence, beginning at the first methionine, whereas numbers inside refer to % nucleotide homology with the β2 receptor.

Adapted from Hodin *et al.* (364), and Goldberg *et al.* (69).
It comprises five main regions, the most homologous of which is the DNA binding region (or C domain). This domain includes the highly conserved "zinc-finger" region, which contains a number of cysteine residues which bind Zn\(^{2+}\) ions and enable DNA binding (69). Similar structures have been identified in a number of DNA binding proteins (74). Ligand binding occurs in the E domain, which is also significantly conserved between the different receptors in the family, in a manner relating to the similarities in the structure of their ligands. The amino terminal (A/B) domain is the most variable region, and plays an important role in the stimulation of transcription (69). In c-erb-A, this domain contains a site which becomes phosphorylated upon activation of two membrane associated protein kinases, protein kinase C, and cAMP-dependent protein kinase (75). Phosphorylation of this nature is thought to be important in determining specific protein-protein interactions between the receptor and other essential, possibly gene specific, transcription factors, and is therefore putatively involved in the targeting of TH action (69). The D domain is also variable, and believed to act as a hinge region, thereby influencing intramolecular interactions. The final F domain is associated with the hormone binding domain, but is more divergent. It is not strictly present in all the members of the receptor superfamily and, in the oestrogen receptor, does not appear to be necessary for ligand binding (76).

1.5.3 Gene Regulation by the THNR

The regulation of gene expression by TH involves interaction of the TH-receptor complex (as a dimer), with both responder genes, such as the growth hormone (GH) gene, and with the director gene i.e. the THNR gene itself, through which a feedback regulation of THNR levels is thought to operate (77,78).

THNR have been shown, by digestion of DNA using micrococcal nuclease, to be associated with the linker DNA sections between nucleosomes (79).

In these regions, T3-inducible hypersensitive sites have been located, implying the opening up of the chromatin in response to T3. At these sites THNR bind specifically to hormone responsive elements in the DNA, causing intensification, or in some cases, inhibition, of transcriptional initiation of specific gene promoters (69). The hormone responsive elements are therefore cis acting enhancers operating at a
distance from the promoter sequences (typically TATA or CAAT boxes) which
determine the correct initiation and rate of transcription, whilst the THNR are trans­
acting elements in transcriptional regulation. Little detail is known about enhancer
function. There are enhancers which are only active at particular times, for example
during development, and/or ones which are tissue specific. There are also inducible
enhancers to which category hormone responsive elements belong, as they rely on the
binding of the TH-THNR complex to stimulate transcription.

The activity of both enhancers and promoters depends on specific
transcriptional co-factors, which may be present in a cell specific manner (66). In
addition to being cell specific, these factors may be expressed in a temporally related
fashion during development, possibly influencing either the targets, or the intensity of
TH induced gene transcription (66,80).

The regulation of gene transcription by TH is clearly a complex process
involving the binding of hormone to different receptor subtypes; the involvement of
different response elements and specific transcription factors. The outcome may be
either the initiation of, stimulation of, or inhibition of transcription. Binding to the
TH response element of the TSH β subunit gene, for example, results in the
displacement of an essential transcription factor from the TATA promoter, thereby
inhibiting transcription (81). The biological response to TH is therefore largely
mediated through binding to nuclear receptors, with a consequent alteration in the
rate of transcription, and subsequent translation to protein.

Evidence for the influence of TH on levels of specific mRNAs is considerable
(for review, see 66). In the rat, TH have been associated with an increase in the
levels of mRNA's encoding malic enzyme (82), α-2 globulin (83), spot 11 (84), spot
14 (85), cytochrome c (86), PEP carboxylase (87), ornithine aminotransferase (88),
and growth hormone (89). Decreases have been shown for both subunits of TSH
(81).

These mechanisms go some way to explaining how TH manage to exert such a
range of effects during development, and how such effects may be controlled.
However, it is possible that TH may also operate through different mechanisms,
either mediated by different receptor types, or independent of the presence of a
receptor.

37
1.5.4 Extra-Nuclear Receptors for Thyroid Hormones

Although some groups have claimed TH act only at the nuclear level, there is a growing body of evidence to suggest that this is not the case. Binding sites for TH have been demonstrated at several sites outside the nucleus in a number of cells (90). Most notable of these sites are those located in the plasma membrane, which appear to be involved in more than simple internalisation of hormone (91).

Plasma membrane binding sites have been located in many tissues, including human and rat erythrocytes (91,92), human placenta (93), rat thymocytes (94), rat synapses (95), liver (94), and in vitro in GH3 rat pituitary tumour cells (94). Two separate sites have been identified; one exhibiting the classic low capacity, high affinity associated with receptor molecules, with a Kd of 2 nM; the other displaying high capacity, low affinity (Kd 260 nM) TH binding (90). These sites are important in the determination of intracellular TH concentrations, and consequently may play a significant role in determining the ability of a cell to respond to TH (96). Membrane binding sites may also directly influence the transport of glucose, amino acids and nucleosides, in addition to stimulating the cooperative activity of acetylcholinesterase and Na+/K+ ATPase (90,97,98,99,92]. In the anuclear erythrocytes, interaction of TH with surface membrane receptors may be the only mechanism through which a response to the hormone may be elicited.

Sterling and coworkers first demonstrated the presence of specific TH receptors on the inner mitochondrial membrane which were associated in an immediate increase in oxygen consumption, implying a direct affect not requiring the synthesis of new protein (100). It has been suggested that this binding site is the enzyme adenine nucleotide transferase, responsible for the transport of ATP from mitochondria to the cytoplasm, and of ADP in the opposite direction (101).

Cytosolic TH binding sites may act as regulators of TH availability to organelles, including the nucleus, by providing a storage and supply function (102). Sites have been reported as possessing different affinities in different cell types, and are thought to contain more than one binding site per molecule (103). More recent work has provided evidence for inducible cytosolic T3 binding proteins (CTBP) (104). Such sites are activated either by NADPH, or by NADP together with DTT, producing different forms suggested to operate differently in the transport of T3 to the
nucleus. The NADPH activated form is thought to act as a cytoplasmic reservoir of T3, whilst the NADP activated form is translocated to the nucleus, where specific binding sites exist for the complex.

In addition to action mediated directly through receptors, TH are proposed to have a variety of specific effects on enzyme activity, the most notable example of this function being the influence of T3, rT3, and T4 on the operation of types II and III deiodinase. It seems probable that other biological processes may also be regulated in this manner.

1.6 Biological Effects of Thyroid Hormone Action

Clinical and biochemical studies have revealed how the effects of TH can be divided into three categories; effects on growth and development, effects on metabolism, and feedback regulation of their own production. Although to some degree these effects may be interrelated, the role played by TH in ontogenesis is clinically the most critical, as malfunctions in this process may result in irreversible damage to the developing nervous system.

The specificity of TH control of development is reflected in tissue specific differences in dependence on TH. The most TH sensitive tissues have been shown to be: the developing brain (105); the lung, again, especially during development (106); the skeleton, particularly growing cartilage (107,108); and the liver, at all stages of life (109). Several other tissues, including the cornea, thymus and lymphatic system, also demonstrate TH dependence to varying degrees (2).

In the fetal lung, thyroidectomy of rabbit or sheep results in a decrease in protein synthesis, with corresponding reduction in cell size, but no fall in cell number (110). T4 is also involved in the accumulation of lung surfactin (possibly in cooperation with glucocorticoids), and deficiency due to fetal hypothyroidism can contribute to respiratory distress syndrome (111,112).

In the fetal rat liver, both glucose-6-phosphatase and cytochrome c oxidoreductase are upregulated by T4 administration in the latter stages of gestation (113). During the third postnatal week, a different set of enzymes can be shown to be TH dependent, including malate:NADP oxidoreductase and pyruvate kinase (114). These are all specific induction effects of enzymes involved in energy metabolism,
and are also mostly effected in cooperation with other stimuli, such as growth hormone. Similarly, TH and cortisol cooperate in the regulation of production of the growth regulator IGF (insulin like growth factor 1) (115). The carrier protein for this growth factor is also under TH control at an early stage of development (116).

Cartilage production during development is partially mediated by TH action, explaining many of the growth defects and bone malformations associated with severe iodine deficiency diseases like cretinism, and also with hyperthyroid associated disorders. A lack of TH at critical points of chondrocyte development results in ultrastructural deficits in many categories, including depleted quantities of endoplasmic reticulum and Golgi apparatus (117), causing disruption of extracellular matrix formation. Subsequent glycogen deposition, and deterioration of chondrocytes results in premature calcification, resulting in stunted and deformed bones. Thus it is apparent that TH exert their influence primarily on the maturation of bone, these effects often being permanent and irreversible. As with the liver, secondary effects, especially on growth, are also a possibility due to stimulation of the production of, and synergistic actions with, GH and somatomedins (2). Conversely, hyperthyroidism results in accelerated skeletal development (2).

TH also affect the development of the peripheral nervous system, where afferent systems such as sensory functions; efferent systems, such as the development of the sciatic nerve, innervation of the diaphragm, iris and heart; and autonomic systems including sweating, body temperature and faecal water content are all TH sensitive (117). By far the most work, however, has been conducted on the CNS.

1.7 Thyroid Hormone Effects on the Developing CNS

The brain is critically dependent on TH for normal development. These hormones are required for the morphological, biochemical, functional and behavioural maturation of the CNS, and operate as signals for the synchronisation of developmental sequences, with resulting long-term effects (117). An enormous body of work has been dedicated to the identification of facets of neural development influenced by TH, and a range of animal models, clinical observations, organ and tissue culture systems have been applied (for reviews see 117-120). The majority of studies have compared the effects of hypothyroidism (clinical, thyroidectomy-
induced, or in vitro) with euthyroid controls. Rather fewer have assessed the effects of hyperthyroidism, either because the system is more difficult to reliably induce, or because lack of the hormone tends to be a greater clinical problem than excess.

1.7.1 Morphological Effects of Thyroid Hormones

Hypothyroidism causes a range of morphological defects in the brains of all vertebrate species so far studied. Generally, a reduction in overall brain weight is observed, coupled with decreases in cell number and cell size (119). Often most apparent is the hypoplasticity of the neuropil, observed by Eayrs in the cerebral cortex, and Legrand in the cerebellum (105,121). Both the number and length of dendrites is reduced, and the degree of branching is limited. Similarly, axonal development is disturbed, reducing axon length, and consequently inter-neuronal contact and electrical activity (119). Such interference with membrane associated events may also contribute to reductions in levels of neurotransmitters and their metabolic enzymes (117). Synaptogenesis is impaired in all regions, either directly, or as a consequence of the hypoplasticity of the neuropil (117). Additionally, the specification of receptors are altered in a region-specific manner, with hyperthyroidism resulting in an increase in muscarinic receptors in the cortex, but not the cerebellum (119). A reduction in the number of cerebral capillaries has been reported, as have disruptions in the cerebellar cell cycle, and gliogenesis is inhibited (117). These observations have led to two models for the influence of TH on brain development, the catastrophic model, where an altered rate of neurite outgrowth leads to desynchronisation of development; and the pleiotypic model, implying a multi-target, multiple effect role for hormone action. It seems likely that both of these models may operate to varying extents in vivo (120).

During normal rat brain development, the total cell number of the cerebrum increases by 50% between birth and full maturity (122). The location of proliferation of neurons is restricted to defined germinal sites, such as the subependymal layer of the forebrain ventricles, and the majority are present by birth. Neuroglia mostly originate from dispersed stem cells. The increase in cell numbers is even more pronounced in the cerebellum, where it begins with a phase of slow division in the first few postnatal days, followed by rapid division. In this region, most neurons are
produced postnatally, with the main exceptions being the Purkinje and Golgi cells (122).

The reduction in cell number caused by hypothyroidism seems unlikely to be caused by a lack of stimulation of cell division, as no satisfactory evidence exists to support this view. It is more likely that alterations in the rates of cell migration and differentiation, together with reduced cell survival are responsible for the effect.

In the cerebellum, hypothyroidism causes a delay in the disappearance of the epigranular layer (EGL) (123). Under normal conditions, cerebellar development is due to the migration of cells from the EGL, and their subsequent differentiation, until by postnatal day 21-25, the EGL has disappeared, and adult cell numbers are reached (122). This process is considerably lengthened under hypothyroid conditions, and the resulting suppression of cell migration and differentiation impairs the establishment of normal contacts, and consequently increases neuronal death (124,125).

Each cell type has its own chronology of proliferation and differentiation. In the cerebellum, for example, the peak rate of basket cell differentiation occurs between postnatal days 2 and 6, that for stellate cells is achieved at day 13, while granule cells differentiate mostly between days 15 and 21 (122). Optimal levels of TH are required for the correct integration of all of these processes. A general suppression of the maturation of nerve cells occurs in the absence of TH, causing a secondary interactive failure between neural cells. The specific damage inflicted by inappropriate levels of TH depends to a large extent on timing and duration, as different cell types and processes are affected. This is apparent in the cerebellum, where Purkinje cell maturation suffers more damage than granule cell development, which occurs later in development (126). In addition, TH is, for example, necessary for the migration of some cells and the differentiation of others.

In many cases, hyperthyroidism causes opposite effects to hypothyroidism, however, these may not be reflected in brain structure, demonstrating the need for optimal TH levels for normal development. For example, excess TH prematurely accelerates the disappearance of the EGL in the cerebellum, yet due to the desynchronising effect, still prevents the formation of normal connections, resulting in neuronal death (117,120).

Many workers have attempted to identify critical periods of TH action on the
developing brain. Initially these were proposed to be the last trimester of pregnancy and first postnatal year in the human, and days 10 to 12 in the rat, as it is chiefly during these periods that many TH-sensitive events take place, including myelogenesis, axonal and dendritic arborisation, synaptogenesis, glial proliferation, and neuroblast proliferation in the cerebellum (127,128). In fact, sensitive periods have been demonstrated to be more extensive, as well as age and region-dependent, suggesting an organisational role in early development. The differentiation of apical spines in pyramidal cells from the auditory and visual cortex has been employed as a measure of TH responsiveness in rats (129). Animals thyroidectomised at day 10, and given T4 replacement commencing at various subsequent days, developed normally if treatment began at day 12, but irreversible damage resulted if T4 therapy was delayed until day 20. This implied a critical period for TH action of between days 10 and 20 for this cell type, after which the potential for maturation was lost. Similar experiments on the maturation of cerebellar Purkinje cells also reveal a critical period before 21 days, and evidence has been presented which suggests that rat plasma TH concentrations rise during this period (130). It also seems likely that certain cell types are vulnerable to the effects of TH deprivation at a much earlier stage of brain development, as Wolter et al. have reported that the debilitating effects of fetal hypothyroidism cannot be compensated for even if T4 supplements are instituted at birth (131).

1.7.2 Biochemical Effects of Thyroid Hormones

The TH exert control over a host of biochemical systems in the developing CNS. In gross terms hypothyroidism is associated with a rise in concentration (but not necessarily the total quantity) of DNA, and a fall in the levels of RNA, the latter due to increased turnover without a corresponding increase in synthesis (132,133). The overall protein/DNA ratio is diminished, as protein synthesis falls (134). This effect on protein synthesis is probably mediated at both the transcriptional and translational levels, to varying degrees, but may also be partly the result of alterations in the permeability of cell membranes to amino acids (134,135). This possibility has been suggested to be either a direct effect on amino acid transport, or else the result of a change in the blood-brain barrier, possibly involving reduced vascularisation (136).
Amino acid metabolism decreases generally, as reflected by conversion of $^{14}$C-leucine to proteins and lipids, whilst certain pathways are favoured, such as the TCA cycle, indicating the possibility of changes of a qualitative as well as a quantitative nature (137). This has been referred to as delaying the metabolic compartmentation of the brain, and demonstrated by a retardation of the age-related increase in glutamate/glutamine specific activity (138).

Changes in amino acid metabolism may also contribute to the deficits noticed in the complement of neurotransmitters (117). Tyrosine is necessary for the synthesis of catecholamines, and tryptophan for serotonin, whilst glutamate, GABA, and glycine all play direct roles in neurotransmission.

The reduction of neurotransmitter activity associated with hypothyroidism may be mediated by TH control of the production of the enzymes involved in the turnover of transmitters and their receptors. TH deficiency has been linked with a fall in the number of catecholamine, muscarinic, β-adrenergic, acetylcholine and GABA receptors in neonatal brain (139-141); and a decline in the activities of GABA transferase, acetylcholine esterase, glutamate decarboxylase, and monoamine oxidase, whilst increases in the activity of cholineacetyltransferase have been reported (134,137,142-144). A synergistic action of TH with growth hormone has been shown to increase the activity of ornithine decarboxylase in the neonatal rat, with implications for the synthesis of polyamine neuromodulators (145). Other enzymes such as glutamate dehydrogenase remain unchanged, thereby emphasising the specificity of TH action relating to the enzymes involved in neurotransmitter metabolism (137).

Similar specificities apply to more general metabolic enzymes. Succinic dehydrogenase, aspartate aminotransferase, glucose-6-phosphate dehydrogenase, neuronal acid phosphatase, and NADH-diaphorase have all been shown to decrease in activity in hypothyroidism, as has Na$^+$/K$^+$ ATPase, although Mg$^{++}$ ATPase is unaffected. Neither alanine aminotransferase nor lactate dehydrogenase are affected, whilst the hydrolytic glial acid phosphatase increases in activity (see 137 for review). That lactate formation is suppressed in hypothyroidism is due therefore to an impairment of glucose uptake and of enzyme activity earlier in the chain. Energy metabolism is clearly affected by lack of TH, and oxygen consumption is reduced, particularly in the cerebral cortex (117,137).

The TH play a major role in the control of myelination, both in terms of the
extent of myelination, and the chemical composition of the myelin itself (146). Under hypothyroid conditions, most brain regions, including the cerebellum, demonstrate a reduction in the levels of myelin constituents, including cerebrosides, phospholipids, sulphatides and cholesterol, together with their associated enzymes (147). Cholesterol synthesis is reduced to 20% of normal in hypothyroidism, whilst total levels are also reduced in hyperthyroidism, as a result of stimulation of catabolic processes, and both a deficiency in the oligodendrocytes per se at early postnatal stages, and diminished enzyme activity contribute to these deficits (117). Timiras has pointed out that although a full complement of myelin is eventually reached, "even a temporary disruption of myelinogenesis may be sufficient to desynchronise the normal pattern of development", implying that long term functional damage may result (117).

TH control of cytoskeletal formation and stability impinges upon both morphological and biochemical aspects of brain development. TH action has been implicated in the production of the microtubule associated proteins MAP1 and MAP2, and τ, factors which stabilise the polymerised form of α and β-tubulin (120,148,149). This is important in the formation of axons and dendrites, and is involved in all major CNS developmental events. Disruption of the chronology of the expression of these factors may have serious long term effects on cell function (150).

1.7.3 Functional Actions of Thyroid Hormones

Impaired synaptogenesis, transmitter synthesis, myelination, and changes in the temporal co-ordination of development inevitably lead to defects in the ontogenesis of interrelated evoked electrical control systems. This is reflected in abnormalities in the EEG, and neurological defects, such as impaired neuromuscular co-ordination and delayed locomotive ability (117). The late maturation of many inhibitory control systems also contributes to an array of behavioural abnormalities in hypothyroidism, which include decreased stress response, motor activity, exploration, habituation and learning difficulties (148). The severity of the neurological dysfunction relates to the degree of deprivation (or excess) of TH during critical developmental periods. PTU-induced hypothyroidism in the neonatal rat delays evoked cochlear electrical activity, a deficit which can be prevented by the administration of physiological concentrations of T4, but only during a critical period spanning from 3 days before birth, to 5-10
days postnatal (123).

In summary, TH appear to influence a wide range of interrelated systems during brain development. The failure of TH to exert initially small actions may therefore have catastrophic consequences on development. Critical periods for TH action are region, and possibly cell type-specific, and consequently appear to extend over most stages of development. Studies of TH action at the cellular level have begun to identify some of the mechanisms by which these hormones primarily operate, and how such operation may relate to the development of the entire system.

As it is apparent that TH play an enormously important role in the normal ontogenesis of the fetal brain, it is perhaps surprising that so little attention has been paid to the possibility that maternal thyroid hormone may traverse the placenta and exert important effects on early CNS development before the onset of fetal thyroid function. Certainly, an enormous amount of CNS development occurs before the fetal thyroid begins to secrete hormone, which as previously mentioned is at the end of the first trimester in the human, and approaching birth in the rat (day 17-18). The neglect of this topic has, up until recently, been due to the precept that TH do not cross the placenta in quantities sufficient to exert a biological effect. The evidence relating to this questionable belief will be reviewed.

1.8 Are thyroid hormones targeted on the feto-placental unit?

If maternal TH are to have any effect upon fetal brain development, it must first be established whether or not they are targeted on the feto-placental unit. If this is the case, do TH cross the placenta from mother to fetus in sufficient quantities to elicit a biological response?

In order to address these problems it is necessary to understand the mechanism of plasma transport of thyroid hormones. Like steroid hormones, the vast majority of TH are transported in the blood stream mostly bound to plasma proteins (Table 2). The proportion of each hormone bound to each protein reflects the affinity of the protein for the TH, and its abundance in the serum. Consequently, although TBG is present in relatively small amounts, its high affinity for TH ensures that most hormone is transported bound to this protein. Proportionate T3 binding is uncertain, but is likely to broadly reflect that for T4 (151).
Table 2: Properties of the thyroid hormone binding proteins.

<table>
<thead>
<tr>
<th></th>
<th>TBG</th>
<th>TBPA</th>
<th>Albumin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration (μM)</td>
<td>0.27</td>
<td>4.6</td>
<td>640</td>
</tr>
<tr>
<td>Hormone distribution (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T4</td>
<td>68</td>
<td>11</td>
<td>20</td>
</tr>
<tr>
<td>T3</td>
<td>38</td>
<td>27</td>
<td>35</td>
</tr>
</tbody>
</table>

TBG - thyroxine binding globulin; TBPA - thyroxine binding prealbumin. Data from Robbins & Bartalena (151).

Since the discovery of TBG in 1952 (152), its role in TH transport has been the subject of much controversy. It has been noted that abnormal levels of TH binding proteins, including total absence of TBG, appear to result in no physiological consequence. It has also been repeatedly observed that the metabolic status of individuals relates more closely to the level of free TH in the serum rather than the total or bound hormone level. When taken together with the fact that free hormone tissue permeation rates far exceed those for the protein hormone complex, and that the pituitary-thyroid feedback mechanism is sensitive to free hormone, it is not surprising that this has led to general acceptance of the "free hormone hypothesis" of delivery of TH (and steroid hormones) to tissues. This theory encompasses all these observations, dictating that,"hormone utilisation in target tissues correlates with, and is determined by the free hormone concentration in serum measured under equilibrium conditions" (153).

Data supporting this view came notably from two sources. Although both agreed that free hormones were taken up by the tissue, the results of Robbins and Rall (154,155) implied that dissociation of hormone from hormone protein complex was not rate limiting during delivery to tissue, so that for every molecule of hormone which was transported into the tissue, at least one molecule dissociated from the binding hormone. At the other end of the scale, Tait and Burstein (156) claimed to
have found no dissociation of hormone from complex whatsoever during delivery, so that free hormone levels gradually fell during passage through the tissue. It should be noted that the free hormone hypothesis does not preclude the dissociation of bound hormone during tissue passage contributing to the biologically active free hormone concentration.

As it stands, this theory fails to explain the role of hormone binding proteins and, as will be seen later, may not hold true under all circumstances. As a result of this, a number of alternative ideas have arisen to challenge the orthodox view. Some of these have attempted to confer a direct biological role on the hormone binding protein, whilst others have promoted more refined versions of the physico-chemical free hormone hypothesis.

Some evidence has suggested the presence of intracellular corticosteroid binding globulin and sex hormone binding globulin (157), and a model of hormone delivery has been presented involving entry of hormone-protein complex into the tissue prior to dissociation, with targeting being dependent upon capillary protein permeability (158). However, concrete physiological evidence for this is wanting. In addition, clinical evidence does not bear out the logical consequence of such a proposal; i.e. that hormone delivery would be largely dependent on levels of hormone binding proteins.

It has been suggested more recently that binding proteins bind specifically to cell surface receptors in capillaries, which catalyse the release of hormone. According to this theory, the free hormone then enters the tissue. In proposing this solution, Pardridge et al. point out that dissociation constants in vitro do not match those which they obtained in vivo. They describe their data with the aid of an equation (equation 1, below), which includes the parameters of tissue permeability and capillary transit time (159).

Employing liver perfusion techniques, with a single pass of a bolus of labelled TH in TBG free medium, they found tissue permeability to be relatively low. They were unable to fit their data accurately to their equation using the established affinity constants of the plasma proteins. Allowing an increased affinity gave an improved fit and a low estimate of tissue permeability. The relatively high uptake in the presence of certain binding proteins was assumed to arise from cell surface catalysis of ligand dissociation, followed by internalisation. It is argued that target specificity is
therefore governed by tissue specificity of such catalysis (159).

\[ FE = 1 - e^{-\frac{k_p t}{1 + K[P]}} \]

Where:
- \( FE \): Fractional Efflux
- \( k_p \): permeation constant
- \( t \): time
- \( K \): affinity constant
- \( [P] \): protein concentration

Ekins et al. (160), challenge the validity of Pardridge's approach. In particular, the inclusion of albumin in ostensibly binding protein free medium for permeability studies has been criticised. Despite the rapid dissociation rate of TH from albumin, it is argued that the levels included limit the supposed levels of "free" hormone available for transfer. In addition to this, it is pointed out that as efflux of hormone from tissue to capillary is likely to be considerable, and so employing a labelling technique capable of tracking only net movement into tissue is likely to severely underestimate the true rate of flux across the capillary wall, thereby giving values for \( K_{pt} \) which are considerably lower than is really the case.

Consequently, Ekins suggests that if permeability rates were an order of magnitude higher than calculated by Pardridge (24-240 cf 0.5-6), then all observed effects on hormone-protein dissociation could be explained using equation 2 as a model (160). Indeed, using the simplified equation 3, Pardridge's data has been reworked, the theoretical calculations broadly agreeing with the experimental results.

As no independent data exists supporting low rates of permeability, then the Ekins hypothesis may apply, implying that under certain circumstances, hormone may dissociate sufficiently fast to supply the tissue with large quantities of hormone. There is therefore no requirement for an unsubstantiated TBG-receptor interaction to explain tissue delivery of TH under these conditions.
Equation 2

\[ FE = 1 - e^{\frac{-k_p t}{1 + K[P] + \frac{k_p}{k_d} R m I_0(mR)}} \]

Where:
- \( I_0, I_1 \) Bessel functions of order 0 and 1
- \( R \) radius of capillary
- \( k_d \) dissociation rate constant
- \( m \) \((ka[P]/D)^{0.5}\)
- \( k_d \) association rate constant
- \( D \) diffusion/mixing rate constant

Equation 3

\[ FE = 1 - e^{-f \frac{[fH]_w}{[fH]_e} k_p t} \]

Where:
- \([fH]_w\) free hormone concentration at blood/capillary wall interface
- \([fH]_e\) free hormone concentration in bolus at equilibrium

Ekins hypothesis is one that links the known clinical data regarding the importance of free hormone levels, with a proposal explaining the function of hormone binding proteins. Broadly encompassing the models for hormone delivery proposed by Robbins and Rall (155), and Tait and Burstein (156), Ekins theory suggests that several factors govern delivery of hormone to tissues. These are brought together by some fairly involved mathematics, but can be summarised relatively simply.

Ekins points out that permeability constants are not the only factors which may
be rate limiting in hormone delivery, and suggests that the rate of uptake also depends on intra-capillary diffusion, both of free hormone, and of the hormone-protein complex. In addition, dissociation rates will also differ depending on local concentrations of free hormone. Thus at the capillary wall the local free concentration will be depleted by tissue uptake, so local ligand dissociation will increase relative to dissociation rates at equilibrium. Consequently, delivery to tissue depends on permeation rates; transit time; intracapillary diffusion and mixing; the affinity constant of the binding protein; local free hormone concentration; and binding protein concentration (160).

Under normal conditions of low uptake, binding protein concentrations will not effect hormone delivery. However, in an organ exhibiting very high free hormone clearance, bound hormone dissociation may become rate limiting. Such an organ would be characterised by a high permeation rate, relatively low surface area for transfer, and a high metabolic demand for the hormone (160-162). Under such circumstances, an elevated level of binding proteins could compensate for the inadequate dissociation rate. The effect of this would be to ensure targeting of hormone to tissues of this nature, whilst levels supplied to other tissues remained unaffected. It has been suggested that the feto-placental unit is a prime example of such a need for an increased specific demand (161).

If this were the case, then far from limiting the supply of hormone to cells (as has been suggested), TBG (and other serum hormone binding proteins) may act to increase the supply of hormone to specific tissues in response to a stimulatory signal. As lack of TH in development causes serious brain malfunction and malformation, the importance of a constant and sufficient supply of TH may have been of considerable evolutionary significance.

All species of vertebrates have some degree of plasma TH binding, from fish whose plasma binding proteins exhibit no distinction between T4 and T3 in terms of affinity, to the higher mammals, which exhibit the presence of high affinity, low capacity binding proteins, to which most of the TH are specifically bound (151,161). Such a phylogenetic trend seems unlikely to be a coincidence, and clearly points to the conferring of some advantage of considerable evolutionary significance by the possession of greater levels of specific plasma TH binding. Early in the evolution of TH control systems, individuals with a more reliable supply of TH from their own
thyroids may have experienced an advantage, hence the presence of TBG in non-placental animals (e.g. chicken). Under these circumstances, hormone binding proteins may ensure a steady supply to target tissues, evening out any sudden fluctuations which may occur. As the supply of iodine for the production of TH is always likely to have been limited, a system capable of economic and efficient use of the element, encompassing specific targeting and guaranteed supply of hormone to tissues with a requirement, would have been advantageous.

With the evolution of placental mammals, the possibility arose of maternal TH crossing the placenta and influencing fetal brain development before the onset of fetal thyroid function. Given the increase in dependence on TH, both up the evolutionary scale, and in phylogenetically younger brain regions (neocortex cf paleocortex) (163,164), it is possible to suggest that an earlier beginning to TH induced brain development may have conferred further advantage, and that a guaranteed supply of TH to the feto-placental unit, afforded by TBG, would have stabilised, and increased the reliability of such an effect.

There are several pieces of evidence which point to an involvement of TBG with the feto-placental unit. Firstly, it is significant that whilst levels of total TH rise during this period (160), free hormone levels remain more or less unaltered in the maternal serum. The rise in hormone levels is therefore restricted to the bound fraction, with TBG increasing three fold to a peak at 20 - 24 weeks in the human (165). Under the terms of the free hormone hypothesis, such an increase would be physiologically insignificant, (as is certainly the case under normal circumstances) as the concentration of binding protein is not thought to be rate limiting.

It seems unlikely, however, that such events are without any biological importance and, in the absence of any other greatly increased targeting or use by other organs of TH in pregnancy, it appears logical to look to the placenta in an attempt to explain the rise of total TH and TBG during pregnancy, as during this period, this is a totally new, and highly metabolically active organ.

A degree of specificity has been reported in the rise of hormone binding proteins during pregnancy. TBG elevation has been widely assumed to be oestrogen-mediated (166), although this has recently been challenged (167). In any event, the rise is certainly linked to pregnancy, and it has been noted that increases in TBG and CBG have, as yet, only been detected in mammals with either hemochorial
or hemoendothelial placentae (168). This suggests firstly that the increase may be specifically associated (either directly, or indirectly) with the placenta, and secondly that some types of placenta are associated with the need for high levels of binding proteins, whereas others are not. The Ekins hypothesis predicts that the physico-chemical nature of the hormone-binding protein complex may determine hormone supply particularly in placentae whose structures offer relatively smaller surface areas for transfer of materials to the fetus, as is the case with the sinusoidal hemochorial placenta.

Relatively high levels of TH and their metabolites are detected in the placenta and amniotic fluid throughout pregnancy (169-171). Levels of T4 are high in placenta compared to other maternal organs which are considered targets for the hormone (23). In addition, the placenta possesses the enzymes necessary for the metabolism of TH, and there is considerable evidence that TH are indeed metabolised to a large degree in this organ (172).

Many accounts mostly from late in pregnancy or at term, have reported the presence of D-I, which possesses both 5', and 5-deiodinating activity (172). In addition, D-II activity has been demonstrated in placenta (23,173,174), implying the ability of placenta to generate locally the "active" hormone T3 from T4. D-III activity has also been demonstrated by a number of groups (45,175,176).

It has therefore been established that the placenta is both rich in TH, and readily metabolises these hormones to both active and inactive products. This evidence suggests that the placenta meets the criteria of tissues characterised by high rates of metabolism of TH and high tissue permeability rates necessary for a target organ of TBG mediated TH delivery. This would tend to discredit the interpretation of the increase in TBG levels as a device to "lock-up" more TH in the bound form, and therefore restrict the amount available to the feto-placental unit (177).

It has also been argued that, accepting the presence of TH in the placenta, that high rates of TH metabolism act to protect the fetus from the actions of TH before the onset of fetal thyroid function (172). In drawing these conclusions, the common mistake of extrapolating results from late pregnancy to encompass the whole period of gestation has frequently been made. In the rat placenta, a closer look at the development of the different enzyme activities provides further evidence to counter these ideas. Firstly, TH non-responsive maternal organs, such as the spleen are characterised by low TH uptake rates, not high rates, as is the case with the placenta,
indicating that the fetus would be better "protected" by the placenta if it was characterised by low TH metabolism and low tissue permeability.

Secondly, metabolism of TH in the period before the onset of fetal thyroid function is mainly of the D-II form: i.e. causing the generation of the highly metabolically active hormone T3. D-III, on the other hand, generating the metabolically inactive rT3 and iodide, is at its most active as the fetal thyroid begins to function (23,177,178). Such metabolism would only begin to "protect" the fetus from TH at around the time that its own thyroid begins to function.

Bearing all this in mind, many groups now believe that the placenta is a target of TH. There is as yet no direct evidence relating to the effects TH may have on placental function. This may be partly due to the tendency of thyroidologists to regard the placenta merely as a selective barrier, rather than the complex endocrine and regulatory organ it in fact is.

In conclusion, several factors suggest the involvement of TBG in supply of TH to the feto-placental unit. These include the physiology of TH binding to TBG; the concomitance of advanced brain development with increased reliance on TH; the increasing presence of high affinity, low capacity binding proteins on the same scale; and the likely scarcity of iodine for the synthesis of TH throughout evolutionary history, particularly in species characterised by prolonged gestational periods. In addition, the placenta exhibits high levels of T4, T3, and rT3, together with significant levels of all three deiodinase enzymes. There is therefore considerable evidence to indicate that TH are targeted on the feto-placental unit, and it is necessary to examine whether they cross in sufficient amounts to exert control over early developmental events in the fetus.

1.9 Do thyroid hormones cross the placenta?

Although many generalisations regarding transplacental transport are made for the sake of convenience, most of these relate to metabolic substrates, the need for which is unlikely to vary except in a proportionate way. Requirements for oxygen, glucose, and amino acids, for example, are likely to increase with respect to fetal size during gestation. It is therefore possible to make generalisations about their methods of transport throughout pregnancy, with glucose transport being carrier mediated and
concentration dependent; amino acids being actively transported, and so forth (179). Certain other substances such as lipid soluble substances are known to cross very rapidly. This being a purely chemical attribute, it too is unlikely to alter with time unless the maternal-fetal concentration gradient dictates otherwise. Transport of the majority of water soluble substances, such as ionic waste products like urea again are a permanent fixture, as is the requirement for minerals like iron, delivered specifically to placental binding sites by transferrin. Bearing in mind the changes which occur in placental function during gestation, and the co-ordinated involvement of hormones in directing development, it would be unreasonable to assume that transport of hormones necessarily behaves in the same way as transport of more ubiquitous metabolites.

Consequently, when it is stated, as has been the accepted view, that TH do not cross the placenta, it is important to know the period of pregnancy over which the observations which substantiate this view were made. When the literature is studied, it becomes apparent that the vast majority of reports cited as evidence for the non-transfer of TH across the placenta have only taken measurements in the third (or less commonly second and third) trimester of pregnancy, approaching term (180,181). There is no logical reason to assume that these measurements would be representative of the whole period of gestation. Only a few have observed rates of transfer during early pregnancy, and yet the evidence of placental metabolism of TH alone points to this being perhaps the period of most interest.

In addition to the time at which measurements are taken, attention should also be paid to placental type, as it is possible that the different placental types of the experimental models used differ with respect to the degree of transplacental hormone passage permitted. In the same way that not all placentae produce chorionic gonadotrophins, then not all are likely to present the same characteristics relating to hormone transport (179). It is possible that such differences are linked with the observations of placental type-specific increases in the levels of hormone binding proteins during pregnancy.

Considerable debate has centred on the amount of hormone which can be considered to be biologically significant during stages of fetal development (24,161,182). Whilst many reports have taken low fetal serum total TH concentrations to indicate lack of function (24,183,184), it is possible that a
concentration which may be considered relatively insignificant if found in the maternal liver, may be sufficient to elicit a response in the fetus. Binding parameters and uptake into organs may result in apparently low total levels of hormone being significant due to high free hormone levels, high tissue concentration of the active hormone, or localisation of the hormone within specific target cells. It is known, for example, that free TH levels are proportionately higher in the fetus owing to decreased binding to the plasma proteins (185).

Two approaches have been employed to determine whether TH traverse the placenta. The first is direct physico-chemical measurement of trans-placental passage of TH, either by radioactive tracer type experiments, or by direct measurement of TH in fetal tissues before it has the capacity to generate these autonomously. The alternative method has been to study the potential effects of the presence or absence of TH in maternal serum during various stages of pregnancy on the development and biochemistry of the fetus.

