Sex steroid hormone action in fetal rat brain: influence of the early intrauterine thyroid hormone environment

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

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London

1999
Dedication

To my children,
Maisalreem, Mohammad and Ahmad,
the joy of my life for
tolerating all the long hours I had to be away from them;

To the recent addition to our family,
the lovely twins Fajer and Hamad
who were born during the last stages of thesis writing;

To my husband,
Othman,
for his love, encouragement and patience;

To my parents,
Amal and Dawoud,
for their love, care and continuous support;

To all of those who truly believed in me

&

To my country
Kuwait
Thyroid hormones, in particular thyroxine, traverse the placenta from mother to fetus during pregnancy and exert influences critical to normal fetal development. Moreover, sex steroid hormones play a critical role during brain development and thyroid hormone deficiency in postnatal progeny has been shown to affect the expression of sex steroid receptors in some tissues, including the brain. Thus, whether a fetus will develop neurological cretinism at birth may depend upon the degree of intrauterine hypothyroxinemia and the sex steroid concentration and sex steroid receptors in utero and beyond. The main aim of this project was to examine the influence of maternal hypothyroxinemia on estradiol (E) and progesterone (P) levels in maternal, fetal and postnatal tissues, as well as the expression of estrogen and progesterone receptor (ER and PR) isoforms in fetal rat brain. ER and PR mRNA and protein expression were studied using RT-PCR and Western blotting methodologies, respectively. Moreover, the expression of several housekeeping genes was studied to see whether the effect of maternal hypothyroxinemia on transcription is a general or selective effect.

Maternal hypothyroxinemia resulted in impaired fetal and early postnatal growth, however, placental growth was normal. Maternal hypothyroxinemia resulted in decreased maternal serum estradiol levels at 14 dg and elevated amniotic fluid estradiol levels at 21 dg. Moreover, fetal brain and carcass estradiol levels were decreased at 16 dg and elevated in brain and liver at 21 dg in the experimental group. The decreased estradiol levels at 16 dg in fetal brain was accompanied by decreased ERα and β mRNA levels and ERα protein levels in the nuclear fraction. The change in ERα and β mRNA expression, prior to the onset of fetal thyroid hormone synthesis are selective as RT-PCR revealed no effect on the expression of housekeeping genes. Postnatal rats studied were euthyroid however E levels were elevated at 30 dpn in females from TX dams probably due to in utero effect. In contrast, no effect was observed for PR expression in fetal brain or P hormone levels in maternal and postnatal serum, amniotic fluid or fetal tissue.

This thesis has shown that indeed thyroid hormone deficiency in utero has deleterious effects both on E hormone levels and on ER expression before the onset of fetal thyroid function. Moreover, this effect is carried on postnatally so that serum E hormone levels in females were altered.
ACKNOWLEDGEMENTS

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I wish to express my appreciation to Professor Roger P. Ekins for the confidence attitude he continually expressed during my study.

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I would like to acknowledge the Physiology Department, Medical Faculty, Kuwait University, for offering me the opportunity to do my degree.
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<td>Description</td>
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<tr>
<td>5'D-I</td>
<td>type I 5'-monodeiodinase (outer or inner ring)</td>
</tr>
<tr>
<td>5'D-II</td>
<td>type II 5'-monodeiodinase (outer or phenolic ring)</td>
</tr>
<tr>
<td>5D-III</td>
<td>type III 5-monodeiodinase (inner or tyrosyl ring)</td>
</tr>
<tr>
<td>A</td>
<td>absorbance</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>AFP</td>
<td>(\alpha)-fetoprotein</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>AR</td>
<td>androgen receptor</td>
</tr>
<tr>
<td>ARA70</td>
<td>androgen receptor associated protein 70</td>
</tr>
<tr>
<td>ATCC</td>
<td>American type culture collection</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BGLU</td>
<td>(\beta)-glucuronidase</td>
</tr>
<tr>
<td>BMR</td>
<td>basal metabolic rate</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CBG</td>
<td>cortisol-binding globulin</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CBP</td>
<td>CREB-binding protein</td>
</tr>
<tr>
<td>CY</td>
<td>cyclophilin</td>
</tr>
<tr>
<td>dATP</td>
<td>2'-deoxyadenosine 5'-triphosphate</td>
</tr>
<tr>
<td>DBD</td>
<td>DNA binding domain</td>
</tr>
<tr>
<td>dCTP</td>
<td>2'-deoxycytidine 5'-triphosphate</td>
</tr>
<tr>
<td>DEPC</td>
<td>diethylpyrocarbonate</td>
</tr>
<tr>
<td>dg</td>
<td>days of gestation</td>
</tr>
<tr>
<td>dGTP</td>
<td>2'-deoxyguanosine 5'-triphosphate</td>
</tr>
<tr>
<td>DHT</td>
<td>dihydrotestosterone</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxynucleotide triphosphate</td>
</tr>
<tr>
<td>dpn</td>
<td>days postnatally</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>dTTP</td>
<td>2'-deoxythymidine 5'-triphosphate</td>
</tr>
<tr>
<td>E</td>
<td>estradiol</td>
</tr>
<tr>
<td>EcR</td>
<td>ecdysone receptor</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylene diamine terta acetic acid</td>
</tr>
<tr>
<td>EGL</td>
<td>external granular layer</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>ER</td>
<td>estrogen receptor</td>
</tr>
<tr>
<td>ERR</td>
<td>estrogen-related receptor</td>
</tr>
<tr>
<td>ERE</td>
<td>estrogen response element</td>
</tr>
<tr>
<td>FSH</td>
<td>follicle-stimulating hormone</td>
</tr>
<tr>
<td>G3PDH</td>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GH</td>
<td>growth hormone</td>
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<tr>
<td>GLUT</td>
<td>glucose transporter</td>
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<tr>
<td>GR</td>
<td>glucocorticoid receptor</td>
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<td>GRE</td>
<td>glucocorticoid response element</td>
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<td>GRIP170</td>
<td>GR-interacting protein 170</td>
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<tr>
<td>HBD</td>
<td>hormone binding domain</td>
</tr>
<tr>
<td>hCG</td>
<td>human chorionic gonadotrophin</td>
</tr>
<tr>
<td>hER</td>
<td>human estrogen receptor</td>
</tr>
<tr>
<td>HH4</td>
<td>histone H4</td>
</tr>
<tr>
<td>HRE</td>
<td>hormone response element</td>
</tr>
<tr>
<td>HSD</td>
<td>hydroxy steroid dehydrogenase</td>
</tr>
<tr>
<td>hsp</td>
<td>heat shock protein</td>
</tr>
<tr>
<td>kb</td>
<td>kilobase</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>LBD</td>
<td>ligand binding domain</td>
</tr>
<tr>
<td>LH</td>
<td>luteinizing hormone</td>
</tr>
<tr>
<td>LHRH</td>
<td>luteinizing hormone releasing hormone</td>
</tr>
<tr>
<td>LMP</td>
<td>low melting point</td>
</tr>
<tr>
<td>MAE</td>
<td>MOPS acetate EDTA</td>
</tr>
<tr>
<td>MAP</td>
<td>microtubule associated protein</td>
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<tr>
<td>MB</td>
<td>maternal brain</td>
</tr>
<tr>
<td>MIT</td>
<td>mono-iodothyronine</td>
</tr>
<tr>
<td>MMI</td>
<td>methimazole</td>
</tr>
<tr>
<td>MOPS</td>
<td>morpholinopropanesulphonic acid</td>
</tr>
<tr>
<td>MR</td>
<td>mineralocorticoid</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>N</td>
<td>normal; control; euthyroid</td>
</tr>
<tr>
<td>NADH</td>
<td>nicotinamide adenine dinucleotide (reduced form)</td>
</tr>
<tr>
<td>N-CoR</td>
<td>nuclear receptor co-repressor</td>
</tr>
<tr>
<td>NGFI-B</td>
<td>nerve growth factor-induced protein</td>
</tr>
<tr>
<td>od</td>
<td>optical density</td>
</tr>
<tr>
<td>ODC</td>
<td>ornithine decarboxylase</td>
</tr>
<tr>
<td>P</td>
<td>progesterone</td>
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<td>polyacrylamide gel electrophoresis</td>
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<td>PBS</td>
<td>phosphate buffered saline</td>
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<td>Acronym</td>
<td>Definition</td>
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<td>----------</td>
<td>------------------------------------------------</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PLSD</td>
<td>protected least significance difference</td>
</tr>
<tr>
<td>PR</td>
<td>progesterone receptor</td>
</tr>
<tr>
<td>PRA</td>
<td>progesterone receptor form A</td>
</tr>
<tr>
<td>PRAB</td>
<td>progesterone receptor forms A+B</td>
</tr>
<tr>
<td>PRB</td>
<td>progesterone receptor form B</td>
</tr>
<tr>
<td>PTU</td>
<td>propylthiouracil</td>
</tr>
<tr>
<td>RAR</td>
<td>retinoic acid receptor</td>
</tr>
<tr>
<td>Rₜ</td>
<td>relative mobility</td>
</tr>
<tr>
<td>RIA</td>
<td>radioimmunoassay</td>
</tr>
<tr>
<td>RIP140</td>
<td>receptor interacting protein 140</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>rPR1</td>
<td>rat progesterone receptor</td>
</tr>
<tr>
<td>rRNA</td>
<td>ribosomal RNA</td>
</tr>
<tr>
<td>RT</td>
<td>reverse transcription</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>rT3</td>
<td>reverse T3</td>
</tr>
<tr>
<td>RXR</td>
<td>9-cis retinoid X receptor</td>
</tr>
<tr>
<td>SA-PMP</td>
<td>streptavidin-coated paramagnetic particles</td>
</tr>
<tr>
<td>SCC</td>
<td>side-chain cleaving enzyme</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>se</td>
<td>standard error</td>
</tr>
<tr>
<td>SHBG</td>
<td>sex hormone-binding globulin</td>
</tr>
<tr>
<td>SMRT</td>
<td>silencing mediator of retinoic acid and thyroid hormone receptors</td>
</tr>
<tr>
<td>SRC-1</td>
<td>steroid receptor co-activator</td>
</tr>
<tr>
<td>SSC</td>
<td>saline sodium citrate</td>
</tr>
<tr>
<td>SSPE</td>
<td>sodium saline phosphate-EDTA</td>
</tr>
<tr>
<td>T2</td>
<td>3,3'-diiodothyronine</td>
</tr>
<tr>
<td>T3</td>
<td>3,5, 3'-triiodothyronine</td>
</tr>
<tr>
<td>T4</td>
<td>thyroxine</td>
</tr>
<tr>
<td>TAF</td>
<td>transcription activation function</td>
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<tr>
<td>TBE</td>
<td>tris-borate EDTA</td>
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<tr>
<td>TBG</td>
<td>thyroxine-binding globulin</td>
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<tr>
<td>TBP</td>
<td>TATA-binding protein</td>
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<tr>
<td>TBPA</td>
<td>thyroxine-binding prealbumin</td>
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<tr>
<td>TBS</td>
<td>tris buffered saline</td>
</tr>
<tr>
<td>TBS-T</td>
<td>tris buffered saline with Tween</td>
</tr>
<tr>
<td>TCA</td>
<td>trichloroacetic acid</td>
</tr>
<tr>
<td>TE</td>
<td>tris-EDTA</td>
</tr>
<tr>
<td>TEBG</td>
<td>testosterone-estradiol-binding globulin</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>TFIID</td>
<td>transcription factor IID</td>
</tr>
<tr>
<td>TG</td>
<td>thyroglobulin</td>
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<tr>
<td>TH</td>
<td>thyroid hormones</td>
</tr>
<tr>
<td>THNR</td>
<td>thyroid hormone nuclear receptor</td>
</tr>
<tr>
<td>TIF1</td>
<td>transcription intermediary factor 1</td>
</tr>
<tr>
<td>TIF2</td>
<td>transcription intermediary factor 2</td>
</tr>
<tr>
<td>TR</td>
<td>thyroid receptor</td>
</tr>
<tr>
<td>TRAP</td>
<td>thyroid receptor auxiliary protein</td>
</tr>
<tr>
<td>TRE</td>
<td>thyroid response element</td>
</tr>
<tr>
<td>TRH</td>
<td>thyrotropin releasing hormone</td>
</tr>
<tr>
<td>Trip1</td>
<td>thyroid hormone-receptor-interacting protein 1</td>
</tr>
<tr>
<td>Tris</td>
<td>tris (hydroxymethyl)-methylamine</td>
</tr>
<tr>
<td>TSH</td>
<td>thyroid stimulating hormone</td>
</tr>
<tr>
<td>TX</td>
<td>thyroidectomised/hypothyroxinemic</td>
</tr>
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<td>UTP</td>
<td>uridine-5'-triphosphate</td>
</tr>
<tr>
<td>VD₃R</td>
<td>vitamin D receptor</td>
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Chapter 1:
Introduction
1.1 Historical background

In the 2nd century, Galeneos, the ancient Greek physician, described the thyroid as a mucus-producing organ with the purpose of lubricating the pharynx and larynx (cited in Ericson and Fredrikson, 1990). However, in 1543 Vesalius gave a full description, but it was not until 1656 that the organ was named "the thyroid" (after the Greek word, thyreos, meaning oblong shield) by Wharton (cited in Werner, 1971), who believed that the function of the thyroid was solely cosmetic, especially for women, giving the throat region “more beautiful roundness” (cited in Ericson and Fredrikson, 1990).

In 1895, an important step in the understanding of thyroid gland function was taken by Baumann who observed that this was the only organ in mammals that had the capacity to incorporate iodine into organic substances. This observation was supported by Oswald in 1899 (cited in Warner, 1971). In 1912, Gudematsch showed that tadpole metamorphosis could be accelerated by feeding thyroid extract, an early indication of the influence of thyroid hormone on maturation. The biologically active iodine-containing compound, thyroxine, was isolated from the thyroid gland by Kendall in 1915. The structure of thyroxine was elucidated as 3,5,3',5'-tetraiodothyronine (T4) by Harington and Barger (1927). Triiodothyronine (T3), or 3,5,3'-triiodothyronine was identified 25 years later in thyroid extracts and plasma by Gross and Pitt-Rivers (1952) and by Roche et al. (1952), respectively. Later, Roche et al. (1956) reported the occurrence of the biologically inactive substances 3,3',5'-triiodothyronine (reverse T3 or rT3) and 3,3'-diiodothyronine (3,3'-T2) in thyroid tissue and plasma of the rat (Leonard and Visser, 1986).

1.2 Clinical considerations

Over one billion people live under iodine deficient conditions and are at risk from the iodine deficiency disorders (Hetzel, 1983; Bernal and Nunez, 1995). Although severe endemias are found mainly in developing countries, recent surveys show that marginal deficiencies also exist in industrialised nations including Greece, Italy, Portugal, Spain, Germany, Ireland and Belgium (Delange and Burgi, 1989). Cretinism, caused by deficiency of thyroid hormone during early development, is at the extreme end of the scale of dysfunction. Different types of cretinism have been identified, and are distinguished on the basis of their aetiology:

Sporadic cretinism or congenital hypothyroidism:

Individuals are born in areas with an adequate iodine intake, however, fetal thyroid function is impaired due to: a) agenesis or dysgenesis of the thyroid; b) enzyme defect compromising thyroid hormone synthesis; c) thyroid damage, for example, from
irradiation (Pharoah and Connolly, 1989). Major CNS damage is reversible by T4 if given after birth, however, the postnatal thyroid function remains permanently impaired and if children were not on continuous T4 replacement therapy, they will remain permanently hypothyroid (Morreale de Escobar et al., 1997).

**Endemic cretinism:**

Iodine deficiency in defined geographical locations resulting in irreversible somatic and mental abnormalities is known as endemic cretinism. Two forms of endemic cretinism are known where severe iodine deficiency has been suffered in utero and/or postnatally and where the effect on brain function is severe and irreversible:

a) **Myxoedematous cretinism:** the characteristics include hypothyroidism, mental and intellectual deficiency (Stanbury, 1986; Gaitan and Dunn, 1992), retardation of growth and puberty (Boyages, 1997) and dwarfism (Hetzel, 1989). Hearing, speech and motor functions are usually spared and injection of iodized oil late in pregnancy and continuation of iodine supplementation into infancy and adulthood improves the developmental outcome of such cretins (Morreale de Escobar et al., 1997).

b) **Neurological cretinism:** the characteristics include mental retardation, deaf mutism, spastic diplegia and squint (Hetzel, 1989; Pharoah and Connolly, 1989). The offspring are totally euthyroid (Boyages, 1997; Morreale de Escobar et al., 1997) and the postnatal thyroid function shows adaptation to their low iodine intake, however, the thyroid function is normal when iodine intake is adequate (Morreale de Escobar et al., 1997). Maternal iodine deficiency has to be corrected before, or during the first half of pregnancy to prevent neurological cretinism.

Selenium and iodine are both essential for thyroid hormone metabolism and thus play a vital role in the health of man and animals. In iodine-deficient rats, selenium deficiency causes further impairment of thyroid hormone metabolism and could therefore play a role in the aetiology of both myxoedematous and neurological cretinism in humans. At least 13 selenoproteins are described including glutathione peroxidase, phospholipid hydroperoxide and selenoprotein-p which act as antioxidant defences, and specific thyroid tissue damage may occur in states of selenium deficiency (Boyages, 1997). Moreover, type I deiodinase, which catalyzes the 5'-deiodination of both T4 and rT3, is a key selenoprotein. In selenium deficiency, both T4 and rT3 levels rise in the serum as a result (Boyages, 1997).

In adults, hypothyroidism results in a decreased basal metabolic rate, decreased cardiac output, constipation, hypothermia, and cold intolerance in addition to neurological
symptoms including dullness and lethargy, slow speech rate and prolonged reflex time. Patients tend to demonstrate a generalized myxedema (nonpitting edema: because of the gel nature of the excess fluid, it is relatively immobile), which results from the accumulation of a mucus-like material, glycosaminoglycans (mucopolysaccharides; primarily hyaluronic acid and chondroitin sulfate) in the interstitial spaces, hence allowing fluid to be retained (Porterfield, 1996).

Hyperthyroidism due to Grave’s disease, Hashimoto’s thyroiditis and nodular goitre are recognized in which common symptoms include increased basal metabolic rate, increased cardiac output, heat intolerance, hyperreflexia, irritability, anxiety, exophthalmos, diarrhoea, and tremor. The most prevalent form of hyperthyroidism is Graves’ disease which is an autoimmune disorder whereby T lymphocytes become sensitized to antigens within the thyroid gland and subsequently stimulate B lymphocytes to produce IgG antibodies to these antigens. Some of these antigens can mimic the action of TSH on the thyroidal TSH receptors (Porterfield, 1996).

1.3 The thyroid gland

The human thyroid gland is located at the base of the neck below the larynx on either side of and anterior to the trachea. The two large lateral lobes that comprise the bulk of the gland lie on either side of the trachea and are connected by a thin isthmus. A third structure, the pyramidal lobe, which may be a remnant of the embryonic thyroglossal duct, is sometimes also seen as a finger-like projection extending headward from the isthmus. The thyroid gland in the normal human being weighs about 15 to 20 g but is capable of enormous growth, sometimes achieving a weight of several hundred grams when stimulated intensely over a long period of time. Such enlargement of the thyroid gland, which may be grossly obvious, is called a goitre and is one of the most common manifestations of thyroid disease.

The thyroid gland develops from an invagination in the floor of the primitive pharynx at the level of the first and second cartilaginous ring. This invagination, lined by cylindrical epithelial cells, can be distinguished in the human embryo between 16 to 17 days of gestation (dg) (Boyd, 1964) and in the rat at 10 dg (Romert and Gauguin, 1973). The invagination grows downward in front of the primitive pharynx and the developing hyoid bone. This down-growth being accomplished by proliferation of existing cells and recruitment of additional cells from the pharyngeal epithelium (Shain et al., 1972). The cells in the endodermal invagination which can be distinguished from the surrounding pharyngeal epithelium develops into a flask-like vesicle with a narrow neck, the ductus thyroglossus. The vesicle divides and continues its down-growth to the final position on
the front of the upper trachea. In humans, this process is completed by 45 to 50 dg and in rats by 13 to 14 dg (O'Riordan et al., 1985; McDougall, 1992).

By the 7th week of human development, the connection of the thyroid to the pharynx is lost, and the cells of the thyroid are grouped into clusters. At about 11 weeks, a central lumen appears in each cluster, completely surrounded by a single layer of cells (Hifer, 1979; O'Riordan et al., 1985). In the human fetal thyroid, it was thought that the initiation of fetal thyroid hormone synthesis was at 10 - 12 weeks of gestation (Porterfield and Hendrich, 1993), however, recent data obtained from aborted fetuses and premature babies suggest that the fetal thyroid T4 and T3 secretion does not start until mid-gestation (Morreale de Escobar et al., 1997). Rat fetal thyroid, on the other hand, starts to secrete thyroid hormones (TH) at 17.5 - 18 dg (Morreale de Escobar et al., 1989).

The functional unit of the thyroid gland is the follicle. The follicle is composed of epithelial cells (follicular cells) which produce thyroxine and triiodothyronine. These cells are cuboidal in a normal gland, columnar in a highly stimulated gland, and squamous in an inactive gland. In the lumen, there exists a clear viscous material called colloid. The major constituent of colloid is the large glycoprotein, thyroglobulin, which contains the TH within its structure. The parafollicular cells, which are located on or between the follicles, produce the polypeptide hormone calcitonin (Porterfield, 1996).

1.4 The thyroid hormones

Thyroid hormones are iodothyronines, compounds formed by coupling two iodinated tyrosine molecules in an ether linkage. Thyroxine constitutes about 90% of the thyroid hormone secreted from the gland; triiodothyronine about 9% and reverse T3 about 1% (Fig. 1.1). When tyrosine molecules are iodinated, moniodotyrosine and diiodotyrosine are formed. These iodotyrosines are not biologically active. Thyroxine 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 (Silva and Larsen, 1986).

1.4.1 Synthesis and storage of thyroid hormones

The normal daily requirement for iodide has been estimated at 100 to 150 µg/day with higher intakes of at least 200 µg/day during pregnancy due to enhanced maternal requirements, increased renal clearance, and diversion of iodide for fetal thyroid hormone production (Boyages, 1997). Iodine is taken up as inorganic iodide by the thyroid follicular cells. The presence of an active iodide pump in these cells allows the concentration of iodide against a steep concentration gradient. Under normal
circumstances, iodide within the thyroid gland is about 25 times more concentrated than in plasma, but during periods of active stimulation, the iodide concentration in follicular cells may be as high as 250 times that of plasma (Goodman, 1994).

Thyroid cells are typical protein-secreting glandular cells. Thyroglobulin is synthesized on the rough endoplasmic reticulum of the follicular cell, and the glycosylation occurs in the Golgi apparatus. It is then packaged in exocytotic vesicles and extruded into the lumen of the follicle. Each molecule of thyroglobulin contains 140 tyrosine residues, and these are the major substrates that combine with iodine to form the TH within the thyroglobulin molecule.

![Figure 1.1: Structures of T4, T3 and rT3](image-url)
In order for iodide to be incorporated into tyrosine molecules, it must first be oxidized to some higher oxidation state, such as atomic iodine $I^3$. The reaction is catalyzed by thyroid peroxidase which requires hydrogen peroxide (oxidizing agent) which together act as a potent system capable of oxidizing iodides. Thyroid peroxidase is a membrane bound enzyme found on the apical surface (near the lumen) of the follicular cell (Goodman, 1994).

Iodination, is the addition of iodine molecules to tyrosine. This process also occurs at the apical surface of the follicular cells. Oxidized iodine even in the molecular form will bind directly to tyrosine in the presence of an iodinase enzyme allowing organification of iodine to occur within seconds or minutes. Tyrosine is first iodized to monoiodotyrosine and then to diiodotyrosine. Then during the next few minutes, hours and even days, coupling of the diiodotyrosine residues with each other and coupling of one molecule of monoiodotyrosine with one molecule of diiodotyrosine yields thyroxine and triiodothyronine, respectively (Guyton, 1991). Thyroid peroxidase catalyzes this reaction and these products of the coupling reaction remain part of the thyroglobulin molecule. Only about 20% of iodinated tyrosine residues undergo coupling, with the rest remaining as monoiodotyrosine and diiodotyrosine.

The thyroid is unique among endocrine glands in that it stores its product extracellularly (in the follicular lumen) as large precursor molecules. In normal individuals about 30% of the mass of the thyroid gland is thyroglobulin. Each thyroglobulin molecule contains 1 to 3 thyroxine molecules and an average of 1 triiodothyronine molecule for every 10 molecules of thyroxine. The TH are stored in this form in an amount sufficient to supply the body with its normal requirements for 2 to 3 months. Therefore, even when synthesis of thyroid hormone ceases entirely, deficiencies in circulating levels may not be observed for several months.

1.4.2 Thyroid hormone secretion

Under normal conditions, little or no thyroglobulin is found in the circulation. Upon acute stimulation with thyroid-stimulating hormone, long strands of protoplasm (pseudopodia) reach out from the apical surfaces of follicular cells to surround luminal thyroglobulin, which is taken up into endocytic vesicles. These endocytic vesicles then fuse with lysosomes and the fused lysoendosomes migrate towards the basement membrane. Concomitant with lysoendosome migration, lysosomal proteinases digest the thyroglobulin molecule to yield free amino acids, including T4, T3, monoiodotyrosine and diiodotyrosine. Of these, only T4 and T3 are released into the bloodstream, in a ratio of 20:1, perhaps by a process of simple diffusion down a concentration gradient (Goodman, 1994).
Free monoiodotyrosine and diiodotyrosine cannot be utilized for thyroglobulin synthesis and are rapidly deiodinated by a specific thyroid deiodinase in the follicular cells. Virtually all of the iodide released from iodotyrosines is recycled into thyroglobulin (Porterfield, 1996). Deiodination of iodotyrosine provides about twice as much iodide for hormone synthesis as the iodide pump and is therefore of great significance in hormone biosynthesis.

1.4.3 Thyroid hormone transport

On entering the blood, all but minute portions of the T4 and T3 combine immediately with several plasma proteins. More than 80% of T4 and T3 binds to thyroxine-binding globulin (TBG), 15% of the T4 and less than 5% of the circulating T3 bind with thyroxine-binding pre-albumin (TBPA, also called transthyretin), while the bulk of the remainder binds to albumin. All three plasma binding proteins are large enough to escape glomerular filtration. The remaining thyroid hormone, less than 1%, is present free in solution and is in equilibrium with bound hormone (Goodman, 1994).

Plasma proteins provide a substantial reservoir of extrathyroidal hormone thus limiting large or rapid changes in the circulating concentrations with acute changes in the rate of secretion of TH. Thyroid hormones, in particular thyroxine, are released to the tissue cells very slowly because of the high affinity of TBG to the hormones. Half of the T4 in the blood is released to the tissue cells in approximately six days, whereas half of the T3 - because of its lower affinity - is released to the tissues in approximately one day (Guyton, 1991). Once in the cells, TH bind to intracellular proteins, with T4 binding more strongly than T3.

It was believed that serum binding proteins serve to minimize T4 and T3 hormone loss from the vascular compartment (Hoffenberger and Ramsden, 1983). More particularly, the rise in TBG seen in pregnancy had been proposed as constituting a mechanism specifically designed to prevent passage of TH from maternal to fetal circulations (Osrio and Myant, 1960). However, Ekins (1984) proposed that thyroid hormone-binding proteins may specifically serve to ensure adequacy of maternal T4 supply to the fetus before the development of the fetal thyroid. There are several pieces of evidence which point to the involvement of TBG with the feto-placental unit. Firstly, while levels of total TH rise during this period (Ekins, 1986), free hormone levels remain more or less unaltered in maternal serum. However, in pregnancy the higher the binding protein concentration, and thus the bound hormone concentration, the higher the free hormone concentration at the capillary wall (Ekins et al., 1994). Secondly, the anatomical structure within the placenta is one in which a rise in TBG would be expected to enhance maternal T4 transfer to the fetal circulation (Ekins et al., 1994). Therefore, TBG is capable of
1.4.4 Metabolism of thyroid hormones

The released thyroxine and triiodothyronine hormones travel to the target tissues. The biologically active T3 is generated *in vivo* by peripheral monodeiodination of T4 in the liver and kidney, and to a lesser extent in other tissues, catalyzed by deiodinases. At least three different iodothyronine deiodinases have been reported. They are distinguished according to their selectivity of the reactions they catalyze and their susceptibility to inhibition by the anti-thyroid drug 6-propyl-2-thiouracil (PTU). They are known as type I and II 5'-monodeiodinases and type III 5-monodeiodinase (Leonard and Visser, 1986). The first step in the metabolic pathway for T4 determines irreversibly whether an active or inactive hormone is formed. Deiodination of T4 in the liver and kidneys provides about 80% of the T3 in the blood.

Type I 5'-monodeiodinase (5'D-I) is a non-selective enzyme that deiodinates both the phenolic (outer) and tyrosyl (inner) rings and is inhibited by PTU (Leonard and Rosenberg, 1978; 1980; Visser, 1979; Visser *et al*., 1979). It catalyzes 5'-monodeiodination of both T4 and rT3, with rT3 being the preferred substrate. As the Km of this enzyme for T4 and rT3 is much greater than the physiological tissue concentrations, it has been suggested that 5'D-I does not play a regulatory role in TH metabolism, but is engaged in the catabolism of surplus hormone for iodide economy (Leonard and Visser, 1986; Leonard, 1990).

5'D-I deiodinase is found in most body tissues including the thyroid. In rats, it is most abundant in the liver and kidney and less so in the developing pituitary, eye and lung. In the brain, the enzyme activity appears to be present mostly in the glial cells, with little if any activity in neurons (Leonard and Larsen, 1985; Leonard and Visser, 1986; Leonard, 1992). The brain 5'D-I enzyme only catalyzes the degradation of rT3, and thereby removes a potent competitive substrate that could affect T3 generation by type II deiodinase (Leonard, 1992).

Type II 5'-monodeiodinase (5'D-II) is insensitive to PTU and is a 5'-monodeiodinase since it only catalyzes phenolic ring deiodination. It is localised predominantly in the CNS and is responsible for local generation of T3 from T4 by 5'-monodeiodination. 5'D-II is found at high levels in the brain and pituitary (Kaplan and Yaskoski, 1980; Leonard and Visser, 1986). In the brain 5'D-II is mainly localised to the neurons (Leonard and Larsen, 1985; Leonard, 1992). This enzyme has also been identified in other mammalian tissue including brown adipose tissue (Leonard *et al*., 1983) and placenta (Kaplan and Shaw,
Thyroxine appears to be the preferred substrate over rT3, however, rT3 is believed to be a competitive inhibitor of T3 production from T4.

Type III 5-monodeiodinase (5D-III) is also insensitive to PTU, however, it is a 5 monodeiodinase as it catalyzes the tyrosyl ring deiodination. This enzyme is most abundant in the skin (Leonard, 1990), placenta, amniotic fluid and fetal tissues including the brain (Visser and Schoenmakers, 1992). In the brain, 5D-III is present predominantly in glial cells (Cavalieri et al., 1986). It catalyzes the inner ring deiodination of T4 and T3, with some preference for T3 over T4. In the rat central nervous system, the activity of this enzyme is highest in the cerebral cortex than in the cerebellum, brain stem and spinal cord, with intermediate values in other brain areas (Kaplan and Yaskoski, 1980). As 5D-III catalyzes the inner ring deiodination of T4 to rT3 and of T3 to 3,3'-T2, it therefore represents an important pathway for the inactivation of thyroid hormone (Visser and Schoenmakers, 1992).

In early pregnancy, T3 cannot be detected in human fetal tissues other than the brain, suggesting that 5'D-II activity and synthesis are exclusive to the brain at this stage. Detection of T3 in fetal brain has been reported as early as 11 week gestation indicating the presence of 5'D-II (Bernal and Pekonen, 1984). Type 5D-III enzyme activity is also present in the fetal cerebral cortex long before the onset of fetal thyroid function with relatively high activity at 19 - 22 weeks gestation aiding in the binding of T3 and converting it to T2 (Sinha et al., 1997).

In rat brain, 5'D-II has been reported at 17 days gestation increasing 6-fold by term (Ruiz de Ona et al., 1988; Obregon et al., 1991). After birth, there is a decline of 5'D-II activity to low levels by 4 to 5 postnatal days, followed by an increase reaching maximal levels by day 12. Activity then gradually declines to low adult levels (Kodding et al., 1986; Obregon et al., 1991). In addition to 5'D-II, the fetal brain also exhibits 5D-III activity which increases 1.5-fold between 14 and 19 days gestation (Huang et al., 1988), with a more pronounced increase during the first few days of postnatal life. Adult values of this enzyme are reached by 20 days postnatally (Kodding et al., 1986). From as early as 17 days gestation, the brain is able to respond to reduced circulating T4 levels to maintain T3 concentrations by increasing T4 uptake (Morreale de Escobar et al., 1992), down-regulating 5D-III activity (Kaplan, 1986; Huang et al., 1988), and increasing 5'D-II activity (Ruiz de Ona et al., 1988; Obregon et al., 1991).

1.4.5 Extrathyroidal pools

Large extrathyroidal pools of TH exist in the liver and kidney where one third of the body’s T4 is found. The T4 pool is 20 times greater than the T3 pool and has a slower
turnover of 10% per day compared to 70% per day for the T3 pool (Porterfield, 1996). The T4 pool serves to "buffer" acute changes in hormone secretion rate as the size of this pool is large relative to its secretion rate (Porterfield, 1996).

1.5 Regulation of thyroid function

Specific feedback mechanisms operate through the hypothalamus and anterior pituitary gland to control the rate of thyroid hormone secretion. Thyroid-stimulating hormone (TSH), also known as thyrotropin, is an anterior pituitary hormone, a glycoprotein with a molecular weight of about 28,000 (Guyton, 1991). This hormone increases all the known activities of the thyroid glandular cells including blood flow, iodide uptake, oxidation of iodide, organification, and coupling. On prolonged stimulation, TSH increases the height of the follicular epithelium (hypertrophy) by increasing the synthesis of ribonucleic acid (RNA) and cellular proteins and may promote thyroidal deoxyribonucleic acid (DNA) synthesis indicative of cell division (hyperplasia) (Goodman, 1994). Furthermore, both endocytosis of colloid and TG proteolysis increase.

The secretion of TSH by the pituitary gland requires stimulation by the hypothalamic hormone thyrotropin-releasing hormone (TRH) produced in the hypothalamus (and other regions of the brain). Constant levels of thyroid hormone in blood are achieved by negative feedback inhibition of TSH secretion at the level of either the hypothalamus or the pituitary by T4 and T3 (Fig. 1.2). High serum T4 levels and to a lesser extent T3 levels inhibit TSH secretion (Goodman, 1994). However, the actual control within the thyrotrope is a function of the intracellular T3 levels. When intracellular T3 is high, there is down-regulation of the TRH receptors and the pituitary response to TRH decreases. However, if serum T4 levels are low, intracellular T3 levels drop and the concentration of thyrotope TRH receptor increases, thereby, increasing the sensitivity of the pituitary to TRH (Porterfield, 1996).

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 low or unchanged. The possibility of a non-pituitary control of thyroid hormone secretion involving the placenta has been suggested. Human chorionic gonadotrophin (hCG), secreted by the placenta in early pregnancy, may be implicated in a feed-back system interacting with the hypothalamic/pituitary system governing maternal thyroid hormone secretion. It has been suggested that the increase in total serum T4 in the first trimester is due to increased thyroid activity. However, as the level of TSH during that period is depressed, then hCG may be exerting stimulatory effects on the maternal thyroid gland during this period (Sinha et al., 1992a).
Figure 1.2: Hypothalamic-hypophyseal-thyroid axis
1.6 Biological effects of thyroid hormone action

Thyroid hormone produces profound effects on the physiology, metabolism and biochemistry of most tissues and cell types of mammalian organisms (Sokoloff, 1971). The general effect of thyroid hormone is to cause wholesale nuclear transcription of large number of genes. Therefore, in virtually all cells of the body great numbers of proteins, enzymes, structural proteins, transport proteins, and other substances increase. The net result is a generalized increase in functional activity throughout the body. Normal thyroid function is necessary for the attainment of normal body weight, structure and stature, and for proper maturation of nervous and other body systems (Legrand, 1986).

Thyroid hormones control the basal metabolic rate (BMR), which is a measure of oxygen consumption under defined resting conditions. Oxygen consumption in all tissues except brain, testis and spleen is sensitive to thyroid status and increases in response to TH. The BMR decreases in hypothyroidism and increases in hyperthyroidism. In hyperthyroidism the mitochondria increase in both size and number, the complexity of their cristae increases, as does the concentration of the oxidative phosphorylation enzymes (Porterfield, 1996). This may be related to changes in mitochondrial respiratory chain components; for example, cytochrome c, ubiquinone, and the microsomal NADH cytochrome c reductase system are increased in hyperthyroidism but decreased in hypothyroidism (Sokoloff, 1971).

In hypothyroidism, protein synthesis decreases but so does protein degradation, while in hyperthyroidism, both synthesis and degradation increase (Porterfield, 1996). Numerous studies have established that TH stimulate protein synthesis (Wolff, 1964). In tissues such as liver, kidney, and heart (which also respond with increased metabolic rate), thyroid hormone administration to normal animals stimulates the incorporation of amino acids into protein. Furthermore, in vivo studies have shown that thyroid hormone administration stimulates nuclear DNA polymerase activity and the synthesis of rRNA (Tata and Widnell, 1966). These effects result in an increased cellular content of functional ribosomes and therefore ultimately result in an increase in the rate of protein synthesis (Sokoloff, 1971).

