Maternal hypothyroxinemia and the ontogeny of thyroid hormone nuclear receptors and cholinergic and monoaminergic neurotransmitter systems in developing rat brain

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

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1999
This Thesis is dedicated to

my wife and parents
ABSTRACT

Irreversible neurological dysfunction in children as a result of maternal thyroid hormone deficit is a worldwide problem. The effects of thyroid hormone insufficiency on neonatal brain development have been intensively studied, however, little is known regarding thyroid hormone action during fetal brain development. A hypothyroxinemic rat dam model was used to investigate the role of thyroid hormone in early brain development, with particular respect to cholinergic and monoaminergic neurotransmitter metabolic enzymes and receptors, and thyroid hormone nuclear receptors.

Maternal hypothyroxinemia disturbed the ontogeny of choline acetyltransferase, monoamine oxidase and DOPA decarboxylase in fetal brain. During postnatal development, region-specific increases in tyrosine hydroxylase, monoamine oxidase and DOPA decarboxylase activities were observed, together with decreased β-adrenergic and D2 dopaminergic receptor binding sites.

The fetal ontogeny of thyroid hormone receptors was disturbed in experimental progeny, but only in preparations of whole nuclei, which exhibited increased receptor binding sites at 16 and 21 days gestation. No differences in binding kinetics were apparent in salt-extracted receptor preparations from experimental progeny and controls. Analysis of thyroid hormone receptor isoform mRNA levels in fetal brain indicated that the non T3-binding variant, α2 (TRα2), was thyroid hormone regulated whereas the α1 and β1 receptor isoforms were not.

This thesis provides further evidence supporting a role for maternal TH in fetal brain development. Three candidate genes are identified, whose expression may be directly affected by T3 in fetal brain, namely choline acetyltransferase, monoamine oxidase (isoform A) and TRα2. Furthermore, these effects may be exacerbated by a general increase in the inhibition of thyroid hormone and retinoic acid-mediated gene transcription as a result of increased thyroid hormone receptor homodimer formation. The disturbed ontogeny of these proteins, amongst others, may underlie the region specific biochemical abnormalities exhibited in hypothyroxinemic dam progeny.
### LIST OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Title Page</td>
<td>1</td>
</tr>
<tr>
<td>Dedication</td>
<td>2</td>
</tr>
<tr>
<td>Abstract</td>
<td>3</td>
</tr>
<tr>
<td>List of Contents</td>
<td>4</td>
</tr>
<tr>
<td>List of Figures</td>
<td>9</td>
</tr>
<tr>
<td>List of Tables</td>
<td>13</td>
</tr>
<tr>
<td>Abbreviations used</td>
<td>15</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>19</td>
</tr>
<tr>
<td>Chapter 1: Introduction</td>
<td>20</td>
</tr>
<tr>
<td>1.1 Historical review</td>
<td>21</td>
</tr>
<tr>
<td>1.2 Development of the thyroid</td>
<td>23</td>
</tr>
<tr>
<td>1.3 Thyroid hormone synthesis</td>
<td>24</td>
</tr>
<tr>
<td>.1 Iodide accumulation</td>
<td>24</td>
</tr>
<tr>
<td>.2 Iodination of thyroglobulin</td>
<td>26</td>
</tr>
<tr>
<td>.3 Thyroid hormone release and secretion</td>
<td>26</td>
</tr>
<tr>
<td>.4 Regulation of thyroid function: the hypothalamic-pituitary axis</td>
<td>26</td>
</tr>
<tr>
<td>1.4 Thyroid hormone transport and cellular uptake</td>
<td>27</td>
</tr>
<tr>
<td>.1 Thyroid hormone cellular uptake</td>
<td>29</td>
</tr>
<tr>
<td>1.5 Thyroid hormone metabolism</td>
<td>30</td>
</tr>
<tr>
<td>.1 Type I 5'-deiodinase</td>
<td>31</td>
</tr>
<tr>
<td>.2 Type II 5'-deiodinase</td>
<td>33</td>
</tr>
<tr>
<td>.3 5-Deiodinase</td>
<td>33</td>
</tr>
<tr>
<td>.4 Regulation of deiodinases</td>
<td>34</td>
</tr>
<tr>
<td>.5 Non-deiodinative thyroid hormone metabolism</td>
<td>35</td>
</tr>
<tr>
<td>1.6 Thyroid hormone action</td>
<td>36</td>
</tr>
<tr>
<td>.1 Superfamily receptor structure</td>
<td>37</td>
</tr>
<tr>
<td>.2 Thyroid hormone receptor isoforms</td>
<td>37</td>
</tr>
<tr>
<td>.3 Mechanisms of thyroid hormone receptor action</td>
<td>41</td>
</tr>
<tr>
<td>.1 TR-mediated activation and repression of transcription</td>
<td>42</td>
</tr>
<tr>
<td>.2 Orphan receptors</td>
<td>43</td>
</tr>
<tr>
<td>.3 Phosphorylation of thyroid hormone receptors</td>
<td>44</td>
</tr>
</tbody>
</table>
Chapter 2 : Materials and Methods