All of the following reports measured TH in the fetus, or fetal circulation during the latter stages of pregnancy. No transfer was detected in the sheep (186,187) or the human (188-190), whilst other workers detected transport, but considered the levels involved to be too low to be biologically significant, both in the human (191), the rabbit (192,193), and the monkey (194). In the rat, again late in pregnancy, Gray and Galton reported limited placental exchange of TH (195). Of the more recent reports, most have drawn data solely from late in pregnancy, and have reported minimal transfer of T4, and total absence of transfer of T3 in the rat (197) and human (24,196). Many groups record degrees of transplacental movement of TH, but do not comment on the biological significance of the amounts involved. Examples of species investigated include: guinea pig (198); dog (199); rabbit (200); rat (201); monkey (202); and man (203).

In the rat, Knobil and Josimovich (204) stated that T4 more readily traversed the placenta, whilst Raiti et al. (205) clearly demonstrated placental transfer in women using large doses of T3, though noting that such transfer was subject to wide variation. They also urge caution when interpreting data produced before the advent of modern immunoassay techniques in the mid 1960's.

Of the data supporting lack of transfer in the first trimester of pregnancy, the most frequently quoted are the reports of Myant (in the human) (206) and Osario and
Myant (rabbit) (183). Both of these reports predate the innovation of immunoassay, and although this in itself does not necessarily invalidate them, it does draw into question the degree of significance attached to them. If low levels of hormone can be biologically significant, then any error in measurement could lead to a complete misinterpretation of the importance of TH transport across the placenta.

Opposing the opinions above is a growing body of evidence in favour of transplacental passage of TH, particularly in early pregnancy, and suggesting a crucial role for this process in the neurological development of a range of higher mammals. This data draws significantly on recent findings, employing the modern techniques of RIA and IRMA.

In the first study of tissue localisation of TH in early developing embryos, Sweney and Shapiro demonstrated that T4 became concentrated in the developing rat palate before the onset of fetal thyroid function (207) thereby revealing that the fetus was capable of concentrating small amounts of hormone which must have crossed the placenta from the mother. Such localised samples would not be revealed by the estimation of TH in serum samples or in whole fetus homogenates.

Working independently, using the rat model, the groups of Morreale de Escobar and Ekins have produced a large quantity of information regarding TH materno-fetal transfer (23,161,177,178,182,208). The first group demonstrated the presence of TH in fetal tissues before day 17 of gestation, at which stage the fetal thyroid begins to function (208). Similar work was also performed by Woods et al., who reported that in the rat, early uptake of TH was predominantly of T4, and although low, was biologically significant (177). When expressed in terms of uptake per µg of protein, the levels were equivalent to, or greater than those found in a number of maternal target organs (heart, ovary, spleen, brain). They also reported that fetal T3 was predominantly generated by local deiodination of T4 in the fetus in early pregnancy. The levels of T3 traversing the placenta and found in the fetal tissues were less than all maternal target organs studied. This situation was found to continue until approximately the 15th day of pregnancy, whereupon a switch in the activities of the deiodinating enzymes appeared to occur. Until this period D-II (generating T3) had predominated, from day 14 onwards D-III took the major role. Thus transfer of T4 and T3 were restricted from around the time that the fetal thyroid began to function.

In late pregnancy T4 was still found to be delivered to the placenta in large
quantities, only during this period the enzyme activity in the placenta converted a high proportion to rT3 and free iodide, as reported by other groups (172), the iodide being rapidly taken up by the fetus. It is argued that such a function acts as a concentrated local supply of iodine to the fetus for use in its autonomous production of TH. In this way the maternal thyroid is active in supplying iodine to the fetus, rather than competing with the fetus, as is frequently suggested (181,172). Clearly, this makes much greater sense in evolutionary terms, as whilst the maternal tissues can withstand a period of hypothyroidism with only small consequence, deprivation of TH for even a short period during fetal development may have catastrophic consequences.

As summarised by Ekins (23), fetal and placental iodide levels rose from day 10 to day 20 of gestation. In addition, although T4 levels in fetal brain were lower than fetal carcass and liver, the liver/brain ratio was 20 times higher than the adult value, suggesting a significant concentration in this organ.

There is much corroborative evidence supporting these views. Cooper et al. (209) emphasise the need for iodide from TH as a fetal source for its own TH production, and the maintenance of an autonomous fetal TH economy. A study on TH transport in goats, undertaken after the onset of fetal thyroid function (65 days to term, compared to the development of the functional thyroid at 50 days in this species) failed to detect any transplacental passage of T4 during this period (210). However, a very high proportion of $^{125}$I, which had $[^{125}$I]T4 as its only source, was transferred to the fetus in the hours immediately following administration, thereby supplying indirect evidence for sequence of events proposed by Ekins et al.. Church et al. noted a rise in fetal free hormone levels subsequent to administration of T4 to pregnant rabbits (211).

Morreale de Escobar (212) reports the detection of TH in both 9-10 day old embryotrophoblasts, and in 13-14 day old embryos and placentae. Clearly the only possible source of TH at this time would be the maternal thyroid. Indeed, maternal thyroidectomy reduced the TH found to undetectable levels.

The second body of evidence concerns the transfer of TH to the fetus, namely a range of clinical and biochemical data on the effects of lack of, or supplements of maternal TH during fetal development. Much of the clinical data involved relates to human subjects in areas of moderate to severe iodine deficiency. The importance of iodine deficiency, and the extent to which it occurs, is easily forgotten in iodine replete regions in the west, such as the UK, and the USA, but it must be remembered
that even in these countries, iodine deficiency was widespread in the not so distant past. Women, for reasons yet unknown, appear to be more seriously affected, with up to 100% of young women in some seriously affected areas being goitrous, and exhibiting depressed levels of serum T4. Cretinism, although far less common, may still reach levels of 8-10% in such regions (213).

Pharoah *et al.* working in Papua-New Guinea first noticed that the administration of iodised oil to pregnant women eliminated the incidence of neurological cretinism in the children, but only if dosing was commenced early in the first trimester of pregnancy (214). At first this was attributed to an unexplained effect of elemental iodine on fetal brain development before the onset of fetal thyroid function, but later it was recognised in view of the fact that the women in this study were hypothyroxinemic, but possessed normal serum T3 levels (ie were clinically euthyroid), that the observed neurological deficits would be better explained by deprivation of T4 to the fetal tissues at this stage in pregnancy (215). Clearly this evidence is in agreement with the proposal that T4 traverses the placenta in the first trimester of pregnancy in physiologically significant quantities, and exerts an important influence on fetal brain development.

As previously stated, it is proposed that this study represents the extreme end of a range of deficits caused by fetal TH deprivation during early pregnancy. A long term clinical follow-up study of children born to mothers of different age and thyroid state has been performed by Man *et al.* (216,217) involving a large number of pregnancies in the USA. These studies were of normal uncomplicated pregnancies and carefully controlled to minimise any bias of the results due to either genetic or environmental influences. The results pointed to a range of relatively minor deficits, including reduced performance in IQ tests, by the children of hypothyroxinemic mothers when compared to the control sample. Children of mothers who had been given adequate replacement of T4 during later pregnancy displayed fewer differences from the control group. This suggests long lasting effects which could only have arisen as a result of maternal hypothyroxinemia, as the offspring of hypothyroxinemic women displayed no signs of hypothyroidism themselves. Despite plasma total T4 being reported as low in early pregnancy - 25 nM at 16 week (shortly after the onset of fetal thyroid function), compared to 120 nM at term (218) - free hormone levels may be relatively high, and localised tissue levels (not measured)
may be sufficient to influence development

Recent years have seen the more widespread use of animal models of maternal hypothyroidism. These allow the identification and quantification of individual biochemical alterations arising in the progeny as a result of the disorder. Much of this work has a direct bearing on the results presented here, and will be discussed later.

1.10 What mechanisms control passage of TH from mother to fetus?

It may be assumed that if maternal TH are necessary for the direction of fetal development, then their availability may be regulated in a manner likely to bring about the appropriate hormonal environment in the fetus. The degree of damage suffered by the developing fetus as a consequence of maternal hypothyroidism would therefore depend on both the levels of TH crossing the placenta, and on differing capacities of individuals to make effective use of the hormone.

The observed clinical evidence would seem to bear this out, as no direct relationship exists between the extent of maternal TH deficiency and the degree of neurological damage found in the children, as might be expected if maternal TH concentrations alone governed delivery to the fetus. In populations suffering severe iodine deficiency, where many, if not all women are hypothyroxinemic, only a proportion of offspring are neurological cretins (219). Similarly, in the study of Man et al. only 36% of the children of hypothyroxinemic mothers fall into the dull normal range of IQ (216).

It is likely that an individual's genotype plays a significant part in determining TH sensitivity, as illustrated by the wide range of clinically normal values for TH levels in the human serum (for total T4, a value in the range of 62 nM to 165 nM is considered normal). In addition, however, the placenta itself may exert considerable influence over TH availability, and it is possible that placental dysfunction may result in incorrect amounts of hormone reaching the fetus at crucial stages of development.

Because of the rapid appearance, development, and decline of the placenta during gestation, it is not automatically possible to measure its performance regarding any one function at one particular point in time, and extrapolate this to indicate a generalised characteristic of the organ, as is possible, for example with the mature
liver. The placenta performs a synchronised sequence of functions specifically related to fetal development, some autonomous, others depending to varying extents on maternal and fetal control, and cannot be regarded in isolation (220). This is emphasised by the predominance in its structure of tissue of fetal origin, particularly in the case of hemochorial and hemoendothelial placentae (179). It has therefore been proposed that the developing fetus may exert control over the supply of TH, and that this is at least initially centred in the placenta (23,161).

Evidence that this hypothesis may be the case comes primarily from the measurement of levels of hormones in the fetus, and from studies of the deiodinase enzymes previously described. In the fetal plasma, the T3/T4 molar ratio is approximately 0.2, compared to a placental level of 0.1, and a maternal value of 0.02, suggesting a placental role in the generation of T3 for transfer to the fetus (182).

Placental deiodinases exhibit a time difference in relation to peak activities. Woods et al. found D-II in rat placenta to be highly active as early as day 10 of gestation, and then to decline rapidly from day 15 to term (177). D-III activity, on the other hand, is first reported at day 14, peaking at day 17 and slowly declining to term (176). The physiological importance of this timing has been the subject of debate. Some groups have suggested that D-II acts to supply the placenta with local T3 for its own metabolism and development, and that D-III acts as a barrier mechanism to prevent TH reaching the fetus (181). Others have argued that on the contrary, the high D-II : D-III ratio in early gestation may assist the transfer of maternal TH to the fetus, and the function of the build up of D-III towards the onset of fetal thyroid function is to guarantee the supply of iodide to the fetus for its own TH synthesis (177). It has been suggested that the switch in enzyme activity may be the result of an as yet undetected fetal signal, perhaps related to the onset of fetal thyroid function (161). In evolutionary terms, where iodine was likely to have been scarce, such systems may have operated to maintain a local iodide gradient across the placenta, even when the mother was slightly hypothyroid.

Placental control of TH transport has also been proposed to operate at an even more fundamental level. It has been noted that whilst levels of TH rise in pregnancy, this rise does not appear to be a result of increased stimulation by the normal thyroid stimulator, pituitary TSH (thyroid stimulating hormone). Indeed levels of this hormone have been reported to drop during pregnancy (221,222). It has therefore
been suggested that the placenta itself secretes a thyroid stimulator. The existence of this substance, designated hCT (human chorionic thyrotropin), was claimed by several groups, who reported that it was immunologically distinguishable from, but structurally related to TSH (222,223). Repeating these results has, however, has proved difficult, and a related idea has arisen suggesting that hCG itself may be responsible for increased maternal thyroid activity.

In common with other glycoprotein hormones, hCG exhibits a microheterogeneity of molecular structure, particularly of the glycosylated section of the molecule. This results in the existence of a family of isohormones, each with slightly different characteristics of biological effect. Thus it may be that one or more of these isoforms are responsible for the increase in thyroid activity found in pregnancy. Evidence for this possibility includes the clinical detection of certain chorionic tumours which are associated with increased secretion of acidic variants of hCG and with increased thyroid activity (224). In addition, an alteration in the apparent molecular weight of serum hCG due to increased glycosylation occurs after the first trimester, suggesting that the hormone may fulfill more than one role during pregnancy (225,226).

Another aspect of placental control of TH transfer has been revealed through the detection of plasma membrane binding sites for T3 in the organ (93). As yet it is unknown precisely what role these sites play, but it is conceivable that they are involved in transfer of TH from mother to fetus, or at least into placental tissue from the sinusoidal endings of the maternal circulation.

It is also possible that the placenta assists directly in the development of fetal thyroid control systems. Fetal TSH secretion, for example, appears to be independent of both maternal and fetal hypothalamic activity (227-229), and it has been suggested that the placenta may interact with the fetal pituitary-thyroid axis to influence its development (226).

In conclusion, the placenta is a complex endocrine unit which interacts with both the fetus, and the mother. It has been proposed that the organ acts in a disciplined manner to regulate the amount of maternal TH reaching the fetus. This level may alter according to fetal needs in different stages of pregnancy, and a degree of intercommunication between fetus, placenta and mother must occur to support the optimal supply of hormone to developing fetal tissues.
Figure 5: Conceptual model of thyroid hormone economy during pregnancy

**EARLY PREGNANCY**

- **UTERUS**
  - Fertilisation/Implantation
  - Trophoblastic invasion
  - Rapid T4 uptake maintains fall in FT4 in early pregnancy
  - 5'-Deiodinase activity
  - T4 transfer in early pregnancy

**PLACENTA**

- T4 transfer in early pregnancy
- T4 → T3 + Iodide

**FETUS**

**LATE PREGNANCY**

- T4 in circulation

**MATERNAL CIRCULATION**

- Iodide economy
- Iodide + rT3

**PLACENTA**

- 5'-Deiodinase
- T4 → T3 (prevents hyperactivity)
A model for integration of the major events of maternal and fetal thyroid hormone economy during pregnancy is presented in Figure 5.

1.11 Rationale

Many factors point to maternal thyroid hormones playing an important role in the development of the fetal nervous system. The data up to the commencement of this work were almost entirely of a general or clinical nature, describing symptoms rather than investigating possible causes. The main aims of this project were to identify mechanisms through which TH may exert an influence on the development of the fetal brain.

Initially, this involved a general examination of the biochemistry of the developing fetus, with special emphasis on the brain, and entailed the use of an animal model of maternal hypothyroxinemia selected to reflect the conditions found in human neurological cretinism. Both treatment of the pregnant dams with anti-thyroid drugs, such as methimazole, and the use of iodine deficient diets presented the danger of fetal hypothyroidism, and were considered unacceptable. Rat dams were therefore rendered hypothyroxinemic by partial thyroidectomy (parathyroid-spared), leaving them with euthyroid levels of T3. The offspring were completely euthyroid with respect to both T3 and T4.

Placental type was also taken into account when deciding upon an animal model, and for this reason the rat was chosen, as it possesses a hemochorial placenta, similar to the human. In addition, the rat is a convenient experimental animal to study, the necessary manipulation of environmental conditions being relatively straight-forward.

The purpose of this approach was to gain an insight into any large scale biochemical deficits in the progeny, analogous to those recorded in hypothyroid animals, which may result from maternal hypothyroxinemia. It was also intended to follow up any significant deficits in the hope of identifying systems adversely affected by deprivation of maternal TH, which could be involved in the neurological deficits apparent in clinical studies.

Problems of identifying the precise targets and initial actions of TH in development are inevitable using in vivo models. Labelling experiments, for
example, face the problems of differing growth rates of different organs. A drop in brain uptake of a particular metabolic precursor (amino acids, sugars, etc) may be due to increased uptake in the liver, rather than any intrinsic alteration in brain metabolism. It can therefore be difficult to draw conclusions from age-related differences in these experiments.

Probably the most important disadvantage of *in vivo* work, however, is the inability to attribute TH effects to specific cell types within the developing brain. Over recent years, work on isolated cell types, notably neurons, astrocytes and oligodendroglia grown in tissue culture has allowed closer examination of TH action at this level, providing information on TH modulation of cell development. Studies performed *in vitro* have included studies on: TH metabolism (230); neurotransmitter synthesis (231); cytoskeletal structure (149); uptake and incorporation of amino acids and sugars (90,232); enzyme activities (233); and synergistic effects with other hormones (234,234), as well as a number of other parameters (117).

The more controlled environment of tissue culture was therefore employed in the second series of experiments, in an attempt to identify more precisely the biochemical nature of the disturbances noticed in the *in vivo* work, and to attribute to it a degree of cell type specificity. Primary cultures of astrocytes, and to a lesser extent, neurons were examined with respect to the effect of TH on the incorporation of $^{3}$H-fucose into glycoproteins. Particular attention was paid to the cell surface localisation of these glycoproteins, as these include a number of cell surface markers, important for the direction of neural development (236). Astrocytes were used, as they are known to be involved in such cell-cell interactions, through which they are critical for the formation of appropriate neural connections (237).

It was hypothesised that TH may be directly involved in glial, as well as neuronal development, and for this reason, a concurrent study was performed to examine the development of cellular mechanisms of TH action, with special reference to glial cells, in order to determine whether these cells possessed sufficient nuclear receptors to suggest the possibility of direct TH action. This required the detailed examination of the ontogeny of fetal rat THNR in neuronal and glial nuclei from different brain regions. A knowledge of the cell specific binding of TH is essential to the understanding of TH action in the developing brain, and although this was conducted for practical reasons during the postnatal period, conclusions drawn from
the results also have implications for potential compartmentalisation of receptor expression in early gestation.

Briefly, the results indicated that the progeny of hypothyroxinemic rat dams suffered a range of biochemical deficits during the neonatal period in most of the organs studied. Closer examination of the brain of these progeny revealed specific deficits in a number of Con A binding glycoproteins, which altered with age. TH stimulated the incorporation of fucose into glycoproteins in developing astrocytic cultures, and this stimulation was found to be predominantly associated with the cell membrane. Nuclear T3 binding studies revealed that despite low levels in adults, neonatal glial nuclei possess high levels of T3 receptors, which vary on a regional basis, and decline with age, indicating the possibility for direct TH action on developing glia.
CHAPTER 2 : MATERIALS AND METHODS
2.1 General

2.1.1 Materials

General laboratory chemicals were purchased from BDH Ltd (Dagenham, Essex) and were of AnalaR grade wherever possible. All fine chemicals were obtained from Sigma Chemical Co. Ltd (Poole, Dorset), and radiochemicals from Amersham International (Amersham, Bucks). Sprague-Dawley rat dams were obtained partially thyroidectomised (parathyroid spared) from Charles River Ltd (Margate, Kent), whilst normal animals were bred in the medical school's own animal house facilities. Details of any chemicals or equipment obtained elsewhere are given in the text.

2.1.2 Protein Determination

Protein was normally estimated by the standard method of Lowry et al. (238), in which alkaline copper sulphate reacts quantitatively with compounds containing two or more peptide bonds to produce a violet colour, in proportion to the number of peptide bonds present. Absorbance was read at 500 nm in a Pye-Unicam spectrophotometer. The standard curve was constructed using bovine serum albumin (BSA; 1 mg/ml) as the standard. All homogenate samples were assayed in duplicate, where the intra-assay variation was <5%. Where this limit was exceeded, the assay was repeated.

Where sample was estimated to be <10 µg/ml, the dye binding method of Bradford was employed (239). This relies on the quantitative binding of Brilliant Blue G250 (Coomassie Blue) to protein, the product possessing an absorbance maximum of 595 nm. The method is more sensitive than that of Lowry et al., and can be measured almost immediately. The standard curve (again constructed with BSA (100 µg/ml) as a standard was not completely linear. This problem was overcome with the aid of a computer generated polynomial equation for the curve from which accurate estimations could be made. The dye was made up in the laboratory until a reliable commercial preparation became available (Protein Assay Reagent; Pierce Ltd, Luton, Beds).
2.1.3 DNA Determination

2.1.3.1 Extraction

Nucleic acids were extracted from tissue homogenates using a modification of the method of Schneider (1945) (240). Firstly homogenate was precipitated with an equal volume of 20% (w/v) TCA on ice for 20 minutes, centrifuged (2,000 g; 20 min), and the pellet washed with 10% TCA (5 vol) and treated as before. The resulting pellet was resuspended in 1.5 ml 5% TCA, and boiled for 20 minutes, cooled and centrifuged. The supernatant was collected, the extraction procedure repeated, and the supernatants combined. Extracts were stored frozen at -20°C.

2.1.3.2 Measurement

DNA was usually determined by the colorimetric diphenylamine reaction (241). Deoxyribose, released from DNA by acid hydrolysis reacts with diphenylamine under acidic conditions to form a blue product with an absorbance maximum of 595 nm. In order to distinguish between RNA and DNA, a reading is taken at 650 nm, which is subtracted from one taken at 610 nm. The difference is proportional to the quantity of DNA in the solution. A standard curve was constructed using calf thymus DNA in 5% TCA. Extracts were assayed in duplicate, and readings were found to agree to within 5%.

Where levels were estimated to be <20 μg/ml, or sample limited, DNA was measured using a slightly modified version of the fluorimetric method of Setaro and Morley (1976)(242). Samples were extracted in the manner described. The extracted deoxyribose reacts with 3,5-diaminobenzoic acid (DABA) under acidic conditions to yield a product which fluoresces with a maximum peak at 520 nm.

DABA solution (30 mg/ml) is acidified dropwise by concentrated HCl, until fully dissolved. To either various concentrations of standard (calf thymus DNA; 100 μg/ml), or tissue extracts (final volume, 1 ml), 1 ml of DABA solution is added. The mixture is heated at 60°C in a water bath for 30 min, cooled, and read in a Perkin-Elmer fluorimeter, at 420 nm excitation, 520 nm emission. The standard curve constructed was linear between DNA concentrations of 0.5 and 30 μg/ml.
2.1.4 Statistical Analysis

All statistical analyses performed in the first two sections of the results were by unpaired Student's t-tests. Variances between populations were not significantly different.

2.2 In Vivo Studies

2.2.1 Animal Model

Partially thyroidectomised rat dams were mated with normal males when the total serum concentration of T4 (measured by RIA) fell approximately 10 nM (compared to approximately 45 nM in normal controls). Animals were housed at 37°C in a 12 hour light dark cycle. Pregnancies were allowed to continue to term, and no induction of parturition was performed. Animals received a normal, iodine replete diet (estimated to result in an average uptake of 34 µg iodide per day), whilst thyroidectomised dams were provided with 0.1% calcium lactate in drinking water. Water and food were available ad libitum, and average food intake was measured at 17 g per day during pregnancy in both groups. These conditions were designed to approximate as closely as possible the changes in TH levels occurring in pregnant women in areas on endemic iodine deficiency (243,244).

2.2.2 Litter Handling

After birth, litters were allowed to suckle for 3 weeks before weaning, when they were rehoused under standard conditions (22°C) in groups of not more than 5 to a cage.

Owing to the discrepancy in litter sizes between the two groups (See Results 1), and the shortage of samples, a schedule was arranged to normalise litter sizes in a manner which made best use of all available litters. This operated as follows. All litters (control, and the progeny of thyroidectomised dams - TD) were, where necessary, initially normalised to 9 animals. No litters were used where the litter size was lower than 5 animals. As far as possible, matched control litters were normalised to that of the TD progeny. Where this was not possible, and to ensure
comparability of the results, smaller litters were sampled at a later date, and compared with animals from litters where some progeny had been sampled at earlier stages and had therefore received similar competition for suckling position. No more than 3 animals were taken from any one litter at any of the sampling days. This produced a sampling chart as follows (Table 3). Animals taken at other days were from control matched litters. Notes were kept of maternal identity, date of birth, length of gestation, and size of litter.

Table 3: Sampling chart

<table>
<thead>
<tr>
<th>Litter Size</th>
<th>No. Sample / day</th>
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<tbody>
<tr>
<td></td>
<td>day 5</td>
</tr>
<tr>
<td>10</td>
<td>4</td>
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<tr>
<td>9</td>
<td>3</td>
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<td>2</td>
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<td>1</td>
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<tr>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
</tr>
</tbody>
</table>

Litters >10, culled to 10; litters <5 not used. Samples for other days had matched control litters.

2.2.3 Organ Sampling

Progeny of both TD and control dams were killed by concussion followed by exsanguination at comparable postnatal stages, from day 1 up to day 35. Animals were weighed whole, then in all animals, brain and liver were removed and frozen immediately in liquid nitrogen. They were then weighed, and stored in a deep freeze at -20°C. Although efforts were concentrated on these two organs, other organs were
also removed for assessment, including; lung, kidney, heart, and spleen, all of which underwent similar treatment.

2.2.4 Tissue Processing

Frozen organs were pulverised in a specially designed chamber, and the resultant powder homogenised in a hand-held homogeniser (clearance 0.1 mm) in 0.32 M sucrose and made up to a suitable volume to give a 10% (w/v) homogenate. This was aliquoted and stored frozen at -20°C. The storage period did not exceed 6 months during which time, protein degradation was monitored and found to be small. As a precaution, and where possible, matched samples were stored for similar periods before use.

2.2.5 Enzyme Activity Measurement

2.2.5.1 Acid Phosphatase (EC 3.1.3.2)

Construction of a pH profile for this enzyme, using citrate buffers with pH's ranging from 2 to 10.5, revealed the pH optimum to be pH 5.5. Measurement of this and all other lysosomal enzymes was conducted by the assay of 4-methyl umbelliferone (4-MU). 4-MU is released from the substrate, a conjugate of 4-MU with the appropriate substrate for enzymic hydrolysis (in this case, 4-MU-phosphate), and fluoresces under alkaline conditions (pH 10.4), with an emission maximum of 444 nm (245).

The assay performed was based on the methodology of Sinha and Rose (246). Samples were measured in duplicate, with a third tube acting as a reagent blank, where homogenate was added after the stopping buffer. 1 ml of homogenate was added to 1 ml of reagent (4-MU-phosphate (12 μM) in citrate buffer (100 mM; pH 4.5), and incubated in a water bath with shaking at 37°C for 15 min. The reaction was terminated by the addition of 3 ml of glycine/NaOH buffer (0.5 M; pH 10.4), and the fluorescence obtained read in a fluorimeter at 360 nm excitation, 444 nm emission. After subtraction of the substrate blank, the activity of acid phosphatase was expressed as nmol umbelliferone released/mg protein/hour.
As very high or very low levels of protein are likely to result in non-linearity of the standard curve, an initial experiment was performed to determine the acceptable range of protein concentrations. Volumes of homogenate used in the assay were then adjusted to fall within the linear part of the standard curve.

2.2.5.2 β-D-Galactosidase (EC 3.2.1.23)

β-galactosidase is another lysosomal enzyme with a pH optimum of 3.5. In the rat brain, it commonly hydrolyses terminal galactosyl residues from an array of glycoconjugates. Its activity was determined in the same manner as acid phosphatase, using 4-MU-galactoside (120 μM) as the substrate. All other parameters were identical.

Concentration and pH profiles were performed as with acid phosphatase, and homogenate volumes adjusted accordingly.

2.2.5.3 β-D-Glucosidase (EC 3.2.1.21)

This enzyme hydrolyses glucosyl residues, and has a pH optimum of 4.5. The activity was estimated in the same manner as acid phosphatase, using 4-MU-glucoside (120 μM) as the substrate. Volumes were adjusted to ensure that the protein content fell within the linear section of the concentration profile.

2.2.5.4 β-N-Acetyl-D-glucosaminidase (EC 3.2.1.30)

This enzyme hydrolyses N-acetylglucosamine residues, and has a pH optimum of 4.5. The activity was estimated in the same manner as acid phosphatase, using 4-MU-glucoside (120 μM) as the substrate. Volumes were adjusted to ensure that the protein content fell within the linear section of the concentration profile.

2.2.5.4 Arylsulphatase (EC 3.1.6.1)

This enzyme has both acidic and neutral/alkaline components, and consists of at least 2 isoenzymes, A and B. It hydrolyses sulphatide residues to galactoceramide.
and sulphate. In these experiments, only the acidic component of arylsulphatase A was estimated, as it is the most commonly studied form in the literature (247), although a later pH profile indicated the alkaline component to be more active in adult rat brain (248). Activity was determined in the same manner as acid phosphatase, using 4-MU-sulphate as the substrate.

2.2.5.5 Na⁺/K⁺-ATPase (EC 3.6.1.3)

This ubiquitous membrane enzyme is essential for the maintenance of membrane potential, of critical importance in the generation of action potentials in excitable tissues. In addition, the high intracellular potassium concentration, generated by the energy dependent exchange with sodium, is essential for the operation of a number of metabolic processes, including protein biosynthesis and the optimal operation of numerous enzymes (for example pyruvate kinase in glycolysis). The expression of its component peptides has been reported to be directly influenced by TH (249).

The activity of Na⁺/K⁺-ATPase is assessed by its catalysis of the release of inorganic phosphate from 30 mM Tris-ATP. In order to correctly assess the activity of this particular enzyme, it is necessary to specifically block the activity of Mg²⁺ ATPase with the cardiac glycoside, ouabain (3 mM). The reaction takes place in 30 mM Tris buffer at pH 7.4 (37°C; 15 min), following the procedure of Baron and Khan (250). After termination of the reaction with 30% TCA, the inorganic phosphate released is measured by the standard procedure of Martin and Doty (251). This well established assay depends on the reaction of phosphate with ammonium molybdate to form phosphomolybdic acid, which is extracted from the aqueous phase with butanol-benzene, reduced with stannous chloride to give a blue colour read in the spectrophotometer at 730 nm. Samples of homogenate were assayed in duplicate, with an additional tube for the ouabain control. Values were compared with a standard curve (0 - 0.6 μmol Pi: Na₂HPO₄ as standard) and, after blank subtraction, expressed as μmol inorganic phosphate liberated/mg protein/hour.

Preliminary experiments were performed to establish the acceptable protein concentration range and the optimal duration of experiment.
2.2.6 Radioimmunoassay

Blood was taken from control and thyroidectomised dams after the lactation period by venipuncture into heparinised tubes. Blood was removed from progeny at the time of death. Plasma was centrifuged at 2,000 g for 10 min and the serum separated and frozen until assay. Measurement of total T3 and T4 were performed using the Amerlex kit from Amersham International, following the standard protocols supplied.

2.2.7 Separation of Subcellular Compartments

Homogenate was separated into membrane and cytosolic fractions by ultracentrifugation. The procedure used is described below, and is standard (252).

Homogenate (0.5 ml; 50-150 mg protein) was diluted with an equal volume of 0.32 M sucrose, and ultracentrifuged (45,000 g; 20 min) to precipitate nuclei and organelles, including mitochondria and lysosomes. The supernatant was collected and centrifuged (104,000 g; 60 min). The supernatant contained the soluble proteins, whilst the pellet consisted of membranes and microsomes. Proteins in the membranes were liberated by incubation overnight in Triton X-100 (0.5%) overnight at 37°C.

2.2.8 Glycoprotein Isolation by Concanavalin A Chromatography

Concanavalin A (Con A) is a plant lectin which specifically binds glucosyl and mannosyl residues. When these form the terminal units of glycoconjugates, it is possible to use this procedure to separate this family of glycoconjugates in a highly specific manner.

2.2.8.1 Sample Preparation

Homogenates were separated into membrane and cytosolic fractions as above, and solubilised with Triton X-100 (0.5%). They were then dialysed overnight in 1 mM phosphate buffer (pH 7.4) and freeze dried, before being made up at similar
protein concentrations (1 mg/ml). These samples were then applied to the Con A column following the protocol below.

2.2.8.2 Con A Chromatography

Concanavalin A was covalently bound to Sepharose 4B (Pharmacia, Milton Keynes, Bucks), following the procedures recommended by the manufacturers. A 10 cm column was then packed to a gel volume of 6.3 ml, and allowed to settle. Sample volume applied to the top of the column was 2-3 ml. Elution was performed in three stages at a flow rate of 8 ml/hour. The first step washed through the unbound fraction with PBS (Na$_2$H/NaH$_2$ 1 mM, NaCl 0.15 M; pH 7.4), with 2 ml samples being collected for 3 hours. Secondly, the same buffer, but containing methyl-D-glucoside (0.2 M) was used to elute the bound fraction for 3-4 hours (2 ml fractions). Finally, this process was repeated using methyl-D-glucoside (1 M). Protein concentrations in the sample and in each fraction by the method of Lowry et al., and an elution profile plotted.

2.2.9 SDS-Polyacrylamide Gel Electrophoresis

2.2.9.1 Sample Preparation

Fractions collected after separation were concentrated and transferred to a suitable phosphate buffer initially by dialysis against (Na$_2$H/NaH$_2$, 0.1 M; pH 7.1) phosphate buffer and freeze drying, and subsequently by the use of Centricon 10 microconcentrators (Amicon Ltd, Stonehouse, Glous.). These are molecular filters which retain molecules above a certain molecular weight (in this case, 10 kD), but allow the remaining molecules and the solute to pass through under the influence of centrifugation. This permits the sample to be transferred to the required volume of a different buffer. Once accomplished, the sample was then made up to 2 mg/ml, or where this was not possible, matched controls were employed. SDS (12 mg/ml in 0.1 M phosphate buffer, pH 7.1) was added in a protein SDS ratio of 1:4. Proteins were reduced by the addition of 2.5% (v/v) 2-mercaptoethanol and denatured by immersing in a boiling water bath for 2 minutes. 5 µl of bromophenol blue (0.8%)
were added just prior to running to mark the electrophoresis front.

2.2.9.2 Gel Preparation

Polyacrylamide gels were prepared in phosphate buffer (0.2 M; pH 7.1) containing 0.4% SDS (w/v), according to the procedure described by Weber & Osborn (253). 8% acrylamide gels were used containing 2.6% cross-linker (ammonium persulphate), and care was taken to thoroughly degas the solution prior to polymerisation. Gels were stored overnight in a humidified chamber, and prerun at 150 mA for 30 min.

2.2.9.3 Electrophoresis

10µl of SDS treated sample solution containing 20 µg protein was applied to each slot of a 20 slot flat bed gel. Six lanes were used for molecular weight markers (high and low), two at each end of the gel, and two in the centre, to ensure that the gel ran evenly. Gels were run at constant current of 20 mA for the first 10 min, and then at 200 mA for 4 hours, or until the marker dye (bromophenol blue) ran off the gel. Gels were then immediately fixed in fixing solution (TCA (11.4%), sulphosalicylic acid (3.4%), methanol (33.3%)) for 1 hour, washed thoroughly and stained (1 hour) with Coomassie Blue (0.3%) in a solution containing methanol (45%) acetic acid (9%). They were then destained overnight in the destaining solution (30% ethanol, 10% acetic acid). When required, gels were preserved in destaining solution containing 10% glycerol.

2.2.9.4 Molecular Weight Determination

Molecular weight of samples was determined using a plot of relative mobility versus log_{10} molecular weight of the known standards. The standards were of two types: high molecular weight standards, including various polymerised states of a 56 kD monomer, up to 336 kD; and low weight markers, which were individual proteins comprising of a single polypeptide chain of known molecular weight, from 12.5 kD (cytochrome c) to 76 kD (ovotransferrin).
2.2.10 Densitometry

After destaining, gels suitable for densitometric quantification were sectioned into lanes, and the start point and end point of the run measured, as were the positions of major bands. Lanes were then loaded into a Pye-Unicam densitometer, and scanned at a wavelength of 540 nm. Gels were zeroed on a blank portion of the gel (commonly before the start point of the run). Preliminary experiments revealed that optimum resolution was obtained at a scanning speed of 0.4 mm/s, using a chart speed of 20 s/cm. This enabled direct calculation of the true distances migrated by the polypeptides.

Measurements were made using the maximum value of each peak as an estimation of abundance, rather than attempting to calculate absolute values on the basis of areas under the curve.

2.3 *In Vitro* Studies

2.3.1 Materials

L-[5,6-3H]-Fucose (45-70 Ci/mmol) was purchased from Amersham International (Amersham, Bucks). Fetal, and newborn calf serum, media, antibiotics and hormones were supplied by Gibco Ltd (Paisley, Scotland). L-T3, and its analogues T4, rT3 and TRIAC were obtained from Henning (Berlin, West Germany). All other chemicals were of analytical grade.

2.3.2 Cell Culture

2.3.2.1 Astrocytes

Culture conditions were modified from those described by Sensenbrenner *et al.* (254) Brains from 1 day old rats were removed, cleaned, and chopped, then dispersed with a sterile pipette. Cells were then plated at a density of approximately 2 x 10⁶ /ml in 64 cm² Sterilin petri-dishes (R&L Slaughter, Upminster, Essex) in modified Eagle's medium (10% fetal calf serum (FCS)), containing L-glutamine (2
mM), penicillin (100 U/ml), streptomycin (100 μg/ml), fungizone (2.5 μg/ml), and supplemented with a mixture of hormones (insulin (10 μg/ml), hydrocortisone (10 nM), transferrin (5 μg/ml), glycine-L-histidyl-L-lysine (10 ng/ml), and somatostatin (10 ng/ml)). The dishes were incubated in a humidified atmosphere of CO₂-air (5%:95%) at 37°C. The medium was changed after 2-3 days, and again after a further 4.

2.3.2.2 Neurons

The procedure used was that described by Pickard et al. (232). Fetuses were removed from culled dams on the 16-17th day of pregnancy, the brains removed, cleaned, and chopped, then dispersed with a sterile pipette. After filtration through sterile bolting nylon cloth (110 μm mesh size), cells were then plated at a density of approximately 2 x 10⁶ /ml in 64 cm² Sterilin petri-dishes in Coon's modified F-12 medium, containing L-glutamine (2 mM), minimum essential medium Eagle nonessential amino acids, penicillin (100 U/ml), streptomycin (100 μg/ml), fungizone (2.5 μg/ml), and supplemented with a mixture of hormones (insulin (10 μg/ml), hydrocortisone (10 nM), transferrin (5 μg/ml), glycine-L-histidyl-L-lysine (10 ng/ml), and somatostatin (10 ng/ml)). The dishes were incubated under the same conditions as were the astrocytes. Initially the medium contained 2.5% newborn calf serum. After 4 days, the medium was changed for serum free medium, in order to select for neuronal cells. Microscopic examination revealed that, after a further 4 days, the majority of cells present were neurons.