During development, TH are known to affect hepatic enzymes, pituitary growth hormones, bone growth, brain and skin maturation (Sokoloff, 1971). Bone growth is promoted indirectly by the actions of TH on both the pituitary gland and the hypothalamus (Goodman, 1994). In addition, the production of cartilage is partially controlled by thyroid hormone, and this may explain the growth defects and bone malformation observed in cretins (Timiras, 1988). Furthermore, many reports indicate a correlation between thyroid function and sexual maturation. A reduction in the number of maturing
ovarian follicles and atrophy of the ovaries and uterus has been reported in hypothyroid rats and rabbits (Parrot et al., 1960; Hagino, 1971).

1.7 Thyroid Hormones and Brain Development

The rat brain can be studied as long as equivalent human and rat developmental stages are compared. At 10 days postnatally, the rat brain is at the developmental stage equivalent to the human brain at birth (Bass et al., 1977), and the rat brain at birth is at the same stage as the human brain at 5 - 6 months of gestation (Eayrs, 1968). Consequently, stages of brain development that occur in the last trimester of human development occur postnatally in the rat. By birth, rat cerebral cortex neurogenesis is essentially complete with the bulk of the neurogenesis occurring between 12 days gestation and birth (Balzas, 1973; Berry, 1974; Stein et al., 1989). Most of the increase that occurs in cells from cerebrum postnatally is from glial cell proliferation, although some formation of interneurons is apparent. In the rat gliogenesis begins at birth and continues into adult life (Berry, 1974), whereas in the human, cerebral neuronal proliferation is nearly complete by the seventh month of pregnancy (Zamenhof and Marthens, 1971) and gliogenesis begins in utero (Dobbing and Sands, 1973). The rat cerebellum develops later than the cerebral cortex and 77% of the neurogenesis occurs after birth (Balazs and Richter, 1973) while in humans cerebellar neurogenesis occurs late in fetal development.

The maturation of the nervous system during the perinatal period has an absolute dependence on thyroid hormone. During this critical period, thyroid hormone must be present for normal development of the brain (Dussault, 1989). Thyroid hormones appear to regulate those processes associated with terminal brain differentiation such as dendritic and axonal growth, synaptogenesis, neuronal migration, and myelination (Oppenheimer and Schwartz, 1997). Deficiency or excess of TH leads to disturbances in brain development which are irreversible if thyroid status is not promptly corrected (Dussault, 1989). The precise role of TH in brain development is unknown. However, a variety of in vivo and in vitro experimental models have been used to evaluate the effect of dysthyroid states (hypothyroidism in particular) on the developing brain. Behavioural, anatomical and biochemical findings are discussed in detail below.

1.7.1 Behavioural effects of thyroid hormones

There is a close relationship between the behavioural performance of the hypothyroid rat and the age at which the thyroid is destroyed. The impairment has proved to be severe in animals in which the thyroid is destroyed on the day of birth and to a less extent when hypothyroidism is induced 10 days postnatally. Similarly, thyroid hormone replacement therapy in animals made hypothyroid at birth is effective only when begun during the first...
10 days of postnatal age (Hamburgh, 1969). Male and female offspring of hypothyroid rat dams have mental deficiencies as exemplified by lack of memory, due to their inability to learn the Lashley maze, and hyperactivity, as demonstrated by increased spontaneous movements in the stabilimeter cage (Hendrich et al., 1984). In a more recent study, maternal thyroidectomy was found to cause significant malbehaviour and reduced activity in adult rat progenies where such animals take longer to emerge from a box placed in an open field and exhibit reduced activity within the open field (Attree et al., 1992). Female progeny demonstrate decreased baseline locomotor activity and when a novel object is introduced into the open field, only male progeny exhibit deficient locomotor behaviour.

In humans, hypothyroid children and cretins show behavioural abnormalities including low IQ, specific learning disabilities, attention deficits, speech and language disturbances and a range of neurological problems (Pharaoh et al., 1980). However, a normal IQ, in congenitally hypothyroid children, can be achieved when the thyroid hormone treatment is initiated within the first few months after birth (Smith, 1981). All these observations point to an important role of the TH during the critical period of central nervous system development where changes in thyroid hormone level have a great effect on neural maturation. On the other hand, adult hypothyroidism causes a range of psychological disorders, behavioural morbidity and motor dysfunction, where general slowing of psychomotor function and changes in mood and memory correlate significantly with general hypothyroid state (Mennemeir et al., 1993). Adult hypothyroidism has also been shown to be associated with dementia, which can be alleviated by replacement therapy with TH (Haupt et al., 1991). The hypothyroid state in human has also been correlated with depressive illness, and successful treatment of manic depression with high doses of T4 has been reported (Sinha et al., 1994). In contrast, hyperthyroidism correlates with certain personality disorders and behavioural dysfunctions, such as increased anxiety, nervousness, irritability, depressiveness and agoraphobia (Rockel et al., 1987; Weller, 1984).

1.7.2 Morphological effects of thyroid hormone

The first anatomical indication of developmental abnormalities resulting from abnormal thyroid function, was the brain size and shape. In rats made hypothyroid at birth, a reduction in brain water content has been reported (Sokoloff, 1971), as well as brain weight loss and alteration in shape (Eayrs, 1960). Legrand reported in 1976 that the maturation of the cerebellum is greatly affected by neonatal hypothyroidism. Furthermore, neonatal hypothyroidism was found to inflict reductions in the size of the cerebellum as well as deficit in basket cell number, a preponderance of undifferentiated neurones, a reduction in synaptic density, and an increase in glial cell number (Balazs et al., 1968; Hetzel and Querido, 1980; Smith, 1981). Postnatal hyperthyroidism, on the other hand,
accelerates differentiation, particularly in the cerebellum, in such a way as to terminate cell replication prematurely and changes the relative proportion of different cell types with fewer neurones in general and a major deficit in basket cells (Smith, 1981). During normal development in most brain regions, neuronal proliferation is terminated at birth, whereas glial cells continue to proliferate in the early postnatal period. In the cerebellum, neuronal proliferation begins after birth in the extragranular layer (EGL) and continues for two weeks. Cell proliferation lasts for a longer time in the hippocampus while it may continue to the adult stages in other zones (Nunez, 1984). Many studies have shown that normal cell proliferation activity can be immensely disturbed by changes in thyroid hormone levels. For example in the cerebellum, the EGL proliferation process is normally completed by day 20 after birth but hypothyroidism was reported to cause a delay in the cell proliferation in the EGL (Nunez, 1984), and severe damage to the maturity of Purkinje cells, but the granular cells were less affected (Legrand et al., 1976). After a period of mitotic activity, cells from the EGL normally migrate towards the inner granular layer, differentiate in the process and establish contacts with afferent mossy fibres and Purkinje cells to yield the normal "wiring pattern". Neonatal hypothyroidism delays the disappearance of the inner granular layer and decreases the number and density of synaptic contacts with Purkinje cells and short parallel fibres, resulting in a permanent impairment of neuronal connectivity. The critical period for this stage of development appears to be the first 21 days after birth (Dussault and Ruel, 1987).

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

Perhaps the most noticeable morphological signs of neural differentiation is the formation of the myelin sheath around the axon. It has been reported that in hypothyroid animals, myelinoogenesis of the developing brain is retarded and the total amount of myelin in some tracts is reduced. This may be related to the reduction in enzyme activities responsible for
myelin synthesis and lack of axons as a result of thyroid hormone deficiency (Hamburgh, 1969). Therefore the timing and duration of TH deficiency plays a major role in determining the specific damage inflicted either on brain region or cell type.

1.7.3 Biochemical effects of thyroid hormone

Thyroid hormones exert control over many biochemical systems in the developing CNS. During normal brain development, the content of protein, DNA and RNA all rise. However, these changes are delayed and reduced in magnitude by thyroid hormone deficiency at, or shortly following, birth (Geel and Timiras, 1967; Balazs et al., 1968). In the brain of the hypothyroid rat protein synthesis is depressed (Geel and Timiras, 1967). Moreover, decreases in protein and RNA per unit DNA have also been reported in rats made hypothyroid after two weeks of postnatal life (Balazs et al., 1968). Rat neonatal hypothyroidism also causes a significant reduction in the DNA content of the postnatal cerebellum but not the forebrain, while, adult-onset hypothyroidism has no effect on the content or concentration of nucleic acid or protein (Ahmed et al., 1993). Alteration in thyroid state also causes a depression in glucose uptake and metabolism as well as a severe reduction in amino acid uptake and synthesis in the brain of the hypothyroid neonatal rat, which may contribute to the depression in the normal rate of protein synthesis (Geel et al., 1967; Balazs et al., 1968; Hamburgh, 1969; Hendrich et al., 1984).

With regard to adult brain, abnormalities including protein and DNA concentrations/ratios, acetylcholine esterase and certain lysosomal enzyme activities, were apparent in 7 month old progeny which were not detected at earlier stages (Pickard et al., 1997). In rapidly developing tissues, the glucose transporter isoform, GLUT1 is expressed at high levels (Mueckler, 1994). Maternal hypothyroxinemia results in a compromise in glucose uptake in fetal brain parenchyma directly due to deficient GLUT1 expression (Pickard et al., 1995). As for GLUT1 expression, ornithine decarboxylase activity (ODC), a highly sensitive marker for the detection of abnormalities in perinatal brain maturation (Slotkin et al., 1986), was reduced in 15 day gestation fetal rat brain (Pickard et al., 1997).

During the differentiation period, the neurones undergo dramatic changes in cell shape. Neurites develop and then differentiate into axon or dendrites. For this neurite outgrowth microtubules are required (Nunez et al., 1992). These microtubules are composed of tubulin (representing about 70% of the neurite proteins) and a number of microtubule associated protein (MAPs) which have been shown to act as promoters of microtubule assembly (Fellous et al., 1979). A reduction in microtubule number and function has been noticed in developing hypothyroid rat axons and dendrites (Stein et al., 1991).
Furthermore neonatal hypothyroidism has been reported to cause changes in MAP composition which in turn impair neurite outgrowth and decrease the rate of microtubule assembly (Fellous et al., 1979).

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

1.8 Sex steroid hormones

Steroids are lipoidal compounds which possess the basic structure of four carbon rings known as the cyclopentanoperhydrophenanthrene ring, or the steroid nucleus. They are synthesized from cholesterol, a C_{27} steroid. Cholesterol in turn is synthesized from acetate (acetyl-coenzyme A) produced via glycolysis or via fatty acid oxidation.

1.8.1 Biosynthesis of sex steroids

Biosynthesis of steroid hormones in cytological term is a complex affair involving mitochondrial events as well as the smooth endoplasmic reticulum (SER). The first step in the biosynthesis of sex steroids from cholesterol occurs in the mitochondrion and involves side chain-cleaving enzyme (SCC) to yield a C_{21} intermediate, pregnenolone. Pregnenolone is then converted in the SER, by 3β-hydroxy-Δ^5-steroid dehydrogenase (3β-HSD), to progesterone (C_{21}). Progesterone conversion to C_{19} androgens and C_{18} estrogens requires re-entry of progesterone into the mitochondria (Table 1.1).

The enzyme 3β-HSD works on a variety of steroid substrates. If it acts early in the sequence by converting Δ^5-pregnenolone to Δ^4-progesterone, the subsequent enzymatic transformations of progesterone are referred to as the Δ^4-pathway. However, if pregnenolone is not first converted to Δ^4-progesterone, it is enzymatically transformed along the Δ^5-pathway (Norris, 1996). Conversion of testosterone into 5α-dihydrotestosterone (DHT) requires the enzyme 5α-reductase. Testosterone is converted by the enzyme complex aromatase to estradiol (Fig. 1.3).
### Table 1.1: Some cytochrome P-450 enzymes involved with steroid hormone synthesis

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Cell location</th>
<th>Organ location</th>
<th>Reactions catalyzed</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-450&lt;sub&gt;sec&lt;/sub&gt;</td>
<td>Mitochondria</td>
<td>Adrenal, gonad, liver</td>
<td>Cleaves cholesterol side chain to yield pregnenolone</td>
</tr>
<tr>
<td>P-450&lt;sub&gt;c17&lt;/sub&gt;</td>
<td>SER</td>
<td>Adrenal, gonad</td>
<td>Performs 17α-hydroxylase reaction necessary for androgen synthesis</td>
</tr>
<tr>
<td>P-450&lt;sub&gt;aro&lt;/sub&gt;</td>
<td>SER</td>
<td>Gonad</td>
<td>Performs aromatization of A-ring necessary for conversion of testosterone to estradiol</td>
</tr>
</tbody>
</table>

### Figure 1.3: Biosynthesis of steroid hormones (Modified from Norris, 1996)
At the time of puberty in males, testosterone secretion increases under the influence of the pituitary gonadotrophin luteinizing hormone (LH), while the development of spermatogenesis at the time of puberty is under the influence of the pituitary gonadotrophin follicle stimulating hormone (FSH) and testosterone (produced by the Leydig cells of the testis). In females, androgens are produced by both the adrenals and the ovaries. Estrogens are produced by the ovaries which are under the control of pituitary FSH and LH. During pregnancy, the corpus luteum secretes progesterone under the influence of LH. Moreover, the placenta and adrenals are all sources of progesterone synthesis. The release of both LH and FSH is in turn regulated by a common hypothalamic releasing hormone (LHRH). Estrogens, progesterone and testosterone participate in a feedback-inhibition loop by which they suppress further secretion of gonadotropins at both the hypothalamic (inhibition of LHRH) and hypophyseal levels. Inhibin, secreted by the granulosa cells, selectively inhibits adenohypophyseal secretion of FSH in response to hypothalamic LHRH.

1.8.2 Transport of steroid hormones in blood

Steroids are nonpolar compounds and are not very soluble in aqueous solutions such as blood. Steroids are bound with plasma-binding proteins and are retained at higher concentrations for longer times in the circulation. These proteins reduce the removal of active steroid hormones by the liver and kidney and their excretion through the urine (Norris, 1996). Estrogens and androgens in blood are largely bound to plasma proteins, primarily albumin and a specific carrier protein referred to as testosterone-estradiol-binding globulin (TEBG) or sex hormone-binding globulin (SHBG). Only 1 - 3% of estrogens and androgens are free in the circulation and are considered to be biologically active. Progesterone is bound to cortisol-binding globulin (CBG), albumin and various other proteins leaving only a small fraction free and biologically active.

Steroid hormones are metabolized primarily by the liver which possesses a series of enzymes capable of altering the specific steroids and rendering them biologically inactive and water-soluble. Metabolism of steroids involves removal of side chains or attached groups or both, as well as conjugation to a glucose as a glucuronide or conjugation with sulfate. The conjugates are released into the blood and excreted in the urine while some of the metabolized steroids are added to the bile and excreted through the intestinal route (Norris, 1996).

1.8.3 Sex steroid hormones and the brain

Steroid hormones play an important role not only in basal physiology, but also during the crucial periods of gestation: conception, nidation, embryonic development, and fetal
Sex steroid hormones play a critical role in the development and proper function of many organs, including the brain. They have access to the brain through the blood-brain barrier. These hormones have many effects on the nervous system that extend beyond their actions of regulating gonadotrophin and prolactin secretion and modulating sexual behaviour. They affect areas of the brain that are not primarily involved in reproduction, such as the basal forebrain, hippocampus, caudate putamen, midbrain raphe, and brainstem locus coeruleus (McEwen et al., 1997). Estrogens and androgens affect verbal fluency, performance on spatial tasks, verbal memory tests, and fine motor skills (Hampson, 1990; Kimura, 1992; Sherwin, 1994; Sherwin and Tulandi, 1996). In addition, estrogens influence the co-ordination of movements in animals (Smith, 1991) and affect the symptoms of Parkinson’s disease and tardive dyskinesia in human subjects (Bedard et al., 1977).

The hypothalamus and preoptic area have been traditionally used for the study of ovarian steroid receptors and their role in the control of reproductive function. However, many of the effects of sex steroids imply that the regions of the brain involved are outside these areas. Research has shown that estrogen and progestin receptors are indeed present in other brain regions, albeit in smaller numbers, including the amygdala, hippocampus, cingulate cortex, locus coeruleus, midbrain raphe nuclei, and central grey matter (McEwen et al., 1997).

In rodents, sexual differentiation of the preoptic area of the brain depends on perinatal exposure to estrogens. The rat fetal testis secretes testosterone perinatally, and this hormone travels to the brain where it is converted to estradiol by aromatization. Estradiol in turn binds to its receptors and as a result there are permanent changes in neuronal growth and certain enzyme systems, possibly related to neurotransmitter turnover (Greenstein and Adcock, 1985). Certainly estrogen receptors have developed in the diencephalon of rodents in time for the release of testosterone by the testis (Barley et al., 1974; MacLusky et al., 1979).
1.9 Sex steroid hormones in pregnancy

The human fetus from midgestation has all the enzymatic systems necessary for the biosynthesis of steroid hormones, such as testosterone, corticosterone, cortisol and aldosterone, but "placental progesterone" is required for this biosynthesis. The placenta synthesizes most of the progesterone and estrogens produced during pregnancy. For this purpose, progesterone uses cholesterol of both fetal and maternal origin, and pregnenolone and pregnenolonic sulfate produced mainly by the fetus (Pasqualini and Sumida, 1986).

In pregnant rats, the serum levels of progesterone reaches an initial peak on day 6 and then remain stable until day 12 where levels then increase to reach a second peak on day 16 (Taya and Greenwald, 1981; Escalada et al., 1996) then abruptly fall after day 18 (Taya and Greenwald, 1981). Serum estradiol level remains unchanged from days 2 through 12 of gestation and then increase gradually until day 22 (Taya and Greenwald, 1981). Serum testosterone remains unchanged from day 2 to 12 of pregnancy and then increases progressively until day 18 followed by a significant decline by day 22 (Taya and Greenwald, 1981). The increase of testosterone levels on day 14 of gestation and the peak attained between days 18 and 20 parallels the rising estradiol levels (Taya and Greenwald, 1981).

The high levels of progesterone during mid-pregnancy may be involved in the suppression of follicular maturation, probably by lowering basal levels of serum LH secretion (Taya and Greenwald, 1981). During days 14 - 18 of pregnancy, the maximum values of serum progesterone correlate with minimum values for serum LH. This suggests that the basal serum levels of LH between days 14 and 18 are insufficient to support optimal thecal cell synthesis of androgen and thus follicular estradiol secretion (Taya and Greenwald, 1981). Gibori et al. (1979) suggested that serum androgen during pregnancy is from an extraovarian source, probably the feto-placental unit.

In contrast to the human placenta, which plays a prominent role in producing progesterone and estrogen, the rat placenta does not produce estrogen (Townsend and Ryan, 1970) and secretes only small amounts of progesterone (Matt and Macdonald, 1984). In early and late pregnancy in rats, the ovaries secrete significant amounts of testosterone and the ovarian synthesis of testosterone is dependent on the availability of LH (Sridaran et al., 1981). In the second half of pregnancy (days 13-18), the ovaries secrete only a limited amount of testosterone, independent of LH, while the conceptuses becomes an important source of testosterone (Sridaran et al., 1981). The placenta at this stage begins to secrete androgens (Chan and Leathem, 1975; Chan and Leathem, 1977; Sridaran et al., 1981; Matt and Macdonald, 1984). The inability of the placenta to secrete
large amounts of androgen before midpregnancy is because of the inadequate levels of active 17α-hydroxylase and 17, 20-lyase enzymes (Warshaw et al., 1986). As the placenta lacks aromatizing enzymes (Sybulski, 1970), the androgens serve as substrates for estradiol biosynthesis in the corpus luteum (Jackson and Albrecht, 1985). Estradiol, aromatized locally in luteal cells, thus act to sustain progesterone synthesis (Gibori and Keyes, 1978). The placental Δ⁴-androstenedione (Δ⁴A) is the predominant androgen utilized in the formation of estradiol, a steroid hormone that in turn maintains luteal function during the second half of rat pregnancy (Jackson and Albrecht, 1985).

The ovary develops an increasing ability to aromatize both Δ⁴A and testosterone during the second half of rat pregnancy, however it has a much higher concentration of endogenous Δ⁴A compared to testosterone and preferentially uses Δ⁴A as the androgen substrate for estrogen formation. At the same time that there is an increase in the ability of the placenta to produce Δ⁴A formation (Jackson and Albrecht, 1985). This may explain the increase in peripheral serum concentration and corpora lutea content of estradiol during the second half of rat pregnancy (Waynforth and Robertson, 1972; Taya and Greenwald, 1981). Thus, although the rat placenta secretes only small amounts of progesterone and does not produce estradiol at all, it sustains the ovarian production of both progesterone and estradiol. These findings have led investigators to reconsider the placenta as an important steroidogenic organ (Macdonald and Matt, 1984; Matt and Macdonald, 1984; Jackson and Albrecht, 1985).

1.10 Molecular mechanisms of thyroid and sex steroid hormone action

1.10.1 Steroid/thyroid nuclear hormone receptor superfamly

The nuclear receptors of steroid and TH belong to a family of ligand-activated transcription factors that exhibit remarkable structural and functional similarity (Evans, 1988). This family also includes receptors for glucocorticoid (GR), mineralocorticoid (MR), vitamin D (VD₃), retinoic acid (RAR), 9-cis retinoid X (RXR) and ecdysone (EcR) (Tsai and O’Malley, 1994). Cloning has also identified an increasing number of receptors with no known ligands, these have been termed “orphan receptors”. The primary structure of nuclear receptors has six regions (denoted A-F) (Fig. 1.4). Progesterone receptor does not possess region F (Gronemeyer, 1993). The DNA binding (C) domain is the most conserved between nuclear receptors and consists of 66-68 amino acids, twenty of these, including nine cysteines (C1 to C9), are conserved throughout the family (Umesono and Evans, 1989). The C domain contains two zinc finger structures named CI and CII which are responsible for DNA recognition and dimerization (Krust et al., 1986; Green et al., 1988; Tsai and O’Malley, 1994). The A/B domain, implicated in
transactivation, and the hinge (D) region are poorly conserved between the various subfamilies (Laudet et al., 1992). The ligand binding domain (LBD or E region) is located carboxy-terminal to the D region and is functionally complex. It usually contains regions important for heat-shock proteins association, dimerization, nuclear localization, transactivation and most importantly ligand binding (Gronemeyer, 1993; Tsai and O'Malley, 1994).

Members of the nuclear receptor superfamily are structurally related and can be grouped based on DNA sequence recognition (Umesono and Evans, 1989). Group I, the GR subfamily, which includes the MR, progesterone receptor (PR) and androgen receptor (AR) and group II, the estrogen (ER)/TR subfamily, which includes the ER, TRα, TRβ, RARα, RARβ, VD3, nerve growth factor-induced protein (NGFI-B), v-erbA, ear2, ear3, knirps, knirps-related, estrogen-related receptor (ERR)1 and ERR2 (Umesono and Evans, 1989). These authors have localized two non contiguous “boxes” in the DNA binding domain, one proximal (P) and one distal (D) and with respect to the amino acid sequence of the P box, all members of this receptor superfamily can be classified as members of either the GR or ER/TR subfamily (Table 1.2). As can be seen from the table, the P box shows sequence conservation while the D box shows sequence divergence. Group II, show conservation not only in the P box of the DNA binding domain, but also in the hydrophobic heptad repeats of the C-terminal region (Forman et al., 1989; Forman and Samuels, 1990). The heptad repeat domain is not found in group I suggesting that the group II factors may have evolved to interact via homo- and heterodimer formation on related response elements. Therefore, the DNA element plays a critical role in determining whether group II receptors bind as monomers or as homo- or heterodimers (Forman et al, 1992).
Table 1.2: Structures of P and D boxes in the GR and ER/TR subfamilies

<table>
<thead>
<tr>
<th>Receptor</th>
<th>P Box</th>
<th>D Box</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>GR subfamily</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GR, MR, PR</td>
<td>GSCKV</td>
<td>AGRND</td>
</tr>
<tr>
<td>AR</td>
<td>GSCKV</td>
<td>ASRND</td>
</tr>
<tr>
<td><strong>ER/TR subfamily</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ER</td>
<td>EGCKA</td>
<td>PATNQ</td>
</tr>
<tr>
<td>TRα</td>
<td>EGCKG</td>
<td>KYDSC</td>
</tr>
<tr>
<td>TRβ</td>
<td>EGCKG</td>
<td>KYEGK</td>
</tr>
<tr>
<td>RARα, RARβ</td>
<td>EGCKG</td>
<td>HRDKN</td>
</tr>
<tr>
<td>VD₃R</td>
<td>EGCKG</td>
<td>PFNGD</td>
</tr>
<tr>
<td>NGFI-B</td>
<td>EGCKG</td>
<td>LANKD</td>
</tr>
<tr>
<td>TR-2</td>
<td>EGCKG</td>
<td>RGSKD</td>
</tr>
<tr>
<td>v-erbA</td>
<td>EGCKS</td>
<td>TYDGC</td>
</tr>
<tr>
<td>ear2</td>
<td>EGCKS</td>
<td>RSNRD</td>
</tr>
<tr>
<td>ear3</td>
<td>EGCKS</td>
<td>RANRN</td>
</tr>
<tr>
<td>knirps</td>
<td>EGCKS</td>
<td>KNEGK</td>
</tr>
<tr>
<td>knirps-related</td>
<td>EGCKS</td>
<td>KNNGE</td>
</tr>
<tr>
<td>ERR1</td>
<td>EACKA</td>
<td>PASNE</td>
</tr>
<tr>
<td>ERR2</td>
<td>EACKA</td>
<td>PATNE</td>
</tr>
</tbody>
</table>

(Adapted from Umensono and Evans, 1989)

Nuclear receptors regulate the expression of specific cellular genes by interacting with distinct DNA sequences termed hormone response elements (HRE) in the target gene promoter. The HRE are structurally related but functionally distinct. The response elements for GR (GRE), ER (ERE), and TR (TRE) have been characterized in detail. They consist of two DNA hexamers, termed “half-sites” (Evans, 1988). GRE and ERE are similar except for two nucleotides per half-site (Klock et al., 1987), while ERE and TRE have identical half-sites but their spacing is different (Glass et al., 1988).

Structures of palindromic HRE for GRE, ERE and TRE.

GRE: AGAACA nnn TGTTCT
ERE: AGGTCA nnn TGACCT
TRE: AGGTCA --- TGACCT
Recent investigations have shown that nuclear proteins may influence the expression of target genes by binding to the receptor without themselves binding directly to the DNA (Burris et al., 1995, Chen and Evans, 1995; Onate et al., 1995). Since such proteins may function in a positive or negative fashion, they have been designated as "co-activators" and "co-repressors". Upon binding of agonist, the receptor changes its conformation in the ligand-binding domain enabling recruitment of co-activators and allowing a more efficient interaction of the receptor with the basal transcriptional machinery and thus activating transcription. In contrast, binding of antagonists induces a different conformational change in the receptor and although some antagonist-bound receptor can dimerize and bind to its cognate DNA element, it fails to dislodge the associated co-repressors, resulting in a nonreproductive interaction with the basal transcriptional machinery (Shibata et al., 1997). The TR and RAR associate with co-repressors in the absence of ligand, thereby resulting in a negative interaction with the transcriptional machinery that silences target gene expression.

A number of proteins have been identified which interact with the LBD of nuclear hormone receptors in the presence of ligand and have been proposed to act as co-activators in hormone-regulated gene transcription. The steroid receptor co-activator (SRC-1) appears to be a general co-activator for all steroid receptors. This co-activator enhances transactivation of steroid hormone-dependent target genes. Many more putative co-activators have been reported including the SRC-1 related proteins, transcription intermediary factor 2 (TIF2) and GR-interacting protein 170 (GRIP170), and other putative and unrelated co-activators such as androgen receptor associated protein (ARA70), thyroid hormone-receptor-interacting protein 1 (Trip1), receptor interacting protein 140 (RIP140) and transcription intermediary factor 1 (TIF1). Moreover, another co-activator, CREB-binding protein (CBP), has been shown to enhance steroid receptor-dependent target gene transcription (Shibata et al., 1997). Similarly, co-repressors for TR and RAR include: silencing mediator of retinoic acid and thyroid hormone receptors (SMRT) and nuclear receptor co-repressor (N-CoR). The unliganded TR and RAR have been shown to inhibit basal promotor activity; this silencing of target gene transcription by unliganded receptors is mediated by these co-repressors (Shibata et al., 1997).

It is generally believed that the DNA-bound hormone-receptor complexes stimulate the activity of the RNA polymerase II transcription machinery at the target promotor by interacting either directly or indirectly with one or more of the basal transcription factors (e.g. TFIID, TFIIA, TFIIB). Transcriptional activation by nuclear hormone receptors is achieved through autonomous transcription activation functions (TAFs) in the NH2-terminal region A/B (TAF-1) and in the HBD (TAF-2) located within the COOH-terminal domain (Gronemeyer, 1992). The TFIID binds to the TATA sequence located typically 25 nucleotides upstream from the transcription start site. TFIID is composed of many
subunits; that responsible for recognizing the TATA sequence is called TATA-binding protein (TBP). Once the TFIIID is bound to this DNA site, the other general transcription factors, along with RNA polymerase II are added (Alberts et al., 1994; Tsai and O’Malley, 1994) and transcription is initiated.

Members of the receptor superfamily can be divided into two subgroups according to their functional properties. Group A consists of the larger steroid receptors GR, AR, PR, MR and ER, which have longer A/B domains. In the absence of hormone these receptors exist as 8 - 10S complexes associated with heat-shock proteins (Denis et al., 1988). Upon binding hormone, heat-shock proteins dissociate, the receptors sediment as a 4S complex, and they are able to dimerize, bind to DNA, and transactivate a target gene. In contrast, Group B receptors such as TR, RAR, VDR, RXR and most (if not all) of the orphan receptors have short A/B domains and do not bind heat-shock proteins. In addition, they are able to bind DNA in the absence of ligand (Tsai and O’Malley, 1994).

1.10.2 Action of thyroid hormones

Thyroid hormones enter target cells where they bind to nuclear receptor proteins. This is followed by the initiation of nuclear gene transcription resulting in synthesis of new proteins (Lazar, 1993; Norris, 1996). Nuclear receptors for TH have greater affinity for T3 than for T4, supporting the hypothesis that conversion of T4 to T3 is a requisite for thyroid hormone action (Norris, 1996). The binding of occupied thyroid receptors to TRE in or near specific genes in the target cell nuclei is variable. Monomers of occupied receptor may bind directly to TRE but they are poor activators of transcription. Similarly, occupied TR homodimers bind for such a short time that they also produce little transcription. However, formation of a unique heterodimer between a monomer of TR and a thyroid receptor auxiliary protein (TRAP) produces maximal transcription following binding to a TRE. TRAPs are regular features of the nuclei of thyroid hormone target cells (Norris, 1996). Among TRAPs, RXR have been extensively studied, and TR binds with the RXR on the TRE half-sites to form heterodimers (Kleiwer et al., 1992, Glass, 1994; Oppenheimer and Schwartz, 1997).

The TR exhibits two activities, transcriptional repression in the unliganded state, and transcriptional activation in the liganded state (Ribeiro et al., 1995). These activities are thought to be mediated through hormone-dependant interactions with other proteins. The unliganded TR is thought to bind a co-repressor protein involved in repression of transcription (Chen and Evans, 1995). Ligand binding appears to induce both a dissociation of this protein with a relief of repression, and an association with another protein(s) that participates in the activation of transcription.
At least two major isoforms of nuclear TR proteins (TRα and TRβ) have been demonstrated in humans, mice, and rats, suggesting there may be a separate functional role for each type (Norris, 1996). The TR has been characterized as the cellular homolog of the v-erbA oncogene product (Weinberger et al., 1986). Multiple TR isoforms exist, encoded by the cellular erbA (c-erbA-α and -β) genes. Alternative splicing of the 3’-most exon of the c-erbA-α gene, coupled with the usage of alternative splicing acceptors, results in the production of mRNAs encoding three proteins: TRα1, TRα2, and TRα3. The non-coding strand of the cerbAα gene locus is also expressed producing the Rev-ErbAα protein, which lacks TH binding activity (Lazar, 1993). Alternative 5’-exon usage of the c-erbAβ gene gives rise to two products, TRβ1 and TRβ2. The three proteins TRα1, TRβ1, and TRβ2 all bind TH and thereby regulate target gene expression. TRα2 binds no known hormone. Its function is unclear, although it has been suggested that it represses TRα1 functions (Koenig et al., 1989; Katz et al., 1995; Oppenheimer and Schwartz, 1997). In the rat, the two isoforms, TRα1 and TRβ1, are found in all tissues, whereas TRβ2, is limited to the pituitary, hypothalamus and other brain regions (Norris, 1996; Oppenheimer and Schwartz, 1997). The predominant TR isoform in fetal liver and brain is TRα, with a very small fraction of TRβ, while in adult tissue TRβ predominates, especially in the liver (Schwartz et al., 1992; 1994).

In addition to the direct measurements of TH in fetal tissues the presence of TR further indicates that the fetus has the potential to utilise TH early in pregnancy. TR were identified in whole rat embryos as early as 13 days gestation, in brain at 14 days gestation and in liver, heart and lung from 16 days gestation onwards; all before the onset of fetal thyroid function (Perez-Castillo et al., 1985). In addition, TR gene c-erbA-α, was found to be expressed in rat CNS by day 14; that is well before the onset of fetal thyroid function (Porterfield and Hendrich, 1992). Concentrations of c-erbA-α increased between 19 days gestation and birth reaching maximum levels by 4 days postnatally (Strait et al., 1990). However, after day 10 there was a decline with adult values only slightly higher than those at 19 days gestation. In contrast, c-erbA-β mRNA levels were low at 19 days gestation, increasing to reach adult values by 10 days postnatally (Strait et al., 1990). With respect to humans, nuclear receptors have been detected in the brain of a 10-week-old human fetus and in 7-week-old whole embryo (Bernal and Pekonen, 1984). Therefore, the hormone and its receptor are present in fetal brain before the onset of fetal thyroid function as the secretion of T4 by fetal thyroid appears at 16 - 17 weeks of gestation, and that of T3 during the last trimester (Fisher and Kline, 1981). It is well known that the initial step of TH action is its binding to TR and these findings indicate that TH is available to fetus at this stage of pregnancy. Therefore, the presence of these
TR in fetal tissues, particularly in the brain early in pregnancy, suggests that TH may play a role in neurological development.

To determine the roles of the individual TR in mediating the effects of TH, transgenic mice lacking a TR gene (TR gene knockouts) have been developed. Mice from which a functional TRα1 gene was deleted, but which still expressed the splice variant, TRα2, and the related orphan receptor, rev-erbAα has been developed (Wikstrom et al., 1998). These mice exhibit bradycardia, reduced body temperature and exhibit mild hypothyroidism, but with a normal overall behaviour and reproduction suggesting that TRα1 plays a role in regulating physiological functions. TRβ knockout mice have goitre, elevated thyroid hormone and TSH levels, and a functional auditory defect. In contrast, mice with both TRα1 and TRα2 inactivation have thyroid hypoplasia, low serum thyroid hormone levels, growth arrest and delayed small intestine maturation (Hsu and Brent, 1998).

1.10.2.2 Extra-nuclear receptors for thyroid hormones

Non nuclear receptors, although less intensively studied, have been reported. In the brain, these include, synaptic membrane binding sites, cytosolic binding sites and mitochondrial binding sites. Binding sites for TH are also notable in plasma membranes of human and rat erythrocytes (Holm and Jacquemin, 1979), human placenta (Alderson et al., 1985), rat synapses (Mashio et al., 1983) and in liver (Gharbi-Chihi and Torresani, 1981). As for membrane binding sites these may directly influence the transport of glucose, amino acids and nucleosides, in addition to stimulating the cooperative activity of acetylcholinesterase and Na+/K+ ATPase (Goldfine et al., 1975).

Sterling and co-workers (1977) 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. 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 (Sterling, 1986).

Cytosolic TH binding sites may act as regulators of TH availability to organelles, including the nucleus, by providing a storage and supply function (Francon et al., 1985). Sites have been reported as possessing different affinities in different cell types, and are thought to contain more than one binding site per molecule (Lennon et al., 1983). In the brain T3 (Geel, 1977) and T4 (Lennon et al., 1980) binding sites have been detected in the cerebellum and cerebrum.
1.10.3 Action of sex steroid hormones

A number of behaviours - sexual, aggressive and maternal - are dependent on the actions of gonadal and adrenal steroids in circumscribed areas of the central nervous system. There are two theories for steroid hormone action: First, the steroids penetrate the cell by diffusion and bind to intracellular receptor proteins. This complex attaches to the genome resulting in alteration of gene expression and de novo protein synthesis (Greenstein, 1986). Second, the steroids may diffuse directly to the nucleus before binding to its receptor (Welshons et al., 1984) and since steroids are highly lipophilic, there seems to be no reason to suppose that they do not become distributed throughout the cell.

The primary role of the steroid receptor complex is to bind to specific loci in the genome and regulate rates of target gene transcription, but in addition steroids can modulate the expression of genes posttranscriptionally (Parker, 1986). Steroid hormone receptors do not bind to DNA in the absence of ligand in vivo. Once the ligand binds, the occupied nuclear receptors behave like transcription factors, bind to specific HRE or sites on the DNA, and initiate new mRNA synthesis. Once a receptor is occupied, it becomes phosphorylated and forms a dimer prior to binding to an HRE associated with promoter region of the gene. A second phosphorylation event occurs after binding to the DNA (Norris, 1996). The resultant synthesis of new proteins by the target cells brings about the events classically associated with the actions of these hormones. Ligand-induced dimerization is important for high-affinity binding of PR to a progesterone response element (PRE) in vitro. In contrast, the ER forms dimers in the absence of ligand, which may explain the efficient binding of non-liganded ER to estrogen response elements (EREs) in vitro. ER dimerization is further stimulated by estrogen. Two dimerization domains are apparently present in steroid receptors, a weak one in the C-terminal zinc finger of the DBD and a strong one in the HBD. However, efficient DNA binding of the PR requires additional sequences located in the A/B region, which most likely provide an additional dimerization interface (cited in Gronemeyer, 1993).