2.1 General

.1 Materials
.2 Animal model
.3 Sample preparation

2.2 Enzyme assays

.1 Acetylcholinesterase (EC 3.1.1.7)
.2 Choline acetyltransferase (EC 2.3.1.6)
.3 Dihydroxyphenylalanine (DOPA) decarboxylase (EC 4.1.1.28)
.4 Tyrosine hydroxylase (EC 1.14.16.2)
.1 Partial purification of DDC from hog kidney 76
.2 Assay procedure 76
.5 Monoamine oxidase (EC 1.4.3.4) 77

2.3 Neurotransmitter receptor binding studies 77
.1 Crude membrane preparation 77
.2 β-adrenergic receptor binding assay 77
.3 Muscarinic cholinergic receptor binding assay 78
.4 Dopaminergic D2 receptor binding assay 78

2.4 Nuclear T3-binding studies 78
.1 Nuclei isolation 78
.1 Receptor extraction 79
.2 Binding assay: whole nuclei 79
.3 Binding assay: salt-extracted receptors 79

2.5 Receptor binding analysis 80

2.6 Expression of TR isoform mRNA in fetal brain 80
.1 Agarose gel electrophoresis 82
.1 RNA electrophoresis 82
.2 DNA electrophoresis 82
.2 RNA isolation and standardisation 83
.1 Estimation of RNA concentration 83
.3 Northern blotting 83
.4 Preparation of cDNA probes 84
.1 Transformation and culture 84
.2 Plasmid purification 84
.3 Plasmid digestion 86
.5 Probe labelling 86
.6 Hybridisation 87
.1 Washing 88
.2 Autoradiography 88
.3 Image analysis 89
.7 Reverse transcription and polymerase chain reaction 89
.1 Reverse transcription 89
.2 Primer design 90
.3 Polymerase chain reaction 92
.4 Oligonucleotide probe design and hybridisation 98

2.7 Statistical analysis 98
Chapter 3: The influence of maternal hypothyroxinemia on feto-placental and neonatal growth

3.1 Animal model
3.2 Maternal thyroid function
3.3 Litter number
3.4 Gross parameters of growth in prenatal progeny
   .1 Fetal body and brain weights
   .2 Fetal brain protein
   .3 Fetal brain DNA and protein:DNA ratio
3.5 Gross parameters of placental growth
   .1 Placental weight
   .2 Placental protein
   .3 Placental DNA and protein:DNA ratio
3.6 Gross parameters of growth in postnatal progeny
   .1 Postnatal body weights
   .2 Postnatal brain region weights
   .3 Postnatal brain region protein
   .4 Postnatal brain region DNA
   .5 Postnatal brain region protein:DNA ratio
3.7 Summary of results

Chapter 4: The effect of maternal hypothyroxinemia on cholinergic and catecholaminergic neurotransmitter systems in developing brain

4.1 Neurotransmitter metabolic enzyme activities
   .1 Cholinergic metabolic enzymes
      .1 Choline acetyltransferase (ChAT) activity
      .2 Acetylcholinesterase (AChE) activity
   .2 Catecholaminergic metabolic enzymes
      .1 Tyrosine hydroxylase (TyrH) activity
      .2 DOPA decarboxylase (DDC) activity
      .3 Monoamine oxidase (MAO) activity
   .3 Maternal hypothyroxinemia and metabolic enzyme activities at 14 dg
4.2 Neurotransmitter receptor binding studies
   .1 Muscarinic cholinergic receptor binding
   .2 β-Adrenergic receptor binding
Chapter 5: The effect of maternal hypothyroxinemia on thyroid hormone nuclear receptors in developing brain.