2.3.3 Serum stripping of TH

The procedure used was a modification of the resin method of Bernal et al. (255). [¹²⁵I]T₄ was added to FCS to give a count of 1000 counts/min/100 μl serum, and allowed to equilibrate for 30 min. AG 1x8 200-400 mesh anion exchange resin (chloride form; Bio-Rad, Watford, Herts) was stirred with labelled serum (250 mg resin/ml serum), at a controlled temperature of 37°C, for 3 hours, after which the serum was filtered (Whatman No. 1), and the procedure repeated using fresh resin. Serum was then filtered through paper (Whatman No. 1), and glass fibre filters
(Whatman GF/D; GF/F), and sterilised through micro-filters (cut off point 0.2 μm) (Flow Ltd, Rickmansworth, Herts). Resin was regenerated using alternative acid and alkaline washes. Counting of an aliquot of stripped serum revealed stripping efficiency to be in excess of 99%.

2.3.4 Immunocytochemistry

To determine the purity of cultures, immunocytochemistry was performed on 15 day old astrocytic cultures grown on cover slips in the presence of FCS. An antibody to the astrocyte-specific marker GFAP was used, in combination with an immunoperoxidase. Rabbit anti-rat GFAP antibody (1:400 dilution) was donated by Mr. P Witney in the department of Histopathology at UCL, and the assay performed according to the protocol supplied.

Cells were fixed in fresh acetone, before being transfered to Tris buffered saline (TBS; pH 7.6). To this, newborn swine serum (NSS) was added (final 1:10; 10 min) then tipped off and the cells washed. Rabbit anti-rat GFAP (1:500; 30 min) was applied, then washed off with TBS, in preparation for the second antibody, sheep anti-rabbit IgG (1:100; 30 min). After further washing in TBS, rabbit peroxidase anti-peroxidase (1:1000; 30 min) was added and the cells subsequently washed. The substrate solution (5 mg 3,3-diaminobenzidine hydrochloride in Tris-HCl buffer (0.2 M; pH 7.6), hydrogen peroxide (1%)) was then added for 10 min. Washing followed in TBS, then in tap water. Cells were counterstained in Mayer's haematoxylin, differentiated in acid alcohol, and "blued" in alkaline tap water, dehydrated, mounted, and viewed under the microscope. Sites of immunoperoxidase activity (indicating the site of GFAP) are stained brown, whilst nuclei are stained blue.

2.3.5 Cell Viability

Two tests for cell viability were performed. Routinely used was the trypan blue exclusion test, where cells were mixed with an equal volume of 0.2% trypan blue, incubated for 3 min at 37°C to facilitate uptake of dye by dead cells, and counted immediately.

Alternatively, viability was assessed using the acridine orange-ethidium
bromide fluorescence test. Acridine orange stains live cells green, whilst ethidium bromide stains dead cells brown (256).

A stock solution (100x strength) was prepared using 50 mg of ethidium bromide and 15 mg of acridine orange in 1 ml of ethanol (95%), made up to 50 ml with distilled water. This was aliquoted (1 ml) and stored at -20°C. The working solution consisted of 1 ml stock in 100 ml phosphate buffered saline (PBS), which was used 1:1 with the cell suspension. The mixture was viewed under ultra-violet light and cells counted promptly, as the stain is cytotoxic.

2.3.6 Radioimmunoassay

RIAs were performed to confirm the concentrations of TH in media containing TH-stripped FCS. The assay for total T3 was performed using the Amerlex kit (Amersham International), whilst free T3 was estimated using in house procedures.

2.3.7 Liquid Scintillation Counting

Both aqueous samples, and samples solubilised with NCS (Amersham International, Amersham, Bucks) were added to scintillation vials containing 3 ml of a sensitive scintillation fluid resistant to excessive quenching. During the duration of the study, regulations on usage and disposal of this fluid meant that three different types were used (ES299 and Picofluor 40 (Canberra-Packard, Pangbourne, Berks); Ecoscint A (National Diagnostics, Aylesbury, Bucks.). However, strict quality assessments were performed on each one to ensure comparison of results was valid. It was found that, after quench correction and some small procedural alterations, it was possible to compare results from all fluids used.

2.3.7.1 Quench Correction

This section of experiments required the use of two different β-emitting isotopes, ^3^H and ^1^4^C. Two quench correction curves were therefore prepared in the following manner.
Figure 6: Quench curves for scintillation counting.

8a. $^3$H

$y = -4.9960 + 80.397x - 56.455x^2 + 17.721x^3$ $R^2 = 0.987$

8b. $^{14}$C

$y = 69.026 + 64.759x - 132.89x^2 + 91.397x^3$ $R^2 = 0.978$
A range of standards were prepared, each containing the same amount of radiolabelled compound ([3H]-fucose and [14C]orotic acid) in 3 ml scintillation fluid. To each of these (performed in triplicate), a measured amount of quenching agent was added as follows: water (0.1 - 1 ml); TCA (0.05-0.5 ml); NaOH (0.05-0.5 ml); and chloroform (0.05-0.3 ml). In addition to these, controls were prepared containing no quenching agent, to determine the counting efficiency of the machine (LKB Rackbeta); and containing no label, to assess background count. Samples were left overnight in a dark cupboard to diminish any chemiluminesence. After blank subtraction, efficiency was plotted against the standard channels ratio to produce a quench curve. A computer graphics package (Cricket Graph 1.3), was employed to obtain a polynomial equation representing the best fit line. Using this it was therefore possible to convert count data to dpm, thereby rendering the results more directly comparable.

Where results fell outside the range of the quench curve, data was disregarded and experiments were repeated. Separate quench curves were prepared for each of the scintillation fluids used. Typical examples for 3H, and 14C are illustrated in Figure 6.

2.4 Fucose Incorporation into Astrocytes

2.4.1 T3 Dose Response

Experiments were begun soon after the second medium change. Medium was changed, substituting fetal calf serum stripped of TH by the resin method (255), and incubated under standard conditions for 24 hours. Cells were then incubated (37°C; 6% CO2) for 48 hours in the same medium containing L-[5,6-3H]-fucose (2 μCi/dish in 1 μM cold carrier). To this a range of concentrations of T3 (1 x 10^-12 - 1 x 10^-7 M) or vehicle controls was added. The experiments were terminated at zero time (control) by the addition of ice cold stopping medium (SM; 50 mM fucose in MEM) and at 48 hours by placing on ice. All subsequent procedures were conducted on ice. Aliquots were taken to determine total count, the remaining medium was discarded, and dishes were washed with SM. Cells were then gently harvested using a rubber or nylon policemen, centrifuged (700 g; 10 min), and washed.
Figure 7: Neuronal cells after removal from culture.

Cells were harvested with a nylon policeman and photographed under phase contrast (×1200)
Harvesting the cells in this manner caused little disruption of cells, which round up on removal (Figure 7). The supernatant was discarded and cells lysed in distilled water and sonicated. After the removal of aliquots for the determination of protein and DNA, the remaining protein was precipitated with 10% TCA, pelleted (2,000 g; 20 min), and solubilised for β-counting with NCS. Samples of both the acid precipitable and acid soluble fractions were counted, and results expressed as dpm/μg protein. Initially, TCA precipitated pellets were subjected to lipid extraction (1 ml chloroform: methanol (3:1 v/v)). Counts of the resultant pellets and extracts revealed >95% of the radioactivity to be located in the pellet, and the step was subsequently omitted.

2.4.2 Time course

All procedures were as described above, except that T3 concentration was fixed at 1 x 10^-8 M (with corresponding vehicle controls), and sampling time ranged from 2 to 120 hours. All reactions were terminated using ice cold SM.

2.4.3 T4 Dose Response

This was performed identically to the T3 dose response experiments, except that T4 (1 x 10^-9 M - 1 x 10^-7 M) was added to the medium instead of T3.

2.4.4 Analogue Studies

For comparison with the effects of T3 and T4 listed above, the effect of the non-biologically active TH derivatives, TRIAC and rT3 were studied at fixed concentrations of 1 x 10^-8 M.

2.5 Fucose Incorporation into Neurons

2.5.1 Time course

After 6 days culture as described in section 2.3.2.2, the medium was changed
for one containing L-[5,6-3H]-fucose (2 μCi/dish in 1 μM cold carrier). To this, T3 (1 x 10^{-8} M) or vehicle control was added. Incubation and processing of cells was identical to the procedure for astrocytes, described in section 2.4.1, except that the stopping medium consisted of Coon's F12 medium containing 50 mM L-fucose.

2.6 Membrane Isolation

Pure plasma membranes from astrocytic cultures were isolated according to an established methodology (Fig. 8) (257). 100 plates of astrocytes were cultured as previously described (section 2.3.2.1), the medium was aspirated, and plates washed twice with Tris-HCl buffer (100 mM; pH 7.6). The washings were discarded, and the plates frozen at -20°C for 48 hours. After this period, plates were thawed, and gently washed, then rinsed with 1 ml of buffer. The material remaining on the plates (surface adherent fraction; SAF) was collected with a nylon policeman. The washings of all the plates were combined and centrifuged (37,000 g; 30 min), the supernatant being discarded. Pellets were resuspended in 2 ml Tris buffer, and layered onto 20 ml sucrose (1 M in Tris buffer), and centrifuged (104,000 g; 3 hours). This resulted in a diffuse opaque layer below the surface, and a pellet fraction (PF), which was retained. The first 3-4 ml of the gradient were discarded, and the next 4 collected into two 2 ml aliquots, each of which was diluted into 20 ml Tris buffer, and centrifuged (37,000 g; 1 hour). The pellets were washed in Tris-HCl (0.0625 M; pH 6.8: 37,000 g; 30 min). These were the membrane fractions (MF).

2.6.1 Identification of Fractions

Quantification of the purity of plasma membrane prepared on the basis of marker enzymes with specific cellular locations was not possible due to insufficient sample. Na⁺/K⁺-ATPase was measured, but failed to produce results in any of the fractions (MF, PF, or SAF), perhaps as the result of enzyme inactivation during processing. However, protein values and distributions between fractions agreed closely with values reported in the literature for similar separations, suggesting that the separation had isolated relatively pure plasma membranes (257,258).
Figure 8: Semi-diagramatic representation of the method for purification of astrocyte plasma membranes.

1. Dishes washed (100 mM Tris; pH 7.6)
2. Freeze-thaw (48 h)
3. Dishes washed
4. Washings combined and centrifuged (37,000 g; 30 min)
5. Pellet resuspended in 2 ml Tris
6. Pellet scraped SAF
7. Resuspended pellet
8. 1.0 M sucrose
9. Centrifuged (104,000 g; 3 h)
10. Opaque layer
11. 2 x 2 ml + 20 ml Tris
12. Centrifuged (37,000 g; 1 h)
13. Pellets combined and washed (37,000 g; 30 min)

Based on procedure of Mersel et al. (258)
2.6.2 Cellular distribution of fucosylated glycoproteins stimulated by 10 nM T3

This experiment was performed in a similar manner to the fucose incorporation dose response and time course experiments, with a few modifications. T3 (10 nM) was added after 24 hour preincubation in TH-stripped serum containing the carrier concentration of unlabelled fucose. In this case, the specific activity of the [3H]-fucose had been doubled by decreasing carrier fucose to 0.5 µM. Label was then added, and incorporation allowed to proceed for 72 hours. Plasma membranes were then prepared in the manner described, combining 50 plates for both T3 and control groups. Samples were then precipitated with 10% TCA (w/v), solubilised with NCS (0.1 ml) and counted. Results were expressed as dpm/µg protein in each fraction.

2.6.3 α-Fucosyltransferase activity

The assays employed for α-fucosyltransferase were based upon those described in the literature for different cell types (259-261). After a number of variations, the procedure arrived at was as follows. Astrocytes in MEM containing TH-stripped serum were preincubated with T3 (1 x 10^-8 M) or vehicle control for 48 hours in the presence of 1 µM L-fucose. Plates were then washed in fucose-free medium, and cells harvested into MOPS-buffered saline (MBS: NaCl (0.1 M), morpholinosulphonic acid (5 mM), MgCl₂ (5 mM); pH 6.7). Two plates were combined per tube, then centrifuged (700 g; 10 min), and resuspended in 0.5 ml MBS. Samples were sonicated on ice, and 50 µl removed for protein determination. Enzyme activity was then measured.

A 400 µl aliquot of sample was added to 100 µl of MBS containing Triton X-100 (0.3%, final volume), BSA (4 mg/ml), asialofetuin (4 mg/ml), ATP (2.5 mM), and 0.125 µCi GDP-[¹⁴C]-fucose. The mixture was vortexed and incubated with shaking (37°C; 3 h). The reaction was terminated by placing on ice, and the fucose acceptor separated using a Centricon 10 micro-concentrator. Preliminary experiments determined that a minimum of 6 volumes were needed to completely wash through all unbound substrate. Membranes were then removed and placed in scintillation vials together with 3 ml Ecoscint A for counting.
2.7 Nuclear Binding Studies

2.7.1 Materials

$[^{125}]$-T3 at a specific activity of $>2800$ Ci/g was purchased from Amersham International (Amersham, Bucks). Dowex AG-1x8 anion exchange resin (chloride form, 200 mesh) was purchased from Bio-Rad Ltd (Watford, Herts). Animals used were the progeny of normal female Sprague-Dawley rats bred in our own animal house or, on occasion, supplied by Charles River Ltd (Margate, Kent).

2.7.2 Isolation of Neuronal and Glial Nuclei.

The method employed is a modification of the procedure described previously for adult rat brain (164,262), and extends the exploitation of differing densities of neuronal and glial nuclei to the developmental period. For each preparation, the brains of 3-6 animals were combined. Animals (aged 5, 11, 15 and 21 postnatal days) were killed by decapitation and the brains removed and cleaned of blood and pia matter. All procedures were performed at $4^\circ$C. Whole brains were soaked for 10 minutes in sucrose (0.25 M) containing MgCl$_2$ (10 mM), chopped finely and soaked for a further 10 minutes, then homogenized in the same solution. The homogenate was filtered twice through nylon bolting cloth (110 pm), and once through steel gauze (39 pm), and the filtrate centrifuged at 700 $g$ for 10 min. The crude nuclear pellet obtained was resuspended in 2.0 M sucrose (approx. 5 vol.) and an aliquot (8 ml) layered onto a discontinuous sucrose gradient of 2.2 M sucrose (8 ml) and 2.0 M sucrose (8 ml).

The gradient was centrifuged (104,000 $g$; 30 min), after which four distinct layers were apparent (Figure 9). These were identified as: cell debris (myelin, organelles, etc); neuronal nuclei with contaminating capillary endothelial nuclei; mature neuronal nuclei; and the pellet, which contained predominantly glial nuclei. The criteria on which these judgements were made, and assessments of purity are described in Chapter 5. Microscopic analysis was performed as previously described (263,264). The technical basis for this method of separation has been reviewed (252).
brain soaked, and minced on ice

filtered

homogenised (0.25 M sucrose, 10 mM MgCl$_2$)

centrifuged (700 g; 10 min)

pellet resuspended in 2.0 M sucrose

centrifuged (104,000 g; 30 min)

debri

capillary endothelial nuclei

neuronal nuclei

glial nuclei
2.7.3 Extraction of Nuclear Receptor

Binding was performed in extracts of separated nuclei from 5 and 21 day old animals, for comparison with the intact nuclear binding studies. Extraction was performed as follows: purified nuclear fractions were washed (4°C) in sucrose (0.25 M, containing MgCl₂(10 mM)) and resuspended in extraction buffer containing KCl (0.4 M), MgCl₂ (1 mM), DTT (1 mM) in Tris-HCl buffer (10 mM; pH 7.4). Extraction was performed at 0°C for 45 min, after which the suspension was centrifuged (50,000 g; 30 min). The pellet was saved for determination of DNA and the supernatant used directly in the binding assay. The procedure was based on that previously described (265).

2.7.4 Regional Distribution

Experiments were performed to determine the regional distribution of neuronal and glial T3 binding at two stages of development. The number of differentiated nuclei is small in the cerebellum before day 15, leading to problems with identification and estimation of purity. Consequently it was decided to compare regional binding at day 15 and day 21. Brains from six rats were dissected into three regions: cerebral cortex, cerebellum and the remainder - a broad region including brain stem, midbrain, amygdala, pons, hypothalamus and other subcortical regions, for these purposes termed brain stem. The purified nuclei from two gradients were combined, and T3 binding assays performed in duplicate as described below.

2.7.5 T3 Binding Assay

T3 displacement binding assays were performed, with minor modifications, by a published procedure (264). Washed nuclear fractions were resuspended in binding buffer (0.25 M sucrose, MgCl₂ (3 mM), DTT (1 mM) in Tris-HCl buffer (20 mM; pH 7.4). Replicate aliquots (200 µl) of the suspended nuclei were incubated with tracer [¹²⁵I]-T3 (1 x 10⁻¹⁰ M) and a range of concentrations of unlabelled T3 (1 x 10⁻¹⁰ - 1 x 10⁻⁷ M) for 30 min at 37°C in 0.5 ml Eppendorf tubes. Non-specific
binding, determined in the presence of $1 \times 10^{-7}$ M unlabelled T3 was subtracted in each case. Under these conditions, endogenous T3 dissociation is regarded as complete, and thus no corrections are made for possible endogenous T3 interference in the assay. At the end of the incubation, the reaction was terminated by placing the tubes on ice and adding 200 µl ice cold 2% Triton X-100. After 15 min, nuclei were centrifuged in a Beckman microfuge ($14,000 \times g$; 1 min; 4°C), washed and recentrifuged. The supernatant was aspirated and the tips containing the washed nuclei severed and counted in a gamma-counter to determine the amount of bound $[^{125}I]$-T3. DNA was extracted and measured (with minor modifications) according to the fluorometric method of Setaro and Morely (242).

In experiments using extracted receptors, binding conditions were as above, except that a range of both labelled ($2.5 \times 10^{-11} - 1 \times 10^{-10}$ M) and unlabelled ($2 \times 10^{-10} - 5 \times 10^{-9}$) concentrations of $[^{125}I]$-T3 were included, with non-specific binding for each labelled concentration being determined in parallel tubes containing $1 \times 10^{-7}$ M unlabelled T3. Reactions were terminated by placing on ice and separation of bound from free T3 performed using the AG-1x8 anion exchange resin binding method (255).

2.7.6 Analogue Studies

The relative binding affinities of THNR's for the analogues TRIAC, T4 and rT3 were determined in intact neuronal and glial nuclei prepared from both 5 and 21 day old animals. Briefly, 200 µl aliquots of nuclear suspension were incubated with a fixed concentration of $[^{125}I]$-T3 ($1 \times 10^{-10}$ mol/l), and concentrations of analogue ranging from $1 \times 10^{-10}$ to $1 \times 10^{-7}$ mol/l. The relative binding affinities were expressed as percentage of $[^{125}I]$-T3 bound in the presence of increasing concentrations of analogue.

2.7.7 Released Receptor Assay

In some experiments, the degree of receptor leakage from intact nuclei was determined. After completion of the receptor binding assay, and before the addition of Triton X-100, selected assay tubes were centrifuged, and aliquots of the supernatant removed for separation of bound and free T3 by the resin method (255).
2.7.8 Data Analysis

Binding parameters (Kd and MBC) were determined using transformed experimental data displayed as Eadie-Hofstee plots (related to Scatchard analysis). Linear regression analysis was used to determine the best fit between points. MBC was assessed from the intersect of the regression line with the y-axis, whereas Kd was derived from the slope of the line (B = MBC - Kd.(B/F)). Statistical analysis was performed using unpaired two-tailed t-tests, with significance defined as \( P < 0.05 \).
CHAPTER 3 : EFFECTS OF MATERNAL HYPOTHYROXINEMIA ON THE GROSS BIOCHEMICAL CHARACTERISTICS OF DEVELOPING RAT PROGENY
3.1 Introduction

In the absence of any specific data regarding the putative long-term effects of maternal hypothyroxinemia on fetal development, the investigation began with a general study of the physical characteristics and gross biochemistry of a number of tissues in the neonatal progeny of partially thyroidectomised rat dams. In fetal or neonatal hypothyroidism, it has been demonstrated that tissue weights, and contents of DNA, protein and the activities of a wide range of enzymes are adversely affected (see Chapter 1). It was therefore necessary to examine whether such effects pertain to animals which had been deprived of TH only during the period of gestation up to the onset of fetal thyroid function. This was accomplished with the hypothyroxinemic rat dam model.

In order to assess the spectrum of any resultant dysfunction, a range of organs was studied, but in view of the severe effects of neurological cretinism on the brain, special attention was paid to this tissue. Results were compared with control progeny, raised under identical conditions (see Chapter 2).

3.2 Maternal Thyroidectomy

3.2.1 Maternal and Fetal Thyroid Hormone Levels

Rat dams of breeding age were partially thyroidectomised (parathyroid spared) at least three weeks prior to mating. After this period, serum was taken from a representative sample of dams and assayed for the presence of T3 and T4 by RIA. Dams were then mated with normal males, and the pregnancy allowed to continue to term. After parturition, a number of progeny were sacrificed and bled to obtain serum for assessment of T3 and T4 levels. The results were compared with control animals (Table 4).

It is apparent that whilst rat dams are severely hypothyroxinemic during pregnancy, they are only marginally deficient in T3. This is thought to be due to a switchover from T4 to T3 synthesis on thyroglobulin, aided by peripheral deiodination of the little remaining T4, affording the mother a degree of compensation (266). Although TSH was not measured, the progeny were euthyroid with respect to
both T3 and T4, and supply of elemental iodine from mother to fetus remains more than adequate due to sufficient dietary supply. This suggests that the thyroids of the progeny suffer no permanent damage resulting from maternal hypothyroxinemia. Any differences in gross biochemical differences between the progeny of thyroidectomised dams (TD) and controls must therefore result from the deficiency of maternal thyroid hormone, specifically T4, prior to the onset of fetal thyroid function.

**Table 4**: Serum levels of triiodothyronine (T3) and thyroxine (T4) in thyroidectomised rat dams and their progeny.

<table>
<thead>
<tr>
<th>Concentration (nM)</th>
<th>Control</th>
<th>TD</th>
<th>n</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dam T4</td>
<td>44.7 ± 20.3</td>
<td>13.5 ± 8.7</td>
<td>5</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>T3</td>
<td>1.22 ± 0.36</td>
<td>0.84 ± 0.25</td>
<td>5</td>
<td>NS</td>
</tr>
<tr>
<td>Progeny T4</td>
<td>47.0 ± 12.2</td>
<td>47.3 ± 13.0</td>
<td>6</td>
<td>NS</td>
</tr>
<tr>
<td>T3</td>
<td>1.15 ± 0.32</td>
<td>1.11 ± 0.37</td>
<td>6</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values are mean ± SD; significance was determined by unpaired Student's t-tests.

3.2.2 Size of Litters

There was a significant decrease in average litter size of thyroidectomised dams, as well as a slight increase in the length of gestation when compared with control dams (Table 5). Many of the TD group experienced difficulties during parturition. No parturition-inducing drugs were administered, as a precaution against affecting the progeny or causing maternal stress, and consequently a considerable number of dams
had to be sacrificed at this point in order to avoid unnecessary suffering. The loss was increased by the low rate of successful matings in this group, making necessary the use of progeny from small sized litters. This was, however carefully controlled by the introduction of the system described in Chapter 2. Despite these steps, several experiments had to be curtailed or abandoned as a result of a shortage of TD progeny.

**Table 5**: Effect of maternal hypothyroxinemia on litter size and gestation length.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>TD</th>
<th>n</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Animals / litter</td>
<td>13 ± 3</td>
<td>8 ± 3</td>
<td>11</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Length of gestation (days)</td>
<td>21.5 ± 0.5</td>
<td>22.5 ± 0.5</td>
<td>10</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values are mean ± SD; Significance was determined by unpaired Student's t-tests

### 3.3 Weight

**3.3.1 Whole Body Weight**

The progeny were allowed to develop for various periods of time before being sacrificed. Significant decreases in total body weight of TD progeny were observed at birth and at postnatal day 14. These differences did not persist past day 30, even tending after this stage to be elevated over control values (Figure 10). The control values were found to correspond to the quoted normal growth rates for the Sprague-
Figure 10: Effect of maternal thyroidectomy on the whole body weight of the progeny.

Except where otherwise stated, n ≥ 5; Values are means ± SD

* $P < 0.01$; ** $P > 0.001$
Dawley rat (267).

3.3.2 Organ Weight

The wet weights of a range of organs removed from TD and normal dam progeny various ages were compared. The organs taken demonstrate a range of sensitivity to TH action during development, and included: brain, liver, lung, kidney, and heart, and the spleen, the last thought to be relatively TH unresponsive. The results are shown in Figure 11.

From these figures, it is apparent that almost all the organs studied demonstrate a significant decrease in their average wet weight at some stage during development. Both the heart and the lung reveal consistent and extensive weight deficits as a result of maternal hypothyroxinemia. These are significant in the heart (Fig. 11 e) at days 11 (22% lower than the control value; \( P < 0.001; n=5 \)), 14 (39%; \( P < 0.01; n=5 \)), and 35 (25%; \( P < 0.001; n = 3 \)); and at days 11 (24%; \( P < 0.01; n = 5 \)), 14 (33%; \( P < 0.005; n = 5 \)), and 35 (28%; \( P < 0.005; n = 5 \)) in the lung (Fig. 11 d). The brain, whilst not attaining statistically significant values, shows a trend towards lower weights in the TD progeny (Fig 11 a). The only exception is the kidney, where no difference is observed (Fig 11 c). Liver weights in the developing progeny are less predictable (Fig. 11 b). Although TD offspring exhibit decreases in liver weight at days 14 (21%; \( P < 0.05; n = 7 \)) and 35 (18%; \( P < 0.05; n = 8 \)), weights are actually increased at day 30 (37%; \( P < 0.05; n = 5 \)).

The presence of apparently anomalous results (liver day 30), and isolated statistically significant results (spleen day 14; Fig. 11 f, for example) serve to emphasise the high potential for variation in this type of measurement, where sample size is limited. This factor was of importance in all the measurements taken for the studies of the gross biochemistry of the progeny, and results from the inevitable variability within any biological population. Ideally, it would have been preferable to take a far greater number of progeny for all the experiments performed in order to confirm results already significant, and to obviate the problem of outlying points. Unfortunately this was not possible, mainly as a result of the type of model used, and the strict enforcement of its criteria. The poor reproductive performance of the hypothyroxinemic dams severely limited sample availability, a fact which contributed
Figure 11: Effect of maternal hypothyroxinemia on the wet weight of various organs in the progeny during the neonatal period.

11 a. Brain

11 b. Liver

Except where stated otherwise, values are mean ± SD of at least 5 experiments; significance was calculated by unpaired Student's t-test.
to the change to a more readily controlled system, that of tissue culture. Other possible problems associated with the model will be discussed at a later stage.

3.4 Organ Protein Content

3.4.1 Total Protein

As TH are known to operate at least in part by stimulating protein synthesis, the possibility that maternal hypothyroxinemia may have long lasting effects on the protein content of fetal organs was investigated (Figure 12). The number of recordings taken from spleen were insufficient to construct a developmental profile.

Total levels of proteins in control animals were found to be in excellent agreement with reported data in all the organs studied, lending confidence to the procedures used (267). One minor exception to this was found in the kidney, where the reported early lag in development was not clear.

Levels of protein in the TD progeny were found to be diminished at some stage in all of the organs studied except the kidney. With the exception of the lung at day 35, all reductions were restricted to the early neonatal period. At day 11, statistically significant total protein deficits in the TD progeny were found in the brain (Fig. 12 a: C: 83.8 ± 11.4 mg, TD: 64.4 ± 6.1 mg; \( P < 0.02; n=5 \)), the liver (Fig. 12 b: C: 114 ± 12, TD: 80 ± 13; \( P < 0.01; n=5 \)), the lung (Fig. 12 d: C: 45.8 ± 2.8, TD: 34.1 ± 5.6; \( P < 0.01; n=5 \)), and the heart (Fig. 12 e: C: 31.0 ± 3.1, TD: 11.6 ± 1.4; \( P < 0.001; n=5 \)). During the later stages of development, values frequently appeared to be elevated in the TD progeny. These increases are significant at day 35 in the brain (Fig. 12 a: C: 172 ± 20 mg, TD: 213 ± 21 mg; \( P < 0.05; n=5 \)), and at day 30 in the liver (Fig. 12 b: C: 456 ± 111, TD: 615 ± 24; \( P < 0.05; n=5 \)). In the lung, however, the TD progeny continue to show diminished levels of total protein content, which is significant at day 35 (Fig. 12 d: C: 113 ± 19, TD: 79 ± 3; \( P < 0.05; n=5 \)).

As with weight measurements, considerable individual variation was encountered, particularly in the kidney and heart. Inefficient homogenisation may have been partly responsible for such scatter.
Figure 12: Effect of maternal hypothyroxinemia on the total protein content of various organs in the progeny during the neonatal period.

12a Brain

- Control
- TD

* P < 0.05
(n = 3)

Age (days)

Total Protein (mg)

12b Liver

- Control
- TD

* P < 0.05
** P < 0.01
(n = 3)

Age (days)

Total Protein (mg)

Except where stated otherwise, values are mean ± SD of at least 5 experiments, significance was calculated by unpaired Student's t-test.
12 c Kidney

![Graph showing total protein (mg) vs age (days) for 12 c Kidney with control and TD groups.](image)

12 d Lung

![Graph showing total protein (mg) vs age (days) for 12 d Lung with control and TD groups.](image)
12 e Heart

![Graph showing total protein levels over age (days)]

- **Control**
- **TD**

*P < 0.01 (n = 3)
3.4.2 Protein Concentration

Because of the individual variations in wet weight recordings, it is also useful to examine the protein data in terms of concentration (expressed as mg protein/g wet weight), calculated on an individual basis for each organ assessed. This data from the brain, liver and kidney, and lung provide a more representative picture of the rate of protein acquisition (Figure 13 a-d). The brain profile reflects that found for total protein in terms of an apparent early lag, significant at day 11 (31%; $P < 0.05$), followed by an over-compensation in the later stages of development, indicating a proportionally greater deficit in the early neonatal period in TD progeny. The liver also appears to demonstrate an initial lag in the acquisition of protein, although the significant elevation noticed in total levels is abolished when expressed as a concentration. Whilst noting these changes, the general picture is not one of large-scale deficits in protein accumulation. No significant change in protein concentration was apparent in the kidney at any of the developmental stages studied.

3.5 Organ DNA Content

3.5.1 Total DNA

Although a degree of polyploidy in some brain cell types (e.g. Purkinje cells) has been reported (268), later isolation of purified cells appeared to rule out this possibility (269). The measurement of total DNA in the organs of the progeny of TD dams therefore provides a broad indication of cell number.

Total organ DNA in controls accurately matched quoted values for developing rat organs (267), although again, a neonatal lag in growth in the kidney was not clearly demonstrated. Sample loss prevented the construction of DNA acquisition profiles for the heart and spleen.

The values for total organ DNA content generally show little or no effect of maternal hypothyroxinemia on cell number in the neonatal rat. Where exceptions to this occur, they appear to be against the general picture of the developmental profile.
Figure 13: Protein concentrations in the organs of TD and control progeny.

Values are mean ± SD; significance determined by unpaired Student's t-tests.
* P > 0.05
Figure 14: Effect of maternal hypothyroxinemia on the total DNA content of various organs in the progeny during the neonatal period.

14 a Brain

![Chart showing DNA content in brain samples over time (ages 5, 11, 14, 21, 30, 35 days).]

14 b Liver

![Chart showing DNA content in liver samples over time (ages 5, 11, 14, 21, 30, 35 days).]

Except where stated otherwise, values are mean ± SD of at least 5 experiments; significance was calculated by unpaired Student's t-test.
Total DNA (mg)

Age (days)

10
5
11
14
21
30
35

(n = 3)

* P < 0.05

TD

Control

14 c Kidney
This is apparent in brain at day 30 (Fig. 14 a: C: 2.086 ± 0.462 mg, TD: 0.992 ± 0.317 mg; P < 0.01; n = 5), and at day 14 in the liver (Fig. 14 b: C: 4.23 ± 0.26 mg, TD: 2.63 ± 0.49 mg; P < 0.05; n = 4), and kidney (Fig. 14 c: C: 3.89 ± 0.57 mg, TD: 2.73 ± 0.32 mg; P < 0.05; n = 4).

Values calculated for the lung were found to be considerably diminished, suggesting a decrease in cell number in TD progeny persisting into the late neonatal period. This may be the result of the limited sample available for this organ, and it is therefore difficult to assess the importance of these results. Data for the lung is represented in the DNA concentration developmental profile for the lung in Figure 15.

3.5.2 DNA Concentration

The decrease in wet weight recorded in the brain and liver of the TD progeny during the early neonatal period is unlikely to be the result of decreased cell number. DNA concentration in the brains of TD progeny is increased by 38% at day 5 (P < 0.05) and by 69% (P < 0.01) at day 11 over control progeny, whilst total DNA levels remain unaffected (Figure 15 a). This indicates that whilst organ weight is diminished in the TD progeny, the DNA content remains broadly unaffected. The trends are repeated in the liver, although differences are not significant (Figure 15 b). No differences were apparent in the kidney (Figure 15 c), whilst the DNA concentration in the lung suggests a severe deficit in cell density during development as a result of maternal hypothyroxinemia (Figure 15 d).

3.5.3 Protein:DNA Ratio

The ratio of protein to DNA provides an estimate of cell size, as wet weight can be influenced by variations in organ water content. Mean values of for each category were therefore combined in a ratio to provide an indication of the development of cell size in the four main organs studied (Figure 16).

In the brain, there is an initial decrease in protein/DNA in the TD progeny compared to the controls, which is reversed over the subsequent days of the neonatal period. Consequently, TD cell size apparently increases after early deficits, whilst that of the controls remains relatively stable (Figure 16 a).
Figure 15: DNA concentrations in the organs of TD and control progeny.

Values are mean ± SD; significance determined by unpaired Student's t-tests.

* P > 0.05
** P > 0.01
Figure 16: The effect of maternal thyroidectomy on the protein/DNA ratios of the organs of progeny.

a) Brain and Liver.

- Brain (C)
- Brain (TD)
- Liver (C)
- Liver (TD)

Protein/DNA vs. Age (days)

a) Kidney and Lung.

- Kidney (C)
- Kidney (TD)
- Lung (C)
- Lung (TD)

Protein/DNA vs. Age (days)
This pattern is reflected to a lesser extent in the liver and kidney. The lung of the TD progeny apparently exhibits a far greater cell size throughout development (Figure 16b), reflecting the data obtained from the limited total DNA sample in this organ.

3.6 Cellular Protein Distribution

3.6.1 Cytosolic and membrane proteins

Data on the gross biochemistry of the offspring of rat dams hypothyroxinemic during early pregnancy suggested that abnormalities occurred in a range of organs including the brain. Although certain trends were noticeable, suggesting more severe deficits in the early neonatal period, differences were often marginal, and it was concluded that a more specific effect on particular proteins may be involved. As defects in the brain are likely to be directly responsible for many of the symptoms of neurological cretinism found in clinical studies, it was decided to concentrate on this organ in a closer examination of the biochemical parameters which may influence the nature of the disorder.

Membrane proteins play important roles in many developmental processes, and include a range of protein subtypes, including enzymes and cell surface markers (270). Deficits in specific proteins within these groups could help to explain the various neurological defects associated with neurological cretinism. It was therefore decided to examine the distribution of proteins between membrane-associated and cytosolic fractions.

Whole brain homogenates were separated into subcellular fractions by ultracentrifugation (see Chapter 2), and the amounts of total protein in TD and control progeny determined in the cytosol and total membrane fractions. The organellar fraction, incorporating the nucleus, mitochondria and lysosomes, and comprising around 80% of the total protein was not used in these experiments.

In the cytosol, the levels of total protein were diminished (significantly at day 35; \( P < 0.05 \)) in the TD progeny at all stages studied. Protein levels were also reduced in the membrane fraction, where an age-related increase in control values appears to be diminished in the TD progeny. (Table 6). In both the membrane and cytosolic fractions, reduced levels of total proteins found in the early neonatal period
persist until day 35, demonstrating that these subcellular regions are more severely affected than the brain as a whole, where compensation of total protein levels took place (Fig. 11 a).

Table 6: Levels of total protein in cytosol and membrane fractions separated from whole brain in TD and control progeny.

<table>
<thead>
<tr>
<th></th>
<th>Total protein (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cytosol</td>
</tr>
<tr>
<td>Day 14</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>21.5 ± 4.0 (5)</td>
</tr>
<tr>
<td>TD</td>
<td>17.3 ± 4.3 (4)</td>
</tr>
<tr>
<td>Day 30</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>25.2 ± 4.3 (4)</td>
</tr>
<tr>
<td>TD</td>
<td>18.8 ± 5.6 (4)</td>
</tr>
<tr>
<td>Day 35</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>23.2 ± 2.8 (3)</td>
</tr>
<tr>
<td>TD</td>
<td>16.4 ± 1.4 (3)*</td>
</tr>
</tbody>
</table>

Values are mean ± SD; significance was determined by unpaired Student's t-tests, and n is given in parentheses.

* P < 0.05.

This therefore provides evidence that the overall distribution of cellular proteins may be affected in the brains of TD progeny, with cytosolic and membrane fractions apparently suffering more severely than the brain as a whole. It is possible that particular subtypes of protein may be more susceptible to early gestational TH
deprivation, as TH are known to regulate the expression of specific proteins in both brain and liver cells (see Chapter 1). However, it is likely that the changes in the levels of such proteins are masked in measurements of total protein, and it remains doubtful whether TH stimulation of specific protein synthesis would be sufficient to account for the large differences observed in this study. It is therefore possible that TH acts as a general trophic growth factor during early development, either independently, synergistically with other growth factors, or through the stimulation of growth factor production.