In the absence of ligand, steroid receptors are inactive in vivo as the unoccupied receptors are associated with several proteins including heatshock proteins. Heatshock proteins were first described in the fruit fly Drosophila about 20 years ago and named because they were synthesized in greater amounts following a sudden increase in temperature (Norris, 1996). Some of the other auxiliary proteins are known as molecular chaperones because they are involved in the folding process following translation of the receptor protein (Norris, 1996). Various molecular chaperones are associated with each of the steroid receptors (Table 1.3). Association of heatshock proteins with the receptor prevents the steroid-receptor complex from binding with DNA, but binding of the specific ligand
causes dissociation of these proteins from the receptor and allows the ligand-receptor complex to bind to DNA.

Table 1.3: Molecular chaperones and steroid hormones (Adapted from Norris, 1996)

<table>
<thead>
<tr>
<th>Proteins</th>
<th>Androgen receptor</th>
<th>Estrogen receptor</th>
<th>Progesterone receptor</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsp90</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>hsp70</td>
<td>+</td>
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<tr>
<td>p60</td>
<td>+</td>
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<td>+</td>
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<tr>
<td>p23</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Immunophilins</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FKBP52 (hsp56)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>FKBP54</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CyP40</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

1.10.3.1 *The estrogen receptor*

Estrogens, through their receptors, are important mediators of sexual development and the attainment of puberty (Greenstein, 1978; Greenstein and Adcock, 1985; Greenstein, 1992). ER are expressed in the anterior hypophysis, preoptic area, amygdala, cerebral cortex and cerebellum of adult female rats (Hirata *et al.*, 1992). Moreover, ER is expressed in the hippocampus (Bettini *et al.*, 1992; O'Keefe *et al.*, 1995), hypothalamus (Rainbow *et al.*, 1982; Hirata *et al.*, 1992) and limbic nuclei (Rainbow *et al.*, 1982). During rodent fetal life, estrogens cause the sexual differentiation of specific brain areas known to be involved in the control of sexual function and behaviour (Greenstein *et al.*, 1995). Estrogen, derived from circulating testosterone, masculinizes the developing preoptic area. The ER mRNA is detected within the rat preoptic area by 18 days gestation (DonCarlos, 1996), coincident with the reported onset of the critical period for testosterone-dependent masculinization of this region (Weisz and Ward, 1980). The high testosterone levels at this age, coupled with the presence of aromatase, presumably lead to the formation of higher estrogen levels in the male brains, and subsequently to the estrogen-dependant sex differences observed in this region.

Since the cloning of the rat ER by Koike *et al.* (1987), there has been the general acceptance that only one ER existed. However, the cloning of a gene encoding a second type of ER from the rat (Kuiper *et al.*, 1996), human (Mosselman *et al.*, 1996) and mouse (Tremblay *et al.*, 1997), has prompted the revaluation of the estrogen signalling system.
This ER subtype, now referred to as ERβ, is highly homologous to the classical ER protein (now referred to as ERα) particularly in the DBD (95%) and in the C-terminal LBD (55%) (Kuiper et al., 1996; Mosselman et al., 1996; Tremblay et al., 1997). The molecular weight of ERα is 67 kDa and this is consistent with the prediction from cDNA sequence that it contains 595 amino acids (Greene et al., 1984). The rat ERβ cDNA encodes a protein of 485 amino acid residues with a calculated molecular weight of 54,200 (Kuiper et al., 1997).

Our knowledge of the distribution of ERβ is limited at this stage. However, high levels of ERβ mRNA is detected in the ovary as well as in the prostate of the rat (Kuiper et al., 1996) and mouse (Couse et al., 1997). In addition, the existence of ERβ mRNA has also been shown in the hypothalamus of rat (Shughrue et al., 1996) and mouse (Couse et al., 1997). There is a moderate to high expression of ERα in the rat uterus, testis, pituitary, ovary, kidney, epididymis and adrenal and of ERβ in rat prostate, ovary, lung, bladder, brain, uterus and testis (Kuiper et al., 1997). The expression of both ER isoforms in the rat ovary was also demonstrated by Byers et al. (1997).

1.10.3.2 The progesterone hormone receptor

Progesterone receptors exist in two molecular forms commonly designated as “A” (80 - 94 kDa) and “B” (108 - 120 kDa) forms, the relative proportion of which can vary among species. Both forms are expressed from the same gene; PR form A (PRA) is an N-terminally truncated naturally occurring variant of PR form B (PRB). In humans, PRA and PRB are transcribed from two promoters, one directing initiation of transcription at +1 and +15 (PRB), while the other promoter produces major transcripts with 5' ends at +751, +761 and +842 which lack AUG1, and are therefore unable to encode PRB (Kastner et al., 1990). For the chicken PR, it has been also proposed that the two isoforms arise from a single class of mRNA by alternate initiation from two in-frame AUGs (Conneely et al., 1988; Conneely et al., 1989). Initiation of translation at AUG128 yields PRA, while initiation of translation at AUG1 yields the larger PRB (Conneely et al., 1989).

In rodents the two isoforms are detected in various tissues (Ilenchuk and Walters, 1987; Schott et al., 1991). In rat reproductive tissue both isoforms PRA and PRB are found, however, PRA is the predominant one (Ilenchuk and Walters, 1987). Using binding assays and gel filtration experiments, it has been shown that rat brain contains both PR, however, PRA is localized in the hypothalamus and preoptic area, and is regulated by estrogen, while PRB, which is present in the midbrain and cerebral cortex, is relatively insensitive to elevated plasma estrogen levels (MacLusky and McEwen, 1978). Estrogen
does not induce the cortical PR and this may be due to the predominance of PRB mRNA (Kato et al., 1994) in this region which, as mentioned earlier, has little or no estrogen-inducibility.

The PR appear around birth in rat brain and increase in development in a region specific manner. Using the DNA sequence of the human PR, Kato et al. (1993) designed two primer sets to use in reverse transcription-polymerase chain reaction (RT-PCR). One primer set flanked the steroid-binding domain (PRA + PRB; total PR mRNA [PRAB]), and the second primer set flanked the part of the 5'-untranslated N-terminal domain (PRB). The developmental pattern of the total PR mRNAs (PRAB) in the cerebral cortex is similar to that of the cortical PRB protein at early stages, but, surprisingly, unlike the receptor, the messages remain high at the later stages from day 12 and thereafter (Kato et al., 1993). The results thus demonstrate that gene expression of PRB is predominantly "turned on" 2 days before birth, followed by PRA mRNA expression around days 8 - 12 in the postnatal female rat brain, especially the cerebral cortex (Kato et al., 1993; Kato et al., 1994). A mismatch exists between PRA protein and its mRNA levels, suggesting the regulation of the PRA protein at post-transcriptional level (Kato et al., 1994).

The intracerebral distribution of PRB and PRAB was studied in 8-week-old female rat brain using RT-PCR (Kato et al.; 1993). PRAB mRNA was found to be widely distributed with relatively little variability throughout the anterior hypophysis, hypothalamus-preoptic area, amygdala, cerebral cortex, and cerebellum. In contrast, PRB mRNA was differentially distributed, with very high levels in the cerebral cortex and low levels in the cerebellum.

Park and Mayo (1991) used rat rPR-1 cDNA to probe polyadenylated RNA isolated from rat liver, brain and uterus. No hybridization to liver RNA was observed, a 4.6 kb transcript was observed in the brain, and multiple transcripts (6.8, 6.2, 4.6, 3.1 kb) were observed in uterine RNA. A similar study in mice was carried out and Northern blot analysis for PR using cDNA-derived probes failed to show PR transcripts in mouse liver (Schott et al., 1991). However, in both mouse and rat, the mRNA transcripts were more abundant in the uterus as compared to the levels present in the vagina (Schott et al., 1991).

As mentioned earlier, the PR is unique among steroid receptors in that it is composed of two hormone binding proteins, A and B. In many instances the action of progesterone and of its receptors require the prior exposure of the tissues to estradiol. An important action of estradiol in this respect is its stimulant effect on PR synthesis (Milgrom et al., 1973; Leavitt et al., 1974; Kraus and Katzenellenbogen, 1993). While estradiol stimulates PR synthesis, progesterone appears to have an inhibitory effect on its own receptors.
During pregnancy the rat hypothalamic cytosol PR, measured by binding assays, are apparently raised in number. This could be due to a decrease in the affinity of the receptors for progesterone in an attempt to shield the brain from high circulating levels of the hormone (Al-Khoury and Greenstein, 1985).

1.10.3.3 Extra-nuclear receptors for sex steroid hormones

Nontranscriptional actions of estrogen and progesterone have been observed in a variety of tissues including brain, pituitary, tumor cells, vascular smooth muscle cells, human prostate cells, spermatozoa, and breast cells. Specific estradiol binding has been detected in cytoskeletal elements (Puca and Sica, 1981). A rapid nontranscriptional effect of estrogen is the reorganization of target cell cytoskeleton, especially with respect to apical microvilli (Szego, 1991). A specific estradiol binding site on rat pituitary membranes has also been demonstrated (Bression et al., 1986) which may play a role in effecting the rapid secretion of prolactin (Zyzek et al., 1981). Progesterone-augmented ion fluxes, protein synthesis, and phosphorylation events can be detected in enucleated oocytes which are mediated by an acute increase in calcium uptake (Wasserman et al., 1980) and ultimately resulting in germinal vesicle breakdown (an exocytotic event). Similarly, sperm are responsive to cell surface progesterone, resulting in calcium uptake and another exocytotic event, the acrosome reaction (Osman, et al., 1989). In rat brain, progesterone acting through a membrane receptor has been found to decrease dopamine release (Dluzen and Ramirez, 1989 a,b).

1.11 Maternal hypothyroxinemia

Maternal thyroid status during pregnancy has profound effects on the development of the fetus in general, and the brain in particular. It is now accepted that placental transfer of maternal thyroxine occurs in a number of species, including man (Vulsma et al., 1989) and rat (Obregon et al., 1984; Woods et al., 1984; Porterfield and Hendrich, 1992). In early pregnancy and following injection of $[^{125}\text{I}]$ T4 to pregnant rat dams at 10 dg, the tissue distribution of accumulated T4 in the fetus was found to be relatively favourable to the brain (Ekins et al., 1986). Recent study by Porterfield and Hendrich (1992) have also indicated the presence of T4 and T3 in fetal brain in early fetal life; before the onset of fetal thyroid function. Earlier studies by Morreale de Escobar et al. (1989) in normal rats showed that both T3 and T4 were present in embryonic samples well before the onset of fetal thyroid function. In contrast in embryonic samples from totally thyroidectomized mothers, T4 and T3 concentrations were below the limits of detection in all tissues (including placenta) at least up to 17 days of gestation (Morreale de Escobar et al., 1985). This was followed by a marked increase in both tissue T4 and T3 concentrations due to the onset of fetal thyroid hormone secretion at 18 days gestation (Morreale de Escobar et al., 1985).
Maternal T4 is available to the fetus during the critical period of neurogenesis and, therefore, an adequate intrauterine thyroid hormone environment is necessary for normal brain development (Nunez, 1984; Woods et al., 1984; Ekins et al., 1986; Ekins et al., 1989; Dussault and Ruel, 1987; Hadjzadeh et al., 1990; Pickard et al., 1993). Absence of TH during development leads to irreversible brain damage (Ford and Cramer, 1977; Morreale de Escobar et al., 1983). However the timing at which the developing brain becomes sensitive to TH is not well known. Although maternal thyroidectomy was found to have little effect on brain levels of T4 and T3 in mid- and late-gestation (Ruiz de Ona et al., 1988), it was found to significantly reduce the brain T4 and T3 levels at 13 and 16 dg (Porterfield and Hendrich, 1992).

Thyroid hormones are also essential for normal fertility and pregnancy as it has been demonstrated that hypothyroidism results in a range of reproductive compromises, including prolonged gestation, small litter size, increased stillbirth, increased fetal resorption and smaller fetuses (Parrott et al., 1960; Porterfield et al., 1975; Hendrich et al., 1984). Maternal thyroidectomy in rats has also been shown to cause reduced body and brain weights early in gestation (Porterfield and Hendrich, 1982; Morreale de Escobar et al., 1985; Pickard et al., 1993).

The influence of maternal thyroidectomy on early rat brain development has been studied. Fetal brain DNA content and total protein content were found to be reduced at 15 and 19 dg (Pickard et al., 1993). Near term, however, DNA content appeared normal, whereas DNA concentration (cell density index) was elevated and the protein:DNA ratio (cell size index) was decreased, suggestive of delayed neuronal maturation. Other workers have reported that maternal thyroidectomy results in a reduction in both cell number and size, as well as RNA content in fetal brain at 22 days of gestation. However these parameters were found to be normal by adulthood (Porterfield and Hendrich, 1982).

The polyamines are important regulators of cellular growth and differentiation. The activity of ornithine decarboxylase (ODC) can be used as a marker for the detection of abnormalities in perinatal brain maturation (Slotkin and Bartolome, 1986). In prenatal brain, maternal hypothyroxinemia resulted in reduced ODC activity at 15 days gestation normalizing by 19 days gestation (after the onset of fetal thyroid hormone synthesis) with elevated levels near term (Pickard et al., 1993).

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

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

An electron microscopic study of brain from severe neurological cretins in China has
revealed changes in the appearance of the myelin sheath (Ma et al., 1986), which serves
to insulate the axon of neurones and thereby facilitate conduction (Norton, 1981). The
influence of maternal thyroid state on myelination has been studied in the rat. The
concentration of cholesterol, a major constituent of myelin lipid, did not change in adult
progeny of maternally hypothyroxinemic dams, however, the activities of the marker
enzymes 2',3'-cyclic nucleotide, 3'-phosphohydrolase, and 5'-nucleotidase, were
significantly reduced in medulla and midbrain (Hajzadeh et al., 1990). The activity of
oleate esterase, which may play a role in myelin degradation (Hirsch et al., 1977), was
increased however in paleocortex (Hajzadeh et al., 1990). Furthermore the concentration
of an important myelin-specific galactolipid, cerebroside sulphate, is reduced in the
midbrain and paleocortex (Sinha et al., 1992). Together, these findings indicate
deficiencies in the normal pattern of myelogenesis, with possible impairment of neural
function.
The attributes of a number of neurotransmitter metabolites have been studied in the CNS of hypothyroxinemic dams progeny. Sinha et al. (1992b) have studied the activity of two acetylcholine metabolic enzymes in adult brain progeny. Choline acetyltransferase, the synthetic enzyme, was found to be increased in activity in paleocortex, whereas the activity of acetylcholinesterase, the degradative enzyme, was found to be decreased in paleocortex, midbrain, and cerebral cortex of adult progeny.

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

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

### 1.12 Effect of thyroid hormones on estrogen and progesterone receptors

Thyroid hormone is closely associated with maturation and sexual differentiation of the brain. It has a direct effect on the expression of the estrogen receptor gene in many tissues. Using RT-PCR analysis, it has been shown that cortical ERα mRNA show no significant change with PTU-induced hypothyroidism in 8 day old female rats (Hirata et al., 1994) in agreement with the absence of changes in the ER protein (Kato et al., 1984). On the other hand, Barbanel and Assenmacher (1982) reported, using binding assay experiments, that neonatal hypothyroidism (PTU-induced) markedly suppressed estrogen receptor development, especially in the pituitary gland, and this was independent of serum estrogen levels. In adult rats, however, TH influence the levels of cytoplasmic estrogen
receptor in the pituitary without any effect on hypothalamic estrogen receptor content (Cidlowski and Muldoon, 1975).

Thyroidectomy has no significant effect on the ER protein level in adult uterus of surgically thyroidectomized rats (Cidlowski and Muldoon, 1975; Eriksson and Freyschuss, 1988) but causes a substantial reduction of the receptor content in the liver (Eriksson and Freyschuss, 1988; Freyschuss et al., 1991) and kidney which can be restored by substitution with triiodothyronine (Eriksson and Freyschuss, 1988). Using solution hybridization, Freyschuss et al. (1991) also showed a decrease in ER mRNA level in the livers of these rats. Growth hormone, and other hormones that influence the serum level of growth hormone also indirectly affect the expression of the liver estrogen receptor gene (Freyschuss et al., 1991). In animals subjected to adrenalectomy plus thyroidectomy, the liver estrogen receptor concentration is reduced. Adrenalectomy or thyroidectomy alone causes a decrease with 14% and 66%, respectively (Freyschuss et al., 1991).

Thyroid hormone may be one of the factors affecting gene expression of PR, particularly form B in the developing cerebral cortex of the female rat. Neonatal hypothyroidism (PTU-induced hypothyroidism) causes a drastic decrease in the levels of progesterone receptor protein (Kato et al., 1984) and mRNA (Kato et al., 1993; Hirata et al., 1994) in the cerebral cortex, with no changes in those in the hypothalamus-preoptic area of the neonatal rat brain, suggesting a regulatory role of TH in the developing rat brain cortex. The thyroid hormone may act on either the PR gene directly via the HRE or other gene(s) resulting in the expression of inhibitory factor(s) (Kato et al., 1993).

1.13 Rationale

Although several studies have been concerned with the effect of the maternal thyroid state, most were concentrated on hypothyroidism (Hendrich et al., 1984; Morreale de Escobar et al., 1985, 1987, 1992; Calvo et al., 1992; Porterfield and Hendrich 1993) in which animals were totally thyroidectomized either surgically or by treatment with $^{131}$I. In addition, administration of either methimazole or propylthiouracil in drinking water or food of pregnant rat dams will block the oxidation of iodide to iodine and consequently block the formation of T4 and T3 in maternal thyroid (Oppenheimer and Schwartz, 1997). Since these drugs readily pass the placenta barrier and are also transmitted to the suckling pups in the mother’s milk, the fetus and neonate also become profoundly hypothyroid. These methods do not represent maternal hypothyroxinemia and do not reflect conditions found in neurological cretinism in man. Therefore, a more appropriate model of partially thyroidectomized rats (parathyroid-spared) was used. In this study, the rat was used
because it possesses a haemochorial placenta, similar to human. In addition, it allows easy manipulation in the environmental and nutritional conditions.

Endemic cretinism serves as the human model of disease for understanding the actions of prenatal thyroid hormone on the developing brain and underscores the important contribution of maternal thyroid hormone to this process. Until recently it was thought that maternal TH were neither available to the human fetus nor necessary for fetal brain development. However, these views have become invalid since the demonstration that maternal TH are available to the fetus in a variety of mammalian species including man (Vulsma et al., 1989) and rat (Obregon et al., 1984; Woods et al., 1984; Porterfield and Hendrich, 1992). Therefore, as maternal TH crosses the placenta during early pregnancy, the fetal brain has access to T4 and is dependent on maternal T4 supply for normal brain function (Porterfield and Hendrich, 1993).

Some field observations and anecdotal evidence suggests that in iodine deficiency endemias, neurological cretinism is more prevalent among males, but iodine deficiency goitre is of predominantly female distribution. In some areas endemic goitre affects upwards of 65% of women of child bearing age but only a few males. The preponderance of goitres among females has been explained in terms of continuous menstrual loss of iodine and pregnancy load. This however, cannot be true, since large goitres are also very common among postmenopausal women well past child bearing age! Adult onset hypothyroidism in females and amenorrhea is of course a well established correlate. Adult onset hypothyroidism in males, although may result in a degree of loss of libido (anecdotal) has never been shown to cause reproductive compromise. Untreated congenital hypothyroidism, however, affects sexual maturation in a similar fashion in both sexes resulting in precocious menarche, auxiliary and pubic hair growth and breast development in females and premature development of secondary sexual characteristics, including early decent of testis to the scrotal sac from the inguinal canals in males. In short, hypothyroidism both in utero and postnatal including adulthood may exhibit gender related permanent and transient phenotypic expression.

It is evident that thyroid hormones do play a programming role in the expression of sex steroid synthesis and/or metabolism or both. This aspect however remains to be investigated in detail. Molecular biological studies have also shown that the steroid/thyroid hormone receptors belong to the same superfamily and there is a degree of structural homology among these receptors. Since both sex steroid and thyroid hormones bind to each other's receptor(s) albeit in limited capacity, there may also be at least a marginal (perhaps critical nevertheless) functional interrelationship. Thyroid receptors can bind to a consensus ERE as the half-site of a consensus TRE DNA sequence is identical to the ERE (section 1.10.1). Therefore, manipulations of thyroid hormones and estrogen
in vitro potentiate or mutually inhibit effects on gene expression (Zhou-Li et al., 1992). This lends a degree of urgency to explore such interaction in view of explaining the gender related phenotypic expression in thyroid hormone pathology. From the discussion above several hypothesis concerning thyroid and sex steroid interrelationships could be constructed. We have selected one of these for investigation in this thesis.

**Hypothesis**

In severe thyroid hormone deficiency *in utero*, feto-maternal estrogen/progesterone hormones protect the fetal brain from neurological cretinism to a certain extent and testosterone may have a predisposing effect. Whether a fetus will develop neurological cretinism at birth will depend upon the degree of intrauterine hypothyroxinemia and the sex steroid concentration and sex steroid receptors *in utero* and beyond.

**Working hypothesis**

Since sex steroid hormones exert their effects through their receptors and the receptors are induced by the hormones themselves, in hypothyroid conditions the sex steroid levels and their receptors will be modulated and deviate from the normal developmental chronology in the brain. In this thesis we have studied that aspect of steroid-thyroid interrelationship.
Chapter 2: Materials and methods
2.1 Materials

General laboratory chemicals were purchased from Merck (Dagenham, Essex) and all fine chemicals were obtained from Sigma Chemical Co. Ltd (Poole, Dorset). Radiolabelled $^{32}$P and $^3$H were purchased from ICN and DuPont, respectively. Hybond-N nylon membranes, Hybond ECL nitrocellulose membranes, Hyperfilm-MP, Hyperfilm ECL and autoradiography cassettes were purchased from Amersham (Bucks). Restriction enzymes were purchased from Promega; all buffers, enzymes and reagents used in reverse-transcription PCR experiments were purchased from Gibco and AmpliWax PCR gem 50 was purchased from Perkin-Elmer. Scintillant fluid (Escoscint) was purchased from National Diagnostics. The sources of all other materials are indicated in the text. Deionized water was used throughout and some of the most common used buffers/solutions are shown in Table 2.1.

2.2 Animal model

Sprague-Dawley rats, bred in the local animal house facilities were used. Partial surgical thyroidectomy (parathyroid-spared) of female rats was performed 'in house'. Animals were allowed two weeks recovery, before collection of blood from the tail vein for serum total T4 determination. When serum T4 levels were < 20 nM, thyroidectomized (TX) rats were mated overnight with normal (euthyroid) males. The control group comprised normal (N) females, also mated with normal males. Mating was verified by the presence of sperm in a vaginal smear; this was designated as day 0 of pregnancy. When pregnancies were allowed to continue to term, N and TX dam litter size was standardized (usually to eight pups per litter). Occasionally, TX dam deliveries were less than eight pups and thus in these cases N dam litters were standardized accordingly. All animals were maintained under normal animal house conditions at a constant temperature of 22 °C on 14/10 h light/dark cycle and fed a standard, iodine-replete diet ad libitum (average iodine intake of 36 μg/day; Ruiz de Elvira et al., 1989). The drinking water of the TX dams was supplemented with calcium lactate (0.1% w/v).

2.3 Tissue collection

Pregnant dams were stunned and killed by cervical dislocation. Blood was taken from dams by cardiac puncture, and uterine horns containing conceptuses were removed and placed immediately on ice. Amniotic fluid was collected, where necessary, and fetuses and placentae were separated. After determination of fetal body weight, fetal brain and liver were dissected and weighted.
Table 2.1: Common buffers/solutions used.

<table>
<thead>
<tr>
<th>Buffer/reagent</th>
<th>Name</th>
<th>Constituent</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x MAE buffer</td>
<td>MOPS acetate EDTA</td>
<td>0.1 M MOPS (3-[N-morpholino]propanesulphonic acid), 40 mM sodium acetate and 5 mM EDTA, pH 7.0-7.5</td>
</tr>
<tr>
<td>20x SSPE</td>
<td>Saline sodium phosphate EDTA buffer</td>
<td>0.2 M phosphate buffer (pH 7.4), 2.98 M NaCl and 20 mM EDTA</td>
</tr>
<tr>
<td>20x SSC</td>
<td>Saline sodium citrate</td>
<td>3.0 M NaCl, 0.3 M sodium citrate, pH 7.0</td>
</tr>
<tr>
<td>5x TBE buffer</td>
<td>Tris-borate EDTA</td>
<td>0.45 M Tris-borate and 5 mM EDTA, pH 8.0</td>
</tr>
<tr>
<td>TE buffer</td>
<td>Tris-EDTA buffer</td>
<td>50 mM Tris-HCl, pH 8.0; 1 mM EDTA</td>
</tr>
<tr>
<td>100x Denhardt's reagent</td>
<td></td>
<td>2% (w/v) Ficol 400, 2% (w/v) polyvinylpyrrolidone and 2% (w/v) bovine serum albumin</td>
</tr>
<tr>
<td>RNA gel loading solution</td>
<td></td>
<td>0.2% (w/v) bromophenol blue, 1 mM EDTA in 50% (v/v) glycerol</td>
</tr>
<tr>
<td>DNA gel loading solution</td>
<td></td>
<td>0.25% (w/v) bromophenol blue and 15% (w/v) Ficoll in water</td>
</tr>
</tbody>
</table>
Male and female postnatal progeny were studied separately. Animals were weighed, stunned then killed by decapitation. Blood was collected from the trunk. Brains and livers were removed to ice and weighed.

All tissues were stored at -70 °C for subsequent analysis. Tissues used for protein analysis were added to cryoprotective agent (10% v/v dimethylsulfoxide, DMSO; Lovelock and Bishop, 1959; MacLusky et al., 1986) before freezing. Blood and amniotic fluid were centrifuged (10,000 g for 10 min) and stored at -20 °C.

2.4 Protein determination

The protein assay method was based on Bradford’s procedure (Bradford, 1976) and employed a commercial reagent (Coomassie Plus Protein Assay Reagent; Pierce Chemical Co., USA). Bovine serum albumin (supplied with the reagent) was used as standard. An equal volume of protein assay reagent (0.5 ml) was added to aliquots (0.5 ml) of standards (1, 2, 4, 6, 8 and 10 µg/ml), diluted samples and sample diluent, mixed well and the absorbance measured at 595 nm. All measurements were performed in duplicate.

2.5 DNA assay: fluorimetric method

The method of Labarca and Paigen (1980) was used with calf thymus DNA as standard. Briefly, 3 ml bisbenzamide reagent (1 µg/ml in 50 mM phosphate buffer saline, pH 7.4) were added to 100 µl sample (diluted as necessary in water), standard (1.5, 3, 6, 15, and 30 µg/ml) and reagent blanks (water). After mixing, tubes were incubated in the dark at room temperature for 30 min, then fluorescence was measured (excitation wavelength of 356 nm, emission wavelength of 458 nm).

2.6 Radioimmunoassay

2.6.1 Determination of thyroid hormone levels

The levels of serum thyroid hormone (total T4 and T3) in N and TX dams and their postnatal progeny were determined by radioimmunoassay (RIA) using commercial kits obtained from North East Thames Region Immunoassay Unit (NETRIA; London, U.K.), following the standard protocols supplied. Briefly, the samples together with [¹²⁵I] T4 (or [¹²⁵I] T3) were incubated with diluted sheep anti-T4 (or anti-T3) serum for 2 h (or overnight for T3). A soluble antibody complex was formed with the sheep anti-T4 (or anti-T3) antibody using donkey anti-sheep IgG antiserum (non-precipitating) and this complex was precipitated by the addition of polyethylene glycol 6,000 (4% w/v). After centrifugation, the radioactivity associated with the antibody-bound fraction (precipitate)
was determined by γ-spectrometry (Nuclear enterprises NE1600 γ-counter). Non-specific binding was determined in tubes without the primary antibody. Quality controls (obtained from BM Browne Ltd) were carried through the entire procedure. The detection limit was 3.19 nM for total T4 and 0.29 nM for total T3.

2.6.2 Determination of steroid hormone levels

Estradiol and progesterone levels were determined in serum and amniotic fluid by RIA using commercial kits (Diagnostics Products Corporation, Gwynedd). In these kits, the anti-estradiol and -progesterone antibodies are immobilised to the walls of polypropylene tubes so that the antibody-bound fraction of the radiolabelled steroid can be determined by simply decanting the tubes. The antibody-bound fraction was determined by γ-spectrometry (as section 2.6.1) and all samples were assayed in duplicate. The detection limit was 0.02 ng/ml for progesterone and 8 pg/ml for estadiol.

Estradiol and progesterone have a strong tendency to adsorb to untreated plastic surfaces. Moreover, progesterone has a higher tendency to adsorb to glass, therefore, all tubes, glassware and pipette tips were siliconized before use. The serum of pregnant rat dams was diluted 1:10 with the diluent supplied by the manufacturers to achieve progesterone levels that fell within the standard curve. Serum and amniotic fluid samples, for estradiol determination, were extracted using diethyl ether according to the manufacturer’s instructions. Briefly, samples were extracted twice with diethyl ether (30 min/extraction). Removal of the upper organic phase was facilitated by freezing the aqueous phase on dry ice. Organic phases were pooled, dried under a stream of nitrogen and stored (-20 °C). Dried samples were then resuspended in diluent supplied by the manufacturers and assayed.

Progesterone and estradiol were also determined in fetal brain. Samples were homogenized in water (4 °C) and high specific activity tritiated steroids were added to determine recovery after extraction. Approximately 2500 dpm tritiated progesterone ([\(^{17}\alpha\)-methyl-\(^3\)H]-promegestone, specific activity 85 Ci/mmol) or estradiol ([\(^{2,4,6,7}\)H(N)]-estradiol, specific activity 85 Ci/mmol) were added to 0.5 ml homogenate and allowed to equilibrate at 37 °C for 30 min. Aliquots were taken for liquid scintillation counting (total counts) and the remainder of the sample was extracted with diethyl ether (as detailed above). Dried samples were resuspended in diluent supplied by the manufacturers and an aliquot was removed and prepared for liquid scintillation counting, to determine recovery. The resuspended sample was then assayed for progesterone and estradiol.
2.7 Preparation of plasmids

The following plasmids were used in this study:

1. rPRl- comprises a 550 bp fragment of rat progesterone receptor cDNA inserted into the BamHl and Sal I ends of the multiple cloning sites of the pGEM 4Z vector (see Appendix A, Fig. A.1). The plasmid was a gift from S. Mahajan, Mayo laboratory.

2. hER- comprises a 265 bp fragment of human (uterus) estrogen receptor (hER) cDNA probe (GB:X03635) inserted into the EcoRI and XhoI ends of the multiple cloning sites of the pBluescript SK- vector (see Appendix A, Fig. A.2). The plasmid was bought from American Type Culture Collection (ATCC).

Plasmids were propagated in E.Coli (subtype HB 101; Promega) then purified as detailed below.

2.7.1 Transformation protocol

Frozen (-70 °C) competent E.Coli cells were placed in an ice bath until just thawed. Cells were gently mixed and 100 µl aliquots were removed to a pre-chilled sterile tube. Approximately 10 ng plasmid (maximum volume 10 µl) were added and, after gentle mixing, tubes were quickly placed on ice. After 10 min cells were heated for 50 sec at 42 °C then immediately placed on ice. After 2 min, 900 µl S.O.C. medium (2% Tryptone, 0.5% Bacto-Yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl2, 10 mM MgSO4, 20 mM glucose, pH 7.0; Gibco) was added and tubes were incubated at 37 °C for 60 min with shaking (225 rpm). A loopful of cells was streaked on to LB agar (Luria-Bertani; Lennox L Agar, Sigma) containing 100 µg/ml ampicillin. Plates were inverted and incubated at 37 °C overnight.

Single colonies were transferred to 10 ml LB broth medium (Lennox L Broth Base; Sigma) containing 0.1% (w/v) glucose and antibiotic (50 µg ampicillin/ml medium). An aliquot (1 ml) was inoculated into LB broth medium (500 ml), containing 0.1% (w/v) glucose and antibiotic (50 µg ampicillin/ml medium), and grown up overnight with shaking. Cells were collected by centrifugation at 10,000 rpm for 5 min. The plasmids were then purified from the cultured cells as detailed below.

2.7.2 Plasmid purification protocol

The plasmid purification employed a commercial kit (Qiagen Ltd). The procedure is based on a modified alkaline lysis procedure, followed by binding of plasmid DNA to an anion-
exchange resin under low salt and pH conditions. RNA, proteins and low molecular weight impurities are removed by a medium salt wash. Plasmid DNA is eluted in a high salt buffer, concentrated and desalted by isopropanol precipitation.

Cells were resuspended in 10 ml resuspension buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 100 μg/ml RNase A), an equal volume of lysis buffer (200 mM NaOH, 1% w/v SDS) was added and, after gentle mixing, incubated at room temperature for 5 min. Then 10 ml chilled neutralization buffer (3 M potassium acetate, pH 5.5) was added and, after mixing, incubated on ice for a further 20 min. Samples were mixed again then centrifuged (20,000 g for 30 min). The supernatant was filtered (Whatman 3MM paper) and the filtrate applied to a Qiagen-tip 500 pre-equilibrated with 10 ml equilibration buffer (0.75 M NaCl, 50 mM MOPS, pH 7.0, 15% v/v ethanol, 0.15% v/v Triton X-100). After washing twice with 30 ml wash buffer (1 M NaCl, 50 mM MOPS, pH 7.0, 15% v/v ethanol), DNA was eluted with 15 ml elution buffer (1.25 M NaCl, 50 mM Tris-HCl, pH 8.5, 15% v/v ethanol). DNA was then precipitated with 0.7 vol. isopropanol (at room temperature to minimize salt precipitation) and collected by centrifugation (15,000 g for 30 min at 4 °C). The DNA pellet was washed with 5 ml 70% (v/v) ethanol, centrifuged (10,000 g for 10 min) then the final pellet was air-dried. DNA was redissolved in 200 μl TE buffer. The plasmid DNA concentration was then determined using spectrophotometry. The DNA concentration was calculated, assuming an absorbance $A_{260}$ nm value of 1 for a 50 μg/ml solution of double stranded DNA (Sambrook et al., 1989).

2.7.3 Linearization of rPR1 plasmid

For preparation of riboprobes (section 2.9.1.1), plasmids were first linearized by digestion with a single restriction enzyme. A restriction enzyme was selected that recognizes a unique site downstream of the insert to be transcribed. Riboprobes were prepared for rPR1 only, and the enzyme EcoRI was used in order to linearize the template. One unit of restriction enzyme was added per μg template. Digestion was performed overnight at 37 °C followed by electrophoresis (section 2.7.5) to check linearization (see Appendix A, Fig. A3). The linearized template was then purified using the Wizard™ clean-up system (Promega) following the manufacturer’s instructions. Briefly, the sample volume was brought up to at least 50 μl with water and then mixed with the clean-up resin. The resin was pipetted into a minicolumn and washed with 2 ml of 80% (v/v) isopropanol then centrifuged (12,000 g for 20 s) to remove any residual isopropanol. To elute DNA, 50 μl prewarmed (65 - 70 °C) DEPC-water or TE buffer was added to the minicolumn, incubated for 1 min at room temperature then centrifuged (12,000 g for 20 s). The DNA concentration was determined and the DNA was stored at -20 °C.
2.7.4 Digestion of rPR1 and hER plasmids

For preparation of rPR1 and hER cDNA probes the rPR1 and hER plasmids were first digested to obtain the cDNA insert, run on a low-melting point (LMP) agarose gel for small fragments (Promega) and purified (section 2.7.6). Labelling of cDNA probes was by Megaprime DNA labelling kit (section 2.9.1.2).

Restriction enzyme digestions contained 2 μg plasmid, 1x restriction enzyme buffer, 20 U of each restriction enzyme and 4 μg acetylated BSA in a final volume of 40 μl. For the rPR1 plasmid, *Bam*HI and *Sal*II were employed, whereas for hER, digestions contained *XhoI* and *EcoRI* with the supplied buffer. The reactions were allowed to proceed for ≥ 4 h at 37 °C and the digests were then run on an agarose gel (see Appendix A, Figs. A3 and A4).

2.7.5 Agarose gel electrophoresis

In order to check that linearization (single enzyme digest) or cDNA excision (double enzyme digest) was complete, agarose gel electrophoresis was carried out. To 2 μl sample (100 ng DNA) or DNA marker (100 bp ladder, Gibco or 1 kb DNA ladder, Gibco and/or Stratagene; as appropriate), 8 μl TE buffer were added, followed by 2 μl DNA loading buffer. Sample (10 μl) was then electrophoresed through a 1% (w/v) Metaphor agarose (Flowgen) gel in 0.5x TBE containing 0.5 μg/ml ethidium bromide. The gel was allowed to run at 80 - 100V for approximately 1 h and the bands were visualized by UV transillumination and photographed.

2.7.6 Recovery of DNA from low-melting-point agarose gels

After checking that the cDNA excision was complete, the remaining reaction mixture was run on a 1% (w/v) LMP agarose gel at 4 °C using a preparative comb (54 x 1 x 4 mm tooth). Standards (1 kb DNA ladder, Gibco and/or Stratagene) were run in adjacent wells. After resolution, gels were visualized by UV transillumination, and the band of interest quickly excised from the gel in order to minimize UV-induced DNA damage. Gel fragments were weighed then 5 vol. 20 mM Tris-HCl-1 mM EDTA (pH 8.0) were added. The tube was incubated for 5 min at 65 °C to melt the gel. The solution was then cooled and 1 vol. phenol-chloroform (premixed with isoamyl alcohol; Amresco) was added and, after mixing, centrifuged (12,000 g for 10 min). The upper aqueous phase was transferred to a fresh tube and re-extracted twice as above. The final aqueous phase was transferred to a fresh tube and 0.1 vol. sodium acetate (3 M, pH 5.2) was added followed by 2.5 vol. ice-cold absolute ethanol. The mixture was stored for 10 min at room temperature and the DNA was recovered by centrifugation (12,000 g for 15 min). The
pellet was washed with 70% (v/v) ice-cold ethanol, air-dried and resuspended in 20 μl water. The DNA concentration was determined by spectrophotometry.