5.1 Thyroid hormone nuclear receptor expression in fetal rat brain
5.2 Triiodothyronine nuclear receptor binding
   .1 Whole nuclei studies
   .2 Salt-extracted receptor studies
5.3 Thyroid hormone receptor isoform mRNA ontogeny
   .1 Northern hybridisation studies
   .2 Semi-quantitative RT-PCR
   .3 Southern hybridisation of PCR products
5.4 Summary of results
   .1 T3-receptor binding assays
   .2 TR isoform mRNA expression

Chapter 6: Discussion

6.1 Animal model
6.2 Maternal hypothyroxinemia and thyroid hormone receptor ontogeny
6.3 Maternal hypothyroxinemia and cholinergic and monoaminergic neurotransmitter systems
   .1 Fetal brain neurotransmitter enzyme ontogeny
   .2 Postnatal neurotransmitter system dysfunction
   .3 Suggested mechanisms of thyroid hormone-induced changes in brain development
6.4 Summary
6.5 Future work

References

Publications
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Structural composition of a follicle, showing distribution of organelles within a representative epithelial cell.</td>
<td>25</td>
</tr>
<tr>
<td>1.2</td>
<td>Thyroid hormone metabolic pathways.</td>
<td>32</td>
</tr>
<tr>
<td>1.3</td>
<td>Schematic diagrams showing comparison of rat thyroid hormone receptor isoforms.</td>
<td>38</td>
</tr>
<tr>
<td>2.1</td>
<td>Schematic diagram of Bluescript KS+ plasmid.</td>
<td>85</td>
</tr>
<tr>
<td>2.2</td>
<td>Schematic representation of the PCR primer positions on the TR isoforms.</td>
<td>91</td>
</tr>
<tr>
<td>2.3</td>
<td>Effect of template concentration on 18S PCR.</td>
<td>94</td>
</tr>
<tr>
<td>2.4</td>
<td>Effect of template concentration on TRα isoform PCR.</td>
<td>95</td>
</tr>
<tr>
<td>2.5</td>
<td>Effect of template concentration on TRβ1 PCR.</td>
<td>97</td>
</tr>
<tr>
<td>3.1</td>
<td>The influence of maternal hypothyroxinemia on serum thyroid stimulating hormone (TSH) in dams throughout pregnancy.</td>
<td>102</td>
</tr>
<tr>
<td>3.2</td>
<td>Serum TSH concentration versus total T4 for normal and partially thyroidectomised rat dams at 16, 19 and 21 dg.</td>
<td>103</td>
</tr>
<tr>
<td>3.3</td>
<td>The influence of maternal hypothyroxinemia on the number of progeny per litter.</td>
<td>105</td>
</tr>
<tr>
<td>3.4</td>
<td>The influence of maternal hypothyroxinemia on progeny body weight.</td>
<td>106</td>
</tr>
<tr>
<td>3.5</td>
<td>The influence of maternal hypothyroxinemia on progeny brain/body weight ratio.</td>
<td>109</td>
</tr>
</tbody>
</table>
4.1 The influence of maternal hypothyroxinemia on the prenatal ontogeny of choline acetyltransferase specific activity in whole brain.

4.2 The influence of maternal hypothyroxinemia on the ontogeny of choline acetyltransferase specific activity in postnatal brain regions.

4.3 The influence of maternal hypothyroxinemia on the prenatal ontogeny of acetylcholinesterase specific activity in whole brain.

4.4 The influence of maternal hypothyroxinemia on the ontogeny of acetylcholinesterase specific activity in postnatal brain regions.

4.5 The influence of maternal hypothyroxinemia on the prenatal ontogeny of tyrosine hydroxylase specific activity in whole brain.