Reductions in the levels of either integral membrane proteins, or cytosolic proteins, resulting from reduced influence (either direct or indirect) of TH, may disrupt a range of developmental events, including: cell adhesion, the establishment of cell-cell interactions during development, intracellular transport, cell communication, and enzyme function. It is conceivable that failure in any of these systems, particularly those affecting the establishment of appropriate neural connections, may be a cause of the symptoms associated with neurological cretinism.

Many of the proteins involved in cell surface interactions are glycoproteins. This class of protein also forms a considerable number of important intracellular enzymes, essential in developing cells. Consequently it was decided to investigate the intracellular distribution of glycoproteins in brain homogenates from TD and control progeny, in order to determine whether maternal hypothyroxinemia may lead to long term disturbances in the levels of these particular proteins in the developing brain.

Subcellular fractions were separated by ultracentrifugation into cytosolic and membrane fractions, as these are the chief locations of interest of the glycoproteins to be studied. Distribution within the organellar fraction was not examined. Samples prepared from 14 and 30 day old brain from both TD and control progeny were dialysed against a buffer appropriate for lectin column chromatography, freeze-dried, and made up at similar protein concentrations. The lectin chosen was Concanavalin A, as its specificity for the glucose-mannose residues renders it selective for the largest group of glycoproteins. Samples were applied to the column, run and collected according to the procedure described in Chapter 2. Results, expressed as the ratio of unbound to bound protein, were only used where recovery of total protein was in excess of 65%.
Figure 17: Elution profiles of proteins extracted from 14 day old rat brain and separated by Con A chromatography.

a) Cytosolic Fraction

b) Membrane Fractions
The resultant elution profiles for a typical experiment at day 14 (Figure 17) clearly show that the proportion of glucose-mannose glycoproteins found in the brain cytosol was greater in the TD progeny than in the controls (Figure 17a), whereas the picture is reversed in the membrane fraction (Figure 17b). A much decreased level of glucose-mannose glycoprotein in the membrane fraction was common to both experiments from day 14. On average, however, the increase in cytosolic values was only marginal, and not significant (Table 7).

At day 30, the proportional increase in glycoproteins in the cytosol of TD progeny was large and significant ($P < 0.01$), albeit from a small number of experiments (Table 7). At this age no difference in membrane associated glycoproteins was discernible between the two groups.

**Table 7 : Separation of brain intracellular proteins by Con A chromatography: Ratio of bound/unbound proteins in TD progeny and controls at two stages of development.**

<table>
<thead>
<tr>
<th>Bound / Unbound Ratio</th>
<th>Cytosol</th>
<th>Membrane</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>0.165 ± 0.070 (3)</td>
<td>0.394 ± 0.224 (2)</td>
</tr>
<tr>
<td>TD</td>
<td>0.228 ± 0.176 (3)</td>
<td>0.111 ± 0.025 (2)</td>
</tr>
<tr>
<td>Day 30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>0.064 ± 0.016 (3)</td>
<td>0.187 ± 0.077 (3)</td>
</tr>
<tr>
<td>TD</td>
<td>0.309 ± 0.035 (3) *</td>
<td>0.223 ± 0.083 (2)</td>
</tr>
</tbody>
</table>

Values are mean ± SD, and n is given in parentheses. ($n=2 - \pm$ difference from mean)

* $P < 0.01$
3.6.2 PAGE analysis of Con A separated glycoproteins from neonatal rat brain

As a result of the possible deficit in incorporation of glycoproteins into membranes in 14 day old TD progeny, a closer analysis of the composition of the glycoproteins involved was undertaken to establish whether the effect was generalised, or confined to certain proteins. Samples separated by Con A chromatography were dialysed and concentrated either by freeze-drying or employing Centricon concentrators (see Chapter 2). Where practical, samples were then made up to 2 mg/ml and 20 μg of protein was loaded into each of the slots on an SDS polyacrylamide gel. If less protein was available, a matched control was employed. The gel was run under suitable conditions (Chapter 2) to establish separation of proteins on the basis of molecular weight.

Molecular weight was determined using a plot of relative mobility versus log_{10} of the molecular weight of known standards (Chapter 2).

The gels revealed that the TD progeny exhibited broadly similar patterns of proteins to the controls in both the cytosol and in the membrane fractions. In the membranes at day 14, both groups showed bands with approximate molecular weights of 60, 45, 33, and 23 kDa. A band corresponding to 16 kDa was only detected in the control group, whereas bands at 200 and 81 kDa were apparently unique to the TD progeny. At day 30 the picture was similar, except that a band at 125 kDa was noticeable in both groups. In addition to the 16 kDa band, the band at 60 kDa was now also absent from the TD progeny. The TD progeny continued to show a protein at 81 kDa not present in the controls, whilst the 200 kDa protein was no longer detected. As is to be expected, these values show a large overlap. All proteins detected have counterparts in the study by Rudge and Murphy of synaptosomal membrane proteins isolated from rat cerebral cortex during development (271), with the exception of the 16 kDa fraction. Interestingly, they found that the 80 kDa protein decreased developmentally, possibly indicating a defect in the timing of this event in the TD progeny.

The glycoprotein fraction from the cytosol also displayed similarities between TD and control samples. At day 14, common bands appeared at molecular weights of approximately 54, 33, 22, 15 and 11.5 kDa. Bands present at 78 and 13.5 kDa in the controls were absent from the TD progeny, which itself exhibited a unique band at around 125 kDa.
By day 30, both groups revealed a protein at 125 kDa, whilst the 11.5 kDa band was now absent from the controls. A 15 kDa glycoprotein in the TD progeny is possibly identifiable with the 13.5 kDa band in the control. Both groups contain a band situated at 78 kDa, in addition to bands at 60 and 40 kDa, not present in either group at day 14.

In general, the results seem to indicate excesses of higher molecular weight species in the TD progeny in the membranes at day 14, and the cytosol at both days. Both groups demonstrate unique glycoprotein species which may either persist over the period studied, as with the 80 kDa protein from the TD progeny membranes, or else exhibit transient differences, as do the 60 kDa membrane species and the 125 and 78 kDa cytosolic species. The existence of temporally expressed glycoproteins which are not affected by maternal hypothyroxinemia is demonstrated by the 40 and 60 kDa species found in both groups, but only at day 30.

Judging by the molecular weights of the membrane glycoproteins, and their binding to Con A, it has been suggested that the 23 kDa protein may correspond to Thy-1, a protein found on a number of different cell surfaces, including astrocytes and neurons (271). The availability of specific antibodies to these proteins would prove useful in enabling their identification.

### 3.6.3 Densitometry

As equal amounts of total protein were applied to the gels for matched TD and control samples, it was possible to attempt to compare the quantities of different proteins separated by PAGE. Gels were sliced into lanes and scanned in a Pye-Unicam densitometer. Comparisons were made by taking the peak reading at 540 nm for each sample as the indication of the abundance of the protein (or proteins).

In the membrane fractions, the relative concentrations of all the glycoproteins common to both groups were diminished in the TD progeny at day 14 (Figure 19a). The 60 and 45 kDa protein fractions in the TD progeny are only 12% and 60% of control values respectively. At day 30, no difference was apparent in the 33 kDa species, and levels of the 23 and 125 kDa glycoproteins were only slightly higher in controls. The species of molecular weight 45 kDa was, however, still severely diminished relative to controls (33%), whilst the 60 kDa species was not detected at
Figure 19: Relative abundance of different protein bands in fractions separated from the brains of TD and control progeny

a) Membranes

Units are proportional to peak absorbance of protein bands at 540 nm.
all in the TD progeny at this stage.

The correct temporal expression of different membrane glycoproteins is known to be of importance in the regulation of a number of developmental processes. With a few exceptions, the pattern of developmental alterations in the controls broadly reflects that reported in synaptic membranes, where protein weights coincide (271). The TD progeny show most alterations from control levels at day 14. As the general picture at day 30 indicates a less disrupted system, a degree of compensation appears to occur, but this may come too late to prevent long term neural dysfunction.

Although the initial experiments with Con A separated proteins from membranes and cytosol suggested the possibility of a failure in incorporation of manufactured glycoproteins into membranes, there is little evidence from the densitometric scans of SDS-PAGE separated samples to indicate a surfeit of any glycoprotein in the cytosol of the TD progeny which corresponds with a deficit of a similar sized membrane glycoprotein (Figure 19 b). Of the cytosolic glycoproteins common to both groups, only the 125 kDa species shows any great difference, with relative levels in the TD progeny being elevated by 100%. This was associated with a deficit of 40% in the corresponding membrane fraction. This suggests a failure of synthesis of proteins destined for the membrane, rather than failure of incorporation. As at day 14, most isolated glycoproteins are affected, it is possible that, as previously suggested, a secondary mechanism is involved, which may include a reduction in membrane synthesis itself, or perhaps the diminished activity of glycosylating enzymes. Some degree of specificity is, however present, as not all proteins are affected to the same degree.

3.7. Lysosomal Enzymes

Another method by which TH could specifically influence brain development is by affecting levels of, or the activities of, important enzyme systems. It is known that TH play an important role in the appropriate expression of a variety of enzymes during development (117). Much work has concentrated on the effect of TH on the activities of anabolic enzymes, and rather less attention has been paid to the involvement of catabolic enzymes, which also have a critical bearing on the structure and function of the brain. The developing brain is a dynamic entity, and cell
populations and their interconnections vary constantly during the growth of the organ. The final structure and its organisation is therefore dependent on the rise and fall of such systems in a correctly co-ordinated manner, a process involving both catabolic and anabolic actions.

The effects of maternal hypothyroxinemia during gestation on lysosomal enzyme activity were therefore examined in the brains of rat progeny at the ages of 14 and 53 days (Table 8). Results indicated that β-D-glucosidase and aryl sulphatase activities were both significantly compromised during the neonatal period in the TD progeny, by 23% and 20% respectively, whilst the other hydrolytic enzymes measured were unaffected. It is suggested that this may impinge on the efficiency of a number of metabolic systems, including those involved in myelin metabolism.

These studies demonstrate specific differences between the two populations of progeny at 14 days of age which result from the absence of maternal thyroid hormone during pregnancy. Although the sample size was small at day 53, it would appear that these differences have been corrected by this stage, pointing to both a quantitative and a qualitative response to maternal hormone in these enzyme systems.
Table 8: The effect of maternal thyroidectomy on the activities of lysosomal enzymes in the brains of:

a) 14 day old progeny

<table>
<thead>
<tr>
<th>Specific activity</th>
<th>Control</th>
<th>TD</th>
<th>n</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(nmol 4-MU/mg protein/h)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acid phosphatase</td>
<td>282 ± 30</td>
<td>317 ± 38</td>
<td>5</td>
<td>NS</td>
</tr>
<tr>
<td>β-D-Glucopyranosidase</td>
<td>15.5 ± 1.9</td>
<td>12.6 ± 1.7</td>
<td>5</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>β-D-Galactosidase</td>
<td>84.5 ± 3.2</td>
<td>80.8 ± 6.0</td>
<td>5</td>
<td>NS</td>
</tr>
<tr>
<td>Aryl sulphatase A</td>
<td>0.79 ± 0.04</td>
<td>0.66 ± 0.10</td>
<td>5</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>β-N-Acetylglucosaminidase</td>
<td>622 ± 47</td>
<td>622 ± 61</td>
<td>5</td>
<td>NS</td>
</tr>
</tbody>
</table>

b) 53 day old progeny

<table>
<thead>
<tr>
<th>Specific activity</th>
<th>Control</th>
<th>TD</th>
<th>n</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(nmol 4-MU/mg protein/h)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acid phosphatase</td>
<td>283 ± 20</td>
<td>261 ± 26</td>
<td>3</td>
<td>NS</td>
</tr>
<tr>
<td>β-D-Glucopyranosidase</td>
<td>5.02 ± 0.61</td>
<td>4.99 ± 0.14</td>
<td>3</td>
<td>NS</td>
</tr>
<tr>
<td>β-D-Galactosidase</td>
<td>41.8 ± 0.9</td>
<td>43.6 ± 4.2</td>
<td>3</td>
<td>NS</td>
</tr>
<tr>
<td>Aryl sulphatase A</td>
<td>0.75 ± 0.05</td>
<td>0.71 ± 0.01</td>
<td>3</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values are mean ± SD; all assays were performed in triplicate with appropriate controls, and significance was determined by unpaired Student’s t-tests.
Studies of the gross biochemistry of the progeny of thyroidectomised rat dams and controls revealed a number of interesting differences, including deficits in protein both relative to brain weight and in terms of the total amount present. Cytosolic and membrane fractions appeared to be more severely affected, and the nature and localisation of Con A binding glycoproteins from TD and control rat brains during the neonatal period was found to be altered. Although this is likely to be a secondary effect of TH, cell surface glycoproteins are themselves instrumental in bringing about the final structure of the brain, and they have been implicated in myelination, cell-cell adhesion and synaptogenesis (237). The pattern of expression of surface glycoproteins is known to vary during development, and it has been suggested that the correct temporal appearance of these molecules is of crucial importance in the establishment of neural circuitry (272). Failure of the suitable operation of these developmental processes could lead to long term neural dysfunction. It was therefore decided to investigate the possibility that glycoprotein synthesis may be under a degree of direct TH control.
CHAPTER 4: THE INFLUENCE OF THYROID HORMONES ON GLYCOPROTEIN SYNTHESIS IN DEVELOPING RAT BRAIN CELLS \textit{IN VITRO}
4.1 Introduction

Techniques for the successful primary culture of neural cells have been improved over recent years, and it is now possible to grow neurons and glia in chemically defined media where verifications of their purity and viability can be made. Primary culture is also considered to be more representative of cells in the developing brain than transformed cell lines (glioma or neuroblastoma). The elimination of maternal and extraneous influences permitted by this system allows the identification of the primary effects of TH. The intention was to identify specific biochemical differences capable of contributing to the known neurological deficits in both animal models and clinical observations.

Neurons respond similarly to TH in vivo and in vitro. This is illustrated by the fact that cultures derived from the cerebellum share the diminished responses to TH demonstrated in vivo(273). TH have been demonstrated to influence a wide range of parameters in neuronal culture, including the temporal expression of cell surface markers, such as N-CAM (thought to indicate the stimulation of synaptogenesis) (274); the regulation of Na+/K+-ATPase production (234); MAP1 synthesis (with coincident neurite formation)(149); and the transport of amino acids (98,232) and glucose (275,276). Transport regulation by TH has been reported to act both directly on the membrane transporters (90), and via a nuclear mediated effect, as upregulation of the mRNA for the glucose transporter protein found predominantly in the brain, BGT3, has been demonstrated in response to T3 in ARL 15 cells (277). This type of regulation may be cell-type specific, as has been shown for other stimulating factors (278).

Although it was initially thought that TH action on glia was secondary to actions on neurons, there is now much evidence to indicate direct glial sensitivity in culture. All three deiodinases (D-I, D-II, and D-III) have been shown to be operative in mixed cell cultures, with D-II activity being up-regulated in response to T3 deprivation (230). This activity was initially assigned a neuronal location (230), but later work showed that glial cells too, possessed both 5-, and 5'-deiodinating activities, and furthermore, that these varied throughout development (279,280)).

Astrocytes were selected for study because of their involvement in a wide range of processes critical to neural development, including: neuronal migration and
survival; neurite formation and outgrowth; and the control of oligodendroglial proliferation and trophic growth (235). The presence of TH in astrocytic cultures has a stimulatory effect on a number of processes including: transport of 2-deoxyglucose and amino acids (281); the intracellular calcium flux, possibly due to translocation of a transporter, rather than by a nuclear mediated mechanism (282); and polyamine synthesis (283). The TH have also been shown to act in co-operation, both synergistically and additively with other modulators of neural cell development. Thyroid hormone acceleration of process formation in cultured astrocytes is potentiated by insulin, as is the accumulation of GFAP, and glutamine synthetase activity increases in an additive manner when exposed to both T3 and hydrocortisone (235). The TH have also been reported to stimulate tubulin synthesis in oligodendroglial culture (284) and to regulate post-translational methylation of oligodendroglial myelin basic protein (285).

Although Con A, used to separate glycoproteins from neonatal brain homogenates, specifically binds glucose or mannose residues from the oligosaccharide chains of the glycoprotein, the extensive metabolism of these sugars prevented direct study of such glycoproteins. Fucose (6-deoxygalactose) is not readily broken down by cells, and therefore has been widely employed in the investigation of glycoprotein synthesis (286). Consequently it was decided to follow the influence of TH on the entry of [3H]-fucose into cultured astrocytes, and its subsequent incorporation into glycoproteins.

The work followed on from a study in the department of the effects of TH on protein synthesis in neuronal culture (232). An optimal concentration of 10 nM T3 was found to stimulate leucine uptake and subsequent incorporation into protein. The effect on uptake was abolished by inhibitors of protein synthesis, indicating a secondary, though very rapid (<1 hour) response. Incorporation was stimulated to a similar extent in both soluble (cytosolic; 127% over controls) and insoluble (membrane associated; 116%) fractions, suggesting a blanket effect, possibly as the result of increased protein synthesis.
4.2 Cell Culture

4.2.1 Astrocytes

Cells were isolated from 1-2 day old normal rat brain and cultured as described in Chapter 2. Growth was allowed to continue without interference, with medium changed every 2-4 days, resulting in confluent cells bearing characteristic cytological features associated with astrocytes. The majority of cells were of the polygonal type, described as epithelioid, with a number of processes (287), whilst a small proportion were in the stellate form. Depending on the initial seeding density and subsequent cell survival, confluence was normally reached between 10 and 15 days after the culture was begun. The cells are pictured after 1, 5 and 12 days in culture (Figure 20). Although the growth medium employed is relatively specific for astrocytes, a small degree of contamination from other neural cell types (oligodendroglia, neurons, and fibroblasts) was always present. Each culture was assessed under phase contrast microscopy, using cytological criteria (287), to determine the extent of such contamination. In most cases, cultures were relatively pure (>95%), with contaminating fibroblasts and oligodendroglia rapidly dying after 4-6 days in culture. If the proportion of astrocytes fell below approximately 90%, the culture was discarded.

4.2.2 Neurons

Neuronal cultures were grown in a medium most suited to neuronal growth, although contamination from other cell types was slightly more extensive than in the astrocytic cultures. This consisted predominantly of fibrocytes and astrocytes (judged by cytological characteristics), composing <10% of total cell number, with levels diminishing towards confluence. Neuronal cultures usually achieved confluence at around 16 days.

4.2.3 Immunocytochemistry

In order to confirm the extent of purity of the astrocytic cultures, some cells
Figure 20a: Astrocytes in culture - 1 day

Astrocytes under phase contrast (x400)
Figure 20b: Astrocytes in culture - 5 days

Astrocytes under phase contrast (x400)
Figure 20c: Astrocytes in culture - 12 days

Astrocytes under phase contrast (x400)
Figure 21: Neurons in culture - 12 days

Neurones under phase contrast (×1200)
were grown on cover slips under identical conditions (see Chapter 2) and stained using an immunoperoxidase-linked antibody raised against rat GFAP, an astrocyte specific cell marker. Results showed that 90-98% of viable cells present stained positively in these tests, indicating relatively pure astrocytic cultures. Comparable procedures were not used for neuronal cultures, where the presence of clear axons was considered a sufficient marker for cell type (Fig. 21).

The nature of these cultures has been previously verified (232).

4.2.4 Cell Viability

Cells were used mostly during the developing period, and consequently it was normally sufficient to assess viability and suitability for study by their density in culture. This was performed by eye, rather than quantitatively, as growth tended to proceed from defined foci rather than in a diffuse manner. Occasionally, viability was checked by the trypan blue exclusion method, which in all cases confirmed very high levels of viability in astrocytic cells ( > 95%), whilst contaminating oligodendroglia were mostly non-viable.

4.3 Effect of TH on the incorporation of [3H]-fucose into astrocytic glycoproteins in vitro.

4.3.1 Time Course of [3H]-fucose incorporation

Preliminary experiments had revealed that if astrocytes were cultured for two or more days whilst deprived of fetal calf serum (FCS), the cultures degenerated rapidly. Consequently, astrocytes were cultured in medium containing FCS stripped of TH and supplemented with either 10 nM T3 or its vehicle as the control (see Chapter 2 for experimental details).

Addition of 10 nM T3 stimulated fucose incorporation into protein fractions by 32% after 48 hours (+T3: 96 ± 24 dpm/μg protein; -T3: 72 ± 26; P < 0.01; n = 14) and 39% after 96 hours (+T3: 209 ± 70; -T3: 150 ± 37; P < 0.05; n = 19) (Figure 22). Incorporation was linear until the final time point (120 hrs).

As results were expressed as dpm incorporated / μg total protein, a blanket increase in protein synthesis as a result of T3 administration can be ruled out, thereby
Figure 22: Effect of 10 nM T3 on the incorporation of $[^3\text{H}]$-fucose into glycoproteins in astrocytes.

Values mean ± SEM; $n$ = range from 6-19.

* $P < 0.02$

** $P < 0.01$
implying a specific effect on glycoproteins. Stimulation of incorporation into glycoproteins was not apparent over shorter time periods, such as up to 24 hours, suggesting a long term, nuclear mediated effect, rather than a direct effect on fucose incorporation. Conceivably, a secondary effect may be operating, for example affecting the levels of synthetic or degradatory enzymes, with a consequent time lag before a difference is noticed in glycoprotein levels. A future study employing inhibitors of protein synthesis would be necessary to clarify this issue, although difficulties may be encountered in culturing cells in the presence of inhibitors for sufficient time to notice a significant effect.

Uptake of $[3^H]$-fucose into the TCA soluble fraction was not affected by the presence of 10 nM. After 48 hours, the recovery of label in this fraction was $14 \pm 4$ dpm/μg protein in the T3 administered samples, compared to $13 \pm 2$ dpm/μg in the controls. This indicates a lack of any direct effect of TH on the transport of free fucose into the cells.

Checks on the recovery of administered fucose, by the measurement of total label taken up by the cells and free label remaining in the medium (pool), typically indicated recoveries of 85 - 110%, always in good agreement with the sums of the soluble and insoluble fractions (data not shown).

4.3.2 Dose Response

The effect of different concentrations of T3 was investigated after 48 and 96 hours. Control experiments were performed with appropriate vehicle concentrations. Variation between control values from all concentrations was small in the 48 hour experiment, and they were therefore regarded together. In the 96 hour experiment, a significant increase in incorporation of $[3^H]$-fucose was only observed at a concentration of 10 nM ($P < 0.05$) (Figure 23). Due to small sample numbers ($\leq 4$), the values for 1 and 100 pM are considered unreliable, a fact borne out by their high degree of variation (shown as SEM).

In the second series of experiments, the effect of a more narrow spread of T3 concentrations was studied after 48 hours, centred on the most active concentration (10 nM).
Figure 23 : Effect of T3 concentration on the incorporation of $[^3H]$-fucose into glycoproteins in astrocytic culture.

(Wide concentration range; incubation period, 96 h)

Values are mean ± SEM; significance was determined by unpaired Student's t-test.

* $P < 0.05$
Figure 24: Effect of T3 concentration on the incorporation of [\(^{3}H\)]-fucose into glycoproteins in astrocytic culture.

(Narrow concentration range; incubation period, 48 h)

Values are mean ± SD; n ≥ 8; significance was determined by unpaired Student's t-tests.

* P > 0.05
** P > 0.01
This resulted in the clear identification of a narrow range of T3 concentrations capable of stimulating fucose incorporation into astrocytic glycoproteins in vitro (Figure 24). Incorporation was significantly elevated compared to controls at three of the concentrations studied, to 125 ± 14 dpm/μg protein at 10 nM T3 (n = 18; P < 0.01), 119 ± 9 at 15 nM T3 (n = 8; P < 0.01) and 119 ± 15 at 5 nM T3 (n=12; P < 0.05), compared to 108 ± 6 dpm/μg protein in controls. Controls for individual T3 concentrations showed no significant differences. At 25 nM T3 a slight, but no longer statistically significant effect was observed.

### 4.3.3 Analogue Studies

In order to discover whether the stimulation of this system by thyroid hormones agrees with accepted criteria regarding their biological activity, the effects of a number of analogues of the thyroid hormones on fucose incorporation were investigated. For accurate comparison of the magnitude of biological effect exerted by each analogue, all experiments were performed using 10 nM T3 as a positive control.

Neither rT3 nor TRIAC at a concentration of 10 nM significantly affected the incorporation of fucose into glycoproteins in culture, whilst T4 caused a rise on a similar scale to that for T3 (Figure 25). The stimulatory effect of T3 was, however, not as great in these experiments as in the time course, possibly as the result of variability within the cultures.

### 4.3.4 T4 Dose Response

Given that T3 is generally regarded as approximately ten times as potent as T4, it was slightly surprising that T4 at the same concentration caused a similar increase in fucose incorporation into astrocytic glycoproteins. Astrocytes and oligodendrocytes in culture primarily convert T4 to T3, 70% of which remains within the cells concerned, some becoming bound to TH nuclear binding proteins detected in culture. On the other hand, rT3 is reported to be a relatively minor product, and is almost all released into the medium (280). This would suggest that the activity is likely to be secondary to conversion to T3, although a direct effect of T4 cannot be eliminated. To further examine this effect, astrocytes were incubated with varying concentrations
Figure 25: Effect of thyroid hormone analogues on the incorporation of \([^{3}H]\)-fucose into glycoproteins in astrocytes in culture.

Values are mean ± SD; n ≥ 11; significance was determined by unpaired Student's t-tests.
* P < 0.05
** P < 0.01
Figure 2b: Effect of T4 concentration on the incorporation of [3H]-fucose into glycoproteins in astrocytes in culture.

Values are mean ± SD; n ≥ 6; significance was determined by unpaired Student's t-test.
* P < 0.05
** P < 0.01
of T4 in order to determine the optimum concentration for the stimulation of fucose incorporation. T3 (10 nM) and vehicle were included as positive and negative controls.

Results demonstrated a dose response profile, but over a wider range of concentrations than was evident for T3 (Figure 26). The optimum T4 concentration for the stimulation of fucose incorporation was 25 nM, where a 12.5% increase was apparent (n = 6; P < 0.01). This compared with a slightly smaller increases of 11.1% at 10 nM (n = 18; P < 0.05) and 11.6% at 100 nM (n = 7; P < 0.02). No increase was apparent at a T4 concentration of 5 nM. The 10 nM T3 produced an increase in incorporation of 20% in these experiments.

4.4 Hormone Availability in vitro

Relatively high levels of hormones are required to produce effects in these experiments, as well as in the majority of similar work in the literature. T3 levels of approximately 10 nM are needed, compared to a normal range of fT3 in human serum of 4-9 pM. Total T3 concentrations, however, are in the region of 1-3 nM. Values are approximately the same in the rat.

As FCS naturally contains proteins capable of binding TH, it was a possibility that much of the hormone was in the bound form, as is found in vivo, where free hormone levels normally govern availability. For this reason, the levels of free and bound T3 were measured in the medium used for astrocytic cultures. This experiment was also intended to monitor any changes in the concentration of total hormone during a period of 48 hours.

Results of the RIAs for T3 indicated that much of the hormone present was indeed in a bound form (Table 9). In medium taken from zero time plates, 99.87% of T3 at a concentration of 10 nM was found to be bound. This implies that the free T3 concentration approximates physiological levels found in serum. As the dynamics of hormone dissociation were not studied, the extent to which bound hormone augments supplies of free hormone could not be discovered. Certainly the levels of total hormone did not decrease significantly within 48 hours.

Analogues of T3 were included in the assay to assess cross-reactivity with the antibody. As expected, TRIAC showed approximately 80% cross-reactivity in the
total T3 assay, whereas T4 and rT3 did not cross-react at all. No T3 was detected in the stripped serum.

Table 9: Distribution of T3 added to culture medium between total and free portions as determined by RIAs for T3.

<table>
<thead>
<tr>
<th></th>
<th>Total T3 (nM)</th>
<th>Free T3 (pM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 hrs</td>
<td>48 hrs</td>
</tr>
<tr>
<td>TH-Stripped FCS</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>rT3</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>TRIAC</td>
<td>7.36</td>
<td>8.54</td>
</tr>
<tr>
<td>T4</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>T3 (1 nM)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>T3 (5 nM)</td>
<td>3.24</td>
<td>5.52</td>
</tr>
<tr>
<td>T3 (10 nM)</td>
<td>10.36</td>
<td>9.54</td>
</tr>
<tr>
<td>T3 (25 nM)</td>
<td>26.41</td>
<td>22.76</td>
</tr>
</tbody>
</table>

Samples were assayed in triplicate; - not determined.

4.5 Effect of TH on the membrane localisation of fucosylated glycoproteins.

Glycoproteins of most interest from the point of view of the formation of
correct neural contacts are cell surface markers. Consequently it was decided to investigate whether any of the fucosylated glycoproteins stimulated by TH in astrocytes were located in the plasma membrane. It was intended to compare the results with those for undifferentiated membrane glycoproteins apparently affected by TH, and separated on a Con A column (Chapter 3).

Plasma membranes were isolated from astrocytic cultures as described in Chapter 2, using a procedure which has been reported to isolate pure plasma membranes. This resulted in the production of three separate fractions: the purified membrane fraction (MF), the pellet fraction (PF), and the surface adherent fraction (SAF). The distribution of proteins between these fractions is shown in Table 10.

**Table 10 : Recovery of protein from fractions separated from astrocytic culture by ultracentrifugation.**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein content (μg)</th>
<th>Exp 1</th>
<th>Exp 2</th>
<th>Exp 3</th>
<th>Mean (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAF</td>
<td></td>
<td>6932</td>
<td>3508</td>
<td>1886</td>
<td>77.5 ± 16.4</td>
</tr>
<tr>
<td></td>
<td>(92.5%)</td>
<td>(79.9%)</td>
<td>(60.0%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PF</td>
<td></td>
<td>529</td>
<td>874</td>
<td>1252</td>
<td>22.2 ± 16.4</td>
</tr>
<tr>
<td></td>
<td>(7.0%)</td>
<td>(19.9%)</td>
<td>(39.6%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MF</td>
<td></td>
<td>34</td>
<td>8.7</td>
<td>23</td>
<td>0.35 ± 0.13</td>
</tr>
<tr>
<td></td>
<td>(0.45%)</td>
<td>(0.2%)</td>
<td>(0.4%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values in parentheses indicate % of total isolated protein.

These results are in good agreement with the reported distribution of protein in similar procedures (257, 258), where recoveries of pure plasma membrane in the membrane fraction were estimated to represent a recovery of approximately 10-30% of total plasma membrane protein, as assessed by the activities of marker enzymes. Similar assays of marker enzymes were attempted after the first separation in order to
verify the assumed purity of the MF. Aliquots were removed for the assay of 
Na\(^{+}/K^{+}\)-ATPase and 5'-nucleotidase (both plasma membrane markers); \(\beta\)-D-glucosidase (lysosomal), and succinic dehydrogenase (a mitochondrial marker), as 
well as for protein determination. However, as protein values in the MF were very 
low, and the volume only 50 \(\mu\)l, it proved impossible to perform all these assays 
satisfactorily given the quantity of sample available. Attempts to measure Na\(^{+}/K^{+}\)-
ATPase were unsuccessful in any of the fractions, suggesting that the enzyme had 
become inactivated during processing.

The separation procedure required the use of large numbers of plates of 
confluent astrocytic cultures (50 plates per sample), and was therefore very time 
consuming. For these reasons, and because the protein distribution achieved was in 
good agreement with those from published studies, it was decided to proceed with a 
piot study to determine whether the stimulation of fucose incorporation into 
astrocytic glycoproteins was preferentially distributed in the plasma membrane 
fraction.

Astrocytic cultures were incubated for 72 hours with \[^{3}\text{H}]\)-fucose, and with or 
without 10 nM T3. Fifty plates were pooled from each category, from which the 
purified plasma membranes fractions were prepared. The inclusion of 10 nM T3 in 
the incubation medium was associated with a 40% increase in the incorporation of 
fucosylated glycoproteins into the plasma membrane of (555 dpm/\(\mu\)g protein in the 
controls compared to 772 dpm/\(\mu\)g protein with 10 nM T3) (Figure 27). Values are 
also elevated to a smaller extent in the other fractions.

It therefore seems likely that TH may stimulate the levels of fucosylated 
glycoproteins in the plasma membranes of cultured astrocytes, although it should be 
remembered that these results were from one experiment, albeit pooled from 50 plates 
per category. Unfortunately the soluble protein fraction from the cytosol is lost in the 
membrane isolation procedure, so it was not possible to compare the relative levels of 
cytosolic fucosylated glycoproteins in cells incubated with or without T3. The data 
available for the astrocytic membrane proteins is, however consistent with the 
decrease in membrane glucose-mannose glycoproteins observed in TD progeny in 
vivo.
Figure 27: Effect of 10 nM T3 on the cellular distribution of \[^3\text{H}\]-fucosylated glycoproteins in astrocytic culture.

Values are from a single determination from samples pooled from 50 plates per category.
SAF = Surface Adherent Fraction; PF = Pellet Fraction; MF = Membrane Fraction.
As the stimulation of fucosylation by 10 nM T3 is relatively small (20%), it is possible that only a proportion of fucosylated glycoproteins in astrocytes are affected by this hormone. It would be necessary in the future to investigate whether TH cause an increase in particular fucosylated glycoproteins, or else cause a general increase in this class of protein, for example by increasing the levels of the main processing enzyme, α-fucosyltransferase. Particular attention should be paid to proteins in the membrane fraction, as this fraction demonstrated a proportionately greater response to T3, and analysis could be performed by electrophoresis and subsequent autoradiography.

4.6 Activity of α-fucosyltransferase.

Although assays for this enzyme have been described for some cell types, notably erythroblasts and cell lines, it had not been previously measured in astrocytes. Considerable differences exist between published procedures applicable to different systems (259-261), and the first priority was therefore to adapt existing methodologies to suit the requirements of astrocytic culture. The procedure finally arrived at is described in Chapter 2. Plates were preincubated with 1 μM fucose, with or without 10 nM T3, and then incubated for various time periods in the fucosyltransferase assay, processed, and the separated product counted.

Activities were very low in all fractions assayed, and were barely higher in 5 hour samples than in zero time controls (Table 11). The controls containing no asialofetuin were lower and considerably divergent. This background is likely to be the result of binding to endogenous fucose acceptors, and it may therefore be of interest that 10 nM T3 induces a 90% increase in the activity of this fraction. This could be the result either of the presence of a larger pool of proteins to be fucosylated, or of a faster rate of fucosylation in the T3 treated cultures.

The procedure was modified on a number of occasions without any evidence of a time dependent increase in binding to the acceptor. This apparent lack of activity, could be the result of very low levels of activity in astrocytes, as these are unknown. However, the presence of fucose incorporation implies the existence of fucosyltransferase activity, and it is therefore possible that the procedure is inappropriate for the enzyme sub-type present in astrocytes. Future work would be
necessary to establish a reliable assay in order to test these hypotheses. The use of a positive control such as the K562 human erythroblastoma cell line, reported to possess high levels of fucosyltransferase activity (260), would be a useful tool in this process.

**Table 11**: Activities recorded in samples of cultured astrocytes assayed for fucosyltransferase.

<table>
<thead>
<tr>
<th></th>
<th>dpm/ mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Acceptor</td>
<td>0 hours</td>
</tr>
<tr>
<td>Control</td>
<td>508 ± 61</td>
</tr>
<tr>
<td>10 nM T3</td>
<td>988 ± 57</td>
</tr>
<tr>
<td>n</td>
<td>2</td>
</tr>
</tbody>
</table>

Cells were preincubated with 10 nM T3 in the presence of 1μM fucose. Values are mean ± SD. (n=2 : ± difference from mean)

4.7 Effect of T3 on fucose incorporation into glycoproteins in neuronal culture.

A limited amount of work was carried out on the influence of TH on fucose incorporation in neuronal culture. Initially, this was performed along the same lines as employed for astrocytes, with a concentration of 10 nM T3. Although this might appear a very high concentration in the absence of any binding proteins in the medium, similar concentrations has been widely employed in neuronal culture to
Figure 28: Effect of 10 nM T3 on the incorporation of $[^3]$H-fucose into glycoproteins in neuronal culture.

Values are mean ± SD; differences were not significant.
produce biological effects (230), and proved to be the optimal concentration for the stimulation of leucine uptake and incorporation in our own experiments (232). Possible reasons for the higher concentrations needed to elicit responses in vitro have been speculated on at length, and include the non-availability of potentiating factors; the capacity for certain cell types to concentrate hormone in vivo, but not in vitro; and lack of cell co-operation in vitro (230,232). When compared to astrocytes, where free levels available to the cells appear to approximate to physiological concentrations, it should be remembered that neurons are far more isolated from serum conditions, and that tissue levels of TH in the brain have been reported to be an order of magnitude higher (288).

Neurons were cultured and experiments performed as described in Chapter 2, with time periods for fucose incorporation of 48 and 96 hours. Results were rather variable at both time points, and a time-related increase in the incorporation of [³H]-fucose into the cultured cells was not demonstrated (Figure 28). Actual levels of incorporated radiolabel were, however, much higher than those incorporated into astrocytic glycoproteins over similar time periods, and the possibility exists that incorporation occurs over a shorter time period in neurons, and is saturable. A T3 concentration of 10 nM appears to have no stimulatory effect on incorporation (Figure 28), or uptake (data not shown) at either time point, although it is possible that the high variation present masks such an effect. It is also possible that the glycoproteins involved in this cell type are different to those affected in astrocytes. Although these results suggest that T3 does not exert an influence over fucose incorporation into neuronal glycoproteins, the possibilities of activity over a shorter time period, or at different concentrations were not investigated, and therefore cannot be ruled out.