2.8 RNA isolation

All solutions used for RNA isolation/analysis were treated with 0.1% (v/v) DEPC and autoclaved. Heat-sensitive compounds were made up in DEPC-treated water and filter sterilized where necessary. Glassware was oven baked (180 °C) overnight and sterile plasticware was used throughout.

2.8.1 Total RNA isolation

The method of Chomczynski and Sacchi (Chomczynski and Sacchi, 1987) was used. Samples were homogenized in denaturing solution (4 M guanidine thiocyanate salt, 25 mM sodium citrate, pH 7.0, 0.5% w/v sarcosyl and 0.1 M 2-mercaptoethanol) using a sterile hand-held homogenizer. To 3.6 ml homogenate, 0.36 ml sodium acetate (2 M, pH 4.0), 3.6 ml citrate buffer-saturated phenol (pH 4.3) and 0.72 ml chloroform:isoamyl alcohol (49:1) were added sequentially, shaking well between each addition. Tubes were vigorously shaken for 15 s after the final addition. Tubes were kept on ice for 15 min, then centrifuged (10,000 g for 20 min at 4 °C). The aqueous phase was removed (avoiding the DNA interface) and an equal volume of ice-cold isopropanol was added. After vigorous shaking, tubes were kept at -20 °C for ≥ 1h. The precipitate was collected by centrifugation (10,000 g for 20 min at 4 °C) and dissolved in 0.3 ml denaturing solution. Nucleic acid was re-precipitated by adding an equal volume of ice cold-isopropanol. After ≥ 1h at -20 °C, the samples were centrifuged (10,000 g for 10 min at 4 °C). The pellet was washed twice with 1 ml 75% (v/v) ethanol (at -20 °C) by suspension/centrifugation. The final pellet was air-dried, then dissolved in 0.5% (w/v) SDS (0.25 μl/mg wet weight tissue) at 65 °C for 15 min. Extracted RNA was stored at -70 °C.

2.8.2 PolyA⁺ mRNA isolation

A commercial system (PolyAtract mRNA isolation system, Promega) was used which employs magnetic-based separation procedure for isolation of polyA⁺ mRNA from total RNA preparations.

Total RNA (0.1 - 1.0 mg) was made up to 500 μl with RNase-free water and heated (65 °C; 10 min). Biotinylated-oligo(dT) (3 μl) and 20x SSC (13 μl) were added, mixed gently, then incubated at room temperature until completely cooled. The biotinylated-oligo(dT) hybridizes specifically to the 3’ polyA⁺ region of mRNA. The entire contents of
the annealing reaction were added to a tube containing washed streptavidin-coated paramagnetic particles (SA-PMP) and incubated at room temperature for 10 min. These particles form complexes with the oligo(dT)/mRNA hybrids via the powerful affinity of biotin for streptavidin. Tubes were placed on a magnetic stand and the supernatant was carefully removed. Captured particles were then washed four times with 0.1x SSC (0.3 ml per wash) by resuspension/capture. The final SA-PMP pellet was gently resuspended in 0.1 ml water to elute mRNA. The SA-PMP were captured and the eluate transferred to a sterile tube. The elution and capture steps were repeated and the eluates were pooled. Finally, the RNA was concentrated by sodium acetate-isopropanol precipitation (section 2.8.2.1) and stored in 75% (v/v) ethanol at -70 °C.

2.8.2.1 Sodium acetate-isopropanol precipitation

To concentrate RNA from dilute samples, sodium acetate-isopropanol precipitation was used. To sample, 0.1 vol. sodium acetate (3 M, pH 5.2) and 1 vol. ice-cold isopropanol were added and then tubes were left at -20 °C overnight. Precipitated RNA was collected by centrifugation (12,000 g for 10 min at 4 °C) and washed with 1 ml ice-cold 75% (v/v) ethanol. The washed pellet was dried in vacuo (15 min) before resuspension in water.

2.8.3 Spectrophotometric standardization

Total (or polyA⁺) RNA sample was diluted with DEPC-water (1 in 500). Blanks were water or 0.5% (w/v) SDS treated in an identical manner to the sample. The absorbance (A) was read at 260 and 280 nm. The RNA concentration was calculated, assuming an $A_{260\text{ nm}}$ value of 1 for a RNA solution of 40 μg/ml (Sambrook et al., 1989). Samples with $A_{260\text{ nm}}/A_{280\text{ nm}}$ ratios ≥ 1.7 were only studied further.

2.8.4 Minigel preparation

In order to check the integrity of total RNA, samples were first subjected to Minigel agarose gel electrophoresis and 28S and 18S rRNAs visualised after ethidium bromide staining.

A 1% (w/v) agarose gel was prepared in 1x MAE buffer to a final volume of 41 ml and boiled. When hand hot, 9 ml formaldehyde was added, mixed then poured into 8 cm x 10.6 cm gel tray and allowed to set. The running buffer was 1x MAE buffer. Total RNA (6 μg), in a 4 μl volume, was added to 16 μl sample mix (0.6x MAE buffer, 8% v/v formaldehyde, 65% v/v formamide and 0.02 μg/μl ethidium bromide), heated at 55 °C for 15 min, then placed on ice. RNA gel loading solution (2 μl) was added and 20 μl sample was loaded into each well. The gel was run at 100 V at room temperature and the bands
were visualised under UV light. Those samples which did not exhibit the expected 28S:18S ratio of 2:1 (Fig. 2.1), were considered to be degraded and discarded. Intact total RNA as well as polyA+ mRNA samples were subjected to Maxigel gel electrophoresis, prior to Northern transfer.

Figure 2.1: 28S and 18S ribosomal RNA showing a ratio of approximately 2:1.

2.8.5 Maxigel preparation and Northern transfer

A 20 cm x 20 cm gel tray was used. The gel was prepared as for minigel preparation (section 2.8.4). RNA marker (0.24 - 9.5 kb RNA ladder; Gibco) and total RNA (20 μg) or poly A+ mRNA (5 μg), in a 12 μl vol, were added to 48 μl sample mix (without ethidium bromide), heated at 55 °C for 15 min, then placed on ice. RNA gel loading solution (6 μl) was added and 60.5 μl marker/sample was loaded into each well. Samples were electrophoresed at 100 V with cooling until the bromophenol blue had travelled approximately 8 cm. RNA marker lanes were removed from the gel and stained with ethidium bromide (0.5 μg/ml in water). After destaining, bands were photographed, with a ruler on the side, for calibration purposes (section 2.12). RNA in the remaining sample lanes was transferred to Hybond-N nylon membranes by capillary transfer using 20x SSPE following a standard method (Sambrook et al., 1989). After overnight transfer, membranes were rinsed briefly in 2x SSPE and air-dried. RNA was fixed to the membrane by UV crosslinking (Spectro Linker x L1000, USA; set at optimal crosslink). Membranes were stored in Whatman paper under vacuum (dessicator) at room temp or at -20 °C. The gels, after transfer, were stained with ethidium bromide (0.5 μg/ml) then destained in water and examined under UV light. All Northern transfers were judged to be successful by this method.
2.9 Hybridization analysis of RNA

2.9.1 Probe labelling

2.9.1.1 Riboprobe labelling

A Maxiscript \textit{in vitro} transcription kit (Ambion Inc.) was used to prepare riboprobes. To 1 \mu g linearized DNA template (section 2.7.3), 1x transcription buffer, 62.5 pmol [\alpha-\textsuperscript{32}P]UTP (800 Ci/mmol; 10 mCi/ml), 10 U T7 RNA polymerase and 0.5 mM each ATP, CTP and GTP were added in a final volume of 20 \mu l. The mixture was incubated at 37 °C for 1 h. Then, 2 U DNase I was added and the mixture was further incubated for 15 min at 37 °C. The reaction was terminated by the addition of 23 mM EDTA and the incorporation was checked by trichloroacetic acid (TCA) precipitation (section 2.9.2.1).

2.9.1.2 cDNA probe labelling

Nonamers are used to prime DNA synthesis on denatured template DNA at numerous sites along its length. The primer-template complex is a substrate for the Klenow fragment of DNA polymerase I. Very small amounts of input DNA can be labelled, enabling very high specific activity DNA probes to be produced with relatively small quantities of added nucleotides.

The Megaprime DNA labelling kit (for use with radiolabelled dCTP; Amersham) was used. To 5 \mu l (25 ng) probe, 5 \mu l primer solution was added, followed by heating to 95-100 °C for 5 min then incubation at 65 °C for 5 min. The mixture was allowed to cool to room temperature, then briefly centrifuged. Then, 10 \mu l labelling buffer (dATP, dGTP and dTTP in Tris/HCl pH 7.5, 2-mercaptoethanol and MgCl\textsubscript{2}), 2 \mu l enzyme (polymerase I Klenow), 23 \mu l water and 5 \mu l (17 pmol) radiolabel ([\alpha-\textsuperscript{32}P]dCTP, 3000 Ci/mmol; ICN) were added, and the mixture was incubated at 37 °C for 30 min.

The specific activity of the probe was determined by TCA precipitation (section 2.9.2.2) and the labelled probe was immediately purified (section 2.9.3) to remove unincorporated \textsuperscript{32}P-nucleotide. The fractions containing radiolabelled DNA were combined and denatured by heating to 95 - 100 °C for 5 min then immediately chilled on ice, before addition to prewarmed hybridization solution.

2.9.1.3 Oligonucleotide probe labelling

The 18S rRNA oligonucleotide probe was used as an internal standard to correct for RNA loading. The sequence is complementary to bases 292 - 321 of rat 18S rRNA (5'-
CCACTGAGATCTATTGGAGCCCGGCTAGCG-3') (Chan et al., 1984; Varghese et al., 1994). This oligonucleotide probe was synthesized by Pharmacia and labelled using a commercial kit (5'-End labelling kit; Amersham). To 5 pmol oligonucleotide probe, 1x phosphorylation buffer, 5.5 pmol [γ-32P]ATP (4500 Ci/mmol) and 5 U T4 polynucleotide kinase were added to a final volume of 5 μl. The mixture was gently mixed and then incubated at 37 °C for 30 min. The enzyme was deactivated by heating at 70 °C for 5 - 10 min. The labelled probe was allowed to cool to room temperature then purified (section 2.9.3).

2.9.2 Determination of incorporation by TCA precipitation

2.9.2.1 Riboprobes

To 1 μl transcription reaction, 199 μl TE-RNA (TE buffer containing 500 μg/ml yeast tRNA; Sigma) was added and mixed well, then 100 μl was spotted onto Whatman GF/C filter (2.5 cm diameter) (total count). Ice cold 10% (w/v) TCA (2 ml) was added to the remaining 100 μl sample and left on ice for 5 min. The precipitated riboprobe was collected by vacuum filtration onto a prewetted (10% w/v TCA) glass fibre GF/C disc and washed (1 x 2 ml ice-cold 10% w/v TCA then 2 x 2 ml with 95% v/v ethanol) (TCA-precipitable count). All discs were then prepared for liquid scintillation counting.

2.9.2.2 cDNA probes

To 2 μl reaction mix, 203 μl water and 50 μl carrier DNA were added, then 5 μl were spotted on a glass fibre GF/A disc (Sigma) (total count). Ice cold 10% (w/v) TCA (2 ml) was added and tubes were left on ice for 15 min. The precipitated DNA was collected by vacuum filtration onto a glass fibre disc and washed (6 x 1 ml ice-cold 10% w/v TCA) (DNA count). All discs were then prepared for liquid scintillation counting.

2.9.3 Column purification of probe

Pharmacia NICK columns were used for purification of probes. Sample was applied to the column in 200 μl with TE buffer. After collecting the elute (200 μl, tube 1), a total of 9 applications of TE buffer (200 μl each) were then eluted and collected (200 μl, tubes 2-10). This was followed by further 3 elutions with TE buffer (500 μl each), and collection (500 μl, tubes 11-13). Radioactivity was counted in a scintillation counter and the specific activity of the probe was calculated.
2.9.4 Prehybridization/hybridization

2.9.4.1 Hybridization with riboprobes

The membrane and hybridization mesh were pre-wet with 2x SSPE. The membrane was placed RNA side up on top of the mesh, both were rolled up tightly and placed in a hybridization bottle containing 2x SSPE (15 ml). The bottle was gently rolled to unwind the membrane/mesh. The 2x SSPE was replaced with 10 ml prehybridization solution (5x SSPE, 2x Denhardt's reagent, 50% v/v formamide, 0.1% w/v SDS and 100 μg/ml denatured salmon testis DNA, in DEPC-water) and the bottles were placed in a rotisserie of a hybridization oven (Hybaid; Teddington, Middlesex) for 2 h at 42 °C. Prehybridization solution was then replaced with 10 ml prewarmed hybridization solution (as prehybridization solution but containing radiolabelled probe at approximately 1 x 10^7 cpm/ml) and the hybridization was carried out overnight.

2.9.4.2 Hybridization with cDNA probes

The process of prehybridization and hybridization was carried out as above (section 2.9.4.1). The prehybridization solution consisted of 6x SSPE, 2x Denhardt's reagent, 50% (v/v) formamide, 0.5% SDS and 100 μg/ml denatured salmon testis DNA. Prehybridization solution was then replaced with prewarmed hybridization solution (as prehybridization solution but containing the denatured DNA probe at approximately 1 x 10^7 cpm/ml) and the hybridization was carried out overnight.

2.9.4.3 Hybridization with oligonucleotide probes

Prehybridization and hybridization mixes (10 ml) consisted of 6x SSPE, 5x Denhardt's reagents, and 50 μg/ml denatured salmon testis DNA. To the prewarmed hybridization solution containing approximately 2 x 10^7 cpm/ml probe (5'-end labelling, section 2.9.1.3) was added. The processes of prehybridization and hybridization were carried out in the same way as earlier (section 2.9.4.1) except that the hybridization temperature was 15 °C below the melting temperature (Tm).

Calculation of Tm:
When hybridizing in 6x SSPE, in the absence of formamide, the equation for Tm is as follows:

\[ Tm (°C) = 81.5 + 0.41 \times (% \text{G+C content}) - % \text{mismatched bases} - (675/\text{probe length}) \]
2.9.5 Washing procedures

2.9.5.1 Washing of Riboprobes

The membrane was rinsed twice with 1x SSPE-0.1% (w/v) SDS at room temperature, then washed sequentially with: 1x SSPE-0.1% (w/v) SDS at 65 ºC for 30 min (one wash); 0.2x SSPE-0.1% (w/v) SDS at 65 ºC for 30 min (two washes); 0.1x SSPE-0.1% (w/v) SDS at 65 ºC for 30 min (one wash). All washes were performed in ‘sandwich boxes’ with gentle shaking and using prewarmed solutions. Radioactivities on the membrane and in each wash solution were monitored with hand-held minimonitor. When washing was complete, the filter was wrapped in Saran-wrap (Dow Chemical Co.), then placed in an autoradiography cassette with an intensifying screen (Hyperscreen) and exposed to preflashed Hyperfilm-MP at -70 ºC.

2.9.5.2 Washing of cDNA probes

After hybridization the membrane was rinsed with 2X SSPE-0.1% (w/v) SDS at room temperature, then washed sequentially with: 2X SSPE-0.1% (w/v) SDS at room temperature for 10 min (two washes); 2X SSPE-0.1% (w/v) SDS at 55 ºC (two washes, 10 min per wash). The filters were then handled as in section 2.9.5.1.

2.9.5.3 Washing of oligonucleotide probes

After hybridization the membrane was rinsed with 6x SSPE at room temperature then washed sequentially with: 6x SSPE at room temperature for 10 min (two washes); 6x SSPE-0.1% (w/v) SDS at the hybridization temperature for 10 min (two washes); 6x SSPE-0.1% (w/v) SDS at 5 ºC below the Tm for 2 min (one wash). The filters were then handled as in section 2.9.5.1.

2.9.6 Membrane stripping

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

2.10 RNA analysis by semi-quantitative RT-PCR

This technique allows the study of genes which are expressed in relatively low amounts. Total RNA obtained from fetal brain is reverse transcribed to obtain a DNA template...
which is then amplified in the PCR reaction and by using primers specific for the gene of interest. This process is known as reverse transcription (RT), hence the name RT-PCR.

2.10.1 Preparation and DNase treatment of RNA samples

Total RNA isolated by the standard protocol was used. However, in order to remove SDS which inhibits the reverse transcriptase, samples were precipitated with sodium acetate-isopropanol (section 2.8.2.1) then resuspended (at ca. 1 μg/μl) in water. The actual concentration was determined by spectrophotometry (section 2.8.3) and the RNA concentration was adjusted to 0.5 μg/μl with water.

In order to remove any contaminating genomic DNA, samples were DNase treated before reverse transcription. The following were mixed on ice, 2 μg total RNA, 40 U RNasin, 1x DNase I buffer (20 mM Tris-HCl pH 8.4, 50 mM KCl, 2.5 mM MgCl₂, 0.1 mg/ml BSA), 2 U RNase-free DNase I (amplification grade) and the mixture was made up to 20 μl with nuclease-free water. The mixture was incubated at room temperature for 15 min and the reaction was terminated by the addition of 2 μl 25 mM EDTA, followed by heating (70 °C ) for 10 min. The sample was then used directly for reverse transcription.

2.10.2 Reverse transcription

Random hexamer priming was used, allowing the reverse transcription of all RNAs, including rRNA. The DNase-treated sample was divided into two 11 μl aliquots, 100 ng random hexamers were added and the mixture was heated at 70 °C for 10 min, immediately chilled on ice for 3 min then briefly centrifuged. With the tube on ice, the following were added, 1x first strand buffer (25 mM Tris-HCl pH 8.3, containing 37.5 mM KCl and 1.5 mM MgCl₂), 5 mM DTT and 500 μM dNTP mix. To one tube (RT+ reaction) 200 U Superscript II RNase H* reverse transcriptase were added, whereas water was added to the other tube (control RT reaction) in a final volume of 20 μl. After gentle mixing, reactions were incubated at room temperature for 10 min then at 42 °C for 50 min. Reactions were terminated by heating at 70 °C for 15 min.

2.10.3 Polymerase chain reaction (PCR)

Initially, the optimal concentrations of magnesium chloride (MgCl₂), primers and deoxynucleotide triphosphates (dNTP) were determined and were found to be 3 mM, 0.3 μM and 0.5 mM, respectively. Optimization of MgCl₂ is important as it may affect all of the following: primer annealing, strand dissociation temperatures of both the template and PCR product, product specificity, formation of primer-dimer artefacts and enzyme activity and fidelity (Perkin Elmer Applied Biosystems division). The enzyme Taq DNA
polymerase requires free magnesium on top of that bound by template, primers and dNTP and therefore optimization is necessary for every primer set. The number of cycles and DNA template were then varied and the optimal cycle number (see Appendix B for figures) and template dilution (see Appendix C for figures) selected for all genes studied.

The PCR reaction was carried out in a programmable thermal cycler (Perkin Elmer, model 9700) using a hot start protocol. The lower reaction mixture consisted of: 1x PCR buffer (20 mM Tris/50 mM KCl), 3 mM MgCl₂, 0.5 mM dNTPs and 0.3 μM each of upper and lower primers in a final volume of 10 μl. An AmpliWax PCR gem 50 wax bead was added and the tubes were incubated at 80 °C for 5 min (to melt the wax), then cooled to room temp (wax solidifies). The upper reaction mixture (15 μl volume) consisting of: 1x PCR buffer, 0.5 μl template (RT+ or RT-) and 1.25 U recombinant Taq DNA polymerase was then added on top of the wax. The PCR reactions were then cycled as follows: 5 min at 94 °C to denature the template (1 cycle); 30 s at 94 °C (denaturation step), 30 s at the appropriate annealing temperature for the primer set under study (annealing step) and 1 min at 72 °C (extension step) for the required number of cycles (see Table 2.2 for annealing temperature and cycle number). Tubes were then incubated for a further 7 min at 72 °C (1 cycle).

When published primer sequences were not used, primers were selected using the Oligo 5.0 (National Biosciences Inc., USA) programme (Table 2.2). The primers chosen where highly specific to the intended target sequence, had similar melting temperatures, were not susceptible to primer-dimer formation and had a stable 5'-end and a less stable 3'-end (an oligonucleotide with a 3'end that is too stable has a greater tendency to false priming).

2.10.4 Analysis of PCR products by agarose gel electrophoresis

After the PCR reaction, PCR products were electrophoresed (section 2.7.5). To 8 μl sample (ca. 80 ng DNA) or 100 bp DNA marker (100 bp ladder, Gibco), 2 μl DNA loading buffer was added. Sample (8 μl) was then electrophoresed through a 2% (w/v) low electroendosmosis (LE; Boehinger Manheim) agarose gel.

2.10.5 Southern transfer

After electrophoresis, 18S rRNA, estrogen and progesterone receptor PCR products were blotted onto Hybond N nylon membranes using capillary transfer and then hybridized to specific oligonucleotide probes (Table 2.3) to check their authenticity (see Appendix D, Fig. D1). The first step in this process was to denature the DNA in the gel by soaking for 30 min in 1.5 M NaCl-0.5 M NaOH, with gentle agitation. The gel was rinsed briefly in deionized water, and then neutralized by gentle agitation in 1 M Tris- HCl -1.5 M NaCl
Table 2.2: Upper and lower primer sequences, annealing temperature, cycle numbers and expected product size for the different genes studied.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequence (5'-3')</th>
<th>Nucleotides of coding sequence(bp)</th>
<th>Annealing temp (°C)</th>
<th>No. of cycles</th>
<th>Expected product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S rRNA (18S)</td>
<td>Upper</td>
<td>GTCCCCCAACTTCTTAGAG</td>
<td>1436 - 1454</td>
<td>52</td>
<td>26</td>
<td>419</td>
</tr>
<tr>
<td></td>
<td>Lower</td>
<td>CACCTACGGAAACCTTTGTTAC</td>
<td>1834 - 1855</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ribosomal protein L19 (RPL19)</td>
<td>Upper</td>
<td>ATCGGCAATGCCAACCTCT</td>
<td>157 - 174</td>
<td>52</td>
<td>33</td>
<td>321</td>
</tr>
<tr>
<td></td>
<td>Lower</td>
<td>GAGAATCCGCTTGTTTTGAAA</td>
<td>457 - 477</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-glucuronidase (BGLU)</td>
<td>Upper</td>
<td>ATCGCCATCAACAACACAC</td>
<td>511 - 530</td>
<td>52</td>
<td>34</td>
<td>529</td>
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<tr>
<td></td>
<td>Lower</td>
<td>TGACGCCTTGGAAAGTAGAAAG</td>
<td>1019 - 1040</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyclophilin (CY)</td>
<td>Upper</td>
<td>CAACCCCAACCTGTCTTCTCG</td>
<td>48 - 68</td>
<td>59</td>
<td>24</td>
<td>369</td>
</tr>
<tr>
<td></td>
<td>Lower</td>
<td>TTGCCATCCAGCACAUSCTGATGAG</td>
<td>396 - 417</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Histone H4 (HH4)</td>
<td>Upper</td>
<td>ACGCCTGTGTCTCTCACTAG</td>
<td>108 - 129</td>
<td>59</td>
<td>39</td>
<td>250</td>
</tr>
<tr>
<td></td>
<td>Lower</td>
<td>GGCGGTCTCCTGTAGAAAGAG</td>
<td>337 - 358</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glyceraldehyde-3-phosphate-dehydrogenase (G3PDH)</td>
<td>Upper</td>
<td>AGGGCTGCTCCTTCTTGTG</td>
<td>135 - 154</td>
<td>57</td>
<td>26</td>
<td>968</td>
</tr>
<tr>
<td></td>
<td>Lower</td>
<td>GGTTGGCTGCAAGAGTTTAC</td>
<td>1082 - 1103</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Estrogen receptor α (ERα)</td>
<td>Upper</td>
<td>TAAGAACCCGAGGAAGATTG</td>
<td>705 - 725</td>
<td>57</td>
<td>38</td>
<td>623</td>
</tr>
<tr>
<td></td>
<td>Lower</td>
<td>TCATGCCGGAATCGACTT</td>
<td>1310 - 1327</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Estrogen receptor β* (ERβ)</td>
<td>Upper</td>
<td>TTCCCGCAGCACCAGTAACC</td>
<td>38 - 58</td>
<td>57</td>
<td>37</td>
<td>262</td>
</tr>
<tr>
<td></td>
<td>Lower</td>
<td>TCCCTTCTGGTTTTGACCTA</td>
<td>279 - 299</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Progesterone receptor B** (PRB)</td>
<td>Upper</td>
<td>ATCCGGGACTCTCCACACA</td>
<td>23 - 42</td>
<td>59</td>
<td>35</td>
<td>228</td>
</tr>
<tr>
<td></td>
<td>Lower</td>
<td>ACGTCGGCAACGCCACTGCTG</td>
<td>232 - 251</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Progesterone receptor A+B** (PRAB)</td>
<td>Upper</td>
<td>CCACTTGCCAAATCCACAGGAGTT</td>
<td>2369 - 2393</td>
<td>69</td>
<td>38</td>
<td>319</td>
</tr>
<tr>
<td></td>
<td>Lower</td>
<td>CCGAAATTCCCACAGCAGTGCCCGG</td>
<td>2664 - 2688</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Published primer sequences (Kuiper et al., 1997).
** Published primer sequences for human (Kato et al., 1993) modified for rat.

All other primer sequences were selected using Oligo 5 programme.
Table 2.3: Oligonucleotide probe sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>Oligonucleotide probe sequence</th>
<th>Nucleotide of coding sequence (bp)</th>
<th>Hybridization temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S</td>
<td>TAAGTGCGGGTCATAAGCTTGCCTGGATTA</td>
<td>1654 - 1683</td>
<td>52</td>
</tr>
<tr>
<td>ER α</td>
<td>GCCTGGCAACTCTTCTCCCGG</td>
<td>711 - 731</td>
<td>61</td>
</tr>
<tr>
<td>ER β*</td>
<td>GGGACTCTTGGAGGTTCTGC</td>
<td>163 - 182</td>
<td>56</td>
</tr>
<tr>
<td>PRAB</td>
<td>TTCGGGAATGGATCAAGGGCAATTGG</td>
<td>2516 - 2540</td>
<td>52</td>
</tr>
<tr>
<td>PRB</td>
<td>CCTTGGCTTGACGCTTGGACCAGGA</td>
<td>80 - 104</td>
<td>60</td>
</tr>
</tbody>
</table>

* Published sequence (Kuiper et al., 1997).

All other primer sequences were selected using Oligo 5 programme.
(pH 7.4) for 30 min. The blotting was then performed overnight in 20x SSPE as for Northern transfer (section 2.8.5). The probes were prepared (section 2.9.1.3), and membranes were then hybridized (section 2.9.4.3) and washed (section 2.9.5.3) as described earlier.

2.11 Protein analysis

2.11.1 Extraction of total, cytosolic and nuclear receptors from tissues

This protocol was based on a published procedure (Schneider et al., 1991). However, slight modifications were applied. Uterus (cytosol) samples from 21 dg dams were used as a positive control. All blots also included a normal 21 dg maternal brain (MB) sample (homogenate, cytosol or extracted nuclear receptor, as appropriate) which served to correct for filter to filter variation.

Tissues were thawed and washed twice with ice-cold isotonic saline. They were then homogenized in ice-cold homogenization buffer (10 mM Tris-HCl pH 7.4, 1.5 mM EDTA and 10% v/v glycerol, 1.0 mM DTT, 1 μg/ml leupeptin, 100 μg/ml bacitracin, 2 μg/ml aprotonin, 1 μg/ml pepstatin A) to yield a 5% (w/v) homogenate. Homogenates were passed through a nylon mesh (110 μm pore size) then through steel gauze (40 μm pore size). An aliquot was removed for protein and DNA assay (sections 2.4 and 2.5, respectively). The tubes were centrifuged (1,000 g for 10 min at 4 °C). The supernatant was further centrifuged (105,000 g for 45 min at 4 °C) and the supernatant (cytosolic fraction) collected. Protein was also analyzed in this fraction.

The pellet, from the initial centrifugation, was washed with 1 ml homogenization buffer and re-centrifuged (1,000 g for 10 min at 4 °C). The pellet was resuspended in 1.0 - 1.5 ml homogenization buffer containing 0.6 M NaCl, incubated on ice for 50 min, then centrifuged (50,000 g for 30 min at 4 °C). The supernatant, which contains isolated nuclear receptors, was collected. An aliquot (100 μg protein) of this fraction was precipitated with 6 vol. absolute ethanol overnight then centrifuged (10,000 g for 10 min at 4 °C). The pellet was then resuspended in homogenization buffer.

2.11.2 SDS-Polyacrylamide gel electrophoresis of proteins

In the presence of thiol reagent (dithiothreitol), sodium dodecyl sulphate (SDS) dissociates proteins into their individual polypeptide subunits. Furthermore, most polypeptides bind SDS in a constant weight ratio (1.4 g SDS per gram of polypeptide) so that the intrinsic charges of the polypeptide are insignificant compared to the negative
charges provided by the bound detergent. Consequently, different SDS-polypeptide complexes have essentially identical charge densities and migrate in polyacrylamide gels according to polypeptide size.

2.11.2.1 Gel preparation

A Hoefer Mighty Small (SE 260) apparatus was used (Hoefer Scientific Instruments, U.K.). Gel sandwiches were prepared using rectangular 10.5 cm x 10 cm glass plates and alumina plates 10.5 cm x 10 cm with 1.5 mm spacers placed between them. The gels were cast in the Hoefer gel caster. Teflon combs (10 wells) were used to create wells for the samples.

To prepare the resolving gel (7.5%), the following components were mixed in a 100 ml Buchner flask: 0.375 M Tris HCl pH 8.8, 7.5% (v/v) acrylamide (Biorad 30% acrylamide:bis acrylamide 29:1), 0.1% (w/v) SDS in a final volume of 52.28 ml water. The mix was degassed for 10 min then 34.5 μl TEMED (NNN’N’-tetramethylethylenediamine, BDH Electran grade) and 262.5 μl 10% (w/v) ammonium persulphate solution (BDH Electran grade) were added to initiate polymerization. The mixture was added to the gel cast containing the glass plate sandwiches then water-saturated butanol (70 μl) was layered on top of the gel solution. When set (about 1 h), the butanol was washed off with water. Gels were stored for up to one week in a humidified sandwich box at 4 °C.

The resolving gels were mounted on the electrophoresis unit. The reservoir was filled with electrophoresis buffer (0.025 M Tris, 0.192 M glycine and 0.1% w/v SDS) and all air bubbles dislodged from the base of the gel. The stacking gel was prepared by mixing the following components in a 100 ml Buchner flask: 0.125 M Tris HCl pH 6.8, 3% (v/v) acrylamide, 0.1% (w/v) SDS in a final volume of 15.96 ml water. The mixture was degassed for 5 min, then 24 μl TEMED and 80 μl 10% (w/v) ammonium persulphate solution were added. After swirling, the mixture was carefully poured on top of the resolving gel with the comb in place. Gels were left for approximately 1 h to set.

2.11.2.2 Sample preparation

Samples (total homogenate, cytosol or salt-extracted nuclei; 50-100 μg protein), tissue standard and molecular weight rainbow markers (14,300 - 220,000 daltons; Amersham) were mixed with loading buffer (0.03% w/v bromophenol blue, 32 mM Tris-HCl pH 6.8, 8% w/v dithiothreitol and 4% w/v SDS), boiled for 3 min, then chilled on ice and centrifuged (10,000 g for 1 min).
2.11.2.3 Electrophoresis

Samples and standards (20 μl) were loaded onto the gel and electrophoresed at a constant current of 20 mA per gel with cooling until the bromophenol blue dye front was approximately 1 mm from the end of the gel. The stacking gel was removed and the distance migrated by the marker proteins and bromophenol blue front were measured. The buffer chamber of the Mighty Small Transphor apparatus (Hoefer) was filled with degassed transfer buffer (25 mM Tris-HCl, 150 mM glycine, 20% v/v methanol and 0.1% w/v SDS, pH 8.3). Gels were equilibrated in transfer buffer then protein was transferred to nitrocellulose membrane (Hybond ECL) with a constant current of 360 mA for 2 h with cooling. After transfer the membranes were dried and stored at 4 °C.

2.11.3 Gel staining and drying

The gel was fixed (7% v/v glacial acetic acid and 40% v/v methanol) for 30 min, washed three times with water, then stained (0.1% w/v Coomassie Brilliant Blue, 50% v/v methanol and 10% v/v glacial acetic acid) for 30 min. Gels were then destained (10% v/v methanol and 14% v/v glacial acetic acid) overnight, placed in preserving solution (10% v/v glycerol) for 30 min, then dried overnight at room temperature between two sheets of cellulose film using a commercial gel drying kit (Promega) according to the manufacturer's instructions.

2.11.4 Immunodetection using the ECL system

Target proteins were detected with specific primary antibodies and horseradish peroxidase-conjugated secondary antibodies. The horseradish peroxidase acts as a catalyst for the oxidation of the substrate luminol, which subsequently emits small but sustained quantities of light. This chemiluminescence is then specifically enhanced, up to 1,000 fold, allowing an image to be recorded on photosensitive film. The ECL system emits a blue light with a maximum emission wavelength of 428 nm. Hyperfilm ECL responds to this wavelength with optimum sensitivity and efficiently captures the signal. The primary and secondary antibodies used in this experiment are shown in Table 2.4.

Membranes were placed in 50 ml Falcon tubes, 10 ml blocking solution (10% w/v non-fat dry milk in TBS-T [20 mM Tris, 137 mM NaCl, pH 7.6 and 0.1% v/v Tween]) were added and the blots incubated for 1 h at room temperature with rotation. The membranes were washed by rinsing twice with TBS-T, followed by two 10 min washes (20 ml/wash). Membranes were then incubated overnight at 4 °C with 3 ml primary antibody solution (diluted in 1 - 5% w/v non-fat dry milk in TBS-T; determined in preliminary experiments). After incubation, the membranes were rinsed twice with TBS-T followed
Table 2.4: Primary and secondary antibodies used for protein detection

### A) Primary antibodies

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Type</th>
<th>Peptide sequence</th>
<th>Company</th>
<th>Abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERα</td>
<td>Mouse monoclonal</td>
<td>amino acids 495 - 595 of bovine steroid binding domain</td>
<td>Insight Biotechnology Ltd.</td>
<td>ERα-S</td>
</tr>
<tr>
<td>ERα</td>
<td>Mouse monoclonal</td>
<td>amino acids 287 - 300 of human hinge region</td>
<td>Bioquote Ltd.</td>
<td>ERα-H</td>
</tr>
<tr>
<td>ERβ</td>
<td>Rabbit polyclonal</td>
<td>amino acids 467 - 485 of rat C-terminus</td>
<td>Affinity Bioreagents, Inc.</td>
<td>ERβ-C</td>
</tr>
<tr>
<td>ERβ</td>
<td>Rabbit polyclonal</td>
<td>amino acids 54 - 71 of rat/mouse and 46-63 of human</td>
<td>Upstate Biotechnology</td>
<td>ERβ-N</td>
</tr>
<tr>
<td>PR</td>
<td>Mouse monoclonal</td>
<td>amino acids 533 - 547 N-terminal to the rat PR DNA binding domain</td>
<td>Affinity Bioreagents, Inc.</td>
<td>PR-N1</td>
</tr>
<tr>
<td>PR</td>
<td>Mouse monoclonal</td>
<td>amino acids 920 - 933 of human C-terminus</td>
<td>Bioquote Ltd.</td>
<td>PR-C</td>
</tr>
<tr>
<td>PR</td>
<td>Mouse monoclonal</td>
<td>amino acids 523 - 536 of chicken</td>
<td>Bioquote Ltd.</td>
<td>PR-N2</td>
</tr>
</tbody>
</table>

ER- estrogen receptor;  
PR- progesterone receptor  
All the above antibodies react with the rat ER or PR as appropriate.

### B) Secondary antibodies

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Source</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-mouse IgG</td>
<td>Sheep</td>
<td>Amersham</td>
</tr>
<tr>
<td>Anti-rabbit IgG</td>
<td>Donkey</td>
<td>Amersham</td>
</tr>
</tbody>
</table>

Secondary antibodies are linked with horseradish peroxidase.
by one 15 min wash and two 5 min washes (20 ml/wash). Membranes were incubated with the appropriate secondary antibody for 1.5 h at room temperature then washed (as after primary antibody incubation but with two extra 5 min washes). Membranes were then covered with detection solution (ECL Plus, 0.125 ml/cm²; Amersham) and incubated for 5 min. Excess detection solution was drained away, the membranes were wrapped in Saran-wrap and then autoradiographed with pre-flashed Hyperfilm ECL. In preliminary experiments, the primary antibody was omitted and filters were incubated with secondary antibody only. No bands were detected with this antibody.

2.11.5 India Ink Staining

After immunodetection, the blots were stained with India Ink to check protein loading (Hancock and Tsang, 1983). The blots were washed twice with buffer (0.15 M NaCl in 0.01 M phosphate-buffered saline, pH 7.2 containing 0.3% v/v Tween) at room temperature (10 min per wash). The blots were then incubated with Pelikan drawing ink A (1 μl India ink/ml buffer) for 1 - 2 h at room temperature with gentle shaking. After incubation the blots were washed twice with distilled deionized water for 15 min each and then allowed to air-dry.

2.12 Determination of protein and mRNA band sizes

In order to determine the size of an unknown band a marker lane was always included when running samples on agarose or SDS gels. The distance migrated by each marker (RNA ladder or protein marker) was measured and a standard curve (Fig. 2.2) was constructed using a plot of log₁₀ molecular mass versus relative mobility (distance migrated by band + distance migrated by dye; Rf). The size of the unknown band was then determined using the equation of the line.