4.6 The influence of maternal hypothyroxinemia on the ontogeny of tyrosine hydroxylase specific activity in postnatal brain regions.

4.7 The influence of maternal hypothyroxinemia on the prenatal ontogeny of DOPA decarboxylase specific activity in whole brain.

4.8 The influence of maternal hypothyroxinemia on the ontogeny of DOPA decarboxylase specific activity in postnatal brain regions.

4.9 The influence of maternal hypothyroxinemia on the prenatal ontogeny of monoamine oxidase specific activity in whole brain.

4.10 Effects of increasing deprenyl and clorgyline concentrations on brain monoamine oxidase activity in 19 dg N dam progeny.

4.11 The influence of maternal hypothyroxinemia on the ontogeny of monoamine oxidase specific activity in postnatal brain regions.
4.12 Scatchard plots of cholinergic muscarinic membrane binding in normal and partially thyroidectomised dam progeny brain regions at 20 pnd.

4.13 Scatchard plots of cholinergic muscarinic membrane binding in normal and partially thyroidectomised dam progeny brain regions at 30 pnd.

4.14 Scatchard plots of β-adrenergic membrane binding in normal and partially thyroidectomised dam progeny brain regions at 20 pnd.

4.15 Scatchard plots of β-adrenergic membrane binding in normal and partially thyroidectomised dam progeny brain regions at 30 pnd.

4.16 Scatchard plots of D2 dopaminergic membrane binding in normal and partially thyroidectomised dam progeny brain regions at 20 and 30 pnd.

5.1 Scatchard plots of T3-receptor binding in whole nuclei from normal and partially thyroidectomised dam fetal brain.

5.2 Scatchard plots of T3-receptor binding in extracted receptor preparations from normal and partially thyroidectomised dam fetal brain.

5.3 Ontogeny of TRα isoform mRNAs in normal and partially thyroidectomised dam fetal brain by Northern hybridisation.

5.4 Northern hybridisation of TRα1 cDNA probe to total RNA from normal and partially thyroidectomised dam fetal brain, after 18 h exposure to hyperfilm.

5.5 Northern hybridisation of TRβ1 cDNA probe to total RNA from normal adult liver, kidney and brain, after 3 nights exposure to Biomax film.
5.6 RT-PCR of TR isoforms in normal and partially thyroidectomised dam fetal brains.

5.7 TR isoform expression ratios/combination in normal and partially thyroidectomised dam fetal brains.

5.8 Southern hybridisation of specific oligonucleotide probes to RT-PCR products from normal and partially thyroidectomised dam fetal brains.
**LIST OF TABLES**

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Plasma concentration and binding characteristics of the TH binding proteins in humans.</td>
<td>28</td>
</tr>
<tr>
<td>1.2</td>
<td>Properties of the thyroid hormone deiodinases.</td>
<td>31</td>
</tr>
<tr>
<td>1.3</td>
<td>Known half-site combinations for TR homo/heterodimers.</td>
<td>41</td>
</tr>
<tr>
<td>2.1</td>
<td>Composition of commonly used buffers and solutions.</td>
<td>81</td>
</tr>
<tr>
<td>2.2</td>
<td>RT-PCR primer pairs.</td>
<td>92</td>
</tr>
<tr>
<td>2.3</td>
<td>Optimised PCR conditions.</td>
<td>96</td>
</tr>
<tr>
<td>2.4</td>
<td>RT-PCR product oligonucleotide probes.</td>
<td>98</td>
</tr>
<tr>
<td>3.1</td>
<td>Maternal serum total 3,5,3'-triiodothyronine and thyroxine in normal and partially thyroidectomised dam pregnancies.</td>
<td>101</td>
</tr>
<tr>
<td>3.2</td>
<td>Fetal brain weight, protein and DNA levels in normal and partially thyroidectomised dam progeny.</td>
<td>107</td>
</tr>
<tr>
<td>3.3</td>
<td>Placental growth and development in normal and partially thyroidectomised dams.</td>
<td>110</td>
</tr>
<tr>
<td>3.4</td>
<td>Postnatal brain region weights in normal and partially thyroidectomised dam progeny.</td>
<td>113</td>
</tr>
<tr>
<td>3.5</td>
<td>Protein concentration in postnatal normal and partially thyroidectomised dam progeny brain regions.</td>
<td>114</td>
</tr>
<tr>
<td>3.6</td>
<td>Protein content in postnatal normal and partially thyroidectomised dam progeny brain regions.</td>
<td>116</td>
</tr>
</tbody>
</table>
3.7 DNA concentration in postnatal normal and partially thyroidectomised dam progeny brain regions.