The modest stimulation of fucosylated glycoprotein synthesis caused by both T3 and T4 in astrocytic culture indicates the likelihood of specific targeting of TH action in this process and it is possible that similar mechanisms may operate in earlier in neural development. Whether these changes result from upregulation of protein precursors or effects on enzyme activity was not discovered, and should be the subject of future investigation. Similarly, much work would be required to establish whether such alterations are capable of contributing to the developmental dysgenesis associated with neurological cretinism.
These and other findings of TH effects on astrocytes in vitro raise the question of whether glial cells are directly responsive to TH during development. It has been acknowledged that in order to identify the precise targets of thyroid hormone during the neonatal period, it is necessary to separate and study nuclei from the different cell types involved (289). It was therefore decided to examine the ontogenesis of glial T3 binding in nuclei separated from neonatal rat brain.
CHAPTER 5 : ONTOGENY OF NUCLEAR BINDING OF THYROID HORMONES IN DEVELOPING RAT BRAIN.
5.1 Introduction

In order to understand the possible mechanisms by which TH of maternal origin may influence the developing brain, it was essential to gain more information about the nature of TH action at the cellular level. Although the study of astrocytes in vitro revealed TH-stimulation of fucosylated glycoprotein synthesis, for this event to have any involvement in the processes of neural development, the capacity for these cells to respond similarly in vivo is a prerequisite. In adult brain, thyroid hormone nuclear binding has been shown to be predominantly localised in the neurons (164,290). It has therefore been assumed that the peak in binding during development is also neuron-associated, and involved with terminal differentiation, with which it coincides (120,289). Thyroid hormone effects on glia at this stage have been considered secondary (120), despite reports of high levels of expression of the (THNR) in such cells in vitro (291,292). In addition, it is known that neonatal thyroid hormone deficiency results in impairment of glial development, including myelination (117). Since the glia undergo rapid division and differentiation in the neonatal rat, it is possible that these processes are under some degree of direct thyroid hormone control.

The ontogeny of the binding of thyroid hormones to nuclear receptors in brain has been investigated in a variety of species, including rat (61,289,293), sheep (294,295) and human (296). A pattern has emerged which indicates a peak in binding capacity at comparable stages in the development of each species. In the rat this occurs at postnatal day 6 and gradually diminishes to adult values (123). This has been taken to indicate the degree of thyroid hormone responsiveness of the tissue over the neonatal period (61,123). As it had not previously been possible to isolate individual nuclear types from the neonatal brain, a reliable method was developed, based on density gradient centrifugation, for the isolation of neuronal and glial nuclei from developing rat brain.

Nuclear binding of thyroid hormones was studied at several stages in neonatal development, both in whole brain and in brains dissected into three major anatomical regions: cerebral cortex, brain stem, and cerebellum, as these areas of the brain differ markedly in their response to TH.
5.2 Nuclear Isolation

5.2.1 Identification

After centrifugation, four distinct layers were apparent. The top layer consisted of cell debris (myelin, organelles, etc) of insufficient density to penetrate dense sucrose after centrifugation. The interface between the homogenate layer and the 2.0 M sucrose yielded mostly small round acidophilic nuclei, with <5% larger ovoid nuclei adjudged to be capillary endothelial nuclei. The layer between 2.0 M and 2.2 M sucrose consisted predominantly of large spherical nuclei with distinct nucleoli (some multinucleolate) and less granular, lightly-stained nucleoplasm. These were considered to be mature neuronal nuclei. The pellet contained smaller basophilic nuclei with granular nucleoplasm and intensely staining diffuse nucleoli adjudged to be predominantly glial nuclei.

5.2.2 Purity and Recovery

Cross-contamination of the neuronal layers with glial type nuclei was <5% and glial with neuronal nuclei <10%. However, due to the developmental period being investigated, blast nuclei in various stages of division were also inevitably present. The majority of these were of glial type and since they resemble the mature glial nuclei they are located in the pellet. As most neuronal development is complete by birth in the rat, blast nuclei of this type are considered to be relatively few, however contamination of the pellet with microneuronoblast nuclei, which are indistinguishable from glial nuclei, cannot be ruled out.

Recoveries of nuclei fell from 70% at day 5 to 45% at day 21 as the proportion of extraneous matter increased, impeding movement down the gradient. In nuclei separated from whole brain, an age-related increase was observed in the glial/neuronal (G/N) ratio, which were estimated from assessment of DNA levels (1.22 ± 0.59 at day 5, compared to 3.62 ± 0.38 at day 21; \( P < 0.001; n = 5 \)). In the regional studies, recovery was highest from the cerebellum, where the mean G/N ratio was 2.39 at day 15, and 8.99 at day 21 (Table 12). These results are to be expected as glial proliferation increases over the neonatal period, and most
development in the cerebellum is postnatal. The relatively high proportion of neuronal nuclei recovered in the cortex and brain stem was, however, surprising. It is assumed that this reflects differential fragility of nuclei from these regions, rather than their overall representation in the tissue. It is possible, for example, that the newly formed glial nuclei are more easily damaged by homogenisation or centrifugation through debris. A lower quantity of extraneous matter was present in the cerebellar preparation and this may explain the higher glial recovery in this fraction.

**Table 12** : Glial/Neuronal ratios of nuclei recovered from regions of neonatal rat at two stages of development.

<table>
<thead>
<tr>
<th></th>
<th>day 15</th>
<th>day 21</th>
<th>n</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cerebral cortex</td>
<td>0.80 ± 0.21</td>
<td>1.81 ± 0.36</td>
<td>5</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Brain stem</td>
<td>0.85 ± 0.15</td>
<td>2.30 ± 0.37</td>
<td>5</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>2.39 ± 0.45</td>
<td>8.99 ± 4.70</td>
<td>5</td>
<td>&lt; 0.05</td>
</tr>
</tbody>
</table>

Values were calculated on the basis of recovery of DNA, and are mean ± SD; significance was determined by unpaired Student's t-tests.

It is possible that the high glial/neuronal ratio in the cerebellum may result from granule cell nuclei contamination of the glial fraction.

**5.3 T3 binding in preparations of neuronal and glial nuclei from developing rat brain.**

**5.3.1 Whole Rat Brain**

Initially, T3 binding was studied in nuclei purified from whole rat brains at 5, 11, 15 and 21 days after birth.
Figure 29: Eadie-Hofstee plots of neuronal and glial nuclear T3 binding at two neonatal stages.

A) Day 5

Eadie-Hofstee plots of mean data from 6 experiments.

B) Day 21
Figure 30: Eadie-Hofstee plot of glial nuclear T3 binding at day 15 illustrating dual receptor model.

Each point represents the mean of 6 experiments. Interpreting the data as indicating the presence of two receptors provides a far better fit of regression lines.
Preparations of both neuronal and glial nuclei demonstrated low capacity high affinity binding at all stages studied. Data were transformed and plotted as B versus B/F. Such plots from days 5 and 21 yielded straight lines (Figure 29), taken to indicate the presence of single classes of high affinity binding sites in both neuronal and glial preparations. Data from day 15 in glia and day 11 in neurons suggested the possible presence of more than one class of binding site, as illustrated in Figure 30. Due to problem of assay sensitivity, however, it was not possible to obtain sufficient data to satisfactorily test this hypothesis.

MBC's for both neurons and glia were found to decline over the neonatal period towards adult values (Figure 31). Glial T3 binding considerably exceeds neuronal binding at day 5 (1774 ± 201 compared to 975 ± 117 fmol/mg DNA), and proceeds to fall away at a greater rate than that of neuronal nuclei over the following days to 557 ± 133 fmol/mg DNA at day 21 (P < 0.05). Allowing for variation, the concentration of neuronal nuclear receptors remains relatively steady over the neonatal period, and the apparent decline to 784 ± 212 fmol/mg DNA at day 21 is not statistically significant.

Glial receptor affinity was significantly greater at day 21 than at either day 5, or 15, as evidenced by a drop in Kd from 3.17 ± 0.40 nM (day 5; P < 0.03) and 3.39 ± 0.24 (day 15; P < 0.01) to 1.83 ± 0.34 nM at day 21. A comparable increase in affinity of neuronal nuclear T3 binding was not detected. It is possible that variation in this category, which was found to be relatively high over the period studied, may mask such a change in affinity (Table 13).

It should be noted that a range exists in reported Kd values, and that although the affinity of the THNR for T3 is usually quoted as 0.5 nM (62), in fact values between 30 pM and 16 nM have been reported (297,298). Differences have also been noted between extracted receptors and intact nuclei (298). Consequently, although the values reported here are at the higher end of the scale, they are by no means unusual.

It should also be noted that there are several possible causes of experimental error associated with transformed data. The most important of these are the displacement of non-specific binding, and mis-classification of bound and free categories after separation. These and other errors may have contributed to the apparent bias illustrated in Figure 30, emphasising the danger of drawing any fundamental conclusions from this observation.

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Values are mean ± SEM of 6 experiments; significance was determined by unpaired Student's t-tests. Significant differences were recorded between neuronal and glial binding at day 5 (* \( P < 0.05 \)), and between glial binding at days 5 and 21 (\( P > 0.01 \)).
Table 13: Dissociation constants for nuclear binding in neuronal and glial nuclei isolated from neonatal rat brain

<table>
<thead>
<tr>
<th></th>
<th>Neuronal (nM)</th>
<th>Glial (nM)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>day 5</td>
<td>2.26 ± 0.40</td>
<td>3.17 ± 0.40</td>
<td>6</td>
</tr>
<tr>
<td>day 11</td>
<td>2.87 ± 0.31</td>
<td>2.77 ± 0.31</td>
<td>6</td>
</tr>
<tr>
<td>day 15</td>
<td>3.57 ± 0.60</td>
<td>3.39 ± 0.24</td>
<td>6</td>
</tr>
<tr>
<td>day 21</td>
<td>2.55 ± 0.58</td>
<td>1.83 ± 0.34*</td>
<td>6</td>
</tr>
</tbody>
</table>

Data were analysed with Eadie-Hofstee plots and results are mean ± SEM. Significant differences (*), determined by unpaired Student's t-test, occurred between days 21 and 5 (P < 0.03), and days 21 and 15 (P < 0.05).

5.3.2 Analogue Studies

In competition experiments, the specificity of T3 binding to receptors was studied in neuronal and glial nuclei from animals at days 5 and 21 (Figure 32). Patterns of binding were similar in neurons and glia from each age. At day 21, the pattern of displacement of [125I]-T3 by unlabeled analogue was TRIAC > T3 > T4 > rT3, as is the case in adult rat brain (164). At day 5 however, preferential binding of TRIAC was less obvious (TRIAC ≥ T3 > T4 > rT3). The TRIAC:T3 affinity ratio was approximately 2:1 at day 5, and 4:1 at day 21, although given the inherent variability of this type of experiment, data was insufficient to draw definite conclusions.
A) Day 5

log Analogue concentration (M)

% [125I]-T3 bound

B) Day 21

log Analogue concentration (M)

% [125I]-T3 bound

- TRIAC
- T3
- T4
- rT3
5.3.3 Regional Binding Studies

Nuclear T3 binding was examined in three brain regions: the cerebral cortex, the cerebellum, and the remainder, classed broadly as brain stem. (Table 14). No significant differences in MBC or Kd between the two age points were observed for either neuronal or glial nuclei from any of the regions studied. Mean Kd's from all regions were 2.46 nM at day 15 and 2.10 nM at day 21 for neurons and 2.02 nM and 2.03 nM for glia (NS), close to those found in whole brain at day 21. Inter-regional differences in Kd were not significant at either day for either population, whilst differences in MBC were highly significant (Table 14).

In neuronal nuclei, the ontogenic decrease in average binding may result from a dilution effect exerted by increasing numbers of cerebellar microneurons and granule cells, which appear to possess fewer nuclear T3 receptors. Cerebellar glial nuclear T3 binding was found to be very low at both days, whilst cortical glia had a MBC for T3 of 2215 ± 147 fmol/mg DNA at day 21 compared to 1111 ± 207 for cortical neurons at the same stage. It is possible that a proportion of the cerebellar glial binding detected here is due to contamination from microneuronoblast nuclei which are developing at this stage and are indistinguishable from glial nuclei using simple histological techniques.

5.3.4 Receptor Leakage

Although not previously noted in rat brain to a large extent, it would be possible to argue that the differences in MBC and Kd observed in whole nuclei during the neonatal period may be due to differential lability or release of the receptors during the incubation period. Possible leakage of the receptors into the incubation medium was therefore assessed.

Results indicated that receptor leakage accounted for less than 5% of total binding in cortical neuronal nuclei at day 15, and was undetectable in neuronal nuclei from brain stem or cerebellum, or in glial nuclei from any region.
Table 13: Distribution of THNR in fractionated nuclei prepared from various rat brain regions at two stages of neonatal development.

<table>
<thead>
<tr>
<th>Region</th>
<th>Nuclear type</th>
<th>MBC (fmol T3/mg DNA)</th>
<th>Kd (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>day 15</td>
<td>day 21</td>
</tr>
<tr>
<td>Cerebral</td>
<td>Neuronal</td>
<td>1046 ± 97</td>
<td>1111 ± 207</td>
</tr>
<tr>
<td>Cortex</td>
<td>Glial</td>
<td>1818 ± 95</td>
<td>2215 ± 147</td>
</tr>
<tr>
<td>Brain Stem</td>
<td>Neuronal</td>
<td>662 ± 16 *</td>
<td>640 ± 40 *</td>
</tr>
<tr>
<td></td>
<td>Glial</td>
<td>452 ± 102 **</td>
<td>619 ± 141 **</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>Neuronal</td>
<td>273 ± 77 **</td>
<td>309 ± 102 *</td>
</tr>
<tr>
<td></td>
<td>Glial</td>
<td>268 ± 26 ***</td>
<td>158 ± 65 ***</td>
</tr>
</tbody>
</table>

Data were analysed using Eadie-Hofstee plots and results are mean ± SEM. Probability values refer to significant differences from cortical levels.

* P < 0.05; ** P > 0.005; *** P > 0.0005 (unpaired Student's t-test)
Figure 33: Eadie-Hofstee plots of TH binding to receptors extracted from neuronal and glial nuclei at two neonatal stages.

Eadie-Hofstee plots of mean data from 3 (day 21), and 4 (day 5) experiments.
5.3.5 Extracted Receptor Binding

Differences in Kd obtained in intact nuclei persisted in extracted receptor preparations (Figure 33; Table 15), falling significantly in neurons from $3.32 \pm 0.49$ nM at day 5 to $1.25 \pm 0.38$ nM at day 21 ($P < 0.03$). Additionally, we observed that MBC's were increased relative to intact nuclei by approximately 3-fold in cortical neurons at day 5 ($4300 \pm 1311$, compared to $1272 \pm 266$ fmol/mg DNA), whilst other values were in broad agreement with intact values. The reasons for this discrepancy are unknown, but are not the result of leakage of receptors from the intact nuclei. When compared to either extracted neuronal receptors, or glial receptors in intact nuclei, an apparently lower stability of the extracted glial receptor was observed. Although not studied in detail, this was most noticeable at day 5, and may partially account for the large variation in glial binding seen in this category (Table 15).
**Table 15**: Maximum binding capacities and dissociation constants of THNR extracted from neuronal and glial nuclei separated from neonatal rat brain.

<table>
<thead>
<tr>
<th></th>
<th>MBC (fmol T3/mg DNA)</th>
<th>Kd (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>day 5</td>
<td>day 21</td>
</tr>
<tr>
<td>Neuronal</td>
<td>4300 ± 655</td>
<td>900 ± 243*</td>
</tr>
<tr>
<td></td>
<td>(4)</td>
<td>(3)</td>
</tr>
<tr>
<td>Glial</td>
<td>1988 ± 698</td>
<td>1111 ± 264</td>
</tr>
<tr>
<td></td>
<td>(4)</td>
<td>(3)</td>
</tr>
</tbody>
</table>

Binding parameters were calculated using Eadie-Hofstee plots. Values are mean ± SEM; n given in parentheses; significance was determined by unpaired Student's t-tests.

* P < 0.01; ** P < 0.03 (neuronal day 5 : neuronal day 21)
CHAPTER 6: DISCUSSION
6.1 Maternal thyroid hormone and gross fetal biochemistry

Thyroid hormone has been recognised as a major factor influencing the metabolic capacity and developmental profiles of almost all vertebrates from fish and amphibians, through birds to mammals. Nowhere is this involvement more important to normal development than in the brain, where the growth and differentiation of neural structure is completed early in life, and is critically sensitive to the presence of appropriate TH levels.

The extent to which a tissue responds to hormone depends on the sufficient delivery of the hormone, coupled with an adequate concentration of active receptors with which the hormone can interact to elicit a biological response. Organs are known to differ in their sensitivity to TH action, and different cell types within those organs also vary in their dependency. Further still, individual organs and cell types are reported to alter their demands for hormone as development proceeds (33). A combination of these factors has been enlisted to explain the complexity of TH control of development, and the critical requirement for the correct level of hormone at the correct time (117, 299).

The primary mechanism of TH action is mediated through the binding of hormone to nuclear receptors, with subsequent effects on gene expression (300). Evidence also supports the existence of a number of extranuclear sites of action, including the cell membrane and the mitochondrion (90, 301). These predominantly metabolic effects are also of importance in the developing organ, where secondary effects on growth and cell survival cannot be ignored.

Clinical and experimental evidence points to the presence of both thyroid hormone and its nuclear receptor in the developing fetal brain during the first trimester of pregnancy (178). In addition, the physiological function of TBG, which in the human rises three fold during gestation, may be to secure an adequate supply of hormone to the fetus before the establishment of independent thyroid function (162). Given the critical influence of TH on brain development, and the clear selective advantage this appears to have conferred, it is not unreasonable to hypothesise that further advantage may have been obtained by the ability to similarly direct brain development at an even earlier stage than permitted by reliance on hormone synthesized by the fetus itself.
This study was commenced to test the hypothesis that maternal hormones may be involved in this capacity, and was one of the first explorations of the effects of maternal hypothyroxinemia on the development of the fetal brain. The aim of the work was to determine the effects of maternal hypothyroxinemia on fetal brain biochemistry, and to progress towards a biochemical model for the transplacental actions of maternal thyroid hormones, specifically T4, in the first trimester of pregnancy.

This required the use of a reliable animal model of the disorder, avoiding the problems previously mentioned (see Chapter 3). Different experimental models may partially account for the lack of uniformity in the literature regarding transplacental effects of TH, especially when it is understood that the disorder is by no means a uniform one itself, displaying a range of severity of outcome (20,22,216,217). Also likely to contribute to variations in severity of effect is the probability that the developing nervous system is polygenetic, being able to achieve, at least to some degree, similar results by different pathways. This plasticity could easily have arisen in an evolutionary system where the price of too rigid a requirement may have been environmental vulnerability. Consequently, although maternal TH appear necessary for the most efficient brain function, it may be possible to compensate for partial absence, the extent of rectification varying between individuals.

Some studies have concentrated on comparing clinical evidence from iodine deficient regions, whilst others have introduced iodine deficient animal models, either by simply eliminating dietary iodide, or by attempting to recreate the diets of iodine deficient regions (20,302,244). More sophisticated approaches have concentrated on the levels of TH themselves, in a more direct manner (303,304). These have generally used totally thyroidectomised rat dam animal models, in which the dam is severely deficient in both T3 and T4, achieved either by surgery, or by the administration of antithyroid drugs such as PTU or methimazole, which interfere with the functioning of the fetal thyroid. The model employed in this study is more subtle, and designed to specifically test the effect of maternal hypothyroxinemia, whilst leaving maternal T3 levels at or near normal values. This is considered to more accurately represent the conditions found in connection with neurological cretinism, and allows easier manipulation and study of individual parameters.

The hypothyroxinemic rat dam model satisfies the requirements in that the dams
used were euthyroid and eumetabolic in terms of T3 levels, although TSH has been shown to be elevated under similar conditions (266). The progeny of hypothyroxinemic dams also exhibited normal circulating levels of T3 and T4, but again hypothyroidism in relation to TSH cannot be ruled out.

It was observed that the hypothyroxinemic dams had a high rate of breeding failure compared to normal dams. The pregnant animals also demonstrated somewhat lengthened periods of gestation and suffered considerable difficulties during parturition. Although severe pregnancy complications and birth difficulties are not common features of iodine deficient regions, except where the levels of maternal T3 are also reduced, similar difficulties have been reported in the literature in iodine deficient animals (170). It may be conjectured that TH status in pregnancy exerts species specific control over the physiological regulatory mechanisms governing birth, including the oxytocin/relaxin and/or prostaglandin systems, either at the level of secretion of these substances, or by sensitising the uterine musculature. In addition, a reduction in both litter size and average weight of the progeny was observed. This reduction may be a result of physiological economy, maximizing the use of the available hormone. Whether control of this phenomenon rests in the mother, or else is due to failure of development of the embryo in very early gestation remains debatable, but since the dams used in our experiments were iodine replete, exhibiting normal T3 levels, it may be argued that mating success; intrauterine fetal development; successful outcome of pregnancy; litter size and neonatal body weight are dependent on the adequacy of thyroxine in the maternal circulation.

The weights of TD progeny are diminished in the early postnatal period, but afterwards appear to match and occasionally exceed those found in the controls. This is in conformity with data from other groups, who have reported diminished fetal and neonatal weight as a result of maternal hypothyroxinemia (170), and work on adult rats where TD progeny were found to be slightly heavier than their normal counterparts (305). These data suggest a lag in development followed by an over-compensation, in both cases indicating long-term effects of in utero TH deficiency.

The degree of weight deficiency was found to be organ specific, with the heart and lung most severely affected, demonstrating significantly lower weights well into the neonatal period. The brains of TD progeny are lower in weight at almost every stage assessed, although not to a statistically significant extent. The kidney was
apparently unaffected. These deficits resemble those found in thyroidectomised neonates, albeit to a lesser degree, even though the deprivation of hormone only occurred during early pregnancy. The observed specificity of effect is likely to be explained by the degree of TH sensitivity expressed by each organ during early pregnancy. The kidney, for example, although highly responsive to TH in the adult, is at a very rudimentary stage of development in early pregnancy, with excretory functions being performed by the placenta. It is therefore not surprising that lack of maternal TH during this period is without effect. The brain, on the other hand, undergoes considerable growth during this period, making it a more probable target for TH action. A similar argument can be applied to the heart, also functional at an early stage. The apparent sensitivity of the lung is more difficult to account for on this basis. It is possible that a particular facet of development is affected in this organ, leading to severe long-term consequences. This specificity of effect may also argue against fetal malnourishment owing to placental insufficiency, as such circumstances are likely to result in more generalised weight loss.

At this stage it is necessary to answer another critical question regarding the validity of the model, namely is lack of maternal TH the causative factor of progeny deficiencies, or is, as has been suggested (306), placental insufficiency caused by maternal hypothyroxinemia, to blame? Certainly it appears that the dams are not functioning normally, despite near normal levels of T3. For this reason, a study of the metabolic state of the placenta at various stages of pregnancy was undertaken (305). The data revealed no apparent alterations in placental metabolism of glucose or the activities of a number of enzymes used as markers of metabolic efficiency. This suggests that the placenta is not significantly affected by hypothyroxinemia in the animal. Further evidence contradicting the hypothesis of placental insufficiency with consequent deprivation of nutrients to the fetus has been provided by Mano and co-workers (302). This group found that in sheep, whereas iodide or T4 administered to the mother reversed the effects on the fetus caused by an iodine deficient diet, DIMIT did not. DIMIT, the non-iodinated TH analogue returned maternal TSH levels to normal, and normalized the activities of tissues dependant on plasma T3. Although an active substitute for T3 in a wide range of maternal tissues (307,308), DIMIT is reported either not to cross the ovine placenta, or else be unstable or ineffective in ovine fetal tissues (309). Consequently, a direct hormonal effect on fetal
development is implicated, rather than a nutritional one. Speculation that maternal hypothyroxinemia may result in impaired transfer of critical metabolites to the fetus therefore seems to be untenable.

Measurements of total protein content and its concentration in a number of organs reflected the data found for organ weights, with almost all organs revealing deficits in total protein content in the early neonatal period which become normalized, or even elevated later in life. Although there is a suggestion of diminished protein concentrations in the early period in brain and liver, the values, rising slightly in brain, and remaining relatively stable in liver, do not differ significantly from the controls. In these experiments no difference was recorded in total DNA, implying that total cell number remains unaffected. The protein/DNA ratio reveals that cell size in the brains of the TD progeny is diminished in the early postnatal period. This reduction is the most likely cause of the reduced brain size. Data from later in the neonatal period shows that both the weight and the total protein content of brain and liver appear to be elevated. This is paralleled by an increase in the protein/DNA ratio indicating an enlarged cell size compared to controls.

Some reports have indicated depleted numbers of cells in the brain as a result of maternal hypothyroxinemia (212). Although our own preliminary results also suggested this to be the case (310), final results presented here show that DNA content in neonatal TD progeny brain is no different from controls. After the first two weeks, however, it is possible that additional cell death may occur, resulting in the lower cell numbers indicated at day 30. This may be the start of a process leading to lower DNA content in the adult TD progeny (161), although the latter data has yet to be substantiated. Likewise, depletions in cell number at the earliest stages of brain development have been reported by several groups (212,311). Both these groups have reported normalizations in cell number (DNA levels) at equivalent gestational stages, i.e. birth in the rat, and the latter stages of pregnancy in the sheep. Porterfield has pointed out that this does not indicate a return to the normal condition, as cells lost in early neurogenesis are likely to be neuronal, whilst even acknowledging the possibility of compensatory formation of some new neurons, the majority of new cell production during the "recovery" period are likely to be glial (304). Additionally, although in the earliest neonatal stages it appears that cell number in the brain is not affected by maternal hypothyroxinemia, the loss of particularly sensitive groups of cells within the CNS is not precluded. This may in turn cause further disruption of
neural development at later stages (312). The ontogeny of the brain consists of both generation and death of cells in a co-ordinated manner. Although overall cell number in this study may be unaltered the actual nature of the cell types present may differ from that comprising the normal population.

In general, with the exception of the lung, no significant alterations were observed in DNA content. In all these experiments, however, sample size, coupled with large errors render interpretation difficult. This includes the lung, where low DNA levels in the TD progeny indicate a considerable cell loss which is apparently sufficient to account for the decreased weight of this organ during development. In addition, lung cell size of the TD progeny is considerably increased, suggesting perhaps, a failure of cell division resulting from diminished DNA synthesis. This finding remains to be confirmed, and it is difficult to explain why such a dramatic effect may occur in the lung, yet not be noticed in other organs, unless local tissue specific factors are involved. Although it is known that TH play a critical role in the early development of the lung (313,314), fetal thyroidectomy has been reported to influence cell size, as opposed to cell number in the sheep and rabbit (24). Despite this, it is possible that a sequence of events leading to normal lung development may be triggered by sufficient maternal hormone very early in pregnancy, lack of such a trigger resulting in the failure of cell proliferation, or cell loss indicated by this work. It is noteworthy that considerable intrauterine acceleration of fetal lung development, both in terms of morphology and biochemistry, has been achieved by the administration of large doses of thyroid hormones to pregnant rabbits (211).

Data for the liver tend to indicate a similar trend to that found in the brain, although the alterations in cell size did not attain statistical significance. The kidney, on the other hand, demonstrated no consistent differences in any of the parameters monitored.

The changes reported here are in good agreement with recent reports from several sources which have examined an array of biochemical parameters in the progenies of either totally, or partially thyroidectomised animals. A range of models have been employed, most commonly, the rat, sheep and rabbit, and despite the differences in placental types between these species (human and rat placentae are hemochorial, whereas the rabbit placenta is hemoendothelial, and sheep placenta syndesmochorial) (179), the following reports show that maternal hypothyroidism
results in reduced weights of whole body, brain, liver, and lung of the developing fetuses.

In the ovine model, a variety of fetal brain abnormalities resulting from maternal iodine deficiency were only partially corrected by the administration of iodine or T4 at the end of the second period of pregnancy (302). Experimental animals from this study possessed fewer, but longer synaptic appositions in the cerebral cortex, and reductions were recorded 10 days prior to parturition in cell number and size in both the cortex and cerebellum. Irreversible effects due to maternal iodine deficiency are clearly implied. That these effects persist in iodine replete, but surgically thyroidectomised animals indicates that it is likely to be lack of maternal T4 which causes the deficits in the fetus (311). In the latter study, parameters such as brain weight, and cell size and number had apparently normalised by birth. Also working with sheep, Smith et al. (315) have shown that PTU induced maternal hypothyroxinemia results in varying degrees of dysgenesis of noradrenaline, dopamine and cholinergic binding sites in fetal and neonatal brain.

Peterson and Young noted that administration of T4, but not T3 to the thyroid deficient guinea pigs (another hemochorial species) during pregnancy prevented fetal goitre (316), and Devaskar et al. found an increase in the rate of glucose metabolism in the progeny of hypothyroid rabbits dosed with T3 or T4 (317). Returning to the rat, Morreale de Escobar et al. have reported developmental and neurological deficits in the offspring of hypothyroxinemic rat dams (170), and Porterfield and Hendricks have shown that brains derived from the progeny of hypothyroxinemic rat dams have compromised patterns of carbohydrate metabolism (318). It has also been observed that, unlike in the sheep, the administration of DIMIT to pregnant rat dams prevents neuronal retardation in the fetuses induced by maternal radiothyroidectomy, clearly implying a transplacental effect (319).

An interesting rat model of endemic cretinism has been developed by Li Jianqun et al., who fed rats on a diet which mimicked the diet of a region of severe iodine deficiency in China (244). The progeny of rats fed in this manner exhibited an array of alterations in neurological development, including decreased neuronal size, increased neuronal cell density, and delayed development of the cerebellum. The pregnant rats were assayed for serum levels of TH, and found to be severely hypothyroxinemic, whilst serum levels of T3 actually rose slightly, although not into
the hyperthyroid range. These data support the observations of Pharoah et al., suggesting that iodine deficiency in early pregnancy effects neuroblast multiplication, differentiation and migration (22), and can equally well be explained by the hypothesis of a feto-placental requirement for T4 during early pregnancy.

The relevance of these studies to the condition pertaining in man is emphasised by the recent clear demonstration of materno-fetal transfer of T4 in cases of congenital hypothyroidism in which a total organification defect prevented the fetus, but not the mother from producing thyroxine. Substantial levels (35-70 nM) were detected in cord serum in late gestation, which could have only originated in the mothers (320). An editorial in the New England Journal of Medicine commented that fetal requirement for hormone may determine supply from the mother, and that sufficient quantities of T4 are transferred to establish normal intracellular levels of T3 in the fetal cerebral cortex (321). As the fetal brain generates the majority of T3 by local deiodination, this requires a supply of T4 and not T3 to the fetus. In this context, it is of interest that the hormone delivery model of Ekins predicts the preferential delivery of T4 rather than T3 to the placenta (161). Several other researchers have noticed a selective effect of maternal T4 rather than T3 concentrations on fetal development (322).

Morreale de Escobar and co-workers (323) and Bonnet and Herrera (324) also suggest that in circumstances of extreme maternal hypothyroidism, and when the fetus has an active thyroid gland, TH efflux from fetus to mother may occur. They showed this to severely diminish levels of TH in fetal tissues relative to controls. The latter group have also provided evidence for materno-fetal passage of TH in the early stages of pregnancy, as they detect an increase in the TH-sensitive fetal growth hormone levels after the administration of T4 to pregnant rat dams, prior to the onset of fetal thyroid function.

Failure of the thyroid control systems, or local alterations in TH metabolism, precipitated by maternal hypothyroxinemia, may also cause detrimental effects on brain development. Porterfield has demonstrated disruptions to the thyroid hormone control system resulting from maternal hyperthyroidism induced by the administration of high doses of T4 or DIMIT (325), and Morreale de Escobar and co-workers have produced a considerable quantity of data regarding the presence and metabolism of thyroid hormones in the developing fetuses of hypothyroid rat dams. After reporting
the presence of TH in normal fetal tissues before the onset of independent thyroid function (208), this group examined and found their absence from the tissues of the progeny of surgically thyroidectomised dams (170), or from the progeny of chronically iodine deficient dams (326). Deficiencies in tissue thyroid hormone content persisted to term. Furthermore, they have clearly demonstrated that although fetal brain 5'D-II is up-regulated in iodine deficient conditions, this is not sufficient to protect the fetal brain from T3 deficiency. They propose that a longer sensitive period during human pregnancy would render even greater damage to the human brain than to that of the rat (327). After birth, iodine transfer in milk mitigates TH deficiency in the offspring (328).

In addition to disruption to the brain biochemistry of neonatal TD progeny, results from our own laboratory also indicate a range of irreversible biochemical deficits in several brain regions of adult progeny of hypothyroxinemic rat dams. It was found that total levels of protein, DNA synthesis, and inorganic phosphate concentration, were all diminished in a region specific manner (161). The region-specific deficit in protein acquisition, indicated by a reduction in protein concentration in the cerebral cortex and the paleocortex may have been due to reduced cell number and/or a reduction in the total synaptic compartment. This emphasises that whilst overall protein levels may be unaffected, some specific regions may be targets of early TH action, and their development may be compromised as a result of maternal hypothyroxinemia.

More detailed examination of the specific biochemical deficits in the neonatal TD progeny revealed persistent and significant reductions in total cytosolic protein, whilst total membrane protein content remained apparently unaffected. Many regulatory enzymes including pyruvate kinase and acetyl CoA carboxylase are located in the cytosol and the failure of such systems may compromise brain cell metabolism. The reduction in cell size noted in early development may be attributable to this effect, although cytosolic protein levels are still depressed later in the neonatal period, when cell size and brain weight have normalised, or even increased above control values. These differences do, however, imply a specificity of effect of maternal hypothyroxinemia.

Effects on glycoprotein expression may be more widespread than is at first apparent. Con A binding glycoproteins are known to be particularly profuse in
synaptic complexes, possibly indicating disruption of synaptogenesis or synaptic function (329), but glycoproteins also form a large number of regulatory enzymes with localization in both intracellular and cell surface membranes, as well as in the cytoplasm and a number of organelles. Glycoproteins have even been implicated in the nucleus as important, temporally-active, transcription co-factors (330). Alterations in the abundance and activities of such enzymes and co-factors may also play a critical role in determining cell function. It was therefore decided to examine the distribution of a subset of these proteins separated by Con A chromatography. Signification alterations were apparent, and at first, it appeared that a straightforward failure of incorporation into the membrane may be involved, but the significant deficit in membrane glycoproteins at day 14 was not matched by a comparable increase in the cytosol. It may be that the effect is a complex one. The evidence obtained from astrocytic culture indicates TH stimulation of $[^3H]$-fucose incorporation into the membrane, but a more comprehensive and a wider study encompassing the measurement of glycoprotein synthesis, degradation and membrane incorporation would be necessary to attempt to clarify this picture.

Clearly, this is evidence for disruption of the normal pattern of protein expression and distribution, and indicates that similar total protein levels may mask a change in the proportions of the individual proteins involved. When a closer examination of this situation was undertaken, changes in the pattern of expression of glycoproteins became apparent. SDS-PAGE analysis, which separates the polypeptides composing the glycoproteins, revealed the absence in membrane fractions prepared from the brains of TD progeny of a 16 kDa glycoprotein at both days studied, and the addition of one with an estimated Mr of 81 kDa. At day 14 the cytosol of the TD progeny lacked a glycoprotein of a similar Mr (78 kDa), although whether this is related to the TD progeny-unique 81 kDa membrane band is unknown. The identities of the proteins involved can only be speculated upon, on the basis of the molecular weights of the polypeptides isolated. Several enzymes, particularly those involved in glycosylation, are glycoproteins, as are a number of receptors, including the nicotinic cholinergic receptor is a pentamer constructed from four species of subunit, each of which is glycosylated.

Cell surface recognition molecules are predominantly glycoproteins, and several glycoproteins are active as transport molecules.

Examination of the relative abundances of the separated glycoproteins reveal
further interesting comparisons. All major membrane glycoprotein fractions from 14 day old TD progeny brain are diminished, although to different extents, whereas cytosolic glycoproteins are not so obviously different. This may hint at a reduction in membrane-associated glycosylation, perhaps arising from depleted levels of the enzymes involved. Diminished glycosylation may result in failure, or reduced efficiency of Con A binding of glycoproteins, and hence their isolation. Alternatively, decreased synthesis of precursor peptides, or an effect on incorporation into the membrane of specific proteins may be responsible for changes in the location or abundance of a glycoprotein. Enhanced degradation of glycoproteins is also possible, though unlikely. The overall deficit in cytosolic proteins does not appear to be paralleled by a decrease in cytosolic Con A binding glycoproteins. The 45 kDa membrane glycoprotein may be identifiable with a 45-49 kDa, Con A-binding glycoprotein detected by Beesley et al. termed gp50. The levels of this protein, as measured by binding to a labelled monoclonal antibody were severely retarded in the cerebellum in thyroid deficient rats at day 14, although compensation occurred by day 28 (329).

Comparing age-related changes in glycoprotein presence, a 60 kDa glycoprotein present at both days in the control is much diminished at day 14, and absent at day 30 in the TD progeny. A comparison between day 14 and day 30 in the membrane fraction also reveals a relative change in the 33 kDa glycoprotein, which is drastically reduced between the two days in the control, but remains the same in the TD progeny. Temporal alterations in the expression of glycoproteins are well known, and their role are known to be of particular importance in cell development (332). This is of particular significance when the glycoproteins concerned are located in the plasma membrane and are involved in cell-cell interactions. It is essential that the correct type of protein is present in the correct proportions at the appropriate time. Alterations in this pattern, as appear to be involved here, may be involved in the disruption of neurogenesis. The results therefore indicate a widespread, but not generalised effect on glycoprotein synthesis, localisation, relative abundance, and temporal and spatial regulation.