2.13 Image analysis

The autoradiographs and photographs obtained were captured using a Kodak DC 40 camera. An optical density (od) step tablet (0 - 3 od units, Kodak) calibrator was used and the images were analyzed using NIH Image 1.61.

2.14 Statistical analysis

All results were analyzed for statistical significance using two-way ANOVA followed by post-hoc analysis using Fisher's Protected Least Significance Difference (PLSD) after fulfilling two criteria, homogeneity of variance (Bartlett's test) and normality. Bartlett’s test was applied using the equation for uneven sample sizes and the table of the F
distribution was used to test the equality of the variances. Where necessary, the data were transformed (logarithmic or square root transformation was used). The variation of the individual observations around the mean of the sample was then examined. Thus, for testing the normality the residuals were arranged in numerical order and the data were plotted to assess the assumption of normality. Chi-square analysis was used to determine whether the number of residual differed from expected. Finally, the analysis of variance was carried out.

All values are expressed as mean ± se and a $p$ value of $< 0.05$ was taken as the minimum level of significance.

Figure 2.2: Plot of \( \log k_b \) vs \( R_f \).
Chapter 3:
Effect of maternal hypothyroxinemia on reproductive performance, prenatal and postnatal growth of progeny and gross placental development
3.1 Introduction

A variety of clinical and biochemical investigations support a role for maternal TH in fetal brain and placental development. Total thyroidectomy results in severe maternal compromise and compromised placental growth. Moreover, in fetal and 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 (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 model. Preliminary characterization of this model has revealed that maternal hypothyroxinemia is associated with a range of behavioural disturbances in adult progeny analogous to that suffered by offspring of hypothyroxinemic women in both iodine-deficient and iodine-sufficient environments. Although changes in the content of brain protein, DNA and RNA have been observed in adult progeny of hypothyroxinemic dams, little is known regarding the influence of maternal thyroid state on these parameters in fetal and early postnatal brain. This study includes the examination of the physical characteristics and gross biochemistry of a number of tissues at different stages of development both preceding and following the onset of fetal TH synthesis.

In view of the severe effects of neurological cretinism on the brain, special attention was paid to this tissue. However, results obtained from placental growth parameters are important to assess the possible involvement of insufficient placenta in the impairment of fetal brain development. In addition, to determine whether the effect of maternal hypothyroxinemia is specific to the CNS only or has a general effect, liver growth was studied as well.

3.2 Maternal thyroid hormone levels

Serum levels of total T4 and T3 in normal and thyroidectomized dams were measured at 14, 16, 19 and 21 dg. Maternal serum T4 was found to be affected by the progression of pregnancy (Table 3.1); levels in N dams remained stable between 14 and 16 dg then decreased markedly (by 36%; p < 0.001) between 16 and 19 dg. A similar decrease in T4 levels with pregnancy has been reported by other groups (Morreale de Escobar et al., 1985; Escobar del Rey et al., 1986; Morreale de Escobar et al., 1988). In TX dams, T4 levels also remained stable between 14 and 16 dg; decreasing between 16 and 19 dg (by 21%; p < 0.05). In contrast to the marked decrease in T4 levels (by 47%; p < 0.001) between 14 and 21 dg in N dams, a less prominent decrease in T4 levels (by 21%; p < 0.05) was observed in TX dams. An expected treatment effect on T4 levels and age-
treatment interaction was observed; when compared with N dams, thyroidectomy resulted in a significant reduction (by 58 - 72%, p < 0.001) in T4 levels at all stages of gestation.

In contrast, total serum T3 level was not affected by age (Table 3.1), while a significant treatment effect was observed. When compared with N dams, serum T3 levels were reduced by 59% at 16 dg (p < 0.001) and by 52% at 19 dg (p < 0.01) in TX dams.

These findings indicate that the thyroidectomized dams used in this study were hypothyroxinemic, rather than severely hypothyroid, as approximately 30% of T4 and 45% of T3 were still present in the circulation. This model closely approximates thyroid status seen in pregnant women in certain iodine-deficient (Connolly and Pharoah, 1989) and iodine-replete (Man et al., 1991) environments.

Table 3.1: Serum total T4 and T3 levels in normal (N) and thyroidectomized (TX) dams

<table>
<thead>
<tr>
<th>Days gestation</th>
<th>Dam</th>
<th>T4 concentration (nM)</th>
<th>T3 concentration (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>N</td>
<td>39.78 ± 2.05 (8)</td>
<td>0.90 ± 0.05 (8)</td>
</tr>
<tr>
<td></td>
<td>TX</td>
<td>11.22 ± 2.21 (5)**</td>
<td>0.62 ± 0.15 (5)</td>
</tr>
<tr>
<td>16</td>
<td>N</td>
<td>39.51 ± 1.51 (19)</td>
<td>1.08 ± 0.14 (21)</td>
</tr>
<tr>
<td></td>
<td>TX</td>
<td>12.98 ± 1.48 (15)**</td>
<td>0.44 ± 0.06 (13)**</td>
</tr>
<tr>
<td>19</td>
<td>N</td>
<td>25.36 ± 0.89 (16)</td>
<td>1.35 ± 0.26 (16)</td>
</tr>
<tr>
<td></td>
<td>TX</td>
<td>10.24 ± 0.86 (18)**</td>
<td>0.65 ± 0.09 (18)*</td>
</tr>
<tr>
<td>21</td>
<td>N</td>
<td>21.04 ± 3.09 (6)b,d</td>
<td>1.06 ± 0.30 (8)</td>
</tr>
<tr>
<td></td>
<td>TX</td>
<td>8.84 ± 0.87 (10)**f</td>
<td>0.70 ± 0.21 (10)</td>
</tr>
</tbody>
</table>

Two-way ANOVA

- **Age**: p < 0.001, NS
- **Treatment**: p < 0.001, p < 0.001
- **Age-treatment**: p < 0.001, NS

Values are mean ± se; numbers in brackets show the number of different rat dams.

- a,b,c,d p < 0.001: N19 vs N14, N21 vs N14, N19 vs N16 and N21 vs N16, respectively;
- e,f p < 0.05: TX19 vs TX16 and TX21 vs TX14, respectively;
- * p < 0.01, ** p < 0.001: TX vs N dam, at the appropriate age (post-hoc analysis);
- NS: not significant
3.3 Feto-placental growth

Fetuses and placentae were collected at 14, 16, 19 and 21 dg. The effect of maternal hypothyroxinemia on litter size, placental weight and fetal body and brain weights was investigated. Fetal liver weights were also measured from 16 dg.

3.3.1 Litter size

There was a significant decrease in the litter size of TX dams compared with control dams. The number of fetuses obtained from N rats averaged approximately 18 per dam. This number was significantly affected by treatment at 16, 19 and 21 dg with litter size being reduced to an average of 13 in TX dams (Fig. 3.1). A reduction in the number of fetuses has been reported for hypothyroid and hypothyroxinemic rats (Hendrich et al., 1984; Morreale de Escobar et al., 1985; Morreale de Escobar et al., 1988; Porterfield and Hendrich, 1991; Ruiz de Ona et al., 1991; Pickard et al., 1993). In contrast, no significant difference in litter size was apparent at 14 dg. However, there was no evidence of an increased number of fetal resorptions in TX dams at this and subsequent stages of gestation. Furthermore, the number of fetuses in N dam litters tended to be lower at 14 dg relative to later ages, which may in part account for the lack of statistically significant difference at this age.

3.3.2 Fetal somatic growth

Fetal body weight, when examined by two-way ANOVA, was found to be significantly affected by age (Fig. 3.1). No significant treatment or age-treatment interaction effects were found, albeit a trend (p = 0.0937) was apparent for the former. Indeed, post-hoc analysis revealed that body weights were slightly lower at 19 and 21 dg in the experimental group (by 9% and 11%, respectively; p < 0.05). Similar body weights were also detected for 19 dg fetuses obtained from normal and surgically thyroidectomized dams (Ruiz de Ona et al., 1991).

3.3.3 Fetal brain growth

As expected, fetal brain weight increased with age and an overall treatment effect was detected using two-way ANOVA, although post-hoc analysis revealed no significant difference at any individual age. Rather, the trend was towards lower weights in the experimental group at all ages (Fig. 3.2). The brain:body weight ratio decreases significantly with age between 14 and 21 dg in both control and experimental groups (Fig. 3.2).
Figure 3.1: Effect of maternal hypothyroxinemia on litter size and fetal body weight. Values are mean ± se. Numbers in brackets show the number of dams.

A) Litter size. Treatment effect: p < 0.001 (two-way ANOVA); * p < 0.001: TX vs N dam, at the appropriate age (post-hoc analysis).

B) Fetal body weights (log plot). Age effect: p < 0.001 (two-way ANOVA). The standard error bars at 14 dg in TX dams is obscured by the box. Body weights increase between 14 and 21 dg in both control and experimental group (p < 0.001, post-hoc analysis).
Figure 3.2: Effect of maternal hypothyroxinemia on fetal brain weight and brain:body weight ratio. Values are mean ± se. Numbers in brackets show the number of dams.

A) Fetal brain weight. Age effect: $p < 0.001$; treatment effect: $p < 0.05$ (two-way ANOVA). Brain weights increase between 14 and 21 dg in both control and experimental group ($p < 0.001$, post-hoc analysis).

B) Brain:body weight ratio. Age effect: $p < 0.001$ (two-way ANOVA). Brain:body weight ratio decreases between 14 and 21 dg in both control and experimental group ($p < 0.001$, post-hoc analysis).
Again maternal hypothyroxinemia was without any effect on this ratio. These results indicate that brain growth sparing is indeed present to counter the possible effect of maternal hypothyroxinemia.

3.3.4 Fetal liver growth

Fetal liver weight showed significant age and treatment-related effects (Fig. 3.3). Liver weights were significantly lower at 16 and 21 dg in the experimental group with no significant difference detected at 19 dg. The liver:body weight ratio was normal at all ages indicating that the decrease in liver weight was due to decrease in overall body and organ weight (Fig. 3.3). A significant age effect was detected by two-way ANOVA for liver:body weight ratio, where the ratio at 19 dg was significantly higher than that at 16 and 21 dg.

3.3.5 Placental growth

Placental weight increases between 14 and 19 dg (p < 0.001) (Fig. 3.4). Maternal hypothyroxinemia was without any effect on placental weight at any age. Therefore, results obtained from placental weights indicate that fetal gross parameters are affected due to maternal thyroid status and not due to insufficient placenta in the experimental group. Using the same model, it has been shown that placentae from hypothyroxinemic and control dams reveal no obvious differences in a range of biochemical parameters including DNA, RNA and protein concentrations (Ruiz de Elvira et al., 1989; Hadjzadeh et al., 1990; Pickard et al., 1993).

3.4 Fetal brain DNA, protein and RNA measurements

3.4.1 DNA concentration

Fetal brain DNA, protein and RNA were measured at 16, 19 and 21 dg. The concentration of DNA provides an indication of cell density (Fig. 3.5). DNA concentration was found to be significantly affected by age and an age-treatment-related effect was apparent. In fetal brain from N dams, the DNA concentration was highest at 16 dg then decreased markedly (by 37%; p < 0.001) by 19 dg. This was not the case in the experimental group where concentrations remained relatively stable throughout the period studied. At 16 dg the brain DNA concentration was significantly lower (by 31%; p < 0.01) in TX dam progeny compared to N dam progeny. In contrast, at 21 dg, the reverse effect was observed where TX dam progeny had slightly higher (by 7%; p < 0.05) DNA concentration. This increased DNA concentration in late gestation was also observed by Pickard et al. (1993).
Figure 3.3: Effect of maternal hypothyroxinemia on fetal liver weight and liver:body weight ratio. Values are mean ± se. Numbers in brackets show the number of dams.
A) Fetal liver weight. Age effect: p < 0.001; treatment effect: p < 0.001 (two-way ANOVA). * p < 0.05: TX vs N dam, at the appropriate age (post-hoc analysis). The standard error bars at 16 dg is obscured by the box. Liver weights increase between 16 and 21 dg in both control and experimental group (p < 0.001, post-hoc analysis).
B) Liver:body weight ratio. Age effect: p < 0.001 (two-way ANOVA). \(a, b \) p < 0.001 N19 vs N16 and TX19 vs TX16, respectively; \(c, d \) p < 0.001 N21 vs N19 and TX21 vs TX19, respectively (post-hoc analysis).
Figure 3.4: Effect of maternal hypothyroxinemia on placenta weight. Values are mean ± se. Numbers in brackets show the number of dams. Age effect: p < 0.0001 (two-way ANOVA). Placentae weights increase between 14 and 21 dg in control group and between 14 and 19 dg in experimental group (p < 0.001, post-hoc analysis).

3.4.2 DNA content

The measurement of total DNA serves as an index of cell number. Overall age and age-treatment interaction effects were observed for this parameter in fetal brain (Fig. 3.5). DNA content increases (by 86%; p < 0.001) between 16 and 21 dg in N dam progeny and between 16 and 19 dg in TX dam progeny (by 171%; p < 0.001). DNA content was lower (by 37%; p < 0.05) in TX dam progeny at 16 dg but higher (by 25%; p < 0.05) at 19 dg relative to controls. This indicates a deficit in cell number at 16 dg but after the onset of fetal thyroid hormone synthesis, over-compensation may occur, normalizing near term.
Figure 3.5: Effect of maternal hypothyroxinemia on fetal brain DNA concentration and DNA content. Values are mean ± se. Numbers in brackets show the number of dams.
A) DNA concentration. Age effect: p < 0.001; age-treatment interaction: p < 0.05 (two-way ANOVA); \(^a\)p < 0.001: N 19 vs N 16, \(^b\)p < 0.001: N21 vs N 16, * p < 0.05, ** p < 0.01: TX vs N dam, at the appropriate age (post-hoc analysis).
B) DNA content. Age effect: p < 0.001; age-treatment interaction: p < 0.05 (two-way ANOVA); \(^*\)p < 0.05: N19 vs N16, \(^b\)p < 0.001: N21 vs N16, \(^c\)p < 0.001: TX19 vs TX16, \(^d\)p < 0.001: TX21 vs TX16, \(^e\)p < 0.001: N21 vs N19; * p < 0.05: TX vs N dam, at the appropriate age (post-hoc analysis).
3.4.3 **Protein concentration**

Protein concentration in fetal brain was found to be significantly affected by age (Fig. 3.6). Protein concentration in fetal brains from the experimental group increased between 16 and 19 dg (by 33%, p < 0.01) then decreased by 21 dg (by 18%, p < 0.05). Maternal hypothyroxinemia was without any effect on protein concentration. These results are similar to those reported by Pickard *et al.* (1993) using a similar animal model.

3.4.4 **Protein content**

When studying the protein content, an overall age effect and age-treatment interaction was observed (Fig. 3.6). Protein content increased between 16 and 21 dg in N dam progeny (by 236%; p < 0.001) and between 16 and 19 dg in TX dam progeny (by 233%; p < 0.001).

Maternal hypothyroxinemia resulted in significantly lower (by 23%; p < 0.05) protein content at 16 dg due to reduced brain weight and protein concentration at this age.

3.4.5 **Protein:DNA ratio**

As wet weight can be influenced by variations in organ water content, the measurement of protein:DNA ratio in fetal brain provides an estimate of cell size. The brain protein:DNA ratio was found to be significantly affected by age (Fig. 3.7). An increase (by 69%, p < 0.01) in protein:DNA ratio was observed between 16 dg and 19 dg in fetal brains from N dams. Maternal hypothyroxinemia was without any significant effect on cell size.

3.4.6 **RNA concentration**

An overall age effect was detected when studying RNA concentration (Fig. 3.7). The RNA concentration was found to be significantly lower at 19 and 21 dg compared to 16 dg in N dam progeny (by 38%, p < 0.01 and 51%, p < 0.001, respectively). In TX dam progeny, RNA concentration at 21 dg was lower (by 45%, p < 0.01) compared to 16 dg. Maternal hypothyroxinemia was without any significant effect on fetal brain RNA concentration.
Figure 3.6: Effect of maternal hypothyroxinemia on fetal brain protein concentration and protein content. Values are mean ± se. Numbers in brackets show the number of dams.

A) Protein concentration. Age effect: p < 0.05 (two-way ANOVA); a p < 0.01: TX19 vs TX16, b p < 0.05: TX21 vs TX19 (post-hoc analysis).

B) Protein content. Age effect: p < 0.001, age-treatment interaction: p = 0.05 (two-way ANOVA); a p < 0.001: N19 vs N16, b p < 0.001: N21 vs N16, c p < 0.001: TX19 vs TX16, d p < 0.001: TX21 vs TX16, e p < 0.01: N21 vs N19; * p < 0.05: TX vs N dam, at the appropriate age (post-hoc analysis).
Figure 3.7: Effect of maternal hypothyroxinemia on fetal brain protein:DNA ratio and RNA concentration. Values are mean ± se. Numbers in brackets show the number of dams.

A) Protein:DNA ratio. Age effect: p < 0.01 (two-way ANOVA); a p < 0.01: N19 vs N16, b p < 0.05: N21 vs N16 (post-hoc analysis).

B) RNA concentration. Age effect: p < 0.001 (two-way ANOVA); a p < 0.01: N19 vs N16, b p < 0.001: N21 vs N16, c p < 0.01: TX21 vs TX16 (post-hoc analysis).
3.5 Postnatal gross body measurements

In immature animals, euthyroidism is a prerequisite for normal growth and development, because deficient thyroid hormone secretion during the perinatal period inevitably causes severe and irreversible damage, especially to the CNS (Chapter 1). Maternal hypothyroxinemia has been shown to result in a wide range of biochemical deficits in brains of immature and adult progeny (Ekins et al., 1989; Ruiz de Elvira et al., 1989). This study was extended to examine the possible effects of maternal hypothyroxinemia on postnatal gross body measurements. Progeny were collected at 1, 10, 15 and 30 days postnatally (dpn). The animals were sexed and the body weights measured. Brains and liver were collected and weighed for both sexes.

3.5.1 Number of live progeny

A significant number of postnatal progeny were found dead at 1 dpn. Whether the mother has actually killed them or whether they were born dead are two possibilities that we have not investigated as some of the births occurred overnight. The number of litter at 21 dg (Fig. 3.1) was 18 ± 0.66 and 13 ± 1.15 for N and TX dams, respectively; the number of live progeny collected at 1 dpn was 11 ± 0.95 and 6 ± 0.73 for N and TX dams, respectively. This implies that at birth the percent survival for N dam progeny was 61% and for TX dam progeny 46%. Similar results for number of live 1 day old progeny from hypothyroid dams have been reported (Hendrich et al., 1997). When the data for number of live progeny was plotted (Fig. 3.8), a significant decrease (by 45%; p < 0.001) in live TX dam progeny was observed compared to controls. To assess whether there was any sex related difference, the number of live progeny of both males and females were plotted individually. Both males and females from TX groups exhibited lower number of live progeny compared to controls (by 40%, p < 0.05 and by 50%, p < 0.01, respectively), and there was no significant difference between male and female progeny number at birth. Stillbirths, exceeding 50% of the litter, have also been reported to occur in the litters of animals whose thyroids had been removed surgically or with ¹³¹I (Parrott et al., 1960).

3.5.2 Postnatal somatic growth

As expected the postnatal body weights were found to increase significantly with age (Fig. 3.9). When body weights were compared an overall age and a treatment effect was detected. At 1 dpn male and female TX dam progeny have significantly lower body weights than their respective controls (by 13%, p < 0.01 and 21%, p < 0.001, respectively). Moreover, N and TX male dam progeny from this age group have higher body weights than females (by 9%, p < 0.05 and 17%, p < 0.05, respectively). Although
Figure 3.8: Number of live postnatal progeny. Values are mean ± se (n ≥ 9).

A) Pooled male and female live progeny. Treatment effect: p < 0.001 (two-way ANOVA); * p < 0.001: TX vs N dam, at the appropriate age (post-hoc analysis).

B) Male and female live progeny. Treatment effect: p < 0.001 (two-way ANOVA); * p < 0.05, ** p < 0.01: TX vs N dam, at the appropriate age (post-hoc analysis).
body weights at 15 and 30 dpn appear lower in the experimental group relative to controls, post-hoc analysis revealed no significant difference. Similar body weights have been reported for 1 dpn N and TX (Hendrich et al., 1997), 10 dpn N and TX (Pickard et al., 1993), 15 dpn N (Barbanel and Assenmacher, 1982) and 30 dpn N and TX dam progenies (Hendrich et al., 1997); the latter group reporting a significant decrease in body weight that continues to 60 days of age.

Figure 3.9: Effect of maternal hypothyroxinemia on postnatal body weight. Values are mean ± se (n ≥ 3). Age effect: p < 0.001; treatment effect: p < 0.01 (two-way ANOVA), ^ p < 0.05: male vs female, at the appropriate age, * p < 0.01, ** p < 0.001: TX vs N dam, at the appropriate age (post-hoc analysis). Error bars at some age points are obscured by the boxes. Male and female body weights in control and experimental groups increase between 1 and 30 dpn (p < 0.001, post-hoc analysis).
3.5.3 Postnatal brain growth

Postnatal brain weights also demonstrated an expected age-related increase and age-treatment interaction was detected (Fig. 3.10). Postnatal male and female progeny from TX dams have significantly lower (by 11%, $p < 0.05$ and 9%, $p < 0.05$, respectively) brain weights than their controls at 1 dpn. In addition a similar difference was observed at 30 dpn when only males from TX dams have lower (by 7%; $p < 0.05$) brain weights than their controls. Maternal hypothyroxinemia was without any effect on brain weight at other ages in agreement with results obtained by the same group (Pickard et al., 1993). Similar brain weights have been reported for 1 and 30 dpn N and TX dam progeny (Hendrich et al., 1997), however, brain weights reported by this group for 30 dpn were slightly higher.

An overall age and treatment effect was also apparent for the brain:body weight ratio (Fig. 3.10). The brain:body weight ratio in normal male and female progeny remained relatively stable between 1 and 15 dpn then declined sharply (by 56% in males and females, $p < 0.001$) due to the greater increase in body weight at this age relative to brain. In TX dam progeny ratios decreased (by 17%; $p < 0.05$) between 1 and 10 dpn, in female progeny only, remained stable between 10 and 15 dpn then fell sharply by 30 dpn (by 60% in females and by 62% in males; $p < 0.001$). Maternal hypothyroxinemia resulted in elevated ratios at 1 dpn (by 17%; $p < 0.001$, females only) and at 15 dpn (by 24%, $p < 0.01$ and by 26%, $p < 0.05$ in males and females, respectively) relative to controls. In addition, females from 1 dpn TX dam progeny have higher (by 17%; $p < 0.05$) brain:body weight ratio than their male littermates.

3.5.4 Postnatal liver growth

Postnatal liver weights were found to be significantly affected by age and treatment, with an additional age-treatment interaction (Fig. 3.11). Maternal hypothyroxinemia resulted in significantly lower (by 29% for males and for females; $p < 0.001$) liver weights at 1 dpn compared to pups from the control group. Similar liver weights have been reported for 1 dpn N and TX dam progeny (Hendrich et al., 1997). At 10 dpn the liver weights for the experimental group catch up with their controls. When liver:body weight ratio was analyzed, an overall age effect was detected (Fig. 3.11) where the ratio decreased between 1 and 10 dpn in N (by 42% and by 44%, males and females, respectively; $p < 0.001$) and TX (by 31% and by 38%, males and females, respectively; $p < 0.001$) dam progenies. Maternal hypothyroxinemia was without any effect on liver:body weight ratio, suggesting that the decrease in liver weight was due to a decrease in overall body and organ weight.
Figure 3.10: Effect of maternal hypothyroxinemia on postnatal brain weight and brain:body weight ratio. Values are mean ± se (n ≥ 3).

A) Postnatal male and female brain weights. Age effect: p < 0.001, age-treatment interaction: p < 0.05 (two-way ANOVA); * p < 0.05: TX vs N dam, at the appropriate age (post-hoc analysis). Male and female brain weights in control and experimental groups increase between 1 and 30 dpn (p < 0.001, post-hoc analysis).

B) Postnatal male and female brain:body weight ratio. Age effect: p < 0.001, treatment effect: p < 0.01 (two-way ANOVA); ^p < 0.05: male vs female, at the appropriate age; * p < 0.05 **p < 0.01, ***p < 0.001: TX vs N dam, at the appropriate age (post-hoc analysis). Male and female brain:body weight ratio in control and experimental groups decrease between 15 and 30 dpn (p < 0.001, post-hoc analysis).
Figure 3.11: Effect of maternal hypothyroxinemia on postnatal liver weight and liver:body weight ratio. Values are mean ± se (n ≥ 3).
A) Postnatal male and female liver weights. Age effect: p < 0.001, treatment effect: p = 0.001, age-treatment interaction: p < 0.001 (two-way ANOVA); * p < 0.001: TX vs N dam, at the appropriate age (post-hoc analysis). Male and female liver weights in control and experimental groups increase between 1 and 10 dpn (p < 0.001, post-hoc analysis).
B) Postnatal male and female liver:body weight ratio. Age effect: p < 0.001 (two-way ANOVA). Male and female liver:body weight ratio in control and experimental groups decrease between 1 and 10 dpn (p < 0.001, post-hoc analysis).
3.6 Thyroid hormone levels in postnatal progeny

After studying the thyroid hormone levels in maternal serum this study was further expanded to assess the long term effect of maternal hypothyroxinemia on the thyroid system of postnatal progeny and to see if any sex related differences were present. Serum samples were collected at 1, 15 and 30 dpn. The serum was assayed for total T4 and T3, however, as there wasn’t enough serum samples from 1 dpn TX dam progeny, the serum was used for measurement of estradiol and progesterone (Chapter 4) and therefore T4 and T3 results for that age group were abandoned.

3.6.1 Serum T4 levels in postnatal progeny

Serum T4 levels in postnatal progeny showed age and treatment related effects (Fig. 3.12). Postnatal T4 levels increased (by 797% in males and by 600% in females; p < 0.001) between 1 and 15 dpn in N dam progeny. A significant decrease in T4 levels was apparent between 15 and 30 dpn in both sexes of N dam progeny (by 37% in males and by 32% in females, p < 0.001) and in females of TX dam progeny (by 50%; p < 0.001) but not their males. This sex-related difference was probably due to the observation that at 15 dpn, female progeny of TX dams had significantly higher (by 38%; p < 0.001) T4 levels than their controls. A similar decrease in T4 levels have been reported to occur between 15 and 30 dpn in N dam progeny (Gayo et al., 1986). Furthermore, at 30 dpn, male progeny of TX dams had higher (by 33%; p < 0.05) T4 levels than their controls. Moreover, at 15 dpn females from TX dam progeny showed higher (by 36%; p < 0.05) T4 levels than their male littermates. The pattern of T4 increase during and just after the suckling period has been reported by others (Morreale de Escobar et al., 1992). T4 values at 30 dpn in both N and TX dam progeny were very similar to those reported by others in adult rats (Hadjzadeh et al., 1990; Pickard et al., 1990) possibly implying that levels remain relatively stable after 30 dpn.

3.6.2 Serum T3 levels in postnatal progeny

Maternal hypothyroxinemia had no effect on T3 levels in postnatal progeny (Fig. 3.12). However, an overall age effect was detected. A significant increase in T3 levels was observed between 1 and 15 dpn in both males and females (by 280% and by 354%, respectively; p < 0.001) of N dams. This pattern of T3 increase during and just after the suckling period has been reported by others (Morreale de Escobar et al., 1992). Again T3 values at 30 dpn in both N and TX dam progeny were very similar to those reported by others in 7 month old rats (Hadjzadeh et al., 1990) indicating that levels remain relatively stable from 30 dpn onwards.
Figure 3.12: Effect of maternal hypothyroxinemia on serum T4 and T3 levels in postnatal progeny. Values are mean ± se (n ≥ 3).

A) Serum T4 levels. Age effect: p < 0.001, treatment effect: p < 0.01 (two-way ANOVA); a,b p < 0.001: 15 dpn vs 1 dpn; c,d,e p < 0.001: 30 dpn vs 15 dpn; f,g p < 0.001: 30 dpn vs 1 dpn; h p < 0.05: male vs female, at the appropriate age, * p < 0.05, ** p < 0.001: TX vs N dam, at the appropriate age (post-hoc analysis).

B) Serum T3 levels. Age effect: p < 0.001 (two-way ANOVA); a,b p < 0.001: 15 dpn vs 1 dpn (post-hoc analysis).
Several studies have been concerned with the effect of the maternal thyroid state on fetal brain development, however, most have concentrated on hypothyroidism (Hendrich et al., 1984; Morreale de Escobar et al., 1985, 1987, 1992; Calvo et al., 1992; Porterfield and Hendrich, 1993) employing animal models in which dams have been totally thyroidectomized either surgically or by treatment with $^{131}$I. Other models include administration of goitrogens (such as methimazole or propylthiouracil) in drinking water or food of pregnant rat dams. These goitrogens block the oxidation of iodide to iodine and consequently block the formation of T4 and T3 in maternal thyroid (Oppenheimer and Schwartz, 1997). Since these drugs readily pass the placental barrier and are also transmitted to the suckling pups in the mother’s milk, the fetus and neonate also become profoundly hypothyroid. Total thyroidectomy also results in severe maternal compromise and reduced placental weight (growth). Moreover, removal of the thyroid gland surgically or by $^{131}$I irradiation, usually results in fewer pregnancies, smaller litters, stillbirths, prolonged gestation and maternal tetany (Parrott et al., 1960). An important consideration is that mothers of neurological cretins are usually ‘hypothyroxinemic’ rather than severely hypothyroid, and impaired neurological development has been described in offspring of hypothyroxinemic women in iodine replete environments. Therefore, a more appropriate model of partially thyroidectomized rats (parathyroid-spared) was used.

In this model, dams were hypothyroxinemic as approximately 30% of T4 and 45% of T3 were present in the circulation. 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 (Porterfield and Hendrich, 1981). This model therefore approximates thyroid status seen in certain iodine-deficient (Connolly and Pharoah, 1989) and iodine-replete (Man et al., 1991) environments. In this study, placental growth was unaffected by maternal thyroid state. Moreover, Hadjzadeh et al. (1990), working with a similar animal model, have shown that placental compromise is not an issue in this model of maternal hypothyroxinemia as no obvious differences were detected in a range of biochemical parameters including DNA, RNA and protein concentrations. Moreover, any differences detected in fetal measurements cannot be attributed to iodine deficiency or postpartum nutritional compromise as both dams and progeny were fed iodine-replete diets and the pups were often cross fostered (Sinha et al., 1991b).

The TX rat dams exhibited reproductive compromise. Reduction in the number of fetuses has also been reported for hypothyroid and hypothyroxinemic rats (Morreale de Escobar et al., 1985; Morreale de Escobar et al., 1988; Porterfield and Hendrich, 1991; Ruiz de Ona et al., 1991; Pickard et al., 1993). Hagino (1971) showed that hypothyroid state
tends to suppress both the percent of ovulation and the number of ova suggesting an influence on neural regulation of gonadotrophin secretion. A contributing factor to small litter size may be fetal resorptions which have been reported with surgically induced hypothyroidism where both the thyroid and parathyroid glands have been removed (Hendrich et al., 1976).

Present data confirm that maternal hypothyroidism results in reduced body and organ weights of fetuses near term (Parrott et al., 1960; Hagino et al., 1971; Morreale de Escobar et al., 1985; Hubank et al., 1986; Morreale de Escobar et al., 1988). In this study, fetuses were characterized by abnormal growth. Fetal body weights at 19 and 21 dg appeared lower in the experimental group. An overall treatment effect was observed for fetal brain weights where a trend towards lower weights in the experimental group was apparent, however, post-hoc analysis failed to reveal a significant difference at any individual age. The brain:body weight ratio decreased as pregnancy progressed again without any significant difference between control and experimental groups, although the experimental group showed a trend to having a higher ratio. Fetal liver weights, on the other hand, were significantly lower in the experimental group at 16 and 21 dg. However, the liver:body weight ratio was normal. Morreale de Escobar et al. (1988) has also shown that fetuses collected at 21 dg from hypothyroid dams had lower body weights, brain weights and liver weights than the corresponding group of fetuses from N dams with a higher brain:body weight ratio and a normal liver:body weight ratio.

These results confirm several previous observations made in this model: hypothyroxinemic dams carry fewer fetuses than euthyroid controls and placenta weight is normal (Pickard et al., 1993). Fetal body and brain weights were previously shown to be reduced at 15 dg and although in our study there is a trend for weights to be lower, statistical significance was not achieved. As mentioned above, other workers have reported similar effects of thyroidectomy in the rat on body and brain growth of progeny (Porterfield and Hendrich, 1982; Hendrich et al., 1984; Morreale de Escobar et al., 1985).

The DNA concentration at 16 dg was lower in fetal brains from TX dams, however, this difference was reversed by 21 dg. Moreover, the cell number was lower at 16 and higher at 19 dg in TX dam progeny consistent with over-compensation resulting from the onset of fetal thyroid function. Maternal hypothyroxinemia was without any effect on protein concentration, although the protein content was affected at 16 dg. Protein:DNA ratio was unaffected by maternal thyroid state and although ratios at 19 and 21 dg were apparently lower in fetal brains from TX dams, statistical significance was not achieved. The reduction in brain weight at 16 dg, although not significant, was accompanied by deficits in the total protein and DNA content, suggestive of abnormal neuroblast proliferation.
Moreover, the increase in DNA concentration and the decrease in protein:DNA ratio near term is consistent with a delay in neuronal maturation (Pickard et al., 1993). Although statistical significance was not attained for DNA and protein content and protein:DNA ratio, other workers have shown that these parameters are decreased in fetal rat brain near term (Porterfield and Hendrich, 1982). The RNA concentration in developing fetal brain was not affected by maternal thyroid status at any stage.

A significant number of postnatal progeny were found dead at birth. Similar results for number of live 1 day old progenies from hypothyroid dams have been reported (Hendrich et al., 1997). A significant decrease in live TX dam progenies compared to controls was observed, however, sex-related differences were not detected. When body weights were analyzed it was found that at 1 dpn TX dam progeny of both sexes appear to have lower body weights than their respective controls and at 15 and 30 dpn body weights appeared lower in the experimental group, however, post-hoc analysis revealed no significant difference. Moreover, females at 1 dpn have lower body weights than males in both control and experimental groups. However, these differences were not significant at all other ages.

Brain weights in TX dam progeny were lower at 1 dpn in both males and females. In addition, at 30 dpn males from TX dams had lower brain weights than the control group. Maternal hypothyroxinemia was without any effect on brain weight at other ages in agreement with results obtained by the same group (Pickard et al., 1993). The brain:body weight ratio revealed that at 1 and 15 dpn the experimental group had a higher ratio than their respective controls indicating that brain growth sparing is indeed present to counter the possible effect of maternal hypothyroxinemia.

As for liver weights, maternal hypothyroxinemia resulted in lower weights at 1 dpn in the experimental group compared to their respective controls. However, when the liver:body weight ratio was analyzed no significant effect of maternal hypothyroxinemia was apparent as both the liver and body weights decreased at that age point. Similar liver weights have been reported for 1 dpn N and TX dam progeny (Hendrich et al., 1997).

T4 and T3 levels in serum of postnatal progeny from N dams increased between 1 and 15 dpn. T4 levels then decreased between 15 and 30 dpn and a similar decrease has been shown to occur in N dam progeny (Gayo et al., 1986). The pattern of T4 and T3 increase during and just after the suckling period has been reported by others (Morreale de Escobar et al., 1992). The effect of maternal hypothyroxinemia on T4 levels was apparent at 15 dpn in female and at 30 dpn in male progenies where the experimental group had higher levels. On the other hand, maternal hypothyroxinemia had no effect on T3 levels in postnatal progeny. T4 values at 30 dpn in both N and TX dam progenies were very
similar to those reported by others in adult rats (Hadjzadeh et al., 1990; Pickard et al., 1990) possibly implying that levels remain relatively stable after 30 dpn.
Chapter 4:

Effect of maternal hypothyroxinemia:
on sex steroid hormone levels in dams, fetuses and postnatal progeny;
and on expression of sex steroid receptors in fetal rat brain
4.1 Introduction

Steroid hormones play an important role during brain development (section 1.8.3) and TH deficiency has been shown to affect sex steroid hormone levels. To assess whether maternal thyroid state affects sex steroid hormone levels, maternal and postnatal serum, amniotic fluid and fetal tissue were examined and sex steroid hormone levels in these samples were determined.

ER and PR expression has also been shown to be affected by TH in some tissues, including the brain (section 1.12); TH have been shown to increase cellular concentrations of ER protein and mRNA (Norstedt et al., 1981; Freyschuss and Eriksson, 1988; Freyschuss et al., 1991), but details of the mechanisms of this regulation are not known. Thyroid hormone may also be one of the factors affecting gene expression of PR, particularly form B, in the developing cerebral cortex of the female rat. Neonatal hypothyroidism (PTU-induced) causes a drastic decrease in the levels of progesterone receptor protein (Kato et al., 1984) and mRNA (Kato et al., 1993; Hirata et al., 1994) in the cerebral cortex, whilst those in the hypothalamus-preoptic area of the neonatal rat brain remain normal, suggesting a regulatory role of TH in the developing rat brain cortex. Sex steroid receptor expression has been poorly studied in fetal rat brain probably due to the low number of transcripts at this age. Therefore, attempts were made to examine sex steroid hormone receptor (PR and ER) expression in fetal brain to see whether receptors are regulated by TH levels during fetal life.

4.2 Sex steroid hormone levels in maternal serum and amniotic fluid

Estradiol and progesterone hormone levels were determined in maternal serum and amniotic fluid at 14, 16, 19 and 21 dg. Collection of amniotic fluid at 21 dg was difficult because of the small volume and high viscosity, therefore, the number of samples for this age group was scarce. For E determination in maternal serum and amniotic fluid, samples were subjected to diethyl ether extraction prior to RIA, as recommended by the manufacturer of the RIA kit.

4.2.1 Estradiol levels in maternal serum and amniotic fluid

A significant age-treatment interaction was observed for maternal serum E levels (Fig. 4.1). In N dams, levels appeared to slightly fall between 14 and 16 dg, and 16 and 19 dg
Figure 4.1: Effect of maternal hypothyroxinemia on serum and amniotic fluid estradiol levels. Values are mean ± se. Numbers in brackets refer to the number of samples.

A) Estradiol levels in maternal serum. Age-treatment interaction: p < 0.05 (two-way ANOVA); * p < 0.001: TX16 vs TX14; * p < 0.05: TX vs N dam, at the appropriate age (post-hoc analysis).

B) Estradiol levels in amniotic fluid. Age effect: p < 0.001, age-treatment interaction: p < 0.05 (two-way ANOVA); * p < 0.001: N21 vs N14; * p < 0.05: N21 vs N16; * p < 0.001: TX21 vs TX14; * p < 0.01: TX21 vs TX16; * p < 0.05: TX19 vs TX16; * p < 0.001: TX21 vs TX19; * p < 0.05: TX vs N dam, at the appropriate age (post-hoc analysis).
followed by an increase in levels near term, however, these changes were not statistically significant. In contrast, serum E levels in TX dams were increased (by 103%; \( p < 0.001 \)) between 14 and 16 dg. In hypothyroxinemic dams, serum E levels appeared reduced (by 46%) relative to controls at 14 dg but increased (by 39%) at 16 dg. However, post-hoc analysis confirmed a significant difference at 14 dg only (\( p < 0.05 \)).

Levels of E in amniotic fluid were 3- to 8-fold higher than those in maternal serum (Fig. 4.1). Significant age and age-treatment interaction was observed for this parameter. A slight but steady increase was detected in N dams with progression of pregnancy; levels were higher by 116% \( (p < 0.05) \) at 21 dg relative to 14 dg and by 93% relative to 16 dg \( (p < 0.05) \). Similar changes were reported for E levels in amniotic fluid with progression of pregnancy \( (\text{de Lauzon et al., 1974}) \). In TX dams, similar results were observed, where levels at 21 dg were higher than at 14 dg and 16 dg \( (193\% ; p < 0.001 \text{ and } 110\%; p < 0.01) \), respectively. At 19 dg amniotic fluid E levels in TX dams were lower \( (34\%; p < 0.05) \) compared to 16 dg increasing \( (219\%; p < 0.001) \) towards term. Maternal hypothyroxinemia was without any effect on E levels in amniotic fluid before 21 dg, however, at 21 dg E levels were significantly higher in TX dams than in N dams.

4.2.2 Progesterone levels in maternal serum and amniotic fluid

Maternal serum P levels exhibit a significant age effect; in N dams levels decreased steadily between 14 and 19 dg from 127 ng/ml to 84 ng/ml \( (p < 0.01) \) then more rapidly to 40 ng/ml at 21 dg \( (p < 0.001) \) (Fig. 4.2). Others have shown a similar decrease in P levels between 14 and 21 dg \( (\text{Pepe and Rochild, 1974; Taya and Greenwald, 1981}) \), between 19 and 21 dg \( (\text{Weisz and Ward, 1980}) \) and between 16 and 21 dg \( (\text{Morishige et al., 1973}) \) although levels reported by the latter group were slightly higher probably due to the different method of assay. Moreover, Gibori and Keyes \( (1978) \) reported similar P levels at 14 dg. In TX dams, a similar though apparently delayed decrease \( (58\%; p < 0.001) \) was seen between 16 and 21 dg. At 14 dg levels appeared reduced \( (24\%) \) in TX dams but increased at subsequent stages of pregnancy, though no overall treatment-related effects were found \( (\text{two-way ANOVA}) \). Maternal hypothyroxinemia was therefore without any significant effect on serum P levels at all ages studied.

When studying P levels in amniotic fluid it was apparent that levels were 25- to 30-fold lower than serum levels \( (\text{Fig. 4.2}) \). No significant difference in amniotic fluid P levels were detected over the study period and maternal hypothyroxinemia was without any effect on this parameter.
Figure 4.2: Effect of maternal hypothyroxinemia on serum and amniotic fluid progesterone levels. Values are mean ± se. Numbers in brackets refer to the number of samples.

A) Progesterone levels in maternal serum. Age effect: p < 0.001 (two-way ANOVA); *p < 0.01: N19 vs N14; b p < 0.001: TX21 vs TX16 (post-hoc analysis).

B) Progesterone levels in amniotic fluid. No significant overall age or treatment-related effects were found.
4.3 Sex steroid hormone levels in fetal tissue

Estradiol and progesterone levels were studied in fetal brain, liver and carcass at 16, 19 and 21 dg. E and P were extracted from fetal tissue prior to RIA with tritiated steroids being added to tissue homogenates prior to extraction for recovery purposes (section 2.6.2).

4.3.1 Estradiol levels in fetal tissue

When studying E levels in fetal rat brain, age and age-treatment interaction effects were detected (Fig. 4.3). In N dam fetuses, levels were at least 2-fold higher at 16 dg compared to levels at 19 and 21 dg (p < 0.001). This ontogenic decrease was not detected in fetal rat brain from TX dams; rather levels remained relatively stable over the study period. Indeed relative to controls, E levels were reduced (by 49%; p < 0.001) at 16 dg but increased (by 96%, p < 0.05) at 21 dg in fetal brain from TX dams. E levels in fetal hypothalamus have been reported by others to be between 290 and 350 pg/g in 21 dg female and male Sherman strain rat fetuses, respectively, with no significant difference detected due to sex (Rhoda et al., 1984).

Estradiol levels in fetal liver were invariant over the study period in normal pregnancy but were significantly influenced by maternal hypothyroxinemia (Fig. 4.3); relative to controls, levels appeared higher at 16 and 21 dg (by 40% and 45%, respectively), but only the latter difference was significant by post-hoc analysis.

In fetal carcasses E levels show a significant age-treatment interaction (Fig. 4.3). E levels in fetal progeny from N dams remain stable throughout the study period. In contrast, levels increased (by 71%; p < 0.05) from 16 to 19 dg in fetal carcasses from TX dams. Maternal hypothyroxinemia had an effect on E levels in fetal carcasses at 16 dg were levels were significantly lower (by 38%; p < 0.05) in the experimental group. This effect of maternal hypothyroxinemia was similar to that seen in fetal brain.

4.3.2 Progesterone levels in fetal tissue

Maternal hypothyroxinemia was without any effect on P levels in fetal brain, liver and carcasses (Fig. 4.4). However, an overall age effect was observed in all tissues studied. In 16 dg fetal brains obtained from N dams, P levels were 2.5-fold higher than levels in livers and carcasses. This observation was similar to that seen for E levels for that age group. In fetal rat brains and carcasses of N dams, levels decreased between 16 dg and 19 dg (by 84%; p < 0.001) and between 16 and 21 dg (by 78%; p < 0.001), respectively. A
Figure 4.3: Effect of maternal hypothyroxinemia on estradiol levels in fetal tissue. Values are mean ± se (n = 4).

A) Estradiol levels in fetal brain. Age effect: p < 0.001; age-treatment interaction: p < 0.001 (two-way ANOVA). *p < 0.001: N19 vs N16; †p < 0.001: N21 vs N16; * p < 0.05, ** p < 0.001: TX vs N dam, at the appropriate age (post-hoc analysis).

B) Estradiol levels in fetal liver. Treatment effect: p < 0.05 (two-way ANOVA). †p < 0.05: TX21 vs TX19; * p < 0.05: TX vs N dam, at the appropriate age (post-hoc analysis).

C) Estradiol levels in fetal carcass. Values are mean ± se (n = 4). Age-treatment interaction: p < 0.05 (two-way ANOVA); *p < 0.05: TX19 vs TX16; †p < 0.05: TX21 vs TX16; * p < 0.05: TX vs N dam, at the appropriate age (post-hoc analysis).
Figure 4.4: Effect of maternal hypothyroxinemia on progesterone levels in fetal tissue. Values are mean ± se (n = 4).

A) Progesterone levels in fetal brain. Age effect: p < 0.001 (two-way ANOVA). *p < 0.001 N19 vs N16; ^p < 0.001: N21 vs N16; †p < 0.001: TX21 vs TX16; ‡p < 0.01: TX21 vs TX19 (post-hoc analysis).

B) Progesterone levels in fetal liver. Age effect: p < 0.05 (two-way ANOVA).

C) Progesterone levels in fetal carcass. Age effect: p < 0.001 (two-way ANOVA); *p < 0.05: N19 vs N16; †p < 0.001: N21 vs N16; ‡p < 0.001: TX21 vs TX16; ‡p < 0.001: TX21 vs TX19; ‡p < 0.01: N21 vs N19 (post-hoc analysis).
similar but slightly delayed decrease (by 71% in brains and 77% in carcasses; p < 0.001) was observed in TX dam progeny between 19 and 21 dg in both brains and carcasses. P levels in fetal hypothalamus has been reported by others to be approximately 5000 and 7000 pg/g in 21 dg female and male Sherman strain rat fetuses, respectively, with no significant difference due to sex (Rhoda et al., 1984). No change in P levels was detected in fetal livers as pregnancy progresses.

4.4 Expression of estrogen and progesterone receptors in fetal brain

Estrogen receptors exist as two isoforms (ERα and ERβ). Little is known about the distribution of the more recently cloned ERβ (Kuiper et al., 1996) in rat tissues. Expression of ERα in 18 dg fetal rat preoptic area has been detected using in situ hybridization (Don Carlos, 1996). Progesterone receptor also exists as two isoforms (PRA and PRB). Both forms are derived from the same gene (section 1.10.3.2) and have been detected in rat brain using RT-PCR methodology (Kato et al., 1993; Kato et al., 1994). In this study, ER and PR receptor expression was studied in fetal rat brain. The initial approach was to obtain RNA (total and poly A+ mRNA) and blot it onto nylon membranes (section 2.8). Membranes were then hybridized with rPR1 RNA and/or cDNA probes to detect PR transcripts, however both attempts failed. Similarly, hybridization with hER cDNA probe to detect ER transcripts was also unsuccessful. This was possibly due to the low number of transcripts in fetal rat brain. Therefore, attempts were also made to study their expression in postnatal brain as levels are expected to be higher. Uterus was used as a positive control but no bands were detected using these probes, probably because the uterus sample was obtained from a pregnant dam where sex steroid levels are high causing down regulation of their own receptors, or probably because the probes used were inappropriate. 18S rRNA cDNA probes were also used on the same blots to check the methodology. In addition, total RNA obtained from postnatal brain samples was used to detect TRα1 mRNA, again to check the methodology. Both attempts proved successful implying that the methodology employed was correct but ER and PR transcripts were below the limit of detection.

Before preparing cDNA probes plasmids were digested (section 2.7.4) to obtain the hER and rPR1 inserts. When run on 1% agarose gel a 265-270 bp hER insert and a 550 bp rPR1 inserts were apparent (Appendix A, Fig. A3). After checking that digestion was satisfactory, the digests were run on a low melting point agarose gel (Appendix A, Fig. A4) and the appropriate band was excised, purified and cDNA probes were prepared (section 2.9.1.2). Linearization of rPR1 plasmid was also checked on 1% agarose gel (Appendix A, Fig. A3) before preparing riboprobes by in vitro transcription (section 2.9.1.1). As mentioned above hybridization of Northern blots was not successful in detecting ER and PR transcripts. However, when the same blots were used to detect the
18S rRNA using cDNA probes, a 1.6 kb band was detected (Fig. 4.5). In addition, using total RNA obtained from postnatal brains, a 2.2 kb band was detected for TRα₂ using specific riboprobes (Fig. 4.5).

When previous attempts to detect ER and PR transcripts using specific cDNA and RNA probes failed, attempts to detect these transcripts using RT-PCR methodology was employed with a successful outcome. The authenticity of the PCR products was later checked using specific oligonucleotide probes after Southern blotting (Appendix D, Fig. D1). As thyroid hormones are thought to have important actions as regulators of both transcription and translation, a suitable control, preferably an mRNA, was examined in place of 18S rRNA to correct for differences between samples in the efficiency of reverse transcription. Therefore, the effect of maternal hypothyroxinemia on transcription in general was investigated. Several housekeeping genes including rat ribosomal protein L19 (RPL19), β-glucuronidase (BGLU), cyclophilin (CY), histone H4 (HH4) and glyceraldehyde-3-phosphate-dehydrogenase (G3PDH) were studied. The 18S rRNA transcript was used as an internal standard for the housekeeping genes. Consequently, final results for the housekeeping genes were expressed relative to 18S rRNA. A housekeeping gene that was not affected by maternal thyroid state was then chosen and used as an internal standard.

4.4.1 Effect of maternal hypothyroxinemia on the expression of housekeeping genes

The housekeeping genes chosen for this study are present in various cell organelles or compartments. RPL19 is a ribosomal protein the function of which is unknown (Chan et al., 1987) and BGLU is a lysosomal enzyme (McKusick and Neufeld, 1983). The protein CY represents 0.1 - 0.4% of the total cytosolic protein of most eukaryotic tissues (Harding et al., 1986), however, its role is unknown. HH4 plays a central role in nucleosome formation (Kornberg, 1977) and is the most highly conserved of the five histones with respect to amino acid sequence (Grimes et al., 1987). Lastly, G3PDH is a key enzyme in the control of glycolysis (Fort et al., 1985).

The optimal MgCl₂, dNTP and primer concentrations were determined in preliminary experiments. The cycle number and template dilution were then verified (see Appendices B and C, respectively). When RNA yield/g brain was studied in fetal brains from N and TX dams (section 3.4.6), it was found that maternal hypothyroxinemia was without any effect on this yield. Therefore, for this experiment it was decided to use the 18S ribosomal RNA as an internal standard. The od measurements obtained for all housekeeping genes were expressed relative to the od obtained from 18S.
Figure 4.5: Hybridization analysis using 18S and TRα2 riboprobes.
A) Fetal brain Northern blot hybridized with 18S riboprobe. A band with an apparent molecular weight of approximately 1.6 kb was detected in fetal brain and maternal 21 dg uterus. N: normal dam; TX: hypothyroxinemic dam; U: maternal 21 dg uterus.
B) Postnatal brain Northern blot hybridized with TRα2 riboprobe. A band with an apparent molecular weight of approximately 2.2 kb was detected in postnatal rat brain. dpn: days postnatally.
Effect of maternal hypothyroxinemia on relative expression of all housekeeping genes is shown in Fig. 4.6. Expression of RPL19 mRNA levels relative to 18S revealed that maternal hypothyroxinemia was without any significant effect on the expression of this gene in fetal rat brain. However, in fetal brains from TX dams, levels appear to be slightly higher especially at 19 and 21 dg. β-Glucuronidase mRNA expression shows a degree of variability among the different ages probably indicating that levels at 19 dg are lower than those at 16 and 21 dg in fetal brains from N dams. However, no significant difference was detected using two-way ANOVA. It seems, therefore, that maternal hypothyroxinemia does not affect the expression of BGLU transcripts in fetal rat brain.

Results obtained when cyclophilin is expressed relative to 18S are quite interesting. Maternal hypothyroxinemia did not show any significant effect on the expression of CY transcripts in fetal rat brain, however, results obtained from TX dams show that levels appear higher at all gestational ages. It would be interesting therefore to increase the number of samples in each experiment and thus get a definite conclusion on whether this effect is real.

Maternal hypothyroxinemia was without any significant effect on the relative expression of HH4 transcripts in fetal rat brain. However, HH4 mRNA levels do seem slightly elevated at 21 dg but the variability is high as shown by the large standard errors. Maternal hypothyroxinemia was without any significant effect on the expression of G3PDH transcripts in fetal rat brain. Expression of this gene in fetal brain showed least variability and results were reproducible. Therefore, sex steroid receptor results from RT-PCR experiments were expressed relative to this housekeeping gene.

4.4.2 Effect of maternal hypothyroxinemia on ERα expression in fetal rat brain

4.4.2.1 Expression of ERα transcripts

The expected 623 bp product was detected in fetal rat brain from N dams as early as 16 dg (Fig. 4.7). However, using the 6-fold dilution, no product was detected at this age in fetal brain from TX dams. Therefore, the PCR reaction was repeated for 16 dg samples using neat and a 2-fold dilution enabling the detection of the PCR product in the TX dam group (Fig. 4.8). The authenticity of the PCR product was verified using a specific ERα oligonucleotide probe after Southern blotting (see Appendix D, fig. D1); a single product was detected after hybridization.
Figure 4.6: Effect of maternal hypothyroxinemia on the relative expression of housekeeping genes in fetal rat brain. The amount of product was expressed relative to 18S. Samples were run in duplicates; results shown are mean ± se (n = 3). No significant difference was detected between groups for all genes studied.
A) Relative rat protein L19 mRNA level. Reactions were performed for 33 cycles at 52 °C.
B) Relative β-glucuronidase mRNA level. Reactions were performed for 34 cycles at 52 °C.
C) Relative cyclophilin mRNA level. Reactions were performed for 24 cycles at 59 °C.
D) Relative histone H4 mRNA level. Reactions were performed for 39 cycles at 59 °C.
E) Relative glyceraldehyde-3-phosphate-dehydrogenase mRNA level. Reactions were performed for 26 cycles at 57 °C.
Figure 4.7: Effect of maternal hypothyroxinemia on the expression of ERα mRNA in fetal rat brain. Reactions were performed for 38 cycles at 57 °C, using standard reaction conditions.
A) Representative ethidium bromide stained gel. The migration of the 100 bp marker (M) is shown on the left-hand side and the calculated size of the signal indicated on the right-hand side of the gel (expected product size is 623 bp). N: normal dam; TX: hypothyroxinemic dam.
B) Relative ERα mRNA level. The amount of product was expressed relative to G3PDH. Samples were run in duplicates; results shown are mean ± se (n = 3). Age effect: p < 0.01; age-treatment interaction: p = 0.0847 (two-way ANOVA); *p < 0.05: N19 vs N16; b p < 0.001: TX19 vs TX16; c p < 0.01: N21 vs N16; d p < 0.001: TX21 vs TX16; * p < 0.05: TX vs N dam, at the appropriate age (post-hoc analysis).
Figure 4.8: Effect of maternal hypothyroxinemia on the expression of ERα mRNA in 16 dg fetal rat brain. Reactions were performed for 38 cycles at 57 °C, using standard reaction conditions.

A) Representative ethidium bromide stained gel. The calculated size of the signal is indicated on the right-hand side of the gel (expected product size is 623 bp). N: normal dam; TX: hypothyroxinemic dam.

B) Relative ERα mRNA level. The amount of product was expressed relative to G3PDH. Samples were run in duplicates; results shown are mean ± se (n = 3). Treatment effect: p < 0.05 (two-way ANOVA); * p < 0.05: TX vs N dam, at the appropriate age (post-hoc analysis).
An overall age effect was detected when the data were analyzed (Fig. 4.7). As for age-treatment interaction, two-way ANOVA showed that there may be a trend for ERα mRNA expression to be affected (p = 0.0847) and by post-hoc analysis, it was revealed that ERα mRNA expression is affected at 16 dg in TX dam progeny. ERα mRNA levels increase significantly from 16 dg to 19 dg in fetal brains from N and TX dams. When the expression of ERα mRNA levels was studied using neat and 2-fold diluted DNA from 16 dg fetal brains, a significant treatment effect was detected where ERα mRNA expression was lower in TX dam progeny (Fig. 4.8).

### 4.4.2.2 Expression of ERα protein

Attempts were made to detect ERα protein in brain fractions (homogenate, cytosol and salt-extracted nuclei). The protein concentration in total homogenates was shown in Chapter 3 (Fig. 3.6) and that for cytosolic and extracted nuclear fractions are shown in Fig. 4.9. Overall age and age-treatment interaction were observed for the cytosolic protein concentration in fetal brain. This increased (by 285%; p < 0.001) between 16 and 19 dg in fetal brains from N dams and between 16 and 21 dg (by 939%; p < 0.001) in fetal brains from TX dams. Maternal hypothyroxinemia resulted in a significantly lower (by 46%; p < 0.01) protein concentration at 19 dg and a higher (by 55%; p < 0.001) one at 21 dg.

The protein concentration in extracted fetal brain nuclear fraction showed an overall age and treatment effect (Fig. 4.9). Protein concentration increased (by 60%; p < 0.01) between 16 and 19 dg and decreased (by 28%; p < 0.05) towards term in fetal brains from N dams. In TX dam progeny, protein concentration increased (by 207%; p < 0.001) between 16 and 19 dg. Maternal hypothyroxinemia resulted in significantly lower (by 61%; p < 0.01) protein concentration at 16 dg.

Because protein concentrations were affected by age and treatment in the different brain compartments, results obtained after image analysis were expressed relative to maternal brain (MB) after correcting for protein concentration in the different compartments as follows:

⇒ mg protein / g wet weight = protein in each compartment (mg) / wet tissue weight (g);
⇒ od value / mg protein = od value x (1000 / µg protein loaded);
⇒ od / g wet weight = (od value / mg protein) x (protein in each compartment (mg) / wet tissue weight (g)).
Figure 4.9: Effect of maternal hypothyroxinemia on fetal brain protein concentration in cytosolic and extracted nuclear fractions. Values are mean ± se (n = 3).

A) Protein concentration in cytosolic fraction. Age effect: p < 0.001, age-treatment interaction: p < 0.001 (two-way ANOVA); *p < 0.001: N19 vs N16; †p < 0.001: N21 vs N16; ‡p < 0.01: TX19 vs TX16; §p < 0.001: TX21 vs TX16; ††p < 0.001: TX21 vs TX19; * p < 0.01, ** p < 0.001: TX vs N dam, at the appropriate age (post-hoc analysis).

B) Protein concentration in extracted nuclear fraction. Age effect: p < 0.001, treatment effect: p < 0.01 (two-way ANOVA); *p < 0.01: N19 vs N16; †p < 0.05: N21 vs N19; ‡p < 0.001: TX19 vs TX16; §p < 0.001: TX21 vs TX16; * p < 0.01: TX vs N dam, at the appropriate age (post-hoc analysis).
When studying ERα protein expression, two antibodies were used. One of these antibodies was raised against the hinge region (amino acids 287 - 300) of ERα (ERα-H) whereas the other was raised against the steroid binding domain (amino acids 495 - 595; ERα-S). Expression of the ERα protein in brain homogenates (total receptors), cytosol and nuclei was studied. Using the ERα-H antibody, a single 67 kDa band was detected in all fractions as early as 16 dg (Fig. 4.10 - 4.12). This species was also detected in cytosolic fraction of maternal uterus. In brain homogenates (Fig. 4.10) and cytosol (Fig. 4.11), a significant overall age effect was detected, however, this was not the case in salt-extracted nuclei due to the variability of results (Fig. 4.12). In N dams, total ERα protein levels in fetal brain increases significantly between 16 dg and 19 dg similar to mRNA levels, however, in TX dam progeny no apparent increase was detected. On the other hand, the cytosolic receptor level showed a steady increase between 16 and 21 dg in both N and TX dam progeny (Fig. 4.11). Maternal hypothyroxinemia was without any effect on total, cytosolic or salt-extracted ERα protein expression in fetal rat brain although levels in extracted nuclei appear lower at 16 dg.

Using the second antibody raised against the steroid binding domain, a 67 kDa and 73 kDa bands were detected in all brain samples (fetal and adult) but not in uterus (Fig. 4.13 - 4.15). The identity of the 73 kDa band is as yet unknown. Analysis of the two bands revealed the following results: the 67 kDa band in brain homogenates, cytosol and salt-extracted nuclei shows a significant overall age effect. In addition an age-treatment interaction was seen in homogenates and cytosolic fraction. The total receptor content showed a significant increase in TX dam progeny between 16 and 19 dg (Fig. 4.13). A similar increase was detected in cytosolic and extracted nuclear receptors both in N and TX dam progeny between 16 and 19 dg with a further increase between 19 and 21 dg in the cytosolic fraction in TX dam progeny only (Figs. 4.14 and 4.15). Maternal hypothyroxinemia showed an effect on total receptor expression at 21 dg where levels were higher in TX dams. However, a different effect of maternal hypothyroxinemia was seen in the cytosolic fraction where levels at 19 dg were lower in TX dam progeny.

The 73 kDa band showed somewhat different results. No significant difference was detected in total receptor (Fig. 4.13). However, an age and an age-treatment interaction was detected in the cytosolic and salt-extracted nuclei fractions (Figs. 4.14 and 4.15). In the cytosol, levels were higher at 21 dg compared to 16 dg in controls while in the experimental group levels increased between 19 and 21 dg. However, in the salt-extracted nuclear fraction, an increase was seen between 16 and 19 dg in TX dam progeny and receptor levels were higher at 21 compared to 16 dg in N dam progeny. Maternal hypothyroxinemia affected the expression of this band at 16 dg where levels were lower in the experimental group. Both bands detected in the homogenate and cytosol show a similar overall change.
Figure 4.10: Effect of maternal hypothyroxinemia on the expression of ERα protein in fetal rat brain homogenates using ERα-H antibody.

A) Representative Western blot. The calculated size of the signal is indicated on the left-hand side of the gel. N: normal dam; TX: hypothyroxinemic dam; MB: maternal brain homogenate; U: uterus cytosol; 50 μg protein was loaded for brain samples and 25 μg for uterus.

B) Ontogenic profile. ERα protein was expressed relative to maternal brain. Results shown are mean ± se (n ≥ 3); age effect: p < 0.05 (two-way ANOVA); a p < 0.05: N19 vs N16; b p < 0.05: N21 vs N16 (post-hoc analysis).
Figure 4.11: Effect of maternal hypothyroxinemia on the expression of cytosolic ERα protein in fetal rat brain using ERα-H antibody.
A) Representative Western blot. The calculated size of the signal is indicated on the left-hand side of the gel. N: normal dam; TX: hypothyroxinemic dam; MB: maternal brain cytosol; U: uterus cytosol; 50 µg protein was loaded for brain samples and 25 µg for uterus.
B) Ontogenic profile. ERα cytosolic protein was expressed relative to maternal brain. Results shown are mean ± se (n = 3); age effect: p < 0.01 (two-way ANOVA); \(^{a}p < 0.05\): N19 vs N16; \(^{b}p < 0.001\): N21 vs N16; \(^{c}p < 0.05\): TX19 vs TX16; \(^{d}p < 0.001\): TX21 vs TX16; \(^{e}p < 0.05\): N21 vs N19; \(^{f}p < 0.001\): TX21 vs TX19 (post-hoc analysis).
Figure 4.12: Effect of maternal hypothyroxinemia on the expression of nuclear ERα protein in fetal rat brain using ERα-H antibody.

A) Representative Western blot. The calculated size of the signal is indicated on the left-hand side of the gel. N: normal dam; TX: hypothyroxinemic dam; MB: maternal brain nuclear receptors; U: uterus cytosol; 100 µg protein was loaded for brain samples and 25 µg for uterus.

B) Ontogenic profile. ERα nuclear protein was expressed relative to maternal brain. Results shown are mean ± se (n = 3); no significant difference was detected.
Figure 4.13: Effect of maternal hypothyroxinemia on the expression of ERα protein in fetal rat brain homogenates using ERα-S antibody.
A) Representative Western blot. The calculated size of the signal is indicated on the left-hand side of the gel. N: normal dam; TX: hypothyroxinemic dam; MB: maternal brain homogenate; U: uterus cytosol; 50 µg protein was loaded for brain samples and 25 µg for uterus.
B) Ontogenic profile of 73 kDa band. ERα protein was expressed relative to maternal brain. Results shown are mean ± se (n = 3); no significant difference was detected.
C) Ontogenic profile of 67 kDa band. ERα protein was expressed relative to maternal brain. Results shown are mean ± se (n = 3); age effect: p < 0.05; age-treatment interaction: p < 0.05 (two-way ANOVA); \(^a\) p < 0.01: TX19 vs TX16; \(^b\) p < 0.01: TX21 vs TX16; * p < 0.01: TX vs N dam, at the appropriate age (post-hoc analysis).
Figure 4.14: Effect of maternal hypothyroxinemia on the expression of cytosolic ERα protein in fetal rat brain using ERα-S antibody.

A) Representative Western blot. The calculated size of the signal is indicated on the left-hand side of the gel. N: normal dam; TX: hypothyroxinemic dam; MB: maternal brain cytosol; U: uterus cytosol; 50 µg protein was loaded for brain samples and 25 µg for uterus.

B) Ontogenic profile of 73 kDa band. ERα cytosolic protein was expressed relative to maternal brain. Results shown are mean ± se (n = 3); age effect: p < 0.001; age-treatment interaction: p < 0.05 (two-way ANOVA); *p < 0.05; N21 vs N16; b p < 0.001 TX21 vs TX16; c p < 0.001: TX21 vs TX19 (post-hoc analysis).

C) Ontogenic profile of 67 kDa band. ERα cytosolic protein was expressed relative to maternal brain. Results shown are mean ± se (n = 3); age effect: p < 0.001; age-treatment interaction: p < 0.05 (two-way ANOVA); *p < 0.001: N19 vs N16; b p < 0.001: N21 vs N16; *p < 0.05: TX19 vs TX16; *p < 0.001: TX21 vs TX16; *p < 0.001: TX21 vs TX19; * p < 0.01: TX vs N dam, at the appropriate age (post-hoc analysis).
Figure 4.15: Effect of maternal hypothyroxinemia on the expression of nuclear ERα protein in fetal rat brain using ERα-S antibody.

A) Representative Western blot. The calculated size of the signal is indicated on the left-hand side of the gel. N: normal dam; TX: hypothyroxinemic dam; MB: maternal brain nuclear receptors; U: uterus cytosol; 100 μg protein was loaded for brain samples and 25 μg for uterus.

B) Ontogenic profile. ERα nuclear protein was expressed relative to maternal brain. Results shown are mean ± se (n ≥ 2); age effect: p < 0.01; age-treatment interaction: p < 0.05 (two-way ANOVA); *p < 0.05: N21 vs N16; b p < 0.001 TX19 vs TX16; c p < 0.01: TX21 vs TX16; *p < 0.01: TX vs N dam, at the appropriate age (post-hoc analysis).

C) Ontogenic profile of 67 kDa band. ERα nuclear protein was expressed relative to maternal brain. Results shown are mean ± se (n ≥ 2); age effect: p < 0.01 (two-way ANOVA); *p < 0.05: N19 vs N16; b p < 0.001 TX19 vs TX16; c p < 0.01: TX21 vs TX16 (post-hoc analysis). The standard error bar at 16 dg is hidden by the box.
When the two antibodies are compared, the striking fact is that the ratios using the antibody raised against the steroid binding domain are much higher than with the other antibody (ca 10x). That is, there may be something extra in fetal brain which is being detected by this antibody or that the epitope in maternal brain is “masked”.

4.4.3 Effect of maternal hypothyroxinemia on ERβ expression in fetal rat brain

4.4.3.1 Expression of ERβ transcripts

The expected 262 bp ERβ product was detected in fetal rat brain from N dams as early as 16 dg and the authenticity of this product was checked using a specific ERβ oligonucleotide probe showing only a single band (see Appendix D, fig D1).

An overall age effect and age-treatment interaction was detected when analyzing ERβ mRNA levels in fetal rat brain. Post-hoc analysis revealed little change in ERβ mRNA levels in normal fetal brain over the period studied, but showed an increase in ERβ mRNA levels between 16 and 19 dg in TX dam progeny. The main effect of maternal hypothyroxinemia was a decrease (by 71%; p < 0.01) in ERβ mRNA levels at 16 dg relative to normal pregnancies (Fig. 4.16). Mean values also tended to increase at 21 dg but this was not found to be statistically significant.

4.4.3.2 Expression of ERβ protein

When studying the expression of ERβ protein, two antibodies were used. One of the antibodies was raised against the C-terminus (amino acids 467 - 485) of the ERβ (ERβ-C) and the second was raised against the N-terminus (amino acids 54 - 71) (ERβ-N). Expression of the ERβ protein in brain homogenates (total receptors) was investigated. Using the ERβ-C antibody, a 59 kDa band (as reported by the antibody supplier) was apparent in fetal rat brain only (Fig. 4.17). However, a higher molecular weight band was detected in maternal brain and this band was used as the internal standard for correction from filter to filter variation. An overall age effect was detected using this antibody (Fig. 4.17). In N dam progeny, ERβ protein levels decreased between 19 and 21 dg. However, in TX dam progeny levels increased between 16 and 19 dg then decreased between 19 and 21 dg. The protein levels do not resemble the mRNA levels although the N progeny show a similar trend. The second antibody that was used detects a 65 kDa band (as reported by the antibody supplier), however, it also detected the 59 kDa band in fetal brain but results obtained using this band were very variable (Fig. 4.18). No significant difference was detected between groups (Fig. 4.18). As for maternal brain only the 65 kDa band was detected, while no bands were detected in uterus cytosol probably because
Figure 4.16: Effect of maternal hypothyroxinemia on the expression of ERβ mRNA in fetal rat brain. Reactions were performed for 37 cycles at 57 °C, using standard reaction conditions.

A) Representative ethidium bromide stained gel. The migration of the 100 bp marker (M) is shown on the left-hand side and the calculated size of the signal indicated on the right-hand side of the gel (expected product size is 262 bp). N: normal dam; TX: hypothyroxinemic dam.

B) Relative ERβ mRNA level. The amount of product was expressed relative to G3PDH. Samples were run in duplicates; results shown are mean ± se (n = 3). Age effect: p < 0.01; age-treatment interaction: p < 0.05 (two-way ANOVA); 1p < 0.001 TX19 vs TX16; 2p < 0.001 TX21 vs TX16; * p < 0.01: TX vs N dam, at the appropriate age (post-hoc analysis).
Figure 4.17: Effect of maternal hypothyroxinemia on the expression of ERβ protein in fetal rat brain homogenates using ERβ-C antibody.

A) Representative Western blot. The calculated size of the signal is indicated on the left-hand side of the gel. N: normal dam; TX: hypothyroxinemic dam; MB: maternal brain homogenate; U: uterus cytosol; 100 µg protein was loaded for brain samples and 25 µg for uterus.

B) Ontogenic profile. ERβ protein was expressed relative to maternal brain. Results shown are mean ± se (n = 4); age effect: p < 0.01 (two-way ANOVA); a p < 0.05 TX19 vs TX16; b p < 0.05 N21 vs N19; c p < 0.01 TX21 vs TX19 (post-hoc analysis).
Figure 4.18: Effect of maternal hypothyroxinemia on the expression of ERβ protein in fetal rat brain homogenates using ERβ-N antibody.

A) Representative Western blot. The calculated size of the signal is indicated on the left-hand side of the gel. N: normal dam; TX: hypothyroxinemic dam; MB: maternal brain homogenate; U: uterus cytosol; 100 μg protein was loaded for brain samples and 25 μg for uterus.

B) Ontogenic profile of 65 kDa band. ERβ protein was expressed relative to maternal brain. Results shown are mean ± se (n = 3); no significant difference was detected.

C) Ontogenic profile of 59 kDa band. ERβ protein was expressed relative to maternal brain. Results shown are mean ± se (n = 3); no significant difference was detected.
of the lower amount of protein loaded or probably because this protein is not detected in pregnant uterus cytosol.

4.4.4 Effect of maternal hypothyroxinemia on PRAB and PRB expression in fetal rat brain

4.4.4.1 Expression of PRAB and PRB transcripts

PRAB and PRB mRNA were detected in fetal rat brain as early as 16 dg (Fig. 4.19 and 4.20). The authenticity of the PCR product was checked using specific oligonucleotide probes and a single product was detected (see Appendix D, fig. D1).

Relative to controls, mean PRAB mRNA levels appeared slightly increased at 21 dg in fetal brains from TX dams but expression was very variable so that no significant difference was detected (Fig. 4.19). An overall age effect was detected when analyzing PRB mRNA levels in fetal rat brain however, no differences were detected with post-hoc analysis (Fig. 4.20). The fetal brain expression of PRAB mRNA was found to be highly variable in the TX dam group at 21 dg as considerable changes and differences from litter to litter are seen at this age which complicates the analysis. PRB mRNA in both N and TX dam groups at 21 dg also show a high degree of variability. The higher PR mRNA levels seen at 21 dg fetal rat brain from TX dams could be in response to the high estradiol levels at this age in the brain (Fig. 4.3) as it is known that estradiol induces PR (Milgrom et al., 1973; Leavitt et al., 1974, 1977; Walters and Clark, 1977; Traish and Wotiz, 1990; Bayliss et al., 1991; Kraus and Katzenellenbogen, 1993; Natraj and Richards, 1993; Kato et al., 1994). Maternal hypothyroxinemia was without any significant effect on PR mRNA expression.

4.4.4.2 Expression of PRAB and PRB protein

Several attempts were carried out to detect the PR proteins using Western blotting. Brain homogenates (100 µg protein) and uterine cytosol (25 µg protein) were loaded and detection was attempted using three specific antibodies. The first antibody used was raised against the N-terminus (amino acids 533 - 547) of the PR (PR-N1), the second was raised against the C-terminus (amino acids 920 - 933) (PR-C) and the third was raised against the N-terminus (amino acids 523 - 536) (PR-N2). Unfortunately, detection of the PR was not successful, possibly due to the low receptor level. Moreover, many researchers when studying PR, inject rats with estradiol as it is known that estradiol induces PR synthesis (Milgrom et al., 1973; Leavitt et al., 1974; Traish and Wotiz, 1990; Bayliss et al., 1991; Kraus and Katzenellenbogen, 1993; Natraj and Richards, 1993; Kato et al., 1994).
Figure 4.19: Effect of maternal hypothyroxinemia on the expression of PRAB mRNA in fetal rat brain. Reactions were performed for 38 cycles at 69 °C, using standard reaction conditions.