The activities of the acid hydrolases were measured in order to determine whether specific enzyme function may be affected as a result of maternal hypothyroxinemia. They are thought to play a developmental role in brain, where
their levels are high throughout the developmental period (333). Results revealed specific deficits in two of the enzymes studied at day 14, but not at day 53, reflecting the disparity of findings between the juvenile and adult progeny.

In the brains of adult progeny of hypothyroxinemic rat dams, acid phosphatase activity was drastically reduced, presumably impinging either directly or indirectly on the homeostasis of the intracellular phosphate pool and affecting the phosphorylation of macromolecules (161). During the neonatal period however, no effect on this enzyme is apparent. \(\beta\)-D-Galactosidase activity was also diminished in the cerebral cortex, paleocortex, midbrain and medulla of the adult progeny, whilst the cerebellar activity remained unchanged. Again, in whole brains from the neonate, this enzyme remains unaffected. \(\beta\)-D-Glucosidase, on the other hand, is significantly diminished in the 14 day old TD progeny, but unaffected in all adult regions. These findings indicate temporally-specific damage to these enzyme systems, possibly caused by the disruption of normal cell type development. Aryl sulphatase exhibited a similar pattern to \(\beta\)-D-galactosidase activity in the adult, and is also depressed in the neonatal TD progeny. As this enzyme is recognized to be preferentially localized in neurons, this may suggest a deficit in specific neuronal types which is present at an early stage, and persists into adulthood. However, it is not possible to deduce whether any particular neuronal type or even specific lysosomal groups are preferentially affected. Similarly, since both galactosidase and aryl sulphatase are involved in galactosylcerebroside and sulphatide metabolism, a significant deficit of these enzymes may place restrictions upon the metabolism of myelin, apart from diminishing general cell defence mechanisms.

Several other enzymes have been found to be adversely affected in the adult progeny. The activity of the neurotransmitter enzyme, acetylcholine esterase, was diminished in most brain regions, whereas choline acetyltransferase and monoamine oxidase remained unaffected (161). Enzymes involved in energy metabolism were also specifically affected; lactate dehydrogenase and Na\(^+\)/K\(^+\)-ATPase were reduced in a number of brain regions, whilst no changes were observed in the activities of succinic dehydrogenase and Mg\(^{++}\)-ATPase (161).

Ruiz de Elvira et al. have reported an increase in the activity of calcineurin (a calmodulin regulated phosphatase) specific to the cerebellum in these progeny at two months of age (334). As this enzyme is associated with the phosphorylation of
microtubule associated factors (such as Tau factor), it is important in neurite formation. This would suggest that the cerebellum may still be undergoing a degree of development at this late stage, implying a delay resulting from maternal hypothyroxinemia.

Damage to the enzyme systems involved in the metabolism of myelin have also been found to be parameter-selective and region-specific. As the general oligodendroglial marker enzyme 2',3'-cyclic nucleotide 3'-phosphohydrolase was decreased in medulla and midbrain, this may point to a deficit in the numbers of active, myelin forming oligodendrocytes in these regions, although neither total cholesterol, nor the levels of the general glial marker, N-acetyl-β-D-galactosaminidase were affected in this study (305).

It is therefore clear that maternal hypothyroxinemia, resulting in "hypothyroidism" in the conceptus in early pregnancy, causes a diversity of biochemical deficits in the brains of the progeny. These differences must be the result of initial disruption of the mechanism by which thyroid hormone governs brain development, and has long-term, irreversible consequences. Although effects are widespread, they are also specific in nature, as some enzymes are affected, whereas others are not; some Con A binding glycoproteins are affected, others are not; and, in the brains of adult progeny, different parameters are affected to varying extents in different anatomical regions (305,161,334). The cumulative effects of a large number of such changes in a number of brain regions is likely to disturb the overall neural efficiency of the affected individual. The presence of specific biochemical deficits noted in this report and elsewhere is commensurate with specific dysfunctions such as strabismus, deaf-mutism, and impaired intellectual ability observed in human cretinism. A recent study of the behaviour of TD progeny has added credibility to the validity of this model, as significant abnormalities were recorded in exploratory behaviour, depth perception, emergence test, and discriminative cognitive function (Skinner box) (335). It is therefore suggested that the biochemical deficits observed may at least in part explain the cognito-motor, behavioural, physical and psychological disorders seen in clinical conditions and iodine deficient endemias.
6.2 Thyroid hormones and glycoprotein synthesis

In view of the observations that TH deprivation in early pregnancy is manifested both in a variety of biochemical deficits in the brains of neonatal and adult progeny, and in the array of clinical dysfunctions previously mentioned, it was necessary to consider plausible mechanisms by which this may occur.

Obviously, differing expression of particular glycoproteins, or enzyme activities during the neonatal period, or at 2 or 7 months after birth cannot be a primary effect of maternal hypothyroxinemia, nor does it seem likely that a sequential alteration of individual gene expression is universally responsible, although it is impossible to rule it out in all cases. Alternatively, a disturbance in neurogenesis during early development may lead to later dysfunction as a result of loss of key cell populations and inappropriate connectivity, as is found in the hypoplastic neuropil of hypothyroid animals (117).

Maternal hypothyroxinemia led to long term perturbations of the levels and distribution of specific glycoproteins, and these molecules are known to be critically involved in the direction of cell-cell interactions in the developing nervous system, from the very earliest stages, in the neuroepithelium (336). The neuroepithelium is involved in the concomitant generation of both neurons and glia (337). This therefore offered one putative mechanism through which direct TH action at specific developmental stages may lead to a long term influence over development. As the effects noticed in vivo may have been secondary to other events, such as reduced membrane synthesis or cyto-architectural alterations, it was first necessary to establish whether glycoprotein synthesis was under direct TH control during "critical periods" of early neural development.

The reported comparable behaviour of neural cells in vitro and in vivo permitted the examination of this possibility in cell culture. Culture conditions in defined media eliminate interference from extraneous sources, permitting identification of the primary effects of TH. The TH are required for normal morphological and biochemical development of cultured neural cells, parallels with in vivo findings including the stimulation of Na+/K+-ATPase, but not Mg++-ATPase activities (223,338) and effects on other enzymes such as cholineacetyltransferase (234).
Glial cells, and in particular astroglial cells, demonstrate considerable $[^3\text{H}]$-fucose incorporation, both in vivo (339), and in vitro (272). Separation procedures have generally indicated that fucose is solely incorporated into glycoproteins, with only limited incorporation into glycolipids or glycosaminoglycans. Some reports have suggested that glial fucose incorporation far exceeds neuronal, but the potential for temporal variation in the expression of different subtypes of glycoproteins must be considered. Other reports claim high incorporation of fucose into neuronal rather than astrocytic glycoproteins (340), and that fucose may be incorporated into non-protein glycoconjugates in astrocytes (340). Data from this thesis indicate high levels of incorporation in both cell types, but suggest the time course, and possibly the mechanisms involved, are cell-type specific in developing cultures of rat brain cells.

The demonstration of TH control of fucose incorporation into astrocytes in developing cultures suggests the possibility that glycoprotein synthesis may be under a degree of direct TH control in the fetal brain. Experiments demonstrated a steady and significant increase in incorporation with time, whilst no immediate difference in free cytosolic fucose was observed, signifying no direct effect of T3 on transport of fucose into the cells. A dose response revealed the optimum concentration to be in the region of 10 nM for T3, and 25nM for T4. The levels of hormones required to stimulate incorporation can be considered to be physiological, as in the medium employed, over 99% of the hormone used proved to be bound to proteins in the FCS.

Incorporation time in vivo appears to be rather quicker than in vitro (341). This may be the result of a co-operative sequestering of the sugar, with a resultant increase in substrate concentration in defined areas of incorporation (specifically on the cell surface) (342). This is probably not possible in the less organised environment of the culture dish. The relatively slow rate of glycoprotein synthesis in astrocytes may, however, reflect the turnover period of 1-2 weeks reported for these proteins in vivo (343).

Studies of the effects of thyroid hormones on glycoprotein synthesis are few and far between. Torres-Pinedo et al. demonstrated a T4 induced increase in the fucose content of microvillae membranes in the rat intestine, an effect potentiated by the presence of cortisol (344). There is however, direct evidence that fucosylated glycoproteins are involved in interactions between glial cells, including astrocytes,
and other cells in development. Steindler and co-workers (272) studied the localisation of $[^3H]$-fucose labelled glycoproteins in glia from the developing mouse brain early in the postnatal period. In the first postnatal week, they found it possible to delineate between developing nuclei in the diencephalon, midbrain, and brainstem using fucose binding lectins. They found that these patterns co-ordinated precisely both with $[^3H]$-fucose incorporation revealed by autoradiography, and with the expression of the astrocyte specific glial fibrillary acidic protein (GFAP). This evidently suggests that glial cells, particularly astrocytes synthesise and express fucosylated molecules which play a major role in pattern formation in the developing brain.

It was possible to detect a fine structure of glycoprotein expression within the transient lectin delineated boundaries found in development, and a temporo-spatial aspect to the expression of these glycoproteins was demonstrated, suggesting that the events involved in such pattern formation and stabilisation may be mediated by a changing glycan code expressed on astrocytic surfaces (272,345). As the plasma membrane was also found to be the dominant location of fucosylated glycoproteins in cultured astrocytes, and the difference between T3 treated samples and controls was greatest in this fraction, it is possible that TH may exert direct influences over the levels of cell surface recognition molecules during the development of astrocytic cultures.

It would have been of interest to monitor how this stimulation altered with age in culture, and to identify effects on specific glycoproteins. Although attempts at the isolation and identification of specific glycoproteins were commenced, time was not sufficient to produce many meaningful results. This would have to be continued in any future work to determine whether specific glycoproteins are affected, or whether an affect on all fucose incorporating glycoproteins is involved. Likely candidates for glycoproteins into which fucose may be incorporated include the cell adhesion molecules (AMOG, L1 and L2/HNK1), extracellular matrix molecules like fibronectin, lamellin or the endogenous lectins (e.g. thrombospondin), or possibly even one of the enzymes responsible for the in situ synthesis of the carbohydrate side chains (also involved in the mechanisms of cell-cell interactions) (345). Another possibility is the 190 kDa fucosylated glycoprotein found by Barbin et al. to be expressed in a region-dependent manner by astrocytes in culture (377).
Attempts were made to clarify the actual mechanism by which TH stimulated fucose incorporation into astrocytic glycoproteins. Stimulation may be due to an increase in the production of a protein precursor, either due to stimulation of the transcription of the particular gene (or genes) involved, or else as the result of stabilisation of the mRNA produced. A second explanation may be that the enzyme (or more correctly, group of enzymes) responsible for fucosylation, \(\alpha\)-fucosyltransferase, is up-regulated, which could be accomplished by an increase in the production of the enzyme in response to TH administration. Attempts to establish a working assay for the measurement of \(\alpha\)-fucosyltransferase activity were unsuccessful, and would be a priority of future investigations. Alternatively, it may be that TH either acts as a co-factor for fucosylation, or stimulates the synthesis of a co-factor. The former of these possibilities would seem unlikely, owing to the time delay of at least 6 hours before any effect becomes noticeable. The final possibility would involve a stabilising effect of TH on existing glycoprotein, effectively blocking turnover.

Assessment of the activities of the enzymes responsible for breakdown of fucosylated glycoproteins, specifically \(\alpha\)-fucosidase, would be necessary to test the possibility of a stabilising effect. In order to supplement this approach to allow for the possibility of a different mediation of stabilisation, pulse-chase experiments could be performed, entailing incubation with \([^{3}\text{H}]-\text{fucose}\), followed by \([^{14}\text{C}]-\text{fucose}\), with the ratio of the two incorporated being compared in T3 treated and untreated cultures. If the effect is due to the blocking of turnover, a lower \(^{14}\text{C}:^{3}\text{H}\) ratio would be expected in the T3 treated samples after an appropriate period.

The question still remains as to whether events which occur in postnatal glial development, associated with the formation of neural patterns, and the delineation of glial boundaries, are of importance in early development, and in what manner defects in these patterns may result in the dysfunctions involved in neurological cretinism. Although such boundaries are more obvious during postnatal development, when the majority of glial proliferation occurs (346), glial cells are also of importance during much earlier stages of embryogenesis, particularly in relation to the establishment of neurons into similarly related units (345). As TH influence the expression of potentially critical glycoproteins during the postnatal period, it is not unlikely that they are involved at earlier stages, and GFAP containing cells have been detected as early
as week 7 in the human (347). If glia, or their neuroepithelial precursors, are the targets of TH action at early stages by such mechanisms, the subsequent effect may be to either restrict neurite outgrowth to the relevant nucleus, or conversely to enable further development. Equally, controls may be exerted on the intrusion of afferents from different regions. As previously suggested, any failure of such mechanisms could have catastrophic consequences.

The long term nature of these putative effects would be mediated by influences on the extensive interaction occurring between brain cells during development. Glial cells, particularly astrocytes, long thought to simply serve homeostatic functions in the brain are now recognised to play a crucial part in neuronal development both in vivo (237), and in culture, where the production of neurotrophic factors has been suggested (348,349).

The potential for astrocytic involvement in precisely timed interactions with other cells, having a critical bearing on the outcome of neural development, is illustrated by what is known about the control of astrocyte ontogeny itself. It is now widely accepted that several different astrocytic types exist (350). In the optic nerve population of astrocytes, Raff et al. have shown that the type 2 astrocyte progenitor (O2A) will develop into a type 2 astrocyte in the presence of FCS, but into an oligodendrocyte in its absence (351). This process is apparently controlled by an amino acid dimer secreted by type 1 astrocytes, plasma derived growth factor (PDGF), which acts as a mitogen for a set duration until its action is prevented in an unknown manner, and differentiation is allowed to commence, stimulated by the release of another peptide associated with ciliary neurotrophic factor (CNTF).

Many different types of interaction may occur in this manner between neuronal and glial cells, and in addition to involving secreted trophic factors, direct surface interactions, or the operation of extracellular matrix molecules such as the neuron-glial adhesion glycoprotein, J1/tenascin (345,377). It is also likely that such actions are region specific, as astrocytes from different regions vary in features of their particular biochemistry, including expression of marker enzymes such as glutamine synthetase (GS), and GFAP (345,377).

Although the notion of a TH effect on neural development mediated via glycosylation is attractive, there are many other mechanisms by which the biochemical and structural deficits associated with maternal hypothyroxinemia could be brought about. TH deficiency may also affect any one, or possibly all, of the
range of direct effects on developing glia, much of which has been referred to previously (Results 2), and that these may contribute to the observed long term defects. The limited studies of fucose incorporation in neuronal culture did not indicate stimulatory effects of TH, suggesting a cell specific targeting of this TH action in developing brain. However, it is possible that differences occur over a different time scale, and a direct effect on neuronal development early in development cannot be ruled out. The sequential expression of a number of glycoproteins has been demonstrated on the surfaces of developing neurons, and influences of TH on this process are possible (352).

Another method by which cell-cell interaction may be mediated other than through the interaction of glycoconjugates, is by modification of regulatory proteins by phosphorylation (353). Such activity is not confined to the membrane, but also acts at the levels of the nucleus, influencing the activities of transcriptional regulators, for example; and in the control of cytoskeleton formation. In this context, it is of interest that 10 nM T3, administered to astrocytes in culture, has been reported to stimulate the phosphorylation of specific proteins (354). Possibly connected with this is the observation that a fast (<4 h) decrease in phosphorylation of a 19 kDa protein in response to T3 is associated with an increase in the detection of a 45 kDa protein, identified as β-actin, in the cytosol of astrocytes, suggesting that the degree of actin polymerisation may, in some circumstances, be temporally slowed upon T3 administration. Incubation for longer periods appears to reverse this phenomenon (354).

It is conceivable that one or any combination of these putative actions of TH may be operative in the early stages of fetal brain development where maternal T4 is the only source of TH. If this is the case, there would be considerable potential for failure in the role of astrocytes in neuronal migration and survival (355), neurite outgrowth (356), neuronal differentiation and proliferation (357), and differentiation of oligodendroglia (358); in addition to possible damage intrinsic to the astrocyte population itself. Such failures may lead to neuronal loss, and/or disruption of the direction of cell growth and metabolism which persist into the neonatal period and beyond. Given the spread of biochemical, morphological, and functional parameters adversely affected in neurological cretinism, it would seem plausible that a "catastrophic" injury mechanism, operating during a critical developmental window
for TH action, may explain many of the observed effects.

6.3 Neonatal nuclear T3 binding

The ontogenesis of the THNRs in the whole fetal brain has been well documented (61,289, 293-6) Although evidence from work in vitro has indicated an affect of TH on glia, it had not previously been possible to examine the in vivo distribution of THNR between the different cell types during the neonatal period. Consequently it has been widely assumed that as THNR levels are low in glial nuclei from adult brain, then this must also be the case in the developing brain (289). Following the results indicating clearly significant effects of TH on astrocytes in culture, it became important to determine whether glial cell types in vivo possessed THNR which actively bound TH at early stages of development. Had this not been the case, then a non-nuclear mechanism for TH stimulation of fucose incorporation would have been indicated, in spite of the long time course involved.

Although it would have been ideal to perform these experiments in animals before the onset of fetal thyroid function, it was impossible to separate distinguishable nuclear types from the brain this early in development. It was already known that whole fetuses and whole brains from later in gestation possess THNR's before the fetal thyroid begins to secrete TH (296,297), and as in very early development, it was not possible to confidently identify individual cell types in sufficient quantities, it was decided that little further information could be obtained from such a study. The advantage of using neonatal animals was that neuronal and glial nuclei could be separated, distinguished, and studied in an ontogenic manner through a period of considerable growth and differentiation.

In due course, it was intended to progress to comparing the distributions and development of receptor concentrations in normal animals with those separated from TD progeny. Certainly, a lack of ability to respond to TH during critical sensitive periods might explain the gross biochemical differences observed in the progeny of hypothyroxinemic rat dams.

The results indicated varying concentrations of THNRs in different cell types, and that cortical glia possess very high levels of binding, despite the paucity of these in the adult (359,360). This confirmed that it is possible for different cell types in
vivo to exhibit TH sensitive periods at specific stages of their development. It is proposed that if this is the case in the neonate, it is possible that groups of cells at very early stages of development, including both neurons and glia, may be critically responsive to TH. The overall binding levels, albeit low found in whole fetus, or whole brain before the onset of fetal thyroid function are merely average values, and are likely to be composed of a small number of highly TH sensitive cells containing a high concentration of THNRs, rather than a large number of cells with just a few receptors.

Support is lent to these findings by studies performed in culture. Astrocytes grown in relatively pure cultures have been demonstrated to possess nuclear T3 receptors, both in binding studies, and by immunocytochemical staining with monoclonal antibodies (292,361). It has been demonstrated that the proportion of cells in culture containing receptors increases over the first two weeks of development from 49% at day 3, to 75% at day 15, and as many as 60% of fibrous astrocytes already had THNR at day 3. As it is known that this type of cell disappears from culture shortly afterwards, possibly becoming polygonal "protoplasmic" astrocytes (362), this emphasises the possibility that TH might act on particular cells at particular developmental stages, giving the appearance of a developmental window for TH responsiveness. Involvement of T3 in such developmental processes is likely to be of importance for the outcome of neural differentiation.

The high capacity of glial nuclei for T3 binding during postnatal development was previously unknown. Glial binding capacity at day five was almost twice that of neuronal nuclei, diminishing until it dropped below neuronal capacity at day 21. Regional studies revealed that cortical glia account for the majority of glial binding, suggesting that the peak in total nuclear T3 binding capacity noticed by many groups at approximately day 5-6 of development (123,182,298) is the result of a high capacity of cortical glia for T3 binding during the neonatal period. The subsequent fall in overall binding capacity in both total brain nuclei, and in glial nuclei separated from whole rat brain, seems likely to be the result the acquisition of glial cells possessing fewer THNR's, particularly in the cerebellum, but also in the midbrain and brainstem. However, as regional variations could not be satisfactorily ascertained before day 15, down-regulation of receptors per nucleus at earlier stages of development cannot be ruled out.
High levels of THNR's in both cortical glia and neurons from whole brain are not retained into adulthood (262). This suggests a dual role for nuclear T3 binding in both cell populations: developmental regulation, requiring the intense stimulation of the protein synthesis necessary for proliferation and differentiation; and a homeostatic role in a fully differentiated cell population. Whether the decrease in capacity between the neonatal period and adulthood is the result of down regulation of receptors, an increase in the proportion of cells containing fewer THNR's, or alterations in receptor function, remain to be investigated.

These findings suggest that levels of THNR in glia are sufficient to account for direct thyroid hormone control of important features of glial development. The postnatal surge in THNR levels may be associated with oligodendroglial proliferation and the onset of myelination, rather than the terminal differentiation of neurons. This runs contrary to the accepted view that TH mediate their effects mostly through neuronal binding in the brain, and that effects on glia are indirect (359). It would also appear that postnatal alterations in the numbers of receptors per nucleus may be smaller than had been previously thought (289).

The possible involvement of TH in myelinogenesis is supported by evidence that oligodendroglia in culture possess high levels of THNR up to the end of proliferation (291) and that myelination both in vivo and in myelinogenic cultures is profoundly affected by hypothyroidism (117). It is likely that the glial nuclear fraction isolated in this study is predominantly oligodendroglial in origin (264), but astrocytic nuclei are inevitably also present. It would be necessary to separate these two types of nuclei to confirm the presence of THNR in each of these populations.

Elevated levels of THNR are associated with neurons in regions responsible for higher mental function in adult rat brain (164), and in species more advanced in evolutionary terms (163). In the neonate, the highest density of receptors was found in both neurons and glia from the cerebral cortex, with the receptor concentrations of both decreasing in a cranio-caudal direction. Arguably, the acquisition of increased thyroid hormone sensitivity in both cell types may have assisted in the refinement of neuron/glia co-operation at critical stages during development, including both the neonatal period and earlier, or else been responsible for important functional changes in the individual cell types. In the cerebellum, which is known to be sensitive to thyroid hormone levels during development (363), and where almost all development
occurs postnatally, relatively low THNR levels are evident. It therefore seems that a widespread and direct effect of thyroid hormones on cerebellar glia during development is unlikely. Although it is feasible that cerebellar dependence on TH may be mediated through some other mechanism than through binding to the nuclear receptor, it has been suggested that a small sub-population of cerebellar neurons, such as the Purkinje cells, are critically responsive to thyroid hormone, and consequently responsible for further cerebellar development (123,363). It is proposed that a similar state of affairs may operate in the fetal brain very early in development.

Several groups have noticed an increase in affinity in nuclei isolated from whole rat brain during the neonatal period (293,298). In nuclei separated from whole brain, the affinity of the glial receptors for ligand appeared to increase abruptly between days 15 and 20. In glial nuclei isolated from separated brain regions over the same period, however, it was not possible to demonstrate a comparable increase in affinity. When the Kd's of glial nuclei from whole brain are regarded together, the data from day 15 seem to be at odds with the pattern of a general increase in affinity over the neonatal period. As the Kd value in glia from day 21 is also significantly lower than that from day 5, it may in fact be the case that the change in affinity occurs more gradually over the postnatal period.

Changes in binding characteristics have been cited as evidence for the operation of functionally different receptors (295). The findings, presented in this thesis, of altered affinity and the suggestion of changes in specificity for T3 analogues between the start and end of the neonatal period might indicate that at least two receptors may be operative in both the neurons and glia during development. It is interesting that Perez-Castillo et al. also found that THNR from 14 day old rat fetuses exhibited identical affinity for T3 and TRIAC (289), even though it is recognised that in adult brain THNR show 2-3 times the affinity of T3 (89). In my experiments, however, despite the presence of a discernible trend, transformed data from the proposed "transitional" periods (around day 15) were insufficient to convincingly demonstrate the presence of more than one class of receptor. It is still possible, however that different THNR subtypes may be involved during development in the brain. In order to appreciate the potential for additional developmental control offered by a variety of THNR isoforms it is necessary to outline some details of the structure and operation of the THNR.
A number of \textit{v-erb-A} related genes have now been identified on human chromosomes 3, 5, 17, and 19, and products are differentially expressed between tissues (364). Two main types of the THNR genes have so far been noted (c-erb-A\(\alpha\) and \(\beta\)), plasmids containing each of these prepared, and mRNA species raised from such clones (67,68,73,364-366). On the basis of the amino acid sequence of the products of \textit{in vitro} translation, both genes have been subdivided into two further subclasses. c-erb-A\(\alpha1\) mRNA has been demonstrated in the skeletal muscle and brown fat of the rat, whilst the \(\alpha2\) product has been found in the brain and hypothalamus (69). The translated protein of c-erb-A\(\alpha1\) mRNA binds TH in competition experiments with a dissociation constant (Kd) of 0.2 nM, which corresponds to the values found \textit{in vivo}, whilst that of \(\alpha2\), the product of alternative splicing of the same mRNA which encodes the \(\alpha1\) receptor, does not bind ligand at all. Despite this, \(\alpha2\) may still play a physiological role in terms of competition for binding to the hormone response element (367).

The c-erb-A\(\beta2\) gene is reported to be pituitary specific and it product differs from the \(\beta1\) only in its amino terminus (364). \(\beta2\), however, is down-regulated by T3 in the pituitary, whilst \(\beta1\) is up-regulated (364). The proportion of which type is produced is thought to be determined by either the operation of alternative promoters, or by post-transcriptional processes. These processes may be tissue, or cell type specific and it seems likely that the two products would differ in their mediation of TH regulation of gene expression possibly acting oppositely, or on different genes (364,368).

It has been proposed that the heterogeneous receptors so far identified may all play physiologically distinct roles (368). Recent work has demonstrated that very small changes in the "zinc-finger" DNA binding domain of the THNR are able to affect target specificity, either directly through response element recognition, or indirectly via protein-protein interactions (368,369). Similarly differences, either in the ligand binding region, or elsewhere, with resulting allosteric changes, may cause an alteration in binding kinetics, such as found in my experiments. It is possible that this is due to the temporally-specific expression of different receptor subtypes. It is therefore of particular interest that very recent work has demonstrated contrasting developmental and tissue-specific expression of the c-erb-A\(\alpha\) and \(\beta\) genes (370). These workers have shown an association between the levels of \(\beta\) mRNA and
development in the brain, whilst proposing a role for the α product in metabolism. However, they do not rule out a developmental role for the α receptor at some point. No cell specificity was determined in these experiments, but the abrupt rise of the level of expression of the β receptor in chick brain occurs at a comparable time to the high levels of glial binding demonstrated in this thesis, giving cause for speculation that this receptor subtype may be glial-specific.

The data from extracted receptors may not support this hypothesis, as declines in binding are only significant in neuronal extracts. It is possible, however, that the absence of chromatin from such extracts alters the binding parameters found in intact nuclei. Several groups have demonstrated that the presence of chromatin is necessary for optimal binding of hormone to the THNR (80,371), and De Nayer and Dozin van Roye have suggested that chromatin associated factors may vary throughout development, influencing the affinity of the receptor in the process (371). It has consequently been suggested that the neonatal fall in Kd is only apparent in intact nuclei, disappearing in receptors extracted from nuclei with high salt (371). In fetal sheep, however, Ferriero et al. also noticed significant alterations in receptor affinity in the brain with extracted receptors (294), supporting the observations in this thesis, but these changes may not necessarily reflect in vivo parameters. It is also of interest that glial extracts exhibited diminished stability of binding relative to the neuronal extracts, possibly indicating cell-specific binding characteristics.

In addition to developmental alterations in the levels of THNRs, many other factors may influence the response of individual cells to TH. Reports suggest that the degree of receptor occupancy varies during development, and is at a peak during the period from 6 to 15 days in nuclei separated from whole brain (372), coinciding with a rise in both plasma T3 concentrations (total and free), and a rise in cytosolic T3 concentration (328). Cerebral T3 and T4 levels rise sharply during this period, also, again peaking at day 15 (328). It has been reported that, in the later stages of brain development, plasma T3 contributes little to brain T3 (31). In hypothyroid conditions, the activity of 5'-DII is much increased in this organ, and it has been noticed that this increase in activity is more sensitive to diminished concentrations of T4 in the brains of the progeny of hypothyroxinemic mothers (178). This could have developed as a response to a lack of maternal T4 in early gestation. T4 concentrations in fetal plasma may therefore be very low in early gestation, indeed too low to
produce an increase in fetal pituitary TSH, yet are still sufficient to normalize brain T3 levels, due to the specific mechanisms responsible for local concentration and generation of the hormone.

Ontogenic variations in the availability of hormone to the nuclear receptor, due to mechanisms such as; cell-specific metabolism of thyroid hormones, cytoplasmic transport, nuclear retention mechanisms (104) and prevailing plasma hormone availability (372,328), are also likely to be involved in the optimisation and specificity of hormone action. However, the fact that levels of receptors themselves have been shown to be up-regulated in hypothyroid, and down-regulated in hyperthyroid rats during the neonatal period, suggests that the level and nature of receptors is a primary indicator of TH sensitivity (373). Post-binding events may also be important in TH control of developmental gene expression, as recent work has suggested the possibility that cofactors may influence binding of the hormone-receptor complex to the hormone response element of the DNA (374).

On a more general level, TH have also been observed to influence chromatin structure on a much larger scale during development. Cortical neurons undergo a shortening of the chromatin repeat length (the average distance between nucleosomes) at the onset of terminal differentiation (375). Deprivation of TH in culture abolishes this transformation, whilst replacement of T3 in the medium results in shortening occurring in the usual way (376). The effect, however, is delayed by a constant period of 15 days, suggesting that the initial exposure to T3 instigates a sequence of dependent molecular events, culminating much later in this change. The significance of the effect is unknown, although chromatin repeat length influences the degree of compaction of DNA, and therefore to some extent, its availability for transcription. If the same time lag pertains in vivo as in vitro, this would place the necessary exposure date to cause the effect at terminal differentiation at approximately fetal day 15. As this is before the onset of fetal thyroid function, only maternal hormone could be responsible.

There is therefore considerable evidence for the involvement of TH in the differentiation and development of all three major neural cell types, both independently, and in co-operation with other hormonal influences. As THNR are present in glia in abundance during development, it seems plausible that they may be present at earlier stages, and the evidence of regional differences in binding show that
it is possible for cell populations to be differentially affected. The developmental alteration in affinity may have physiological significance, and evidence that changes persist in extracts points to differences in the properties of the receptors themselves, although other factors cannot be ruled out. The early presence of THNR; their cell specific, temporal regulation; and the availability of a variety of putative targeting and regulatory mechanisms, all lend support to the argument that the levels of maternal hormone found in the early embryo may be biologically important for development, and rate limiting in terms of subsequent expression of functions in the adult.

6.4 Summary

In conclusion, an experimental model of maternal hypothyroxinemia mimicked the thyroid hormonal levels found commonly in pregnant women in iodine deficient endemias. A range of gross biochemical differences were noted in the rat progeny of partially thyroidectomised dams in a number of organs, including the brain, and the acquisition of protein, both on a total and specific level, was adversely affected.

Glycoproteins, particularly those located in the cell membrane are critical for the determination of cell-cell recognition and interaction in the developing brain. Consequently, although alterations recorded at day 14 could not have been a primary effect of maternal hypothyroxinemia, it was considered plausible that TH may act directly in the control of expression of some of these molecules at earlier stages of development. When investigated in cell culture, this appeared to be the case, with TH at 10 nM stimulating the incorporation of [3H]-fucose into the protein fraction of developing astrocytic cultures. Closer investigation revealed that a high proportion of the labelled sugar became localized in the plasma membrane, and that the stimulation of its incorporation was disproportionally higher in this fraction. It therefore seems reasonable to suggest that the mechanisms controlled by these cell surface molecules are possibly affected by TH at early stages of development, including before the onset of fetal thyroid function.

In a parallel study, the mechanisms via which TH action is mediated were studied in normal neonatal animals. Essentially, this was intended to determine whether glial cells in vivo possessed significant levels of TH nuclear binding during their development, as well as opening the way forward to studying the characteristics
of THNR binding in the progeny of hypothyroxinemic dams. The work showed that THNR levels alter considerably in different cell types during development. During the neonatal period, glial cells particularly in the cerebral cortex, possessed high levels of THNR, although it is known that these are lost later in life. The decline over the study period, however appeared to be due to increases in the proportion of glial cells containing fewer nuclear receptors, rather than a decrease in the numbers of receptors per cell. This implies that cell populations are temporally dynamic in their abilities to respond to TH and vary their demand for TH in a region-specific, as well as time-dependent manner. Measurements of whole brain, and whole body THNR levels may conceal the true nature of TH control, and it is feasible that small populations of cells may be highly responsive to TH action before the onset of fetal thyroid function. It is still of interest, however that THNR are present in tissues well before the onset of fetal function, and have been detected as early as day 13 in whole rat embryo, and day 14 in the fetal rat brain. The fetus therefore possesses the capacity to respond to TH, the presence of the hormone in its tissues, and the ability to concentrate and localize its action.

The alterations in binding affinity noted may suggest the operation of different receptors. Given the known heterogeneity of the gene products of the c-erb-A protooncogene, encoding both active and inactive, as well as organ specific THNR's, it is possible that ontogenic variations in receptor subtype may be involved in the mediation of thyroid hormone action in the developing brain. It is, however, important to remember that a number of other factors also play an important part in a cell's ability to respond to TH, and the nature of the effect elicited. These include differing plasma transport capabilities; levels of metabolism; capacity for localization: or different capabilities at the site of hormone action, including levels of nuclear receptors or transcriptional co-factors. All of these may vary according to genetic (and possibly environmental) differences between individual fetuses.

6.5 Future work

Several avenues of possible future work opened up as a result of these studies. On the further use of the experimental model of maternal hypothyroxinemia, considerably more data could be obtained on a wide variety of biochemical parameters, and the work is being continued. Further work would be desirable on
the areas where limited experimental numbers prevented concrete conclusions from being drawn, such as effects on the developing lung, and on the precise nature of the glycoproteins found to be affected.

The mechanisms by which TH increases incorporation of $[^{3}\text{H}]$-fucose into glycoproteins are yet to be determined. Some essential experiments were begun, and it is necessary to complete these if the affected glycoproteins are to be identified, and their role in development ascertained. Despite this, one putative mechanism has been identified through which TH levels at critical stages in development ("developmental windows") may, at least in part, be responsible for long term, and irreversible effects on the developing brain.

Studies at the molecular level are additionally required if the nature of the receptors at which TH operate are to be fully understood. Alterations in the pattern of expression in terms of total receptor concentrations present in cell types at different stages in development would be likely to affect the ability to respond to TH. Whether long term alterations in receptor levels are present in the TD progeny should therefore be studied. Additionally, now that possible alterations in the expression of receptor subtypes have been identified, it may be possible to examine the expression of these subtypes using the techniques of molecular biology. Probing the developing brain using cDNA's specific to mRNA's for the different receptor isoforms (possibly by \textit{in situ} hybridisation) would be the only way to satisfactorily determine whether different receptors are involved in specific cell types, or brain regions at different stages of development. Work in this area is currently underway in this laboratory.
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9. Hubank M, Sinha AK 1988 L-Triiodothyronine (L-T3) stimulates the incorporation of L-[5,6-3H]-fucose into glycoproteins in developing primary astrocytic cultures. Annales d'Endocrinol 49:139 Abstract.
Nuclear tri-iodothyronine (T₃) binding in neonatal rat brain suggests a direct glial requirement for T₃ during development


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ABSTRACT

Tri-iodothyronine (T₃) binding studies were performed on neuronal and glial nuclei prepared from developing rats brain by discontinuous sucrose gradient centrifugation. Maximum binding capacities (MBC) and dissociation constants (Kₐ) were obtained from Eadie-Hofstee plots of transformed data. An ontogenic study on nuclei prepared from whole brain revealed that on day 5 after birth, glial nuclear MBC was 1774 ± 201 fmol/mg DNA compared with 974 ± 117 fmol/mg DNA for the neurones (P < 0.01). Although diminishing to 667 ± 112 fmol/mg DNA by day 21, alterations in neuronal MBC over the neonatal period were not statistically significant, whereas glial MBC diminished steadily to 557 ± 133 fmol/mg DNA in glial nuclei (P < 0.05). Over the same period, a significant reduction in Kₐ was noted only in the glia, from 3.17 ± 0.40 to 1.83 ± 0.34 nmol/l (P < 0.03). Ligand specificity of the receptor in both nuclear types on day 21 was tri-iodoacetic acid > T₃ > thyroxine > 3,3',5'-T₃ , but this was less clearly demonstrated at day 5.

Regional studies on days 15 and 21 demonstrated that for both neuronal and glial nuclei, receptors are concentrated in the cerebral cortex and diminish in a cranio-caudal direction. Cerebral glial MBC on day 21 was 2215 ± 147 fmol/mg DNA, at this stage still exceeding the cerebral neuronal capacity of 1111 ± 207 fmol/mg DNA. The results indicate that neonatal glia may respond directly to thyroid hormones via nuclear receptor binding, and that receptors are predominantly located in the cortex. Decreases in average MBC in the late neonate may be due to increases in the numbers of cells containing fewer nuclear receptors.