A) Representative ethidium bromide stained gel. The migration of the 100 bp marker (M) is shown on the left-hand side and the calculated size of the signal indicated on the right-hand side of the gel (expected product size is 319 bp). N: normal dam; TX: hypothyroxinemic dam.

B) Relative PRAB mRNA level. The amount of product was expressed relative to G3PDH. Samples were run in duplicates; results shown are mean ± se (n = 3). No significant difference was detected.
Figure 4.20: Effect of maternal hypothyroxinemia on the expression of PRB mRNA in fetal rat brain. Reactions were performed for 35 cycles at 59 °C, using standard reaction conditions.

A) Representative ethidium bromide stained gel. The migration of the 100 bp marker (M) is shown on the left-hand side and the calculated size of the signal indicated on the right-hand side of the gel (expected product size is 319 bp). N: normal dam; TX: hypothyroxinemic dam.

B) Relative PRB mRNA level. The amount of product was expressed relative to G3PDH. Samples were run in duplicates; results shown are mean ± se (n = 3). Age effect: p < 0.05 (two-way ANOVA).
4.5 Sex steroid hormone levels in serum of postnatal progeny

In the absence of any specific data regarding the putative long-term effects of maternal hypothyroxinemia on postnatal progeny sex steroid hormone levels, the investigation was consequently expanded to study serum E and P levels in 1, 10, 15 and 30 dpn males and females.

4.5.1 Serum estradiol levels in postnatal progeny

An overall age effect was observed for E levels in postnatal rat serum (Fig. 4.21). In male progeny of N and TX dams estradiol levels were high at 1 dpn decreasing steeply (by 87% and 83%, respectively; p < 0.001) by 10 dpn. Levels then increased (by 73% in N and by 69% in TX dam progeny; p < 0.05) between 10 and 15 dpn followed by a further marked decrease (by 77% in N and by 78% in TX dam progeny; p < 0.001) by 30 dpn. Furthermore, levels at 30 dpn were lower than those at 10 dpn in both N (59%; p < 0.01) and TX (63%; p < 0.001) dam progeny.

In contrast to male progeny, E levels in female progeny of N and TX dams decreased (by 55% and 46%, respectively; p < 0.05) between 1 and 10 dpn, remained relatively stable between 10 and 15 dpn then decreased markedly (by 82% in N and by 54% in TX dam progeny; p < 0.001) between 15 dpn and 30 dpn. Moreover, levels at 30 dpn were significantly lower (by 76% in N and 52% in TX dam progeny; p < 0.001) than those at 10 dpn. Similar estradiol levels have been reported for 14 dpn control progeny where levels were 216.1 ± 18 pg/ml (Barbanel and Assenmacher, 1982).

TX dam male progeny collected at 10 and 30 dpn have lower (by 57% and 67%, respectively; p < 0.001) E levels than their female littermates. Maternal hypothyroxinemia resulted in significantly higher (by 121%; p < 0.01) serum E levels only in female progeny at 30 dpn relative to their controls, but not at any other age.

4.5.2 Serum progesterone levels in postnatal progeny

An overall age effect was observed for P levels in postnatal serum (Fig. 4.21). Progesterone levels in both male and female progeny of N and TX dams remained stable between 1 and 10 dpn, then increased between 10 and 15 dpn (by 354% in males and by 140% in females from N dams; by 354% in males and by 280% in females from TX dams; p < 0.001) and between 15 and 30 dpn (by 594% in males and by 509% in females from N dams; by 225% in males and 214% in females from TX dams; p < 0.001). No significant difference in P levels was detected between male and female progeny and maternal hypothyroxinemia was without any effect on serum P levels. Weisz and Ward
Figure 4.21: Effect of maternal hypothyroxinemia on serum estradiol and progesterone levels in postnatal progeny. Values are mean ± se (n ≥ 3).

A) Serum estradiol levels. Age effect: p < 0.001 (two-way ANOVA); a, b, c, d, e, f, g, h, i, j, p < 0.001: 10 dpn vs 1 dpn, c, d, p < 0.05: 10 dpn vs 1 dpn, e, f, p < 0.05: 15 dpn vs 10 dpn, g, h, i, j, p < 0.001: 30 dpn vs 15 dpn, a, b, c, d, e, f, g, h, i, j, p < 0.001: male vs female, at the appropriate age, * p < 0.01: TX vs N dam, at the appropriate age (post-hoc analysis).

B) Serum progesterone levels. Age effect: p < 0.001 (two-way ANOVA); a, b, c, d, e, f, g, h, i, j, p < 0.001: 15 dpn vs 10 dpn, e, f, g, i, j, p < 0.001: 30 dpn vs 15 dpn (post-hoc analysis).
(1980) also showed that progesterone levels in 1 to 5 dpn progeny were not statistically different between males and females and that levels during that period remained relatively stable.

4.6 Summary

The literature values of blood steroid levels in rats vary according to various authors and it is therefore difficult to make comparisons with our results. It has been reported that serum estradiol level remains unchanged from days 2 through 12 of gestation and then increase gradually until day 22 (Taya and Greenwald, 1981). Although E levels in our results increase near term, this increase was not statistically significant. Maternal hypothyroxinemia affects serum E levels at 14 dg when values were lower in TX dams. E in amniotic fluid were 3- to 8- fold higher than those in serum and, like serum, levels tended to increase towards term. In hypothyroxinemic pregnancies, this increase was exaggerated so that amniotic fluid E levels were increased relative to controls at 21 dg.

The higher E levels detected in amniotic fluid compared to maternal serum could be due to the presence of \( \alpha \)-fetoprotein (AFP) which binds estradiol. AFP is a fetospecific protein synthesized by the yolk sac and the fetal liver and secreted into the plasma during intrauterine and early postnatal life (Ruoslhati, 1979). It is a glycoprotein which appears in fetal and maternal blood and in amniotic fluid in a number of species. Rat amniotic fluid is a rich source of AFP (Stanislawski-Birencwajg, 1967; Sell et al., 1972; Kerckaert et al., 1975) which has a high affinity for estrone and 17\( \beta \)-estradiol but not for other steroids and nonsteroid estrogens (Uriel et al., 1972). The role of AFP in the developing rat fetus may be to keep estrogens from affecting estrogen sensitive targets, by preventing high levels of hormone from circulating in unbound form. This role, however, does not appear to be ubiquitous because human AFP does not bind estrogens (Swartz and Soloff, 1974).

A steady decrease in maternal serum P levels was detected between 16 and 21 dg in both N and TX dams. Others have shown a similar decrease in P levels in N dams between 14 and 21 dg (Pepe and Rotchild, 1974; Taya and Greenwald, 1981) and between 19 and 21 dg (Weisz and Ward, 1980). It has been reported that serum P levels reach an initial peak on day 6 and then remain stable until day 12 where levels then increase to reach a second peak on day 16 (Taya and Greenwald, 1981; Escalada et al., 1996) then abruptly fall after day 18 (Taya and Greenwald, 1981). Gibori et al. (1977) reported that the normal increase in serum progesterone at the beginning of the second half of pregnancy requires the actions of both placental luteotropin and 17\( \beta \)-estradiol. From midpregnancy, the corpus luteum appears to secrete progesterone at full capacity (Sridaran and Gibori, 1983). During days 14 - 18 of pregnancy, maximal values of serum P correlate with minimal values for serum LH. The high levels of P during midpregnancy may be
involved in the suppression of follicular maturation, probably by lowering basal levels of serum LH. In contrast to the results obtained with E, maternal hypothyroxinemia was without any effect on either serum or amniotic fluid P levels. In non-pregnant euthyroid and thiouracil-induced hypothyroid rats P and E levels have not been reported to be statistically different (Lee et al., 1986).

Amniotic fluid P levels were 25- to 30- fold lower than those in maternal serum. The highest concentrations of progesterone are found in uterine vein blood, while in amniotic fluid and fetal blood the values are twice as small as the peripheral blood values (de Lauzon et al., 1974). The low levels of progesterone in both fetal blood and amniotic fluid support the concept of an ovarian origin of this hormone in the pregnant rat.

In fetal tissue, the highest P and E levels were detected in normal fetal brain at 16 dg. E levels remain relatively stable in livers, however, in fetal brains from N dams, levels tend to decrease between 16 and 19 dg and in fetal carcasses levels tend to increase between 16 and 19 dg in the experimental group. Maternal hypothyroxinemia resulted in lower fetal brain E levels at 16 dg but increased levels at 21 dg. In fetal liver levels were higher at 21 dg in the experimental group and in fetal carcass levels were lower at 16 dg. Interestingly, E levels in 16 dg fetal brain from N dams were 2-fold higher than levels in livers and carcasses. The pattern of change in E levels in fetal brains and carcasses is similar. The increased levels at 16 dg in N dam progeny may indicate that more estradiol is being synthesized or less is being metabolized. In contrast, in the liver the reverse is seen where E levels appear to be increased in the experimental group again implying increased synthesis or reduced metabolism.

A similar pattern of decrease in progesterone levels was detected in fetal brains and carcasses from N and TX dams with progression of pregnancy. This was not the case in fetal liver although levels appeared lower at 21 dg. Nevertheless, maternal hypothyroxinemia was without any effect on P levels in any of the fetal tissues studied. It has been reported that P levels in 17 to 21 dg male and female plasma were not different from one another on any day of gestation, however, they were significantly lower than their mothers on every day of gestation (Weisz and Ward, 1980). Males and females had an identical pattern of steadily declining progesterone titers between 18 and 21 dg similar to the declining levels observed in our experiments in brains and carcasses.

Maternal hypothyroxinemia was without any significant effect on the expression of the housekeeping genes studied in fetal rat brain implying that transcription was not affected. As G3PDH mRNA levels appeared to be the most stable over the study period, it was decided that this gene will be used as an internal standard when studying the expression of estrogen and progesterone receptor mRNA levels. Estrogen and progesterone receptor
mRNA and protein levels were detected using RT-PCR and Western blotting, respectively. Unfortunately, PR levels were not detected using Western blotting probably due to the low protein content in fetal tissue.

Both isoforms of ER mRNA were detected in fetal rat brain as early as 16 dg. An overall age effect was apparent where ERα mRNA levels increase between 16 and 19 dg in both N and TX dam progeny. When using the antibody raised against the hinge region of ERα protein, a 67 kDa band was detected. Cellular protein levels increased between 16 and 19 dg in N dam progeny while cytosolic ERα levels increased in both N and TX dam progeny between 16 and 21 dg. In contrast, no significant change in receptor level was detected in salt-extracted nuclei. Maternal hypothyroxinemia resulted in lower ERα mRNA at 16 dg and although protein levels in the cytosolic and nuclear fractions appeared lower at 16 dg in TX dam progeny, levels failed to attain statistical significance. The decrease observed in ERα mRNA levels and not protein levels at 16 dg due to maternal hypothyroxinemia implies that there may be an increase in translation or a decrease in turnover or both.

Using an antibody raised against the steroid binding domain of the ERα, two bands were detected in brain samples, of 73 and 67 kDa. However, the higher molecular weight band was not detected in maternal uterus. In contrast to the results obtained with the anti-hinge antibody, the expression of the 67 kDa band in total brain homogenates increased between 16 and 19 dg in TX dam progeny. On the other hand, cytosolic receptor levels increased in N dam progeny between 16 an 19 dg and in TX dam progeny between 16 and 21 dg. Moreover, in extracted nuclear fraction receptor levels increased in both N and TX dam progeny between 16 and 19 dg. The effect of maternal hypothyroxinemia was apparent at two age points: at 21 dg in brain homogenates where levels were increased and at 19 dg in the cytosolic fraction where levels were decreased relative to controls. As for the 73 kDa band, the only significant difference was detected in the cytosolic and salt-extracted nuclear fractions. In the cytosol, levels were higher at 21 dg compared to 16 dg in controls while in the experimental group, levels increased between 19 and 21 dg. However, in the salt-extracted nuclear fraction, receptor levels were higher at 21 compared to 16 dg in N dam progeny, while in the experimental group an increase was seen between 16 and 19 dg decreasing near term. Maternal hypothyroxinemia affected the expression of this band at 16 dg in salt-extracted nuclear fraction where levels were lower in the experimental group. Interestingly, both bands detected in the homogenate and cytosol show a similar overall change.

Levels of ERβ mRNA showed an overall age effect and an age-treatment interaction. Maternal hypothyroxinemia resulted in lower ERβ mRNA levels at 16 dg, similar to the result seen with ERα mRNA. Using an antibody raised against the C-terminus, a 59 kDa
band was detected in fetal rat brain only. ERβ protein levels decreased between 19 and 21
dg in N and TX dam progenies. Moreover, levels increased between 16 and 19 dg in TX
dam progeny only and although a similar trend appeared in N dam progeny, this was not
statistically significant. In contrast, using an antibody raised against the N-terminus, two
bands were detected, of 65 and 59 kDa band which could be the product of another ERβ
isoform as it has been demonstrated that rat ERβ mRNA exists in several isoforms
(Petersen et al., 1998). Again the 59 kDa band was only apparent in the fetal rat brain
homogenates. No significant difference was detected between the groups using this
antibody. Maternal hypothyroxinemia was without any effect on the expression of the
ERβ protein levels.

Both isoforms of PR mRNA were detected in fetal rat brain as early as 16 dg. PRB
mRNA levels showed an overall age effect while PRAB mRNA levels showed no
significant change with age. Maternal hypothyroxinemia was without any effect on PR
mRNA expression. PR protein was not detected in fetal rat brain using Western blotting
methodology. The PR from rat tissue is extremely labile and can be easily modified or
destroyed during preparative procedures (Walters and Clark, 1977). Therefore, glycerol,
which interacts with the surface of the receptor molecule in such a way as to favour the
folded native state which preserves high affinity interactions (Ogle, 1983), was added to
the homogenization buffer, however, detection of PR was still not possible.

In many instances the action of progesterone and of its receptors require the prior
exposure of the tissues to estradiol. An important action of estradiol in this respect is its
stimulant effect on PR synthesis (Milgrom et al., 1973; Leavitt et al., 1974; Kraus and
Katzenellenbogen, 1993). Estradiol injection results in an increase in cytoplasmic PR in
the pituitary gland, preoptic area and mediobasal hypothalamus but not in the amygdala
(Clark et al., 1982) suggesting that not all brain ER containing cells are programmed to
synthesize PR (MacLusky and McEwen, 1980).

Estradiol levels in postnatal serum were highest at 1 dpn, decreased steeply by 10 dpn and
reached their lowest at 30 dpn. In immature rats, AFP sequesters circulating estrogen of
maternal origin and keeps levels high, although the free fraction of E is relatively low
during this period. By day 23, AFP declines and the free fraction of estradiol rises
sharply and the total serum concentration of E falls markedly (Puig-Duran et al., 1979),
thus rendering the steroid available to the tissues. The unbound fraction of estradiol rises
at about 18 days of age when the falling levels of high-affinity estradiol binding protein
appear to have reached a critical concentration (Puig-Duran et al., 1979). The effect of
maternal hypothyroxinemia was apparent at 30 dpn where female progeny from TX dams
had higher E levels than their controls. In contrast to E levels, P levels in postnatal

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progeny increased from birth to 30 dpn in both sexes. Maternal hypothyroxinemia was without any effect however on P levels in postnatal progeny.
Chapter 5:
Discussion
5.1 Maternal thyroid hormone state

Maternal hypothyroxinemia, resulting from iodine deficiency, was reported to be associated with increased incidence of neurological disorders in the offspring. There is evidence suggesting that the neurological damage observed in these circumstances is sustained early in fetal life; before onset of fetal thyroid function (Pharoah et al., 1971). Using the rat as an animal model, the term “cretin” has often been applied to those that are made hypothyroid at birth or soon thereafter. However, this may lead to considerable confusion as such rats are not the animal equal of the neurological cretins from iodine-deficient areas, but of sporadic congenital hypothyroid babies. In our experiments, the primary requirement was to set up a model analogous to the hypothyroxinemic state of pregnant women that exists in iodine deficiency endemias, where most of the women have very low circulating T4, high TSH but near normal T3 levels (Hetzel et al., 1983; Man et al., 1991).

In order to avoid any extraneous influences on the maternal and fetal metabolism and to exclude factors other than maternal TH, antithyroid drugs as well as iodine deficiency were avoided. Several studies have been concerned with the effect of the maternal thyroid state, most were concerned with hypothyroidism (Hendrich et al., 1984; Morreale de Escobar et al., 1985, 1987, 1992; Calvo et al., 1992; Porterfield and Hendrich, 1993) in which animals were totally thyroidectomized either surgically (Morreale de Escobar et al., 1985, 1987, 1992) or by treatment with 131I (Hendrich et al., 1984; Porterfield and Hendrich, 1992, 1993). These two methods of thyroidectomy were avoided because they depress the maternal levels of both T4 and T3 leading to a hypothyroid rather than hypothyroxinemic state. In addition, administration of antithyroid drugs such as methimazole (MMI) or PTU in drinking water or food of pregnant rat dams interferes with the functioning of the fetal thyroid and the use of 131I may damage maternal tissue and thus affect pregnancy. Total thyroidectomy also results in severe maternal compromise and reduced placental weight (growth). Moreover, removal of the thyroid gland surgically or by 131I irradiation, usually results in fewer pregnancies, smaller litters, stillbirths, prolonged gestation and maternal tetany (Parrott et al., 1960). These methods result in severely hypothyroid dams and thus do not represent maternal hypothyroxinemia which is seen in mothers of neurological cretins.

Therefore, different experimental models may partially account for the lack of uniformity in the literature regarding transplacental effects of TH, especially when it is considered that the disorder is by no means a uniform one itself, displaying a range of severity of outcome (Pharoah et al., 1971). In the present study, partially thyroidectomized (parathyroid-spared) Sprague-Dawley rats were used. The dams were hypothyroxinemic as approximately 30% of T4 and 45% of T3 were present in the circulation; this model
therefore approximates thyroid status seen in certain iodine-deficient (Connolly and Pharoah, 1989) and iodine-replete (Man et al., 1991) environments.

In humans, thyroid size increases during pregnancy, and serum total T4 and T3 levels can double. The primary basis for the increase in serum TH levels is an estrogen-induced increase in liver TBG production which leads to an increase in hormone binding (Porterfield, 1996). In rats, measurement of maternal plasma T4 and T3 showed that the concentration of these two hormones decreased as pregnancy progressed. The decrease in T4 levels in normal dams near term (at 21 dg), compared to those determined at 16 dg, has been shown by other groups (Morreale de Escobar et al., 1985; Escobar del Rey et al., 1986; Morreale de Escobar et al., 1988). This decrease in T4 level with progression of pregnancy could be due to deiodination of T4 by the placenta to provide iodine for the fetus as the fetal thyroid becomes active. Although maternal thyroidectomy caused significant reduction in T3 level, this reduction was, however, within the euthyroid level.

5.2 Fetal growth

The TX rat dams exhibited reproductive compromise. The results confirm the reported adverse effects of maternal hypothyroxinemia and maternal hypothyroidism on reproductive competence and the number of viable fetuses (Morreale de Escobar et al., 1985, 1988; Hadjzadeh et al., 1990; Hubank, 1990; Porterfield and Hendrich, 1991; Ruiz de Ona et al., 1991; Pickard et al., 1993; Porterfield and Hendrich, 1993). The precise role of TH in this process is not well known yet. The reduction in the litter size number has been explained due to increased resorptions which has been reported with surgically induced hypothyroidism where both the thyroid and parathyroid glands have been removed (Hendrich et al., 1976), however, in this study fetal resorptions were not detected. A reduction in the number of maturing ovarian follicles, and/or atrophy of ovaries, however, may be the cause (Parrot et al., 1960; Hagino, 1971). In addition, maternal thyroidectomy in rabbits was reported to result in an increase in the number of unruptured follicles, enlarged, polycystic ovaries, and a decrease in the number of ovulations (cited in Hendrich et al., 1976); this condition may operate in our model as well.

Present data confirm that maternal hypothyroidism reduces the body and organ weights of fetuses near term (Parrott et al., 1960; Hagino et al., 1971; Morreale de Escobar et al., 1985; Hubank et al., 1986; Morreale de Escobar et al., 1988). Other workers have reported similar effects of thyroidectomy in the rat on body and brain growth of progeny (Porterfield and Hendrich, 1982; Hendrich et al., 1984). In the present study, fetal body weights at 16, 19 and 21 dg appeared lower in the experimental group. Moreover, brains of TX dam progeny were lower in weight at almost every stage assessed, although not to
a statistically significant extent. These deficits resemble those found in thyroidectomized neonates, albeit to a lesser degree, even though the deprivation of hormone occurred during early pregnancy. In a similar study, Pickard et al. (1993) reported a significant reduction in fetal brain weight at 15 dg, after which normalization was attained. However, others working with more severely hypothyroid rat dams have reported a significant reduction in fetal brain weight in late gestation; at 20 and 21 dg (Morreale de Escobar et al., 1985). The brain:body weight ratio decreased as pregnancy progressed again without any significant difference between control and experimental groups, although the experimental group showed a trend of having a higher ratio consistent with some degree of brain sparing. It may be agreed that in mild hypothyroxinemia in utero, CNS growth can be maintained, but in severe hypothyroxinemia irreversible compromise in brain growth and development results. The neurological cretinism in severe iodine deficiency endemias where serum T4 levels are rarely more than 2 nM (but normal T3) lends some support to this assumption.

Reports regarding the effect of maternal TH state on fetal liver growth early in pregnancy are sparse. Available data indicate the presence of maternal TH in fetal liver as early as 16 dg and the amount measured was higher than that obtained in the brain (Porterfield and Hendrich, 1991; Ruiz de Ona et al., 1991; Sinha et al., 1992a). This was found to be significantly reduced with maternal thyroidectomy. In an earlier study, fetal liver TH concentration was found to be increased after the onset of fetal thyroid function, with maximum values at 20 dg for T4 and 22 dg for T3. Maternal thyroidectomy, on the other hand, reduces these levels between 17 dg and 22 dg except for days 19 and 21. These changes were either not observed or occurred to a lesser extent for liver T3; an indication of independent deiodination system (Morreale de Escobar et al., 1985; Ruiz de Ona et al., 1991; Morreale de Escobar et al., 1993). Therefore, the presence of TH in fetal liver before onset of fetal thyroid function may indicate that fetal liver growth may be influenced, at least to a certain degree, by maternal TH deficiency. Data obtained in this study indicated that fetal liver is highly susceptible to maternal hypothyroxinemia as fetal liver weights were significantly lower in the experimental group at 16 and 21 dg. However, the liver:body weight ratio was normal. Liver weights at 19 dg were not affected probably because as the fetus starts to produce its own TH some degree of compensation takes place, however, it could not be sustained and hence the reduced weights seen at 21 dg. It would be relevant to mention that the same observation was reported for fetal liver from hypothyroid rat dams at 20 and 21 dg (Morreale de Escobar et al., 1985). In addition, Morreale de Escobar et al. (1988) also showed that fetuses collected at 21 dg from hypothyroid dams had lower liver weights than the corresponding group of fetuses from N dams with a normal liver:body weight ratio. Reduced liver weights in the experimental group but normal liver:body weight ratios indicate a general somatic growth compromise as well.
The results obtained in this study and others, showed scattered changes in the cellular growth parameters throughout pregnancy suggesting that TH effect may vary depending on the stage of development and the kind of cellular growth. The DNA content, which has been defined as an index of cell number (Dobbing, 1981), was significantly reduced in fetal brain from TX dams at 16 dg but increased at 19 dg relative to controls. This latter effect is consistent with the onset of fetal thyroid function resulting in over-compensation of cell division. This may indicate that maternal TH is crucial for neuronal proliferation, but this effect diminishes when the fetus is able to synthesise its own TH. The reduction in brain weight at 16 dg, although not significant, was accompanied by deficits in the total protein content and DNA content, suggestive of abnormal neuroblast proliferation. However, the RNA concentration was not affected. Moreover, the increase in DNA concentration and the decrease in protein:DNA ratio near term is consistent with a delay in neuronal maturation. Depression in the protein:DNA ratio was also reported in fetal brain from hypothyroid rat at 20 and 22 dg and in sheep between 84 and 125 dg (Potter et al., 1981; Porterfield and Hendrich, 1982; Morreale de Escobar et al., 1985). These changes were found to persist in early postnatal period and in adult progenies from hypothyroxinemic rat (Al Mazidi, 1989; Hubank, 1990). This reduction in the cell size index may suggest a lag in the neurite maturation and in the cellular outgrowth, which may in turn cause retardation in the cellular connections and synaptogenesis. The possible retardation in neurite outgrowth may result in disruption of the myelination process. In fact such changes were reported for adult progenies from hypothyroxinemic rats (Hadjzadeh, 1990), where significant reduction in some of myelin proteins and myelin lipids of certain brain regions were reported.

The early reductions in brain cell number were found to be compensated for postnatally (Porterfield and Hendrich, 1982; Hubank, 1990) or at birth (Potter et al., 1981; Hetzel and Potter, 1983). Fetal brain cell number in rats represents predominantly neuronal cells (except the cerebellum), and cellular proliferation after birth is thought to be primarily glial (Zamenof and Marthens, 1971). Porterfield has pointed out that this normalization in the cell number may be deceptive and may in fact be related to the increase in glial cells rather than a normalization of the neuronal cells. However, a delayed action of maternal hypothyroxinemia was reported for 30 days old and adult rat progenies, in which significant reduction in the cell number was observed (Ekins et al., 1989; Al Mazidi 1989; Hubank, 1990). This late depletion in the cell number may possibly be related to an increase in the rate of cellular death, whereas the observed reduction in the cell number early in gestation (at 16 dg) does not rule out the possible early loss of particular group of cells, which in turn may lead to further disruption in neural development at later stages (Jacobson, 1974).
In normal rats, the maternal contribution of T4 to the fetus is important even after the onset of fetal thyroid function, as 17% of total TH in the fetus is of maternal origin (Morreale de Escobar et al., 1990). Maternal hypothyroidism was reported to considerably reduce maternal contribution of TH to only 10% of normal values (Morreale de Escobar et al., 1985). Besides its possible effect on fetal development, this reduction in maternal TH level may affect placental development as well. It is therefore necessary, at this stage, to answer another critical question regarding the validity of the model, namely is lack of maternal TH the causative factor of neural and somatic compromise in the progeny, or, as has been suggested (Herrera et al., 1980), placental insufficiency caused by maternal hypothyroxinemia, is to blame? Certainly it appears that the dams are not functioning normally, despite near normal levels of T3. For this reason, a study of placental growth at various stages of pregnancy was undertaken.

The effect of TH on placental weight was studied by Spencer and Robinson (1993) who reported that a daily injection of T4 (10μg) into pregnant rat significantly increased (by 20%) the placental weight at 20 dg. In the present study, placental growth was unaffected by maternal thyroid state. Moreover, Hadjzadeh et al. (1990), working on the same animal model, showed that placental compromise in this model of maternal hypothyroxinemia did not occur as no obvious differences were detected in a range of biochemical parameters including DNA, RNA and protein concentrations. Moreover, any differences detected in various measurements cannot be attributed to iodine deficiency or postpartum nutritional compromise as both dams and progeny were fed iodine-replete diets and the pups were often cross fostered (Sinha et al., 1991). In a more severe TH condition, maternal hypothyroidism, occasional reductions in the placental weight was reported between 13 and 21 dg (Morreale de Escobar et al., 1985).

Others have shown that protein concentration remains fairly constant in normal placenta throughout pregnancy, and maternal thyroidectomy was with no effect (Morreale de Escobar et al., 1985; Al-Yatama, 1995). This, however, contradicts the reported decline in protein concentration at 19 dg (Pickard et al., 1993), albeit being an isolated effect. Moreover, in normal placentae, the DNA content and the protein:DNA ratio showed maximum values at 19 dg, indicating that the cellular proliferation / differentiation is complete at this stage of pregnancy (Al-Yatama, 1995). A similar increase in the cell size index in normal rat placentae between 13 and 20 dg was reported by Morreale de Escobar et al. (1985). Maternal hypothyroxinemia has no effect on either DNA content or DNA concentration, indicating normal cell number and density, however, the protein:DNA ratio, was compromised at 19 dg, suggesting delayed cellular differentiation although normalization was achieved by 22 dg (Al-Yatama, 1995). Early work of Pickard et al. (1993) tended to eliminate any effect of maternal hypothyroxinemia on any of these parameters. Therefore, maternal hypothyroxinemia does not affect cellular parameters,
suggesting that the placenta in the hypothyroxinemic condition is likely to function normally.

5.3 Effect of maternal hypothyroxinemia on sex steroid hormones and receptor expression in fetuses

Expression of ER and PR in fetal brain may be dependant upon the availability of TH to the fetus at different stages of fetal brain development. Firstly, the effect of maternal hypothyroxinemia on maternal sex steroid levels was studied. Maternal hypothyroxinemia resulted in lower maternal serum E levels at 14 dg compared with the normal dams. This difference was reversed by 16 dg, however, statistical significance was not attained. It is known that in the second half of pregnancy (days 13-18) the conceptuses becomes an important source of testosterone (Sridaran et al., 1981) which serves as a substrate for estradiol. Moreover, the placenta at this stage also begins to secrete androgens (Chan and Leathem, 1975, 1977; Sridaran et al., 1981; Matt and Macdonald, 1984), however, as the placenta lacks aromatizing enzymes (Sybulski, 1970), the androgens are aromatized to estradiol in the corpus luteum (Jackson and Albrecht, 1985). Therefore, the lower maternal serum E levels in TX dams at 14 dg and the higher ones at 16 dg could indicate a delayed secretion of testosterone by fetuses in TX dams at 14 dg. Lee et al. (1986) have shown that in non-pregnant euthyroid and thiouracil-induced hypothyroid rats E levels are not statistically different. However, the difference detected in E levels in our model could be due to the fact that hormone levels during pregnancy are greatly elevated and thus any slight change could more easily be detected.

E in amniotic fluid were 3- to 8-fold higher than those in serum probably due to the presence of AFP which appears in sera of pregnant rats between 11 and 13 dg (Sell et al., 1972) and which binds 17β-estradiol (Uriel et al., 1972) and protects estrogen sensitive targets in the developing rat fetus, by preventing high levels of hormone from circulating in unbound form. Like serum, amniotic fluid E levels tended to increase towards term and in hypothyroxinemic pregnancies, this increase was exaggerated so that levels were increased relative to controls at 21 dg. This increase may be due to fetal contribution as E levels are higher in fetal brain, liver and carcasses at this age. In addition to the above factors, it is also known that fetal blood contains an average of 350 pg/ml of estradiol (de Lauzon et al., 1974) which could be another contributing factor to the high levels of this hormone in amniotic fluid.

In fetal tissue, the highest E levels were detected in normal fetal brain at 16 dg. E levels remained relatively stable in livers and carcasses, whereas, in fetal brains from N dams levels tended to decrease between 16 and 19 dg. Maternal hypothyroxinemia resulted in lower fetal brain E levels at 16 dg but increased levels at 21 dg relative to controls,
probably indicating that over compensation has occurred after the onset of fetal thyroid function. In fetal liver, levels were higher at 21 dg in the experimental group and in fetal carcasses, levels were lower at 16 dg. Interestingly, E levels in 16 dg fetal brain from N dams were 2-fold higher than levels in livers and carcasses probably indicating the requirement of this tissue for E at this critical stage of development or due to the presence of aromatase activity in this tissue allowing the aromatization of testosterone to estradiol. This was not the case in brains from the experimental group indicating that the brain is deprived from the otherwise normal estradiol level at this age.

After studying the E hormone levels in fetal tissue, the expression of ER mRNA in fetal brain was studied. However, before doing so, a suitable internal standard, preferably an mRNA, had to be investigated. Therefore, expression of several housekeeping genes, from different cell compartments, in fetal rat brain was examined to assess the effect of maternal hypothyroxinemia on transcription in general as it is known that TH act as regulators of both transcription and translation. Maternal hypothyroxinemia had no apparent effect on the expression of the housekeeping genes studied (ribosomal protein L19, β-glucuronidase, cyclophilin, histone H4 and glyceraldehyde-3-phosphate-dehydrogenase) in fetal rat brain implying that transcription was not affected. G3PDH mRNA levels appeared to be the most stable over the study period allowing their use as an internal standard. It would appear therefore that intrauterine TH deficiency does not influence directly the transcription of structural genes. Thyroid hormones, however, have been shown to enhance protein synthesis in a variety of tissues and cells both in vivo and in cell cultures (Hong-Brown and Deschepper, 1992). Since TH does not appear to affect transcription for so many structural genes, the enhanced protein synthetic effects of TH may be due to translational/postranslational effects.

Expression of sex steroid receptors has been poorly studied in fetal rat brain probably due to the low number of transcripts at this age. In this study both isoforms of ER mRNA were detected in fetal rat brain as early as 16 dg with ERα mRNA levels increasing between 16 and 19 dg in both N and TX dam progeny. A 67 kDa band was detected using the antibody raised against the hinge region of ERα protein. Total ERα protein levels increased between 16 and 19 dg in N dam progeny while cytosolic ERα levels increased in both N and TX dam progeny between 16 and 21 dg. In contrast no significant change in receptor level was detected in salt-extracted nuclei. Maternal hypothyroxinemia resulted in lower ERα mRNA at 16 dg and although protein levels in the cytosolic and nuclear fractions appeared lower at 16 dg in TX dam progeny, this difference failed to attain statistical significance. The decrease observed in ERα mRNA levels and not protein levels at 16 dg due to maternal hypothyroxinemia implies that transcription of this gene is compromised but translation is less severely affected.
Thyroid hormone is closely associated with maturation and sexual differentiation of the brain. It has a direct effect on the expression of the estrogen receptor gene in many tissues. Using RT-PCR analysis, it has been shown that cortical ERα mRNA show no significant change with propylthiouracil (PTU)-induced hypothyroidism in 8 day old female rats (Hirata et al., 1994) in agreement with the absence of changes in the ER protein (Kato et al., 1984). On the other hand, Barbanel and Assenmacher (1982), using binding assay experiments, reported that neonatal hypothyroidism (PTU-induced) markedly suppressed estrogen receptor development, especially in the pituitary gland, and this was independent of serum estrogen levels. In adult rats, however, TH influence the levels of cytoplasmic estrogen receptor in the pituitary without any effect on hypothalamic estrogen receptor content (Cidlowski and Muldoon, 1975).

In our series of experiments, somewhat different results were obtained using an antibody raised against the steroid binding domain of the ERα. This antibody detected two bands in brain samples of 73 and 67 kDa with only the latter species present in maternal uterus. The expression of the 67 kDa band in total brain homogenates increased between 16 and 19 dg in TX dam progeny but remained relatively stable in controls. In the cytosolic and salt-extracted receptors from the nuclear fraction this increase was detected in both N and TX dam progeny. However, cytosolic receptor levels continued to increase between 19 and 21 dg in TX dam progeny only. The effect of maternal hypothyroxinemia was apparent at two age points: at 21 dg in brain homogenates where levels were increased and at 19 dg in the cytosolic fraction where levels were decreased relative to the controls. Both these effects occur after the onset of fetal thyroid function probably indicating that some sort of compensation is taking place at least in the total fraction. In addition, the increased receptor level at 21 dg in TX dam progeny may be in response to the increased E level seen in the brain at this age as it has been shown that in the rat liver and pituitary, estrogen treatment causes a significant increase in ER mRNA (Shupnik et al., 1989). Moreover, estradiol injection 2 hours before sacrifice has been shown to increase the nuclear receptor sites of the 2 - 3, 10 - 11 and 25 - 26 day old female cerebral cortex (MacLusky et al., 1979).

Somewhat different results were obtained for the 73 kDa band. Cytosolic receptor levels were higher at 21 dg compared to 16 dg in controls while in the experimental group, levels increased between 19 and 21 dg. In the salt-extracted nuclear fraction, receptor levels were higher at 21 compared to 16 dg in N dam progeny, while in the experimental group an increase was seen between 16 and 19 dg decreasing near term. Maternal hypothyroxinemia affected the expression of this band at 16 dg where levels were lower in the experimental group. Again this effect of maternal hypothyroxinemia is similar to the effect seen on E levels. It is evident therefore that maternal thyroid state does indeed
modulate the physiological programming of sex steroids and their receptors in a selective manner in the brain.

The presence of a second band detected in fetal and maternal brains using the antibody raised against the steroid binding domain is of interest. It has been reported that in human endometrial and breast cancer tissue, multiple isoforms of ER are detected. ER isoforms of relative masses of 50, 65 and 70 kDa have been detected using H222 antibody for both breast (Puddefoot et al., 1993) and endometrial (Marsigliante et al., 1995) cancer tissues. H222 is a primary antibody that binds the C-terminal portion of the steroid binding domain (Greene et al., 1984; Golding and Korach, 1988; Blaustein et al., 1993; Zhou et al., 1995) similar to the antibody used in this study. Moreover, an isoform of the estrogen receptor mRNA that lacks exon 4 (Δ4 ER mRNA) was abundant in rat brain with only trace amounts in uterus (Skipper et al., 1993). Apparently, tissue-specific alternative splicing accounts for these differences in abundance. Exon 4 encodes a part of the steroid-binding domain and therefore the use of H222 antibody, which recognizes the C-terminal portion of the steroid-binding domain, would detect both forms. The possible functions for the putative Δ4 ER protein is that it may act as a ligand independent transcription factor to effect a degree of constitutive (estrogen-independent) regulation of estrogen-responsive genes, whereas the conventional ER may further modulate the expression of the same set of target genes in the cells.

It has also been reported by Golding and Korach (1988) that in the mouse uterus, ER derived from the nuclei appears as a closely spaced doublet having apparent molecular masses of 66.4 and 65 kDa, while ER from the cytosolic compartment has a single band of 65 kDa consistent with our finding for maternal uterus cytosol. The appearance of the nuclear ER doublet was shown to be hormonally inducible (Golding and Korach, 1988), and the relative proportion of the two doublet bands are influenced by the type of hormone treatment, with weakly estrogenic compounds yielding the lower band as predominant while potent estrogens increase the proportion of the upper band. As levels of E are high during pregnancy, then the presence of this band could be due to high hormone levels. However, the absence of this doublet from uterus cytosol cannot be explained unless it could be due to the lower protein loaded or due to the absence of this band from uterine cytosol and its localization in the nuclear fraction (Golding and Korach, 1988). A phosphorylative mechanism has been suggested for the production of the ER doublet as the existence of the upper band which has a higher molecular weight would be consistent with being a phosphorylated species. It has been shown that phosphorylation of mouse ER, upon estrogen binding, has been shown to occur at multiple serine residues (Washburn et al., 1991; Lahooti et al., 1994). However, because of the magnitude of difference between the two bands, it is unlikely that the 73 kDa band is a phosphorylated product.
In addition to the above explanation on the identity of the second band, the antibody may be cross-reacting with a protein that has a similar epitope to the ERα. It is known that AFP displays a molecular weight of 70 kDa (Sell et al., 1972; Kerckaert et al., 1975; Mujoo et al., 1983) and this protein may be binding to ERα antibody. Indeed there is only a 3 kDa difference, this prediction cannot be ruled out using the methods employed in this study and further experiments would be required to test this. Immunoprecipitating the samples with anti-AFP antibody and then reacting with the anti-ERα antibody will show whether this band is indeed the AFP. However, although cross-reaction of the antibody with a protein that has a similar epitope to the ERα is a possibility, it is more likely that there is a new hitherto unreported ERα isoform in rat brain that may have been the result of alternative splicing or the product of phosphorylation.

When studying the expression of the second isoform of ER, it was found that maternal hypothyroxinemia resulted in lower ERβ mRNA levels at 16 dg similar to the results seen with ERα mRNA. Moreover, a 59 kDa band was detected, in fetal rat brain only, using an antibody raised against the C-terminus. A delayed increase in ERβ protein levels was detected between 16 and 19 dg in the experimental group compared to controls. Protein levels decreased between 19 and 21 dg in both N and TX dam progenies. The presence of the 59 kDa band in fetal brain probably implies that it plays a role during fetal brain development and disappears sometime postnatally. This is further supported by the low levels detected at 21 dg. In contrast, using an antibody raised against the N-terminus, two bands were detected, one of 65 and one of 59 kDa. Again the 59 kDa band was only apparent in fetal rat brain homogenates. No significant difference was detected between the groups using this antibody although results of the 65 kDa band do seem to increase with gestation. The high degree of variability and the inability to detect any differences in the expression of the 59 kDa between the groups using the second antibody could be due to the fact that this antibody is specific to detect a spliced form of the ERβ protein (the 65 kDa band) and that it is cross reacting with at least a part of the 59 kDa sequence of a homologous epitope. It has recently been demonstrated that rat ERβ mRNA exists in several isoforms. The most abundant of these mRNA variants has been called ERβ2 which has an in-frame insertion of 54 nucleotides that results in the predicted insertion of 18 amino acids within the ligand binding domain (Petersen et al., 1998). ERβ2 mRNA is expressed at levels equal to those of the previously published ERβ (ERβ1) in ovary, prostate, pituitary and muscle; and in tissues of the nervous system, including frontal cortex, hippocampus, and hypothalamus. ERβ1 was present in a 2- to 6-fold greater abundance than ERβ2 (Petersen et al., 1998). The existence of these isoforms for ERβ may explain the two bands detected in fetal brain homogenates. Maternal hypothyroxinemia was without any effect on the expression of the ERβ protein levels.
The regulation of the ER concentration in different tissues differs substantially. The ER in the uterus seems to be constitutively expressed, the main mode of regulation being downregulation by estrogens (Clark and Peck, 1979; Eriksson, 1982), whereas hepatic ER is mainly dependent on the hormones of the thyroid and pituitary glands (Norstedt et al., 1981; Freyschuss and Eriksson, 1988). Thyroid hormones are important regulators of the ER concentration in the rat liver and in a recent publication by Ignatenko et al. (1992), it was claimed that hepatic ER levels were not affected by thyroidectomy. However, others have shown that thyroidectomy decreases ER (Eriksson and Freyschuss, 1988; Freyschuss et al., 1991) and ER mRNA levels (Freyschuss et al., 1994) in the liver substantially, but not in the uterus (Eriksson and Freyschuss, 1988). Our results suggest that at least one component of the ER system namely ERα in the fetal brain may be regulated by TH environment in utero.

Thyroid hormones are necessary for normal GH synthesis and release (Montes et al., 1977; Shapiro and Sachchidananda, 1982) and probably affect liver ER partly through the GH axis. The action of thyroid hormones on GH synthesis and release seems to be dose-dependent, and hyperthyroidism decrease pituitary GH levels and secretion in rats (Daughaday et al., 1968; Miki et al., 1992). However, the effects of thyroid hormones are partly direct since treatment with T3 or T4 (20 μg/day) have been shown to double liver ER (Eriksson and Freyschuss, 1988) and ER mRNA (Freyschuss et al., 1991) levels in hypophysectomized female rats and partly via regulation of serum growth hormone (Freyschuss et al., 1994). A similar mechanism may possibly be in operation in the brain in our animal model. The change observed in ERα as a function of intrauterine TH deficiency may be an indirect effect mediated through fetal pituitary GH.

As far as P is concerned, a steady decrease in maternal serum P levels was detected between 16 and 21 dg in both N and TX dams. Others have shown a similar decrease in P levels in N dams between 14 and 21 dg (Pepe and Rotchild, 1974; Taya and Greenwald, 1981) and between 19 and 21 dg (Weisz and Ward, 1980). In contrast to the results obtained with E, maternal hypothyroxinemia was without any effect on either serum or amniotic fluid P levels. This is partly in agreement with results obtained by Lee et al. (1986) where they have shown that in non-pregnant euthyroid and thiouracil-induced hypothyroid rats P levels have not been reported to be statistically different. Amniotic fluid P levels were 25- to 30-fold lower than those in maternal serum as the highest concentrations of progesterone are normally found in uterine vein blood, while in amniotic fluid and fetal blood the values are twice as small as the peripheral blood values (de Lauzon et al., 1974). The low levels of progesterone in both fetal blood and amniotic fluid support the concept of an ovarian origin of this hormone in the pregnant rat. In any case, whatever the origin, P values in TX and N fetuses (amniotic fluid) do not change.
Therefore, TH levels in utero does not control P levels in the fetus whereas E levels are modulated by TH environment indicating a parameter selective effect.

In fetal tissue P levels in brains and carcasses decreased as pregnancy progresses in both normal and experimental groups. The highest P levels were detected in normal fetal brain at 16 dg. This was not the case in fetal liver although levels appeared lower at 21 dg. Nevertheless, maternal hypothyroxinemia was without any effect on levels in any of the fetal tissues studied. It has been reported that P levels in 17 to 21 dg male and female plasma were not different from one another on any day of gestation, however, they were significantly lower than their mothers on every day of gestation (Weisz and Ward, 1980). In this latter study, males and females had an identical pattern of steadily declining progesterone titers between 18 and 21 dg similar to the declining levels observed in fetal brain and carcass in the present study.

Both isoforms of PR mRNA were detected in fetal rat brain as early as 16 dg. PRB mRNA levels showed an overall age effect while PRAB mRNA levels showed no significant change with age. Maternal hypothyroxinemia was without any effect on PR mRNA expression. Unfortunately, PR protein was not detected in fetal rat brain using Western blotting methodology. Thyroid hormone may be one of the factors affecting gene expression of PR, particularly form B in the developing cerebral cortex of the female rat. It has been shown that neonatal hypothyroidism (PTU-induced hypothyroidism) causes a drastic decrease in the levels of progesterone receptor protein (Kato et al., 1984) and mRNA (Kato et al., 1993; Hirata et al., 1994) in the cerebral cortex, with no changes in those in the hypothalamus-preoptic area of the neonatal rat brain, suggesting a regulatory role of TH in the developing rat brain cortex. The thyroid hormone may act on either the PR gene directly via the HRE or other gene(s) resulting in the expression of inhibitory factor(s) (Kato et al., 1993).

In many instances the action of progesterone and of its receptors require the prior exposure of the tissues to estradiol. An important action of estradiol in this respect is its stimulatory effect on PR synthesis (Milgrom et al., 1973; Leavitt et al., 1974; Kraus and Katzenellenbogen, 1993). It has been shown that PRA is localized in the hypothalamus and preoptic area, and is regulated by estrogen, while PRB, which is present in the midbrain and cerebral cortex, is relatively insensitive to elevated plasma estrogen levels (MacLusky and McEwen, 1978). In the present study, E levels in brain may not have had a role in the upregulation of PR protein levels as whole brains were studied and the bulk of the brain is the cerebral cortex which has PRB. It has been reported that estrogen does not induce the cortical PR and this may be due to the predominance of PRB mRNA (Kato et al., 1994) in this region which, as mentioned earlier, has little or no estrogen-inducibility. It has been shown that estradiol injection results in an increase in cytoplasmic
PR in the pituitary gland, preoptic area and mediobasal hypothalamus but not in the amygdala (Clark et al., 1982) suggesting that not all ER-containing cells in brain are programmed to synthesize PR (MacLusky and McEwen, 1980). It is assumed therefore that no change in PR in TX fetal brains (although ER and E levels change) may be due to noninducibility of PR by E in the brain at these stages of development.

5.4 Maternal hypothyroxinemia and postnatal progeny

In this series of experiments, smaller numbers of rats were collected but male and female numbers were similar. The percent survival of TX dam progenies at birth was 46%. Similar results for number of live 1 day old progenies from hypothyroid dams have been reported (Hendrich et al., 1997). A significant decrease in live TX dam progenies compared to controls was observed with both sexes being similarly affected. Stillbirths, exceeding 50% of the litter, have also been reported to occur in the litters of animals whose thyroids had been removed surgically or with I$^{131}$ (Parrott et al., 1960). Our results confirm numerous other observations that intrauterine TH levels are critical for postnatal survival rate.

Previous studies conducted with fetuses and young progeny from thyroidectomized rat dams have demonstrated reductions in brain and body weights (Morreale de Escobar et al., 1985; Hubank et al., 1986) whereas no such effects were apparent in the adult progeny (Hadjzadeh et al., 1990). The deleterious effects of an altered thyroid hormone environment in utero on gross brain and somatic growth may therefore have been compensated for by the normal thyroid state of the progeny in later life. In this study, it was found that there was a transient retardation of growth where body weights at 1 dpn in TX dam progeny of both sexes have lower body weights, however, this was corrected at 15 dpn.

Similar to the results obtained with body weights, the brain weights in TX dam progeny were lower at 1 dpn in both males and females. In addition, at 30 dpn males from TX dams had lower brain weights than the control group. Maternal hypothyroxinemia was without any effect on brain weight at other ages is in agreement with results obtained using a similar model (Pickard et al., 1993). Similar brain weights have been reported for 1 and 30 dpn N and TX dam progeny (Hendrich et al., 1997); however, brain weights reported by this group for 30 dpn were slightly higher. When the brain:body weight ratio was analyzed, it was apparent that maternal hypothyroxinemia affected this ratio at 1 and 15 dpn with the experimental group having a higher ratio than the controls indicating that brain growth sparing is indeed present to counter the possible effect of maternal hypothyroxinemia.
The reduction in fetal liver weights in the experimental group persisted even after birth. Liver weights in postnatal progeny were lower at 1 dpn in TX dam progeny compared to their respective controls. However, when the liver:body weight ratio was analyzed no significant effect of maternal hypothyroxinemia was apparent as both the liver and body weights decreased at that age point. Similar liver weights have been reported for 1 dpn N and TX dam progeny (Hendrich et al., 1997). Our study confirms that maternal thyroid may have a critical role in somatic growth (long term) of the progeny.

In rodents, estradiol of both maternal and fetal origin is bound by circulating AFP and, hence, is not readily available to enter target neurons. AFP sequesters circulating estrogen and keeps levels high, therefore, in this study E levels in postnatal serum were highest at 1 dpn, decreasing steeply by 10 dpn and reaching their lowest at 30 dpn. It has been reported that by day 23, AFP declines and the free fraction of E rises sharply and the total serum concentration of E falls markedly (Puig-Duran et al., 1979), thus rendering the steroid available to the tissues. Puberty in the female rat occurs at around 35 days of age and is characterized by the onset of cyclic release of sex steroid and pituitary hormones and of ovulation and sexual behaviour. Puberty in the rat is the result of the appearance in the circulation of physiologically active estradiol after day 20 (Greenstein, 1992). The appearance of unbound estradiol in the plasma of developing female rats after 21 dpn may likewise be responsible for the fall in serum FSH concentrations which occurs at about this time (MacKinnon et al., 1976). Furthermore, by artificially suppressing the free fraction of estradiol, the concentrations of circulating FSH could be kept elevated in the presence of relatively high concentrations of estradiol (Puig-Duran et al., 1979). In contrast to E levels, P levels in postnatal progeny increased from birth to 30 dpn in both sexes.

In immature animals, euthyroidism is a prerequisite for normal growth and development, because deficient thyroid hormone secretion during the perinatal period inevitably causes severe and irreversible damage, especially to the CNS. In this study it has been shown that the progeny of hypothyroxinemic dams were euthyroid and perhaps eumetabolic as well; however, as far as steroid hormones are concerned, maternal hypothyroxinemia resulted in higher serum E levels in 30 dpn female progeny from TX dams relative to their controls. As postnatal progeny were euthyroid then the differences in postnatal E levels are probably due to the in utero effect. In contrast, maternal hypothyroxinemia was without any effect on P levels in postnatal progeny. These results confirm part of our hypothesis that intrauterine TH levels are critical for the correct physiological programming of the sex steroids and their receptors both short and long term.
In severe thyroid hormone deficiency in utero, feto-maternal estrogen/progesterone protect the fetal brain from neurological cretinism to a certain extent. Whether a fetus will develop neurological cretinism at birth will depend upon the degree of intrauterine hypothyroxinemia and the sex steroid concentration and sex steroid receptors in utero and beyond. Therefore, in order to gain a better understanding about the role of maternal hypothyroxinemia on sex steroid hormone levels and receptor expression, an animal model of partially thyroidectomized rat was used. Since sex steroid hormones exert their effects through their receptors and the receptors are induced by the hormones themselves, in hypothyroid conditions the sex steroid levels and their receptors will be modulated and deviate from the normal developmental chronology in the brain. In this thesis we have studied that aspect of steroid-thyroid interrelationship.

The aim was to obtain a hypothyroxinemic maternal state similar to that found in iodine deficient regions. A range of cellular growth parameters were investigated mainly in fetal brain. The results obtained for the placenta showed minimal changes to placental growth, as reflected by normal fetal body weight. Maternal hypothyroxinemia has resulted in abnormal cellular proliferation and maturation in fetal brain. Several housekeeping genes were studied in fetal brain at different stages of development and it was found that maternal hypothyroxinemia did not significantly affect the expression of these genes indicating that transcription in general was not affected.

Maternal hypothyroxinemia affected sex steroid hormone levels, mainly E, in maternal serum, amniotic fluid and fetal tissue. This is the first study that shows the levels of sex steroid hormone levels in fetal rat brain, liver and carcasses. Moreover, ER and PR protein and mRNA levels in fetal rat brain have not been studied before at this early age. In addition, it is the first time that the different isoforms of ER mRNA and protein are shown in fetal brain after the recent discovery of the ERβ isoform. Interestingly, ER and E levels in the brain are reduced in the experimental group before the onset of fetal thyroid hormone. Detection of two bands for the two ER isoforms using antibodies raised against different epitopes is rather interesting, however, the identity of these two bands cannot be explained at this stage. In contrast, no effect was observed for PR in fetal brain or P levels in fetal tissue.

This thesis has shown that indeed thyroid hormone deficiency in utero has deleterious effects on estradiol hormone levels in maternal serum and fetal tissue especially before the onset of fetal thyroid function. Estrogen receptors in fetal brain are also affected by maternal thyroid state again before the onset of fetal thyroid function. The effect of maternal hypothyroxinemia on E levels and ER expression in fetal brain before the onset
of fetal thyroid function proves the hypothesis that sex steroids may indeed play a role in protecting the brain from neurological cretinism. Although E and ER expression are normalized in the experimental group after the onset of fetal thyroid function, the long term effects on brain cannot be ruled out. This is clearly shown in postnatal progeny where the early effect of maternal hypothyroxinemia is carried on after birth where estradiol levels seem to be affected in female progeny. As the rats are euthyroid then any effect observed must be due to the in utero effect.

5.6 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 sex steroid receptor expression, particularly ER expression. This study focused on sex steroid expression in whole brain and thus it could be expanded to include in situ and immunocytochemistry work as well. This can further be extended to postnatal progeny to see the long term changes in ER expression. In situ hybridization and immunocytochemistry experiments will help to delineate discreet brain areas and specific groups of neurons where ER expression is controlled by TH both in utero and at postnatal stages. In addition, the mechanisms behind the changes observed in ER expression can also be studied, that is, whether thyroid hormones regulate ER expression directly or via other pathways.

In order to obtain precise information of the effects of different doses (subcritical to supraphysiological) on particular cell types from particular brain regions (discreet brain nuclei) cell culture models along with the use of differential display technique can profitably be utilized. Part of this work is in progress in our laboratory using this paradigm.

It would also be interesting to know whether the different ER isoforms are expressed in other fetal and postnatal tissue. Moreover, the identity of the second band detected for the ER isoforms can be studied further to reveal whether it is the result of alternative splicing or is a phosphorylated product.

Further work would also be desirable on the areas where limited numbers of experiments prevented concrete conclusions being drawn. Detailed work involving many more housekeeping genes may throw light upon the in utero and long term expression/suppression of various structural components.
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Figure A1: Schematic diagram of pBluescript SK- vectors.

Detail of hER cDNA insert within the multiple cloning site
Figure A2: Schematic diagram of pGEM 4Z plasmid.

Detail of rPR1 cDNA insert within the multiple cloning site

- Ampicillin resistance gene
- pGEM 4Z vector (2746 bp)
- f1(+) origin
- SP6 promoter
- T7 promoter
- ~ 550 bp rPR1 insert

- EcoRI
- Sac I
- Kpn I
- Ava I
- Sma I
- BamHI
- Sal I
- Acc I
- Hinc II
- Pst I
- Sph I
- Hind III
Figure A3: Agarose gel electrophoresis showing hER and rPR1 undigested, digested and linearized plasmids. M: DNA ladder; P: undigested plasmid; D: digested plasmid; L: linearized plasmid.

A) hER undigested and digested plasmid. The undigested plasmid shows multiple bands. After digestion a 265 - 270 bp insert and 2964 bp vector band was apparent.

B) rPR1 undigested, digested and linearized plasmid. A 550 bp insert and 2746 bp vector band was apparent after digestion, only a single band was detected after linearization.
Figure A4: Low melting point agarose gel electrophoresis showing hER and rPR1 digested plasmids. M: DNA ladder.
A) hER digested plasmid. A 265 - 270 bp insert was apparent which was then excised and used to prepare cDNA probes.
B) rPR1 digested plasmid. A 550 bp insert was apparent which was then excised and used to prepare cDNA probes.
APPENDIX B

Optimization of cycle number

The amount of product over a range of amplification cycles was examined and a cycle number that falls in the linear range was chosen. A pooled sample of 16, 19 and 21 dg fetal rat brain reverse transcribed RNA, from N dams was used. This pooled cDNA sample was diluted 1 in 6 allowing the same sample to be used for all cycle number optimization experiments. The RT- sample was used neat. In addition a water sample (W) was used as a negative control. The amount of product was measured and calibrated (section 2.13) and the intensity of the bands (od) was plotted against the cycle number. The MgCl₂, primer and dNTP concentrations were determined in preliminary experiments and were 3 mM, 0.3 μM and 0.5 mM, respectively.

PCR reactions were carried out for an increasing number of cycle steps (two cycle increments). For 18S rRNA, cycle number was increased from 20 to 34 cycles and product was first detected at cycle number 24 increasing linearly up to cycle number 32 (Fig. B1). For RPL19, cycle number was increased from 22 to 36 cycles (Fig. B2). Product was first detected at cycle number 32 and increased linearly up to cycle number 36. For BGLU, cycle number was increased from 24 to 38 cycles, product was first detected at cycle number 34 and increased linearly up to cycle number 38 (Fig. B3). The annealing temperature for 18S rRNA, RPL19 and BGLU was 52 °C. For CY, cycle number was increased from 20 to 34 cycles, product was first detected at cycle number 24 and increased linearly up to cycle number 28 (Fig. B4). For HH4, cycle number was increased from 34 to 48 cycles (Fig. B5). Product was first detected at cycle number 38 and increased linearly up to cycle number 48. The annealing temperature for both CY and HH4 was 59 °C. For G3PDH, cycle number was increased from 22 to 36 cycles and product was first detected at cycle number 24 increasing linearly up to cycle number 32 (Fig. B6). For ERα, cycle number was increased from 32 to 46 cycles. Product was first detected at cycle number 36 and increased linearly up to cycle number 46 (Fig. B7). For ERβ, cycle number was increased from 36 to 50 cycles. Product was first detected at cycle number 36 and increased linearly up to cycle number 42 (Fig. B8). The annealing temperature for G3PDH, ERα and ERβ was 57 °C. For PRAB and PRB, cycle number was increased from 28 to 42 cycles with annealing temperatures of 69 °C and 59 °C, respectively. For PRAB product was first detected at cycle number 34 and increased linearly up to cycle number 38 (Fig. B9) while for PRB product was first detected at cycle number 30 and increased linearly up to cycle number 34 (Fig. B10). From these experiments a cycle number was chosen that yielded sufficient product for ethidium bromide visualization whilst falling well within the linear range.
Figure B1: RT-PCR analysis of 18S rRNA: effect of increasing cycle number. Reactions were performed with a normal cDNA sample (from 16, 19 and 21 dg fetal brain) for various cycle numbers at 52 °C using standard reaction conditions.

A) Ethidium bromide stained gel. Cycle numbers are indicated at the top and bottom of the gel; the migration of the 100 bp marker (M) is shown on the left-hand side and the calculated size of the signal indicated on the right-hand side of the gel (expected product size is 419 bp). RT- and W are reverse transcriptase minus (undiluted) and water controls, respectively. Each dilution of sample was run in duplicate except cycle no. 34 in triplicate. RT- and water samples were run in singlicate for 34 cycles.

B) Product-input relationship. The amount of product showed a linear relationship with cycle number between cycles 24 and 32.
Figure B2: RT-PCR analysis of RPL19: effect of increasing cycle number. Reactions were performed with a normal cDNA sample (from 16, 19 and 21 dg fetal brain) for various cycle numbers at 52 °C using standard reaction conditions.

A) Ethidium bromide stained gel. Cycle numbers are indicated at the top and bottom of the gel; the migration of the 100 bp marker (M) is shown on the left-hand side and the calculated size of the signal indicated on the right-hand side of the gel (expected product size is 321 bp). RT- and W are reverse transcriptase minus (undiluted) and water controls, respectively. Each dilution of sample was run in duplicate. RT- and water samples were run in singlicate for 36 cycles.

B) Product-input relationship. The amount of product showed a linear relationship with cycle number between cycles 32 and 36.
Figure B3: RT-PCR analysis of β-glucuronidase: effect of increasing cycle number. Reactions were performed with a normal cDNA sample (from 16, 19 and 21 dg fetal brain) for various cycle numbers at 52 °C using standard reaction conditions.

A) Ethidium bromide stained gel. Cycle numbers are indicated at the top and bottom of the gel; the migration of the 100 bp marker (M) is shown on the left-hand side and the calculated size of the signal indicated on the right-hand side of the gel (expected product size is 529 bp). RT- and W are reverse transcriptase minus (undiluted) and water controls, respectively. Each dilution of sample was run in duplicate. RT- and water samples were run in singlicate for 38 cycles.

B) Product-input relationship. The amount of product showed a linear relationship with cycle number between cycles 34 and 38.
Figure B4: RT-PCR analysis of cyclophilin: effect of increasing cycle number. Reactions were performed with a normal cDNA sample (from 16, 19 and 21 dg fetal brain) for various cycle numbers at 59 °C using standard reaction conditions.

A) Ethidium bromide stained gel. Cycle numbers are indicated at the top and bottom of the gel; the migration of the 100 bp marker (M) is shown on the left-hand side and the calculated size of the signal indicated on the right-hand side of the gel (expected product size is 369 bp). RT- and W are reverse transcriptase minus (undiluted) and water controls, respectively. Each dilution of sample was run in duplicate except cycle no. 34 in triplicate. RT- and water samples were run in singlicate for 34 cycles.

B) Product-input relationship. The amount of product showed a linear relationship with cycle number between cycles 24 and 28.
Figure B5: RT-PCR analysis of histone H4: effect of increasing cycle number. Reactions were performed with a normal cDNA sample (from 16, 19 and 21 dg fetal brain) for various cycle numbers at 59 °C using standard reaction conditions.

A) Ethidium bromide stained gel. Cycle numbers are indicated at the top and bottom of the gel; the migration of the 100 bp marker (M) is shown on the left-hand side and the calculated size of the signal indicated on the right-hand side of the gel (expected product size is 250 bp). RT- and W are reverse transcriptase minus (undiluted) and water controls, respectively. Each dilution of sample was run in duplicate except cycle no. 48 in triplicate. RT- and water samples were run in singlicate for 48 cycles.

B) Product-input relationship. The amount of product showed a linear relationship with cycle number between cycles 38 and 48.
Figure B6: RT-PCR analysis of glyceraldehyde-3-phosphate dehydrogenase: effect of increasing cycle number. Reactions were performed with a normal cDNA sample (from 16, 19 and 21 dg fetal brain) for various cycle numbers at 57 °C using standard reaction conditions.

A) Ethidium bromide stained gel. Cycle numbers are indicated at the top and bottom of the gel; the migration of the 100 bp marker (M) is shown on the left-hand side and the calculated size of the signal indicated on the right-hand side of the gel (expected product size is 968 bp). RT- and W are reverse transcriptase minus (undiluted) and water controls, respectively. Each dilution of sample was run in duplicate except cycle no. 36 in triplicate. RT- and water samples were run in singlicate for 36 cycles.

B) Product-input relationship. The amount of product showed a linear relationship with cycle number between cycles 24 and 32.
Figure B7: RT-PCR analysis of ERα: effect of increasing cycle number. Reactions were performed with a normal cDNA sample (from 16, 19 and 21 dg fetal brain) for various cycle numbers at 57 °C, using standard reaction conditions.

A) Ethidium bromide stained gel. Cycle numbers are indicated at the top and bottom of the gel; migration of the 100 bp marker (M) is shown on the left-hand side and the calculated size of the signal indicated on the right-hand side of the gel (expected product size is 623 bp). RT- and W are reverse transcriptase minus (undiluted) and water controls, respectively. Each dilution of sample was run in duplicate. RT- and water samples were run in singlicate for 46 cycles.

B) Product-input relationship. The amount of product showed a linear relationship with cycle number between cycles 36 and 46.
Figure B8: RT-PCR analysis of ERβ: effect of increasing cycle number. Reactions were performed with a normal cDNA sample (from 16, 19 and 21 dg fetal brain) for various cycle numbers at 57 °C, using standard reaction conditions.
A) Ethidium bromide stained gel. Cycle numbers are indicated at the top and bottom of the gel; migration of the 100 bp marker (M) is shown on the left-hand side and the calculated size of the signal indicated on the right-hand side of the gel (expected product size is 262 bp). RT- and W are reverse transcriptase minus (undiluted) and water controls, respectively. Each dilution of sample was run in duplicate. RT- and water samples were run in singlicate for 50 cycles.
B) Product-input relationship. The amount of product showed a linear relationship with cycle number between cycles 36 and 42.
Figure B9: RT-PCR analysis of PRAB: effect of increasing cycle number. Reactions were performed with a normal cDNA sample (from 16, 19 and 21 dg fetal brain) for various cycle numbers at 69 °C, using standard reaction conditions.

A) Ethidium bromide stained gel. Cycle numbers are indicated at the top and bottom of the gel; migration of the 100 bp marker (M) is shown on the left-hand side and the calculated size of the signal indicated on the right-hand side of the gel (expected product size is 319 bp). RT- and W are reverse transcriptase minus (undiluted) and water controls, respectively. Each dilution of sample was run in duplicate except cycle 42 which was run in triplicate. RT- and water samples were run in singlicate for 42 cycles.

B) Product-input relationship. The amount of product showed a linear relationship with cycle number between cycles 36 and 42.
Figure B10: RT-PCR analysis of PRB: effect of increasing cycle number. Reactions were performed with a normal cDNA sample (from 16, 19 and 21 dg fetal brain) for various cycle numbers at 59 °C, using standard reaction conditions.

A) Ethidium bromide stained gel. Cycle numbers are indicated at the top and bottom of the gel; migration of the 100 bp marker (M) is shown on the left-hand side and the calculated size of the signal indicated on the right-hand side of the gel (expected product size is 228 bp). Each dilution of sample was run in duplicate except cycle 42 which was run in quadruplicate.

B) Product-input relationship. The amount of product showed a linear relationship with cycle number between cycles 30 and 36.
APPENDIX C

Optimization of template dilution

The amount of template dilution was varied to verify linearity of the reaction with respect to the amount of products. The sample was used neat and at various dilutions: 3, 6, 12 and 24 fold. The RT- sample was used neat and a water sample was used as a negative control. The MgCl₂, primer and dNTP concentrations were 3 mM, 0.3 μM and 0.5 mM, respectively. The amount of product was measured and calibrated (section 2.13) and the intensity of the bands (od) was plotted against the template dilution. From these experiments it was decided that for all genes studied a 6-fold dilution yields enough product and falls within the linear range. This dilution was used for all further experiments alongside the standard conditions and optimized cycle numbers.
Figure C1: RT-PCR analysis of 18S rRNA: effect of increasing template dilution. Reactions were performed with a range of dilutions of pooled normal cDNA sample (from 16, 19 and 21 dg fetal brain) for 26 cycles at 52 °C, using standard reaction conditions. A) Ethidium bromide stained gel. Template dilution is indicated at the top of the gel; the migration of the 100 bp marker (M) is shown on the left-hand side and the calculated size of the signal indicated on the right-hand side of the gel (expected product size is 419 bp). RT- and W are reverse transcriptase minus (undiluted) and water controls, respectively. Each dilution of sample was run in duplicate, RT- and water samples were run in singlicate. B) Product-input relationship. The amount of product showed a linear relationship with template dilution up to 3-fold dilution. A template dilution of 1 corresponds to undiluted sample.
Figure C2: RT-PCR analysis of RPL19: effect of increasing template dilution. Reactions were performed with a range of dilutions of pooled normal cDNA sample (from 16, 19 and 21 dg fetal brain) for 33 cycles at 52 °C, using standard reaction conditions.

A) Ethidium bromide stained gel. Template dilution is indicated at the top of the gel; the migration of the 100 bp marker (M) is shown on the left-hand side and the calculated size of the signal indicated on the right-hand side of the gel (expected product size is 321 bp). RT- and W are reverse transcriptase minus (undiluted) and water controls, respectively. Each dilution of sample was run in duplicate, RT- and water samples were run in singlicate.

B) Product-input relationship. The amount of product showed a linear relationship with template dilution up to 3-fold dilution. A template dilution of 1 corresponds to undiluted sample.
Figure C3: RT-PCR analysis of β-glucuronidase: effect of increasing template dilution. Reactions were performed with a range of dilutions of pooled normal cDNA sample (from 16, 19 and 21 dg fetal brain) for 34 cycles at 52 °C, using standard reaction conditions. A) Ethidium bromide stained gel. Template dilution is indicated at the top of the gel; the migration of the 100 bp marker (M) is shown on the left-hand side and the calculated size of the signal indicated on the right-hand side of the gel (expected product size is 529 bp). RT- and W are reverse transcriptase minus (undiluted) and water controls, respectively. Each dilution of sample was run in duplicate, RT- and water samples were run in singlicate. B) Product-input relationship. The amount of product showed a linear relationship with template dilution over the range studied. A template dilution of 1 corresponds to undiluted sample.
Figure C4: RT-PCR analysis of cyclophilin: effect of increasing template dilution. Reactions were performed with a range of dilutions of pooled normal cDNA sample (from 16, 19 and 21 dg fetal brain) for 24 cycles at 59 °C, using standard reaction conditions.

A) Ethidium bromide stained gel. Template dilution is indicated at the top of the gel; the migration of the 100 bp marker (M) is shown on the left-hand side and the calculated size of the signal indicated on the right-hand side of the gel (expected product size is 369 bp). Each dilution of sample was run in duplicate.

B) Product-input relationship. The amount of product showed a linear relationship with template dilution up to 3-fold dilution. A template dilution of 1 corresponds to undiluted sample.
Figure C5: RT-PCR analysis of histone H4: effect of increasing template dilution. Reactions were performed with a range of dilutions of pooled normal cDNA sample (from 16, 19 and 21 dg fetal brain) for 39 cycles at 59 °C, using standard reaction conditions.

A) Ethidium bromide stained gel. Template dilution is indicated at the top of the gel; the migration of the 100 bp marker (M) is shown on the left-hand side and the calculated size of the signal indicated on the right-hand side of the gel (expected product size is 250 bp). RT- and W are reverse transcriptase minus (undiluted) and water controls, respectively. Each dilution of sample was run in duplicate, RT- and water samples were run in singlicate.

B) Product-input relationship. The amount of product showed a linear relationship with template dilution up to 3-fold dilution. A template dilution of 1 corresponds to undiluted sample.
Figure C6: RT-PCR analysis of glyceraldehyde-3-phosphate-dehydrogenase: effect of increasing template dilution. Reactions were performed with a range of dilutions of pooled normal cDNA sample (from 16, 19 and 21 dg fetal brain) for 26 cycles at 57 °C, using standard reaction conditions.

A) Ethidium bromide stained gel. Template dilution is indicated at the top of the gel; the migration of the 100 bp marker (M) is shown on the left-hand side and the calculated size of the signal indicated on the right-hand side of the gel (expected product size is 968 bp). RT- and W are reverse transcriptase minus (undiluted) and water controls, respectively. Each dilution of sample was run in duplicate, RT- and water samples were run in singlicate.

B) Product-input relationship. The amount of product showed a linear relationship with template dilution up to 3-fold dilution. A template dilution of 1 corresponds to undiluted sample.
Figure C7: RT-PCR analysis of ERα: effect of increasing template dilution. Reactions were performed with a range of dilutions of pooled normal cDNA sample (from 16, 19 and 21 dg fetal brain) for 38 cycles at 57 °C, using standard reaction conditions.

A) Ethidium bromide stained gel. Template dilution is indicated at the top of the gel; the migration of the 100 bp marker (M) is shown on the left-hand side and the calculated size of the signal indicated on the right-hand side of the gel (expected product size is 623 bp). RT- and W are reverse transcriptase minus (undiluted) and water controls, respectively. Each dilution of sample was run in duplicate, RT- and water samples were run in singlicate.

B) Product-input relationship. The amount of product showed a linear relationship with template dilution up to 3-fold dilution. A template dilution of 1 corresponds to undiluted sample.
Figure C8: RT-PCR analysis of ERβ: effect of increasing template dilution. Reactions were performed with a range of dilutions of pooled normal cDNA sample (from 16, 19 and 21 dg fetal brain) for 37 cycles at 57 °C, using standard reaction conditions.

A) Ethidium bromide stained gel. Template dilution is indicated at the top of the gel; the migration of the 100 bp marker (M) is shown on the left-hand side and the calculated size of the signal indicated on the right-hand side of the gel (expected product size is 262 bp). RT- and W are reverse transcriptase minus (undiluted) and water controls, respectively. Each dilution of sample was run in duplicate, RT- and water samples were run in singlelicate.

B) Product-input relationship. The amount of product showed a linear relationship with template dilution between 6-fold and neat. A template dilution of 1 corresponds to undiluted sample.
Figure C9: RT-PCR analysis of PRAB: effect of increasing template dilution. Reactions were performed with a range of dilutions of pooled normal cDNA sample (from 16, 19 and 21 dg fetal brain) for 38 cycles at 69 °C, using standard reaction conditions.

A) Ethidium bromide stained gel. Template dilution is indicated at the top of the gel; the migration of the 100 bp marker (M) is shown on the left-hand side and the calculated size of the signal indicated on the right-hand side of the gel (expected product size is 319 bp). RT- and W are reverse transcriptase minus (undiluted) and water controls, respectively. Each dilution of sample was run in duplicate, RT- and water samples were run in singlicate.

B) Product-input relationship. The amount of product showed a linear relationship with template dilution up to 3-fold dilution. A template dilution of 1 corresponds to undiluted sample.
Figure C10: RT-PCR analysis of PRB: effect of increasing template dilution. Reactions were performed with a range of dilutions of pooled normal cDNA sample (from 16, 19 and 21 dg fetal brain) for 35 cycles at 59 °C, using standard reaction conditions.
A) Ethidium bromide stained gel. Template dilution is indicated at the top of the gel; the migration of the 100 bp marker (M) is shown on the left-hand side and the calculated size of the signal indicated on the right-hand side of the gel (expected product size is 228 bp). RT- and W are reverse transcriptase minus (undiluted) and water controls, respectively. Each dilution of sample was run in duplicate, RT- and water samples were run in singlicate.
B) Product-input relationship. The amount of product showed a linear relationship with template dilution between 6-fold and neat. A template dilution of 1 corresponds to undiluted sample.
Figure D1: Southern blots hybridized with specific oligonucleotide probes
N: normal dam; TX: hypothyroxinemic dam.
A) A Southern blot hybridized with an 18S specific oligonucleotide probe.
B) A Southern blot hybridized with an ERα specific oligonucleotide probe.
C) A Southern blot hybridized with an ERβ specific oligonucleotide probe.
D) A Southern blot hybridized with a PRAB specific oligonucleotide probe.
E) A Southern blot hybridized with a PRB specific oligonucleotide probe.
In all blots a single band can be detected.