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INTRODUCTION

The ontogeny of the binding of thyroid hormones to nuclear receptors in brain has been investigated in a variety of species, including rats (Schwartz & Oppenheimer, 1978; Valcana & Timiras, 1978; Perez-Castillo, Bernal, Ferreiro & Pans, 1985), sheep (Ferreiro, Bernal & Potter, 1987; Polk, Chermocka, Revicszky & Fisher, 1989) and man (Bernal & Pekonen, 1984). A pattern has emerged which indicates a peak in binding capacity at comparable stages in the development of each species. In the rat this occurs on day 6 after birth and gradually diminishes to adult values (Dussault & Ruel, 1987). This has been taken to indicate the degree of thyroid hormone responsiveness of the tissue over this period (Schwartz & Oppenheimer, 1978; Dussault & Ruel, 1987). In adult brain, thyroid hormone nuclear binding has been shown to be localized predominantly in the neurones (Yokoto, Nakamura, Akamizu et al. 1986; Gullo, Sinha, Woods et al. 1987a). It has therefore been assumed that the peak in binding during development is also neurone associated, and involved with terminal differentiation, with which it coincides (Nunez, 1984; Perez-Castillo et al. 1985). Thyroid hormone effects on glia at this stage have been considered to be secondary (Nunez, 1984), despite reports of high levels of expression of the thyroid hormone nuclear receptor (THNR) in such cells in vitro (Yusta, Besnard, Ortiz-Caro et al. 1988; Luo, Puymirat & Dussault, 1989). In addition, it is known that neonatal thyroid hormone deficiency results in impairment of glial development, including myelination (Timiras, 1988). Since the glia undergo rapid division and...
differentiation in the neonatal rat, it is possible that these processes are under some degree of direct thyroid hormone control. It has been acknowledged that in order to identify the precise targets of thyroid hormone during this period, it is necessary to separate and study nuclei from the different cell types involved (Perez-Castillo et al. 1985).

Recent availability of specific probes for the detection of the mRNA subtypes for the THNR have enabled the demonstration of expression of the c-erb-A proto-oncogene in several tissues (Umesono & Evans, 1989). Conventional ligand-binding techniques, however, provide quantifiable information reflecting the function of receptors in tissues. Consequently we have developed a reliable method, based on density gradient centrifugation, for the isolation of neuronal and glial nuclei from developing rat brain, and studied the nuclear binding of thyroid hormones at several stages in neonatal development, both in whole brain and in brains dissected into three major anatomical regions: cerebral cortex, brain stem and cerebellum. A preliminary report of this work has been presented elsewhere (Hubank, Sinha, Gullo & Ekins, 1989).

MATERIALS AND METHODS

Materials

$[^{125}\text{I}]$Tri-iodothyronine (T$_3$) at a specific activity of >2800 Ci/g was purchased from Amersham International (Amersham, Bucks, U.K.). L-T$_3$, L-thyroxine (T$_4$), reverse T$_3$ (rT$_3$; 3,3',5'-T$_3$) and tri-iodoacetic acid (TRIAC) were obtained from Henning (Berlin, F.R.G.). Dowex AG-1 x 8 anion-exchange resin (chloride form, 200 mesh) was purchased from BioRad Ltd (Hemel Hemstead, Herts, U.K.). All other chemicals were of analytical grade.

Animals were the progeny of normal female Sprague-Dawley rats bred in our own animal house or, on occasion, supplied by Charles River Ltd (Margate, Kent, U.K.).

Isolation of neuronal and glial nuclei

The method employed is a modification of the procedure described previously for adult rat brain (Gullo, Sinha, Bashir, Hubank & Ekins, 1987a; Gullo et al. 1987b), and extends the exploitation of differing densities of neuronal and glial nuclei to the developmental period. For each preparation, the brains of three to six animals were combined. Animals (aged 5, 11, 15 and 21 days) were killed by decapitation and the brains removed and cleaned of blood and pia matter. All procedures were performed at 4 °C. Whole brains were soaked for 10 min in sucrose (0-25 mol/l) containing MgCl$_2$ (10 mmol/l), chopped finely and soaked for a further 10 min, then homogenized in the same solution. The homogenate was filtered twice through nylon bolting cloth (110 μm), and once through steel gauze (39 μm), and the filtrate centrifuged at 700 g for 10 min. The crude nuclear pellet obtained was resuspended in 2-0 mol sucrose/l (approximately 5 vol.) and an aliquot (8 ml) layered into a discontinuous sucrose gradient of 2-2 mol sucrose/l (8 ml) and 2-0 mol sucrose/l (5 ml).

The gradient was centrifuged at 104 000 g for 30 min, after which four distinct layers were apparent. The top layer consisted of cell debris (myelin, organelles etc) unable to penetrate dense sucrose after centrifugation. The interface between the homogenate layer and the 2-0 mol sucrose/l yielded mostly small round acidophilic nuclei, with <5% larger ovoid nuclei adjudged to be capillary endothelial nuclei. The layer between the 2-0 mol sucrose/l and 2-2 mol sucrose/l consisted predominantly of large spherical nuclei with distinct nucleoli (some multinucleolate) and less granular, lightly-stained nucleoplasm. These were considered to be mature neuronal nuclei. The pellet contained smaller basophilic nuclei with granular nucleoplasm and intensely staining diffuse nucleoli adjudged to be predominantly glial nuclei. The technical basis for this method of separation has been reviewed (Guroff, 1980).

Cross-contamination of the neuronal layers with glial-type nuclei was <5% and of glial with neuronal nuclei, 10%. However, due to the developmental period being investigated, blast nuclei in various stages of division are also inevitably present. The majority of these are of glial type, and since they resemble the mature glial nuclei they are located in the pellet. As most neuronal development is complete by birth in the rat, blast nuclei of this type are considered to be relatively few; however contamination of the pellet with microneuronoblast nuclei, which are indistinguishable from glial nuclei, cannot be ruled out. Microscopic analysis was performed as previously described (Cragg, 1967; Sinha, Rose, Sinha & Spears, 1978).

Regional distribution

Experiments were performed to determine the regional distribution of neuronal and glial T$_3$ binding at two stages of development. The number of differentiated nuclei is small in the cerebellum before day 15, leading to problems with identification and estimation of purity. Consequently it was decided to compare regional binding on days 15 and 21. Brains from six rats were dissected into three regions: cerebral cortex, cerebellum and the remainder—a broad region including brain stem, midbrain, amygdala, pons,
hypothalamus and other subcortical regions, for these purposes termed brain stem. The purified nuclei from two gradients were combined, and T₃ binding assays performed in duplicate as described below.

**T₃ Binding assay**

T₃ displacement binding assays were performed as previously described (Gullo *et al.* 1987b). Under these conditions, endogenous T₃ dissociation is regarded as complete, and thus no corrections are made for possible endogenous T₃ interference in the assay. DNA was extracted and measured (with minor modifications) according to the fluorometric method of Setaro & Morley (1976).

**Analogue studies**

The relative binding affinities of THNRs for the analogues TRIAC, T₄ and rT₃ were determined in intact neuronal and glial nuclei prepared from both 5- and 21-day-old animals. Briefly, 200 µl aliquots of nuclear suspension were incubated with a fixed concentration of [¹²⁵I]T₃ (1 x 10⁻¹⁰ mol/l), and concentrations of analogue ranging from 1 x 10⁻¹⁰ to 1 x 10⁻⁷ mol/l. The relative binding affinities were expressed as a percentage of [¹²⁵I]T₃ bound in the presence of increasing concentrations of analogue.

**Released receptor assay**

In some experiments, the degree of receptor leakage from intact nuclei was determined. After completion of the receptor-binding assay, and before the addition of Triton X-100, selected assay tubes were centrifuged and aliquots of the supernatant removed for separation of bound and free T₃ by the resin method (Bernal, Coleoni & DeGroot, 1978).

**Data analysis**

Binding parameters dissociation constant (K_d) and maximum binding capacity (MBC) were determined using transformed experimental data displayed as Eadie-Hofstee plots (related to Scatchard analysis). Linear regression analysis was used to determine the best fit between points. MBC was assessed from the intersection of the regression line with the y axis, whereas K_d was derived from the slope of the line (B = MBC - K_d (B/F)). Statistical analysis was performed using unpaired two-tailed t-tests, with significance defined as P < 0.05. Values are means ± S.E.M.

**RESULTS**

**Nuclear isolation**

Recoveries of nuclei fell from 70% at day 5 to 45% at day 21 as the proportion of extraneous matter increased, impeding movement down the gradient. An age-related increase was observed in the glial/neuronal (G/N) ratio, estimated from both nuclear counts and DNA levels (data not shown). These observations were expected, and add confidence to the separation procedure. In the regional studies, recovery was highest from the cerebellum, where the mean G/N was 2.4 at day 15 and 5.3 at day 21. These results are to be expected as glial proliferation increases over the neonatal period, and the cerebellum develops mostly postnatally.

**Whole brain studies**

Initially, T₃ binding was studied in nuclei purified from whole rat brains at 5, 11, 15 and 21 days after birth. Preparations of both neuronal and glial nuclei demonstrated low-capacity high-affinity binding at all stages studied. Data were transformed and plotted as
TABLE 1. Dissociation constant ($K_d$) values for nuclear tri-iodothyronine ($T_3$) binding in neuronal and glial nuclei isolated from developing rat brain. Nuclei were incubated with $[^{125}]T_3$ ($1 \times 10^{-10}$ mol/l) and increasing concentrations of unlabelled $T_3$ ($1 \times 10^{-10}$-$1 \times 10^{-7}$ mol/l) in binding buffer (37 °C, 30 min). Data were analysed using Eadie-Hofstee plots and results are means ± S.E.M.

<table>
<thead>
<tr>
<th></th>
<th>Neuronal</th>
<th>Glial</th>
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<tr>
<td>Day 5</td>
<td>2.26 ± 0.40</td>
<td>3.17 ± 0.40</td>
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<tr>
<td>Day 11</td>
<td>2.87 ± 0.31</td>
<td>2.77 ± 0.31</td>
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<tr>
<td>Day 15</td>
<td>3.57 ± 0.60</td>
<td>3.39 ± 0.24</td>
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<tr>
<td>Day 21</td>
<td>2.55 ± 0.58</td>
<td>1.83 ± 0.34*</td>
<td>6</td>
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* $P<0.05$ compared with day 5; ** $P<0.01$ compared with day 15 (unpaired Student's t-test).

**FIGURE 2.** Mean maximal binding capacities (MBC) of thyroid hormone nuclear receptor in preparations of neuronal (□) and glial (▲) nuclei at different stages of development. Each point represents the mean ± S.E.M. of the number of experiments given in Table 1. Each experiment was performed in triplicate, and intra-assay variation was < 10%.

B versus B/F. Such plots from days 5 and 21 yielded straight lines (Fig. 1), taken to indicate the presence of single classes of high-affinity binding sites in both neuronal and glial preparations. Data from day 11 in glia and day 15 in neurones suggested the possible presence of more than one class of binding site; however, the evidence was insufficient to conclude that this was the case.

Gial receptor affinity was significantly greater on day 21 than on either days 5 or 15, as evidenced by a drop in $K_d$ from 3.17 ± 0.40 nmol/l (day 5; $P<0.03$) and 3.39 ± 0.24 (day 15; $P<0.01$) to 1.83 ± 0.34 nmol/l on day 21. A comparable increase in affinity of neuronal nuclear $T_3$ binding was not detected. It is possible that variation in this category, which was found to be relatively high over the period studied, may mask such a change in affinity (Table 1).

MBCs for both neurones and glia were found to decline over the neonatal period towards adult values (Fig. 2). Glial $T_3$ binding considerably exceeds neuronal binding on day 5 ($1774 \pm 201$ compared with $975 \pm 117$ fmol/mg DNA), and proceeds to fall away at a greater rate than that of neuronal nuclei over the following days to $557 \pm 133$ fmol/mg DNA on day 21 ($P<0.05$). Allowing for variation, the concentration of neuronal nuclear receptors remains relatively steady over the neonatal period, and the apparent decline to $784 \pm 212$ fmol/mg DNA on day 21 is not statistically significant.

**FIGURE 3.** Specificity of tri-iodothyronine ($T_3$) analogue binding to nuclear receptors in separated neuronal nuclei from (a) 5-day-old and (b) 21-day-old rat brain. Increasing concentrations of 1-T$_3$ (▲), tri-iodoacetic acid (□), thyroxine (○), and reverse T$_3$, 3,3',5'-T$_3$ (■) were incubated with nuclei at 37 °C for 30 min with 1 $\times$ 10$^{-10}$ mol $[^{125}]T_3$.

Results are expressed as per cent $[^{125}]T_3$ specifically bound in the presence of analogue. Bound levels in the absence of competitor are designated as 100%.
TABLE 2. Distribution of thyroid hormone nuclear receptor in fractionated nuclei prepared from various rat brain regions at two stages of neonatal development. Nuclei were incubated with $[^{125}I]$tri-iodothyronine ($T_3$) ($1 \times 10^{-10}$ mol/l) and increasing concentrations of unlabelled $T_3$ ($1 \times 10^{-10}$–$1 \times 10^{-7}$ mol/l) in binding buffer (37°C; 30 min). Data were analysed using Eadie-Hofstee plots and results are means ± S.E.M.

<table>
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<th>Region</th>
<th>Day 15</th>
<th>Day 21</th>
<th>Day 15</th>
<th>Day 21</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>MBC (fmol $T_3$/mg DNA)</td>
<td>$K_d$ (nmol/l)</td>
<td>MBC (fmol $T_3$/mg DNA)</td>
<td>$K_d$ (nmol/l)</td>
</tr>
<tr>
<td>Cerebral cortex</td>
<td>Neuronal</td>
<td>1046 ±97</td>
<td>2.35 ±0.34</td>
<td>1111 ±207</td>
</tr>
<tr>
<td></td>
<td>Glial</td>
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<td>2.14 ±0.35</td>
<td>2215 ±147</td>
</tr>
<tr>
<td>Brain stem</td>
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<td>662 ±16*</td>
<td>2.46 ±0.39</td>
<td>640 ±40*</td>
</tr>
<tr>
<td></td>
<td>Glial</td>
<td>452 ±102**</td>
<td>1.86 ±0.50</td>
<td>619 ±141**</td>
</tr>
<tr>
<td>Cerebellum</td>
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<td>2.56 ±0.51</td>
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<tr>
<td></td>
<td>Glial</td>
<td>268 ±26***</td>
<td>2.06 ±0.29</td>
<td>158 ±65***</td>
</tr>
</tbody>
</table>

*P < 0.05; **P < 0.005; ***P < 0.0005 compared with cortical levels (unpaired Student’s t-test).

MBC, maximal binding capacity; $K_d$, dissociation constant.

Analogue studies

In competition experiments, the specificity of $T_3$ binding to receptors was studied in neuronal and glial nuclei from animals on days 5 and 21 (Fig. 3). Patterns of binding were similar in neurones and glia from each age. On day 21, the pattern of displacement of $[^{125}I]$tri-iodothyronine ($T_3$) by unlabelled analogue was $TRIAC > T_3 > T_4 > rT_3$, as is the case in adult rat brain (Gullo et al. 1987). On day 5, however, preferential binding of $TRIAC$ was less obvious ($TRIAC > T_3 > T_4 > rT_3$). The $TRIAC:T_3$ affinity ratio was approximately 2:1 on day 5 and 4:1 on day 21, although given the inherent variability of this type of experiment, data were insufficient to draw definite conclusions.

Regional binding studies

Nuclear $T_3$ binding was examined in three brain regions: the cerebral cortex, the cerebellum, and the remainder, classed broadly as brain stem (Table 2). No significant differences in MBC or $K_d$ between the two age points were observed for either neuronal or glial nuclei from any of the regions studied. Mean $K_d$s from all regions were 2.46 nmol/l on day 15 and 2.10 nmol/l on day 21 for neurones and 2.02 and 2.03 nmol/l for glia, close to those found in whole brain at day 21. Inter-regional differences in MBC were highly significant (Table 2).

In neuronal nuclei, the ontogenic decrease in average binding may result from a dilution effect exerted by increasing numbers of cerebellar microneurons and granule cells, which appear to possess fewer nuclear $T_3$ receptors. Cerebellar glial nuclear $T_3$ binding was found to be very low on both days, whilst cortical glia revealed an MBC for $T_3$ of $2215 ± 147$ fmol/mg DNA on day 21 compared with $1111 ± 207$ for cortical neurones at the same stage. It is possible that a proportion of the cerebellar glial binding detected here is due to contamination from microneuronoblast nuclei which are developing at this stage and are indistinguishable from glial nuclei using simple histological techniques.

Receptor leakage

Although not previously noted in rat brain to a large extent, it would be possible to argue that the differences in MBC and $K_d$ observed in whole nuclei during the neonatal period may be due to differential lability or release of the receptors during the incubation period. Possible leakage of the receptors into the incubation medium was therefore assessed.

Results indicated that receptor leakage accounted for less than 5% of total binding in cortical neuronal nuclei on day 15, and was undetectable in neuronal nuclei from brain stem or cerebellum, or in glial nuclei from any region (data not shown).

DISCUSSION

During the neonatal period, both neuronal and glial nuclei possess high levels of THNRs. Significant decreases in MBCs from whole brain were recorded over the study period in glial nuclei, whilst the neuronal decline was marginal. The receptor concentration is higher in the cerebral cortex for both neuronal and glial nuclei, and remains high over the period studied. The hierarchy of analogue binding to THNRs was identical in both nuclear populations.

This work was demonstrated, for the first time to our knowledge, the high capacity of glial nuclei for $T_3$ binding during postnatal development. Glial binding capacity on day 5 is almost twice that of neuronal nuclei, diminishing until it drops below neuronal...
capacity on day 21. Regional studies revealed that cortical glia account for the majority of glial binding, suggesting that the peak in total nuclear T₃ binding capacity noticed by many groups on approximately days 3–6 of development (Schwartz & Oppenheimer, 1978; Dozin-van-Roye & De Nayer, 1979; Perez-Castillo et al. 1985) is the result of a high capacity of cortical glia for T₃ binding during the neonatal period. The subsequent fall in overall binding capacity in both total brain nuclei, and in glial nuclei separated from whole rat brain, seems likely to be the result of the acquisition of glial cells possessing fewer THNRs, particularly in the cerebellum, but also in the midbrain and brainstem. However, as regional variations could not be ascertained satisfactorily before day 15, down-regulation of the receptor content of each nucleus at earlier stages of development cannot be ruled out.

High levels of THNRs in both cortical glia and neurones from whole brain are not retained into adulthood (Gullo et al. 1987a). This suggests a dual role for nuclear T₃ binding in both cell populations: developmental regulations, requiring the intense stimulation of the protein synthesis necessary for proliferation and differentiation; and a homeostatic role in a fully differentiated cell population. Whether the decrease in capacity is the result of down-regulation of receptors, a proportion of cells containing fewer THNRs, or alterations in receptor function remains to be investigated.

These suggestions are supported by evidence that oligodendroglia in culture possess high levels of THNR up to the end of proliferation (Yusta et al. 1988), and the myelination both in vivo and in myelogenetic cultures is profoundly affected by hypothyroidism (Timiras, 1988). Although the glia comprise a mixture cell types, it is likely that the corresponding nuclear fractions isolated in this study are predominantly oligodendroglial in origin, as the number of oligodendrocytes far outstrips the number of astrocytes at this stage of development (Sinha et al. 1978).

Raised levels of THNR are associated with neurones in regions responsible for higher mental function in adult rat brain (Gullo et al. 1987a), and in species more advanced in evolutionary terms (Wierich, Schwartz & Oppenheimer, 1987). In the neonate, the highest density of receptors was found in both neurones and glia from the cerebral cortex, with the receptor concentrations of both decreasing in a cranio-caudal direction. Arguably, the acquisition of increased thyroid hormone sensitivity in both cell types may have assisted in the refinement of neurone/glia co-operation at critical stages during development, or else have been responsible for important functional changes in the individual cell types. In the cerebellum, which is known to be sensitive to thyroid hormone levels during development (Legrand, 1979), and where almost all development occurs postnatally, relatively low THNR levels are evident. It therefore seems that a widespread and direct effect of thyroid hormones on cerebellar glia during development is unlikely. It has been suggested that a small sub-population of cerebellar neurones, such as the Purkinje cells, are critically responsive to thyroid hormone, and consequently responsible for further cerebellar development (Legrand, 1979; Dussault & Ruel, 1987).

Several groups have noticed an increase in affinity in nuclei isolated from whole rat brain during the neonatal period (Valcana & Timiras, 1978; Dozin-van-Roye & De Nayer, 1979). In nuclei separated from whole brain, the affinity of the glial receptors for ligand appeared to increase abruptly between days 15 and 20. In glial nuclei isolated from separated brain regions over the same period, however, we were unable to demonstrate a comparable increase in affinity. When the Kₐ of glial nuclei from whole brain are regarded together, the data from day 15 seem to be at odds with the pattern of a general increase in affinity over the neonatal period. As the Kₐ value in glia from day 21 is also significantly lower than that from day 5, it may in fact be the case that the change in affinity occurs before day 15. Although this explanation remains speculative, in a separate series of experiments (data not shown), we have also noticed a significant increase in affinity in salt-extracted THNR from both neuronal and glial nuclei.

It is interesting that the apparent increases in binding affinity observed in our experiments coincide with the end of cortical glial proliferation and the onset of myelination (Eayrs & Goodhead, 1959). Changes in binding characteristics have been cited as evidence for the operation of different functionally different receptors (Polk et al. 1989), although they may result from the action of different binding cofactors (Bismuth, Anselmet & Torresani, 1985; De Nayer & Dozin, 1985). Given the known heterogeneity
of the gene products of the c-erb-A proto-oncogene, encoding both active and inactive, as well as organ specific thyroid hormone receptors, it is possible that ontogenic variations in receptor subtype may be involved in the mediation of thyroid hormone action in the developing brain. The true significance of these results must remain open to interpretation until a comprehensive study of the ontogenic expression of THNR isofoms in the developing brain clarifies the issue.

ACKNOWLEDGEMENTS

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REFERENCES

Bernal, J., Coleoni, A. H. & DeGroot, L. J. (1978). Thyroid hormone receptors from liver nuclei: characteristics of receptors from normal, thyroidectomised, and triiodothyronine treated rats; measurement of occupied and unoccupied receptors and chromatin binding of receptors. Endocrinology 103, 403-413.


INTRODUCTION

For a number of years, the consensus view amongst endocrinologists has been that thyroid hormones of maternal origin neither cross the placenta in significant amount, nor are implicated in the development of the fetus in general, or of the fetal CNS in particular. Nevertheless, for a number of reasons (some of which have been discussed in), the validity of this view appeared to us to be open to doubt; we therefore initiated studies (ca 1980) specifically intended to verify or disprove some of the experimental evidence and theoretical postulates on which this view was based.

Results of our preliminary studies immediately confirmed the suspicion that previous work on thyroid hormone (TH) transport from mother to fetus was either irrelevant to the events occurring in early pregnancy (having been confined to animal studies late in pregnancy - ie following development of the fetal thyroid gland) - or, in the case of the relatively few studies conducted during early pregnancy, had been misinterpreted or ignored.

Aside from the questionable nature of past experimental evidence relating to this issue, the principal explanation offered for the supposed absence of placental TH transport appeared equally unsound, albeit superficially persuasive. This relied on the proposition that thyroxine binding globulin (TBG) and the other TH binding proteins (thyroxine-binding pre-albumin (TBPA) and albumin) present in maternal serum prevent passage of TH from maternal to fetal circulations. This concept reflects a view widely held amongst endocrinologists, ie that TBG and other specific hormone binding proteins in serum serve to minimise hormone loss from the vascular compartment and, indeed, that this constitutes their principal physiological role.

The serum concentration of TBG rises approximately 2-3 fold in human pregnancy; the concentrations of some of the specific steroid binding proteins likewise rise, albeit - in certain cases - to a greater factorial extent. Speculation on these proteins' physiological role has therefore inevitably centered on the possibility that they are in some way implicated in governing hormone transport during gestation, the notion that the rise in TBG "protects" the fetus from maternal TH being a prime example of such concepts. Thus although, in the context of this Symposium, the principal questions of interest are: do thyroid hormones traverse the placenta and, if so, are they of importance to the full maturation of the fetal brain?, the role of TBG and other thyroid binding proteins in TH transport - particularly during pregnancy - clearly constitutes an important complementary issue. Coincidentally it is one which, because of its wider relevance to endocrinology and reproductive physiology, has attracted much recent interest and controversy.

In this presentation we hope to clarify the confusion that centers on the role of binding proteins
in hormone transport, showing inter alia that, contrary to common opinion, binding proteins facilitate, and do not inhibit, hormone efflux from the vascular compartment. Indeed we suggest that TBG may have evolved specifically to ensure adequacy of a maternal thyroxine supply to the fetus-placental unit, possibly forming part of a complex feedback system governing the thyroid hormone environment to which the (early) fetus is exposed. However, we also present data relating to the more general hypothesis that maternal thyroxine not only traverses the placenta, but is of crucial importance to the development of the fetal CNS.

**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 constitutes the essential determinant of hormone action. 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, or in consequence of genetic abnormality), endocrine status appears to correlate with the serum free concentration, not the bound. The thyroid/pituitary/hypothalamic feedback system provides the classic example of these propositions, the system appearing to operate in such a way as to maintain free thyroid hormone concentrations at a (near) normal level in the face of wide differences in the concentrations (or compositions) of the thyroid binding proteins present in blood. Similar concepts apply in the case of the steroid hormones.

Such observations underlie the view that measurements of serum free hormone are diagnostically more valuable than measurements of bound (or total) concentrations. Clearly they also imply that both the bound hormone moiety and the binding proteins themselves are physiologically irrelevant, the latter view being sustained by the fact that no physiological consequence attributable to an absence or elevation of these proteins has ever been observed. Nevertheless, the notion that the hormone binding proteins in serum possess no physiological role is difficult to accept. Consequently endocrinologists have frequently sought explanations of these proteins' existence and biological function.

A conventional view - the "vascular retention hypothesis" - has been that they provide an intravascular "buffer store" of hormone, attenuating transient surges of secretion or peripheral demand, and restricting hormone entry into "sensitive" cells. Illustrative of this concept is Osorio and Myant's suggestion that TBG prevents TH transport from mother to fetus, thus serving to isolate or "protect" the fetus from the maternal endocrine system.

**Hormone transport to target tissues: "bound hormone" hypotheses**

Other 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 CBG levels, Keller, Richardson and Yates suggested that certain tissues are permeable to specific binding proteins, 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 micro-circulation". Though Keller et al's hypothesis has subsequently been largely disregarded, similar ideas have more recently been proposed by Sliiter et al who claim to have observed intra-cellular localization of CBG and SHBG, and have suggested that, following their structural modification, these proteins convey hormone directly into the cell nucleus. However, a major difficulty arising with all such concepts is that physiological manifestations of abnormality in binding-protein levels would be expected to be evident; moreover they offer no specific explanation of the characteristic changes of binding protein levels seen in pregnancy.

The ideas of Pardridge and his colleagues in this area have commanded particular attention during the past decade, and are currently the subject of considerable controversy. They center on the proposition that the equilibrium constant governing the protein binding of hormone is altered in the microvasculature of target organs, causing the intracapillary free hormone concentration to be elevated in such tissues, and large amounts of (dissociated) bound hormone to be selectively transported to them. Thus Pardridge claims that "the function of plasma protein binding is the selective..."
delivery of ligands to tissues in a way that varies from organ to organ\textsuperscript{11}, implying - like Keller et al - that variation in binding protein levels 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 all recent challenges to the free hormone hypothesis (including our own), we totally reject Pardridge's ideas, believing them to derive from an oversimplified theoretical analysis of the kinetics of hormone efflux from target organ capillaries, coupled with crucial misinterpretations of experimental data\textsuperscript{13}. However, in part because of the interest Pardridge's views continue to provoke, but more particularly because of the relevance of some of Pardridge's experimental data - when correctly interpreted - to the subject of this Symposium, 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\textsuperscript{14}. It must be emphasized 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\textsuperscript{11}" was originally viewed by Pardridge as contradicting the hypothesis. This misconception constituted the principal foundation of Pardridge's proposal of an "apparent", in vivo, dissociation constant ($K_{D}^{\text{app}}$) deviating from the "absolute" constant ($K_{D}$) estimated in vitro\textsuperscript{10}, and whose value was supposedly given by $K_{D} e^{t/k_p}$, where (t) is the capillary transit time and ($k_p$) the capillary wall permeation constant\textsuperscript{10,11}. The demonstrated manifestation of this effect was elevation of the "apparent" in vivo free hormone level in tissues (eg the liver) characterised 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 (radio-labelled) hormone efflux observed in Oldendorf-type experiments. However, following criticism of these ideas and their mathematical basis\textsuperscript{14}, Pardridge and Landaw formulated, in 1984, a radically altered hypothesis\textsuperscript{15}, albeit the notion of a tissue-specific elevation of an "apparent" dissociation constant continues to feature in it. The revised hypothesis proposes 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\textsuperscript{15}" arising, for example, from "a conformational change in the plasma protein" as it transits the target tissue.

The basis for this new proposal differs from that underlying Pardridge's original hypothesis; it centers on the discrepancy between Pardridge and Landaw's experimental observations on radiolabeled 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\textsuperscript{15}, which takes the form:

$$
F\ E = 1 - e^{-\frac{k_p t}{1 + K[P]}}\quad (1)
$$

where $F\ E$ = fractional efflux of radiolabeled hormone during a single pass through the target organ

$[P]$ = protein concentration

$K$ = affinity constant

$t$ = capillary transit time

$k_p$ = capillary wall permeation rate constant

Pardridge's more recent views depend crucially on the demonstration that increase in the protein content of the injected labeled-hormone bolus used in his organ-perfusion experiments does not cause a reduction in tissue uptake of hormone of the magnitude predicted by Equation 1\textsuperscript{15}. However, it is readily demonstrable that this simplified equation (which is essentially identical to one proposed in our critique of Pardridge's earlier theoretical analysis\textsuperscript{14}) depends on the assumption that all kinetic events occurring within, and adjacent to, the capillary in the course of unidirectional hormone efflux proceed at infinite speed, i.e. that the only constraint on the rate of hormone efflux is the rate constant governing capillary wall hormone permeation.

The invalidity of this proposition can readily be demonstrated by reference to the more correct
efflux equation applicable to Partridge's experiments, assuming (for the sake of clarity) that the extracapillary free (labeled) hormone concentration remains essentially at zero as the tracer bolus transits organ capillaries, and that intracapillary free hormone mixing and diffusion are infinitely rapid (implying that the free hormone concentration across the entire capillary radius is uniform\textsuperscript{13,14}):

\[ \text{FE} = \frac{k_p t}{1 + K[P] + \frac{k_p}{k_d}} \]

where \( k_d \) = dissociation rate constant of bound hormone complex.

Clearly the simplified form of Equation 2 relied on by Partridge reflects the assumption that \( k_d \to \infty \) (i.e., that the protein hormone complex dissociates infinitely rapidly), implying that \( k_p/k_d = 0 \). This assumption is clearly questionable. Moreover, inclusion of a term in the efflux equation reflecting bound hormone dissociation is sufficient to explain all Partridge's experimental observations (see, for example, Figure 1). It is quite evident that the insignificant discrepancies between experimental results and those predicted from Equation 2 provide no justification for the postulation of hitherto unsuspected intracapillary hormone release mechanisms of the kind proposed by Partridge.

A kinetic model of hormone transport

Partridge's experimental results nevertheless confirm a proposition that we have frequently advanced, i.e., that circumstances may arise in a particular target organ in which bound hormone dissociation exerts rate limiting effects on hormone delivery\textsuperscript{14,16}. In such circumstances, variation in the bound hormone level will affect hormone delivery to the organ concerned, as is implicit in Equation 2 and exemplified by the data shown in Figure 1. This proposition underlies our own challenge to the free hormone hypothesis, and our suggested explanation for the evolution and existence of serum binding proteins.

Though Equation 2 is capable of explaining Partridge 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).

\[ K = 212.8 \text{ L/mM} \]
\[ k_p t = 1.30 \]
\[ \frac{k_p}{k_d} = 3.85 \]

Figure 1. Unidirectional extraction of [125I] T3 by rat brain plotted versus arterial albumin concentration (data reproduced from Figure 5, Reference 18). Note the typically good fit of Equation 2 to the experimental data.
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". Our analysis reveals that serum binding proteins assist in sustaining a high free hormone concentration at the capillary wall in the face of hormone efflux, thus enhancing (not, as commonly thought, restricting) hormone efflux from the capillary. Moreover it is demonstrable that a rise in the serum concentration of a binding protein that carries two hormones (as does TBG) is potentially capable of specifically enhancing transport to certain tissues of one relative to the other in consequence of differences in their kinetic characteristics. Thus, in the case of T4 and T3, a rise in TBG is likely to increase T4 delivery to target tissues in which the rate of hormone efflux per unit area of capillary wall is high. This suggests that, although the free hormone concept may broadly apply throughout the body, binding proteins may nevertheless serve a subtle role in directing particular hormones to particular target organs in particular physiological circumstances (albeit for reasons quite different from those previously advanced by other investigators).

The marked changes in the concentrations of many binding proteins seen in pregnancy suggest that the physicochemical phenomena discussed above may specifically operate to regulate hormone supplies to tissues implicated in the reproductive process. Moreover, evidence 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 characterized by hemochorial and hemendothelial placentae. 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 in the alterations in the levels of these proteins arising in pregnancy. In the case of TBG, consideration of T4 and T3 binding kinetics suggests that a TBG rise results in preferential T4 delivery to any organ in which there is a high local rate of capillary efflux of TH. Meanwhile our results on placental transport of T4 indicate that fetal T4 uptake in the rat is relatively high in the first 9 - 10 days of fetal life, but thereafter falls to very low levels. Placental T4 accumulation nevertheless 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) are low. These observations are clearly commensurate with the proposition that TBG is physicochemically designed to supply T4 to particular target tissues.

Nevertheless, although our own data and those subsequently obtained by others (eg) have shown the belief that the feto-placental unit does not require T4 to be questionable, they do not directly confirm the proposition that the presence of large protein bound T4 pool in maternal blood serves to ensure adequacy of a feto-placental T4 supply. Nevertheless, the suggestion that this constitutes the primary role of TBG (and analogous serum binding-proteins in other mammalian species) accords with observations that high levels of protein bound T4 are seen only in mammals, that total TBG deficiency in man is essentially confined to males (and is without apparent physiological consequence), and that the bound T4 level in serum rises in pregnancy. Moreover, 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 characterized by a long gestational period. Likewise, the finding that lack of maternal T4 in early fetal life is associated with irreversible neurological damage, and the recent observation that T4 alone is available to enter the fetal brain, are entirely commensurate with the idea that T4 is of special importance in regard to the differentiation of the fetal CNS.

In the following sections, we present further evidence supportive of this view.

PLACENTAL TRANSFER AND FETOPLACENTAL METABOLISM OF THYROID HORMONES AND IODIDE

Our initial experimental approach was to investigate the placental transfer of TH from mother to fetus, paying particular attention to early pregnancy. In brief, rats were injected with [131I]iodide and either L-[3'-125I]T3 or L-[3',5'-125I]T4 at various stages of pregnancy, and then killed after 1 hour. Radioactivity was determined in total, TCA soluble (iodide and other cleavage products) and TCA
insoluble (iodothyronines) fractions of maternal blood and tissues, placentae and fetal tissues. Due to changes in fetal water content throughout pregnancy, concentrations of radioactivity were expressed as a function of the tissue protein content (radioactivity/g protein) and, in order to standardise results, all values were finally expressed as a percentage of the radioactivity associated with 1 g of maternal plasma protein.

At 10 days gestation, following injection of [\(^{125}\)I]T4, fetal concentrations of radioactivity in TCA insoluble and soluble fractions were 9 and 35% of the maternal plasma concentration respectively\(^{21}\). Placental concentrations were similar at the earliest gestational age at which they were examined (14 days), but whereas they increased throughout the remainder of pregnancy, fetal concentrations declined. Although fetal extraction of maternal [\(^{125}\)I]T3 was also observed in early pregnancy - the respective concentrations of radiolabel in acid insoluble and soluble fractions being 17 and 73% of the maternal plasma concentration - the absolute amounts of T3 transferred represent only a minor supply relative to T4, due to the 100-fold higher concentration of the latter in maternal blood. These findings have been confirmed by independent observations in a variety of animal species\(^{18,22}\).

In order to illustrate the possible relevance of transferred maternal T4 to fetal development, concentrations of radioactivity in various maternal organs and fetus were compared\(^{4,21}\). At 10 days gestational age, the concentration in the fetal iodothyronine fraction was equivalent to, or greater than, that in maternal brain, ovary and heart, but less than that in maternal liver and kidney. The concentration in the fetal TCA soluble fraction was only less than that of maternal kidney, indicating efficient metabolism of accumulated T4. After mid gestation, the fetal iodothyronine concentration was comparable only with that of maternal kidney, whereas TCA soluble metabolites were higher than those in maternal brain and heart. Thus, in early gestation, fetal concentrations were of the same order as those found in many maternal organs, implying that the supply of maternal T4 is sufficient to influence fetal development.

The presence of acid soluble radioactivity (\(^{125}\)I) in fetuses and placentae indicates metabolism of maternal T4. The ratio \(^{125}\)/\(^{131}\)I in the TCA soluble fraction of 10 day old fetuses was greater than that of maternal plasma, demonstrating local deiodination of maternal T4. Chromatography of fetal extracts\(^{21}\) revealed that, in early pregnancy, 35% of transferred radiolabel was present as T3, 22% as the form of rT3 and 43% remained as T4. In late pregnancy only 3% was recovered in the T3 fraction, whereas 66% co-migrated with rT3. In the placenta, relatively little T4 was converted to T3 following the onset of independent fetal thyroid function, most of the radiolabel (ca 70%) appearing as T3. These findings are consistent with our observations of feto-placental 5'-deiodinase; activity of this enzyme is high in early pregnancy but declines after mid gestation\(^{21}\). The associated change in metabolic pattern may serve a dual role: to limit the supply of maternal T4, while at the same time ensuring adequacy of iodide supply for fetal T4 synthesis.

### Table 1. Uptake of \(^{131}\)I by maternal tissues and the feto-placental burden

<table>
<thead>
<tr>
<th>Tissue</th>
<th>n</th>
<th>Ratio</th>
<th>% Dose of (^{131})I/organ</th>
</tr>
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<tbody>
<tr>
<td>Liver</td>
<td>12</td>
<td>46 ± 4</td>
<td>3.43</td>
</tr>
<tr>
<td>Kidney</td>
<td>12</td>
<td>66 ± 5</td>
<td>0.63</td>
</tr>
<tr>
<td>Ovary</td>
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<td>42 ± 12</td>
<td>0.06</td>
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<tr>
<td>Brain</td>
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<td>6 ± 2</td>
<td>0.04</td>
</tr>
<tr>
<td>Heart</td>
<td>13</td>
<td>28 ± 7</td>
<td>0.13</td>
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<tr>
<td>Spleen</td>
<td>13</td>
<td>38 ± 4</td>
<td>0.12</td>
</tr>
<tr>
<td>Thyroid</td>
<td>12</td>
<td>6400 ± 4300</td>
<td>1.17</td>
</tr>
<tr>
<td>Maternal total</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Fetoplacental burden</td>
<td>4</td>
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</table>

\[
\text{Ratio} = \frac{\% \text{ dose/g wet weight}}{\% \text{ dose/ml plasma}} \times 100
\]
We have also investigated placental transfer of maternal iodide throughout gestation, demonstrating substantial $^{131}$I accumulation in both fetus and placenta 1 hour after injection. Iodide accumulation by the feto-placental unit increased throughout gestation, reaching a peak after establishment of autonomous fetal thyroid function. At 20 days gestation, the combined $^{131}$I content of the total feto-placental burden was higher than that in all maternal organs studied (thyroid included) (Table 1). Under normal conditions, although an iodine deficiency state may prevail during pregnancy (due to increased renal clearance), the feto-placental unit is able to compete efficiently with maternal tissues for iodide.

**Placental Control of Feto-Maternal Thyroid Hormone Economy**

The particular relevance of TH with respect to the maturation of a variety of tissues and the organism as a whole, and the especially severe, potentially irreversible damaging effect of their deficiency on developing fetal brain and nervous system, constitute the basis for the concept of a specific placental control of maternal thyroid function. This proposition is consistent with certain indirect evidence suggestive of a nonpituitary regulation of TH concentrations in pregnancy and is centred on the hypothesis of a placenta functioning as an integrated and autonomous endocrine system, interconnected both with the fetus and the mother. As in the case of steroid hormone production (e.g., estrogen) by the fetal adrenals, gonads and placenta, a complex function endocrine role may be postulated for the placenta, the organ controlling TH homeostasis through the synthesis and secretion of a placental thyrotropin, independently from the maternal hypothalamus-pituitary axis. This putative placental control system would be expected to respond to different and specific feedback signals both from the fetal (T4, TSH?) and the maternal (T4, TBG?) compartments, fulfilling the critical role of monitoring the changing feto-placental requirements for T4 and ensuring an appropriate T4 environment for optimal fetal development (Figure 2).

Clinical evidence suggests that the increase in maternal thyroid activity consequent upon higher circulating TBG levels may not be mediated through pituitary TSH, whose circulating levels in early pregnancy have been described, in most reports, as lower or unchanged. The possibility of a non-pituitary control of TH secretion through a putative placental hormone has recently received renewed attention, speculation centering either on the existence of a specific chorionic thyrotropin (hCT) or, alternatively, a secondary biological activity of chorionic gonadotropin (hCG) (which is known to induce clinical hyperthyroidism in patients with hydatidiform mole or choriocarcinoma). While there is no agreement concerning the existence, or chemical and immunological properties, of hCT, the thyrotropic activity of hCG has been demonstrated under several in vitro conditions. The molecular basis of this phenomenon may be found in the significant degree of structural homology with TSH. In our experience, the intrinsic thyrotropic activity of 1 IU of partially purified hCG is equivalent to 0.11 μU of TSH using, as a bioassay system, the functional parameter of iodide uptake in rat thyroid FRTL-5 cells. This implies that hCG concentrations of 50-80 IU/ml normally found in the first trimester of pregnancy would correspond to physiologically significant amounts of TSH, able to suppress pituitary TSH secretion. Nevertheless, speculation regarding the physiological relevance of the thyrotropic effects of hCG...
activity measured in vitro must take into account, among other factors, the observation that hCG, like other glycoprotein hormones (eg TSH, FSH) encompasses a "family" of structurally-related iso hormones with differing biological potencies and half-lives; the molecular composition of hCG can thus influence its interactions with the human thyroid. Although thyroid stimulating activity and hCG immunoactivity coelute in gel filtration²⁴, the occurrence of particular hCG variants (with higher degree of glycosylation or modifications in the carboxy-terminal region) might, in our view, specifically account for a regulation of thyroid function in pregnancy.

Distribution and Metabolism of Thyroid Hormones in Fetal Rat Brain

The transfer of T4 from the maternal circulation to the feto-placental unit indicates that fetal rat tissues are exposed to TH of maternal origin before the onset of an autonomous thyroid function. Comparison of the distribution pattern of radioactivity in fetal and maternal organs, following injection of dams with [¹²⁵I]T4, revealed localisation of activity in fetal brain in early pregnancy (10 days gestation). In the fetus, the concentration of radioactivity associated with the iodothyronine fraction was marginally higher in the liver relative to brain, whereas in the mother, the hepatic concentration was 12-fold higher than that in brain²¹.

Current views regarding TH action centers on the proposition that these hormones mediate their effects by interaction with nuclear receptors. We therefore examined the subcellular localisation of transferred maternal T4 in fetal brain and liver. Large percentages of radiolabelled iodothyronine were recovered in the nuclear fractions of both liver and brain, with substantial amounts also present in the cytosol (Table 2).

Expression of concentrations of radioactivity in the brain relative to the corresponding liver values (brain/liver ratio) for mother and fetus, indicated a more favourable distribution of iodothyronine in subcellular fractions of fetal brain as compared to maternal brain (Table 3). The fetal brain appears both to accumulate T4 and to actively metabolise it to free iodide, T3 and rT3, as confirmed by chromatographic analysis of the subcellular fractions. These observations accord with the demonstration of 5'- and 5-deiodinase activities in fetal rat brain²⁰.

The presence of iodothyronines in organelles other than the nucleus, raises the possibility of extranuclear-mediated effects of TH, at least during development. Nevertheless, it should be emphasised that these observations must be viewed with caution at this stage, since a degree of translocation of TH, due to the subcellular fractionation procedure, cannot be ruled out.

Table 2. Percentage distribution of TCA soluble and insoluble radioactivity in subcellular compartments of fetal liver and brain.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Liver (soluble)</th>
<th>Liver (insoluble)</th>
<th>Brain (soluble)</th>
<th>Brain (insoluble)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclei</td>
<td>20 ± 19</td>
<td>39 ± 22</td>
<td>17 ± 15</td>
<td>28 ± 23</td>
</tr>
<tr>
<td>Lysosomes</td>
<td>9 ± 5</td>
<td>2 ± 1</td>
<td>6 ± 3</td>
<td>3 ± 2</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>17 ± 28</td>
<td>24 ± 19</td>
<td>21 ± 13</td>
<td>16 ± 10</td>
</tr>
<tr>
<td>Microsomes</td>
<td>5 ± 5</td>
<td>3 ± 2</td>
<td>5 ± 8</td>
<td>5 ± 7</td>
</tr>
<tr>
<td>Cytosol</td>
<td>49 ± 25</td>
<td>28 ± 8</td>
<td>51 ± 20</td>
<td>48 ± 32</td>
</tr>
</tbody>
</table>

Table 3. Brain/liver ratios of TCA soluble and insoluble radioactivity in adult and fetal rats

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Adult (soluble)</th>
<th>Adult (insoluble)</th>
<th>Fetus (soluble)</th>
<th>Fetus (insoluble)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclei</td>
<td>0.02 ± 0.03</td>
<td>0.26 ± 0.06</td>
<td>1.75 ± 0.40</td>
<td>0.94 ± 0.07</td>
</tr>
<tr>
<td>Lysosomes</td>
<td>0.05 ± 0.04</td>
<td>0.05 ± 0.01</td>
<td>0.84 ± 0.07</td>
<td>0.70 ± 0.05</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>0.16 ± 0.02</td>
<td>0.05 ± 0.02</td>
<td>2.83 ± 0.22</td>
<td>0.95 ± 0.04</td>
</tr>
<tr>
<td>Microsomes</td>
<td>0.31 ± 0.06</td>
<td>0.03 ± 0.02</td>
<td>0.85 ± 0.05</td>
<td>0.14±0.10</td>
</tr>
<tr>
<td>Cytosol</td>
<td>0.14 ± 0.09</td>
<td>0.04 ± 0.01</td>
<td>1.45 ± 0.12</td>
<td>0.94±0.07</td>
</tr>
</tbody>
</table>
EFFECTS OF EARLY MATERNAL HYPOTHYROXINEMIA ON CELLULAR FUNCTION IN THE CNS OF THE PROGENY

Following our initial hypothesis, we investigated the consequences of maternal hypothyroxinemia in early pregnancy on brain function in progeny at various stages of postnatal life. Dams were partially thyroidectomised (parathyroid-spared) and maintained under normal animal house conditions with food and water ad libitum. The total daily iodine intake was similar to control dams, at 34 μg per day. These animals were severely hypothyroxinemic, but T3 levels were within the normal range. Control (N) and thyroidectomised (TM) dams were mated and pups were standardised to a constant litter size. Both control and experimental progeny were studied at different postnatal ages, from 14 days to 7 months. Serum levels of T4 and T3 in progeny of thyroidectomised animals were found to be within the normal range.

In 14 day old animals, body and brain weight were reduced by 15 and 35%, respectively. Although preliminary data indicated no change in brain DNA content, more recent studies have demonstrated a 18% reduction. The total protein content of brain was reduced by 20%, and subfractionation revealed a severe depression (60%) in cytosolic protein, whereas insoluble protein fractions were unchanged. The glycoprotein content of the cytosolic fraction was found to be increased by 46%, whereas that of particulate fractions was diminished. Acid phosphatase and β-D-galactosidase activities were both reduced by 35%, and a less severe (17%) decrease was observed in aryl sulphatase activity.
In adult animals (7 months), the significant reduction in brain/body weight ratio persisted, but the decrease in brain weight was less than that in 14 d old animals. Protein concentration was reduced in all brain regions studied, but only significantly so in cerebral cortex and midbrain (Figure 3). DNA concentration was diminished in cerebral cortex, midbrain and medulla (by 38, 25 and 55%, respectively) (Figure 4), while no change was observed in RNA concentrations. Protein/DNA ratio (an indicator of cell size) was increased in cerebral cortex, midbrain and medulla, but decreased in cerebellum and paleocortex. RNA/protein ratio (an indicator of protein synthetic capacity) was only significantly changed in paleocortex.

These observations in both young and adult animals are indicative of gross biochemical changes in brain. In particular, reductions in protein concentration may reflect compromised enzyme synthesis. This possibility was investigated in more detail in adult progeny by determination of specific activities of selected enzymes associated with a range of cellular functions.

**Energy metabolism**

Lactate dehydrogenase (LDH) activity was significantly reduced in all regions except cerebellum (Figure 5), and Na⁺,K⁺-ATPase activity was significantly decreased in paleocortex (Figure 6). In contrast, no changes were observed in either succinate dehydrogenase (a mitochondrial marker enzyme) or Mg²⁺-ATPase activities.
Lysosomal enzymes

No significant change in both β-D-glucosidase and aryl sulphatase B activities was observed. Specific activities of β-D-galactosidase (Figure 7) and aryl sulphatase A (Figure 8) were significantly diminished in all regions, except cerebellum. Acid phosphatase activity was also significantly reduced in paleocortex and midbrain.

Neurotransmitter enzymes

Maternal hypothyroxinemia resulted in a differential effect on enzymes associated with neurotransmitter function; acetylcholine esterase (AChE) activity was reduced in cerebral cortex, paleocortex, midbrain and medulla (Figure 9), whereas no changes were observed in choline acetyl transferase activity. Aminergic transmitter function was unaffected, since monoamine oxidase activity remained within the normal range in all brain regions.

Calmodulin Calcineurin Systems

During early postnatal development, calcium-dependent, calmodulin-activated phosphatase activity (calcineurin) appeared to be diminished in TM dams at pH 4.0, whereas a significant increase was observed at pH 7.0. At seven months, a significant increase of the acidic component was observed in cerebral cortex. Therefore, a range of calcineurin dysgenesis at different age points are evident.
Figure 9. Brain AChE activity in adult progeny of N and TM dams

Tissue Culture Systems as Models

Studies on the progeny of thyroidectomised rat dams revealed both gross and specific biochemical deficits in the postnatal brain. However, the in vivo model presents restrictions which make difficult identification of the specific mechanisms responsible. Chief of these is the inability to specifically attribute any biochemical alteration to a lack of availability to the fetus of TH in utero.

In order to obviate these problems, in vitro techniques have also been employed. It is recognised that neural cells in culture behave similarly to cells in vivo in their responses to hormones, nuclear binding of T3, and developmental profiles. The use of tissue culture in a chemically defined medium eliminates interference from maternal and extraneural sources, permitting identification of the primary effects of TH. This approach has often been proposed as a useful model of neurological development and allows the intrinsic properties of neural cell types and the specificity of TH action to be investigated in a well controlled manner.

Initially, the effects of TH on protein synthesis in neurons were examined, revealing that T3 (1-30 nM) stimulated leucine uptake and subsequent incorporation into TCA-precipitable material. Stimulation of both uptake and incorporation was maximal (149 and 35% over control values, respectively) at 10 nM T3. Stimulation of leucine uptake was rapid (<1 hour), but the effect was abolished in the presence of inhibitors of protein synthesis, indicating that it was secondary to a pleiotrophic response of the cells. Although leucine incorporation was stimulated to similar degrees in soluble and insoluble (membrane-associated) protein fractions (respective values were +127 and 116% over controls), it is as yet unknown if this reflects a blanket stimulation of protein synthesis by T3.

Glucose is considered to be the primary energy substrate in the CNS; therefore, any changes in glucose utilisation may profoundly effect brain development. We have previously shown that T3 enhances 2-deoxyglucose uptake in neurons in primary culture, and have also observed similar effects in astrocytic cultures. Neuronal cultures were found to be unresponsive to insulin, indicating that TH may be important in regulation of energy metabolism during the period of neuronal proliferation and early differentiation in utero.

Much in vitro work has concentrated on neuronal, or mixed neural cell cultures, yet the importance of astrocytes, in terms of neuronal migration and survival, neurite outgrowth and differentiation, and in the control of oligodendroglial proliferation and trophic growth (specifically pertinent to early neurogenesis) has been acknowledged. TH are known to regulate the morphogenesis and cytoskeletal organisation of astrocytes, and to control glutamine synthetase activity, with consequent repercussions for inhibitory neurotransmitter availability. Astrocytes are also known to migrate, though the region specific control of their production and mechanism of their migration remain unknown.
Figure 10. Effect of T3 on [3H]fucose incorporation into glycoproteins in astrocytes

Preliminary data obtained in vivo indicated differences in quantity and localisation of Con A binding glycoproteins in the brains of progeny from hypothyroxinemic rat dams. Alterations in the expression of important glycoprotein cell surface markers early in development may have long term consequences resulting from interference with normal cell development, connectivity and establishment of neural circuitry. In addition, many enzymes important in brain development are glycoproteins. We have therefore investigated the effects of TH on glycoprotein synthesis in cultured astrocytes, using the incorporation of 3H-fucose as a marker.

Addition of T3 (10 nM) resulted in significant stimulation of fucose incorporation at 48 and 96 hours (32 and 39% over control values, respectively) (Figure 10). No immediate difference in free cytosolic 3H-fucose was noticed, implying a lack of a direct effect of T3 on uptake, nor was the effect due to a blanket increase in protein synthesis.

The stimulation of fucose incorporation into glycoproteins may be accounted for by an increase in the production of a peptide precursor or precursors, an increase in production of the enzyme responsible for fucosylation, or by a stabilising effect blocking turnover. The long time lag occurring before a significant effect was apparent suggests that, in this case, TH do not act directly on translation or fucosylation, and implies a nuclear mediated effect. Studies currently in progress are intended to answer these questions, and provide information on the specificity of the effect.

Neurological development is well coordinated, both temporally and spatially, and the existence of developmental "windows" for the action of exogenous controlling factors such as TH seems plausible. These "windows" may differ between cell types, and result in long term effects by acting at the levels of the system; controlling the establishment of neural circuitry, or the cell; inducing, for example, a cascade of sequential gene expression.

CONCLUSIONS

In recent years, studies from several laboratories have shown that, at least in early rat pregnancy maternal T4 crosses the placenta and is available to the developing fetus. In late pregnancy, maternal T4 transfer is reduced, coincident with the establishment of independent fetal TH economy. These observations challenge the notion of minimal transplacental passage throughout pregnancy and suggest a possible fetal need for maternal TH during critical phases of fetal growth. The high degree of localisation of transferred T4 in fetal brain, relative to the distribution pattern in the mother, suggests a specific role in development of the fetal neural system. Indeed, the fetal brain appear to possess the capability to utilise maternal T4 in early pregnancy, since both T3 and rT3 have been recovered from subcellular organelles, indicating the presence of functional 5'- and 5-deiodinase enzyme systems.

The predominant pattern of feto-placental T4 metabolism changes from 5'-deiodination in early pregnancy to 5-deiodination in late pregnancy. The observation that this switch precedes the
establishment of independent fetal thyroid function, coupled with the observations that fetoplacental deiodinase activities are predominantly localised within the placenta\textsuperscript{21}, suggest a degree of fetal control over its own TH environment, mediated at the level of the placenta. Since high levels of rT3 may serve to inhibit local T4 to T3 conversion in fetal tissues, this (hypothetical) signal may serve to protect the fetus from high levels of T3 (derived from both maternal and fetal sources), while concurrently maintaining an adequate iodide supply. Consequently, fetal T4 per se may play an important role in late development.

The identification of a thyroid stimulating function in chorionic gonadotropin also suggests an integrated system for TH economy in the mother, fetus and placenta. Control of maternal hypothalamic-pituitary-thyroid status by a placental factor has been indicated in the literature, since placental carcinomatous conditions, hydatidiform mole, hyperplasia and other placental dysfunction has been reported to impinge upon maternal thyroid function.

Use of a rat model has permitted the influence of maternal T4 on fetal brain development to be studied in detail. A wide range of biochemical deficits has been observed in brains from both young and adult progeny of hypothyroxinemic rat dams. Decreases in protein/DNA ratios and DNA concentrations indicate disturbances in cell size and cell loss; in particular, the significant reductions seen in β-D-galactosidase activity are indicative of neuronal loss. Protein content appears to be reduced, and more specific studies have suggested compromised brain cell function in the progeny of hypothyroxinemic dams. Alterations are also observed in the activities of selected enzymes associated with parameters such as energy metabolism, lysosomal function, synaptic function, myelin synthesis and calcium metabolism. These differences persist up to 7 months of age and, therefore, cannot be corrected by a normal thyroid state in the progeny. These observations accord with the existence of specific phases in (early) brain development, characterised by absolute requirements for maternal T4. Failure to provide an adequate supply of TH during such periods may thus result in irreversible brain damage in postnatal life.

Our findings provide a possible explanation for the behavioural and cognitive deficits observed in children born to euthyroid but severely hypothyroxinemic mothers in iodine deficient regions. It should be emphasised that not all enzymes studied were affected in the animal model and changes were region-specific, analogous to the selective dysfunctions observed in humans.

It may be argued that effects observed in the in vivo rat model may be secondary to placental dysfunction or maternal nutritional disadvantage. These possibilities, although clearly pertinent to observations in humans, have been minimised in our experiments, since the rat dams were euthyroid (T3 levels were in the normal range) and food consumption (including iodine intake) was normal. Although an effect of decreased maternal T4 on placental function per se cannot be entirely ruled out, our experiments with primary cultures of neural cells are supportive of a specific role for TH in cellular proliferation and differentiation of the CNS.

In conclusion, we have presented a basic model for detailed investigation of the influence of maternal TH, especially T4, in early neurogenesis and the consequences for the functional efficiency of the adult brain in iodine deficient regions.

REFERENCES

5. Osorio, C. and N. B. Myant, The passage of thyroid hormones from mother to foetus and its


DISCUSSION

BRAVERMAN (Worcester): I would like to make 4 brief comments: 1) Nelson's study on schizophrenia is irrelevant because the data were questionable; 2) You do not take into account the intersitial space; 3) if TBG enhances transport in man, there is no TBG in any of the rodents studied; 4) Cavalieri and others have clearly shown that addition of proteins decreases the entrance of T4 both in perfused liver systems and in hepatocytes.

EKINS (London): Of course when you add TBG or a binding protein acutely you obviously reduce the free hormone concentration of the sample to which it is added and that reduces hormone transport into any target cell. But the homeostatic system which exists in man keeps the free concentration constant. The experiment you are referring to is not appropriate. What happens in man is that the total hormone level rises and the free concentration remains roughly constant. Therefore, the coexistence of a more or less constant free concentration of thyroxine and an increased bound hormone concentration enhances hormone transport. Concerning the possible role of the binding proteins in hormone delivery to the fetus, we must assume that there are species differences in the placentas. I am only suggesting that a high level of bound hormone due to high TBG levels assists hormone transport to the fetal-placental unit.
Differences in Nuclear Triiodothyronine Binding in Rat Brain Cells Suggest Phylogenetic Specialization of Neuronal Functions*

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Department of Molecular Endocrinology, The Middlesex Hospital Medical School, London W1N 8AA, United Kingdom

ABSTRACT. The central nervous system depends on thyroid hormones (TH) in regard to its development, maturation, and maintenance of normal functions. As there is much evidence to suggest that the effects of TH are mainly mediated through specific nuclear binding sites, we have studied the anatomical distribution of T₃ nuclear receptors in different regions of adult rat brain, and the localization of receptors in the fractionated neuronal and glial nuclei of neocortex, paleocortex, and cerebellum. Purified nuclei from the various brain regions were prepared by ultracentrifugation in 2.2 M sucrose. Purified neuronal and glial fractions were obtained by discontinuous sucrose gradient centrifugation in 2.2 and 2.4 M sucrose. The washed nuclear fractions were used for T₃ binding assay at 37°C for 30 min and the data analyzed by least squares nonlinear regression analysis. Nonfractionated nuclei from all regions studied were found to have similar dissociation constant (K_d) values (1.04-1.38 nM) and Eadie-Hofstee plots indicated the presence of an apparently ubiquitous single class of high affinity, low capacity binding sites. The increase in binding from cerebellum (54 ± 24 fmol/mg DNA; mean ± se) to neocortex (666 ± 89 fmol/mg DNA) showed a caudo-cranial pattern. In fractionated neuronal nuclei, the same trend was observed, only to a greater degree (1628 ± 266, 994 ± 76 and 212 ± 29 fmol/mg DNA in neocortex, paleocortex, and cerebellum, respectively); the difference between corresponding values for glial nuclei of neocortex and paleocortex (357 ± 139 and 250 ± 92 fmol/mg DNA, respectively) was not statistically significant, and no specific T₃ binding was found in cerebellar glial nuclei. These data suggest that TH may have an important role in neurons from phylogenetically newer regions, concerned with higher mental functions. The caudo-rostral distribution pattern may also indicate a gradient of TH actions in central nervous system regions. (Endocrinology 120: 2398-2403, 1987)

The importance of thyroid hormones (TH) in the ontogenesis of the central nervous system (CNS) has been well documented (1). In addition to effects on the development of normal cytoarchitecture of the CNS, TH deficiency or excess in the first trimester of pregnancy has been correlated with irreversible dysfunctions, such as neurological cretinism, low IQ, impaired motor coordination, impairment of learning, and affective disorders (2, 3, 4). These dysfunctions point to two distinct effects of the hormone: the impairment of higher mental functions, presumably controlled by the cortex; and cellular and cytoarchitectural deficits, with consequent motor disorders, abnormal posture, auditory dysfunction, and many other defects, due to dysgenesis of lower brain regions.

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* Part of this work has been presented at the 15th Annual Meeting of the European Thyroid Association, 1986, Stockholm, Sweden.
† Wellcome Trust Research Fellow from the Chair of Endocrinology, University of Catania, Catania, Italy.
is, and the development of a technique to isolate neuronal and glial nuclear fractions (8), we have studied nuclear T$_3$ binding in both nonfractionated nuclei of several anatomical brain regions and in fractionated nuclei from neocortex, paleocortex, and cerebellum. We report on the differential distribution of T$_3$ nuclear receptors in CNS regions and cell types and hypothesize that neuronal nuclei from phylogenetically newer regions may be more dependent on TH.

Materials and Methods

Materials

-$\alpha$-T$_3$, -T$_4$, and DL-Dithiothreitol were obtained from Sigma Chemical Co. (England) and rT$_3$ from Henning (West Berlin, Germany). Triton X-100 was obtained from BDH (England). $[^{125}]$T$_3$ at a specific activity higher than 2800 $\mu$Ci/$\mu$g was obtained from Amersham International (Amersham, England). All other chemicals were of analytical grade.

Adult Sprague-Dawley rats were bred locally in the Middle-Tennessee Medical School animal house.

Isolation of brain nuclei

Neuronal and glial nuclei were separated as has already been reported (8). For each nuclear preparation the brains of three Sprague-Dawley rats (250-300 g) were combined. The animals were killed by cervical dislocation followed by exsanguination. The brains were dissected out and the attached pia mater and blood vessels were removed carefully with forceps. The excised brains were chilled on ice and washed three times with 10 mM MgCl$_2$. Neocortex, paleocortex, midbrain, medulla, and cerebellum were then separated and the tissues homogenized in 10 mM MgCl$_2$ by twenty strokes in a glass homogenizer of 1-mmurance. The homogenate was filtered through nylon mesh (110 microns) and the filtrate was centrifuged at 6000 $\times$ g for 10 min.

Total nonfractionated nuclei were purified by resuspending a crude nuclear fraction in 2.2 M sucrose with subsequent recentrifugation at 110,000 $\times$ g for 30 min.

Neuronal and glial nuclei were purified on a 2-step discontinuous sucrose gradient. The crude nuclear pellet was diluted to 4.6 ml in 2.2 M sucrose to obtain an optimal sucrose density for minimum separation of brain neuronal and glial nuclei. A fixed volume of 10 ml of 2.4 M sucrose was loaded into centrifuge tubes of total capacity of 43 ml and a volume of 15 ml crude nuclei; was then placed on the 2.4 mattress. Finally a fixed volume of 10 ml of 2.2 M sucrose was introduced along the side of the centrifuge to form a layer on 2.4 M sucrose and the tubes were ultracentrifuged for 30 min at 110,000 $\times$ g.

Discontinuous sucrose gradient centrifugation of the crude clear fraction yielded four distinct layers: one at the top of the gradient, a second one at the interface of the homogenate and the 2.2 M sucrose, a third layer at the interface of 2.2 and 2.4 M sucrose, and a pellet at the bottom of the tube. The top layer consisted of myelin, microsomes, mitochondria, lysosomes, and other cell debris. The second layer consisted mainly of very small nuclei which were extremely basophilic with diffuse cromatin. This layer was also slightly contaminated with capillary endothelial nuclei. Preliminary light microscopic assessment indicated this layer to be enriched in microneuronal nuclei. The third nuclear layer at the interface of 2.2 and 2.4 M sucrose was composed of relatively large nuclei, spherical in shape, and with a distinct nucleolus. A few of the nuclei were multinucleolated with relatively acidophilic nucleoplasm and less granular in comparison with other nuclear fractions. This fraction comprised the neuronal fraction (13-15). The pellet at the base of 2.4 M sucrose layer consisted mostly of small round basophilic nuclei with intensely stained nucleoli. According to criteria reported by others (13, 16), these nuclei were mostly oligodendrogial. For both neuronal and glial nuclear fractions, the purity was slightly better than 90% with minimal extranuclear contamination obviating the need for gross corrections made in neuron-enriched fractions (10). The extent of cross-contamination of the purified layers was between 6% and 8% (8).

Microscopy

Bulk isolated nuclear fractions were resuspended in 0.15 M NaCl and examined by phase contrast microscopy. Some nuclear fractions were also lightly stained with 0.1% methylene blue in 0.15 M NaCl to examine the histological characteristics of the isolated nuclei. In some experiments resuspended nuclear fractions were diluted with 0.15 M NaCl to convenient volumes, stained with 0.1% methylene blue and counted in a hemocytometer to obtain the total number of nuclei in each fraction and to check for gross contamination.

T$_3$ binding assay

After ultracentrifugation, total nonfractionated nuclei, neuronal nuclei, and glial nuclei were resuspended in 0.32 M sucrose, centrifuged at 2000 $\times$ g for 20 min, and finally resuspended in 20 mM Tris-HCl, 1 mM DTT, 0.32 M sucrose, 3 mM MgCl$_2$, pH = 7.4 (binding buffer). Replicate samples (200 $\mu$l) of the suspended nuclei were incubated with tracer $[^{125}]$T$_3$ (1 x 10$^{-10}$ M) and a range of concentrations of unlabeled T$_3$ (1 x 10$^{-10}$ to 1 x 10$^{-7}$ M) in 500 $\mu$l Beckman tubes. Nonspecific binding, determined in the presence of cold T$_3$ (1 x 10$^{-7}$ M), was subtracted in all cases and ranged from 10-15% of the total. At the end of the incubation period, the reaction was stopped by placing the tubes on ice followed by the addition of 200 $\mu$l Triton X-100 2% (final concentration 1%) for 15 min. The nuclei were then centrifuged in a Beckman microfuge at 8000 $\times$ g for 3 min, the pellet was washed with cold binding buffer and recentrifuged for 1 min. The supernatant was aspirated, the tips were severed, and the radioactivity was counted in a $\gamma$-counter to determine the bound T$_3$ fraction.

In preliminary time-course experiments we found that at 37 C T$_3$ binding, in mixed nuclear population and neuronal and glial nuclei, reached a plateau by 10 min, remaining stable up to 50 min. Thirty-minute incubation at 37 C was subsequently chosen for all the experiments.

DNA was measured, with minor modifications, by the method of Setaro and Morley (17).
NUCLEAR T₃ BINDING IN NEURONS AND GLIA

We next studied the compartmentation of T₃ binding between the different cell populations of which brain is composed, i.e. neurons and glial cells, in various brain areas using a technique developed for the separation of neuronal and glial nuclei from adult rat brain on a discontinuous sucrose gradient (8).

After dissection of the various areas as in our studies on T₃ binding in total nonfractionated nuclei, we separated the neuronal and glial fractions. Binding experiments could only be performed in neocortex, paleocortex, and cerebellum, since recovery of DNA in midbrain and medulla was insufficient.

With the same incubation conditions as used for nonfractionated nuclei, T₃ binding was found to be higher in neuronal nuclei from neocortex than in neuronal nuclei from paleocortex and cerebellum (Table 2). The difference between the number of binding sites in glial nuclei from neocortex and paleocortex was not statistically significant. We were not able to detect specific T₃ binding to cerebellar glial nuclei and this may explain the low number of T₃ binding sites obtained in nonfractionated cerebellar nuclei, given the high ratio of glial cells to neurons (5:1 to 10:1).

Nonlinear least-square analysis of the binding data showed that nuclear T₃ binding was saturable in glial nuclei from neocortex and paleocortex, and neuronal nuclei from all areas studied. The range of Kᵦ for these fractions (0.82-1.25 nM) was similar to that of nonfractionated nuclei. Plots of B against B/F (Eadie-Hofstee plot) yielded straight lines (Fig. 1) suggesting the presence of single classes of high affinity binding sites.

Results

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After dissection of the various areas as in our studies on T₃ binding in total nonfractionated nuclei, we separated the neuronal and glial fractions. Binding experiments could only be performed in neocortex, paleocortex, and cerebellum, since recovery of DNA in midbrain and medulla was insufficient.

With the same incubation conditions as used for nonfractionated nuclei, T₃ binding was found to be higher in neuronal nuclei from neocortex than in neuronal nuclei from paleocortex and cerebellum (Table 2). The difference between the number of binding sites in glial nuclei from neocortex and paleocortex was not statistically significant. We were not able to detect specific T₃ binding to cerebellar glial nuclei and this may explain the low number of T₃ binding sites obtained in nonfractionated cerebellar nuclei, given the high ratio of glial cells to neurons (5:1 to 10:1).

Nonlinear least-square analysis of the binding data showed that nuclear T₃ binding was saturable in glial nuclei from neocortex and paleocortex, and neuronal nuclei from all areas studied. The range of Kᵦ for these fractions (0.82-1.25 nM) was similar to that of nonfractionated nuclei. Plots of B against B/F (Eadie-Hofstee plot) yielded straight lines (Fig. 1) suggesting the presence of single classes of high affinity binding sites.

Discussion

Our observations of T₃ receptor binding in nonfractionated nuclei from adult rat CNS regions indicate a caudo-cranial distribution profile in conformity with those observed in vitro by Schwartz and Oppenheimer (20) and Ruel et al. (10), the cortical binding being the

<table>
<thead>
<tr>
<th>Region</th>
<th>T₃ bound (fmol/mg DNA)</th>
<th>Kᵦ (nM)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neocortex</td>
<td>1638 ± 286</td>
<td>1.25 ± 0.15</td>
<td>6</td>
</tr>
<tr>
<td>Paleocortex</td>
<td>357 ± 139*</td>
<td>1.02 ± 0.09</td>
<td>4</td>
</tr>
<tr>
<td>Medulla</td>
<td>250 ± 92*</td>
<td>0.91 ± 0.50</td>
<td>3</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>212 ± 29</td>
<td>0.82 ± 0.10</td>
<td>4</td>
</tr>
</tbody>
</table>

See the legend to Table 1. The values of T₃ binding in glial nuclei, corrected for 8% neuronal contamination, were 226 and 170 fmol/mg DNA in neocortex and paleocortex, respectively.

* Stat not sign.
of receptors during development has been reported in the cerebellum (22). Second, when we studied fractionated nuclei from different brain compartments, we were unable to detect specific T₃ nuclear binding sites in cerebellar glial nuclei (mainly oligodendrocytes). The observed low receptor concentration in the total cerebellar nuclei may therefore be a dilution effect of a large low affinity, high capacity compartment. Thus, a high receptor concentration in any small group of T₃ responsive cell types, such as Purkinje cells, may escape detection, causing the in vitro specific receptor binding value to be artificially low in nonfractionated nuclei. Another possibility is that the relatively higher 5'-deiodinase activity observed in cerebellum may compensate for the low receptor concentration by making available a larger intracellular T₃ pool (23). Consequently, it remains possible that T₃ has an important role in the functional homeostasis of highly metabolic, electrically active cell populations such as neurons and astrocytes, even in the cerebellum.

We have previously reported that the nuclear T₃ binding capacity of neuronal nuclei from brain (without cerebellum) is higher than that of glial nuclei (8). This concurs with the findings of Haidar et al. (6) and Yokota et al. (7) who report almost the same ratio in chicken and rat brain. Therefore, T₃ binding sites appear to be more numerous in neuronal nuclei than in glial nuclei in a variety of animal species.

With the exception of cerebellar glial nuclei, a saturable T₃ binding was found in all the fractions studied, including the glial nuclei from paleocortex and neocortex, and all had a similar dissociation constant (K_d). We have already reported that in combined neocortex and paleocortex, the binding affinity of various analogs in neuronal and glial nuclei was closely comparable (8), and similar to what has already been described for liver nuclei and total nonfractionated brain nuclei (22).

These findings differ from the recent reports by Kolodny et al. (9) and Ruel et al. (10), who described an absence of specific T₃ receptors in glial nuclei from cerebral cortex, suggesting that the known effect of T₃ on myelination could be mediated through neurons. The reason for this discrepancy is not clear. A possible explanation is that some of the separation procedures used to obtain glial nuclei may have caused a loss of T₃ receptors, rendering difficult their detection given the low number of receptors present in these nuclei.

Glial cells (oligodendrocytes) are involved in myelin synthesis which is known to be influenced by TH (24, 25). The synthesis and activity of glial marker enzymes such as carbonic anhydrase (26, 27) and 2',3'-cyclic nucleotide 3'-phosphohydrolase are reduced in hypothyroid rats and genetically hypothyroid mutant mouse (hyt) (28, 29). This may suggest a role of TH in glial...
yne synthesis and activity, and possibly the need for specific T3 receptor population in glial nuclei.

The apparent absence of specific nuclear T3 receptors in cerebellar glial nuclei is difficult to explain. Cerebellar glial nuclei have different functional characteristics from those in other brain regions. For example, in the rat, the morphology of neuronal differentiation differs between neocortex and the cerebellum (1). Glial cells in cerebellum also appear to be different from those in other areas, with the cerebellar cortex possessing various types of astrocytes, each ultrastructurally distinct from one in other regions (30). White matter is scarcely represented in the cerebellum, and the glial nuclear fraction isolated may be mainly composed of nonmyelinated satellite oligodendrocytes, which are restricted to grey matter (31).

We have recently observed that T3 stimulates protein synthesis in rat neurons in primary culture (32). The requirement for T3 in neurons may be due to the protein synthetic activity of these cells (33–35), rendering synthesis, by neurons, of specific proteins for plasmatic transport (36, 37).

It is presented in this paper that cortical cells may be relatively more dependent in terms of metabolic requirements for cytoarchitectural and functional homeostasis than neurons from phylogenetically older regions such as cerebellum, which are primarily concerned with motor function. It is therefore possible that the acquisition of increased sensitivity to TH by glial nuclei from higher CNS regions may have made important contribution to the increased capacity for the control of mental function.

Ional cells, which are capable of regeneration and do have the high homeostatic demands of the highly and irreplaceable neurons, are likely to be less dependent on TH, explaining the lower receptor number and the absence of an equivalent phylogenetic trend. Nonetheless it is emphasized that receptor concentration, per se, in different nuclear types are not definitive determinants of cell functions.

Binding in oligodendrocytes and astrocytes and in different subtypes of these cells (capillary glia, satellite glia, perineuronal glia, smooth astrocytes, protoplasmic astrocytes, and fibrous astrocytes) remains to be assessed in detail. Further studies along these lines are necessary to clarify the effects of TH on the control of glial functions.

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