3.8 DNA content in postnatal normal and partially thyroidectomised dam progeny brain regions.

3.9 Protein:DNA ratios in postnatal normal and partially thyroidectomised dam progeny brain regions.

4.1 Activity of monoamine oxidase isoforms A and B in normal and partially thyroidectomised dam fetal brain at 16 and 19 dg.

4.2 Whole brain DOPA decarboxylase, monoamine oxidase and choline acetyltransferase specific activities in 14 dg normal and partially thyroidectomised dam progeny.

4.3 Muscarinic cholinergic receptor number and affinity in normal and partially thyroidectomised dam progeny brain regions at 20 and 30 pnd.

4.4 β-adrenergic receptor number and affinity in normal and partially thyroidectomised dam progeny brain regions at 20 and 30 pnd.

4.5 D2 dopaminergic receptor number and affinity in normal and partially thyroidectomised dam progeny brain regions at 20 and 30 pnd.

5.1 Nuclear T3-binding in whole nuclei from normal and partially thyroidectomised dam fetal brains.

5.2 T3-binding in extracted receptor preparations from normal and partially thyroidectomised dam fetal brains.
ABBREVIATIONS USED

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>AChE</td>
<td>acetyl cholinesterase</td>
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<tr>
<td>AF1</td>
<td>activation function 1</td>
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<tr>
<td>AF2</td>
<td>activation function 2</td>
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<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
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<tr>
<td>ATPase</td>
<td>adenosine 5'-triphosphatase</td>
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<tr>
<td>Bmax</td>
<td>number of binding sites</td>
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<td>cAMP</td>
<td>cyclic adenosine 5'-monophosphate</td>
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<td>CBP</td>
<td>CREB binding protein</td>
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<td>cDNA</td>
<td>complementary DNA</td>
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<td>c-erbA</td>
<td>cellular homologue of the avian erythroblastosis virus oncogene</td>
</tr>
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<td>ChAT</td>
<td>choline acetyltransferase</td>
</tr>
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<td>CNPase</td>
<td>2',3'-cyclic nucleotide 3'-phosphodiesterase</td>
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<td>CNS</td>
<td>central nervous system</td>
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<td>COUP-TF</td>
<td>chicken ovalbumin upstream promoter transcription factor</td>
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<td>COX</td>
<td>cytochrome c oxidase</td>
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<td>CREB</td>
<td>cAMP response element binding protein</td>
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<tr>
<td>CSF</td>
<td>cerebrospinal fluid</td>
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<td>CTBP</td>
<td>cytosolic T3 binding protein</td>
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<td>DOPA decarboxylase</td>
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<td>DR</td>
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<td>GABA</td>
<td>γ-amino butyric acid</td>
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<td>hCG</td>
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</tr>
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</tr>
<tr>
<td>hTRβ1</td>
<td>human TRβ1</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>-------------</td>
<td>------------------------------------------------</td>
</tr>
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<td>IDD</td>
<td>iodine deficiency disorders</td>
</tr>
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<td>immunoglobulin γ</td>
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<td>inverted palindrome</td>
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</tr>
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</tr>
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</tr>
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</tr>
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<td>MAP</td>
<td>microtubule-associated protein</td>
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<td>monoamine oxidase</td>
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<td>MBP</td>
<td>myelin basic protein</td>
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<td>MCS</td>
<td>multiple cloning site</td>
</tr>
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</tr>
<tr>
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</tr>
<tr>
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</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>mRPD3</td>
<td>mammalian reduced potassium dependency gene-3</td>
</tr>
<tr>
<td>mSin3</td>
<td>mammalian suppressor interacting gene-3</td>
</tr>
<tr>
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</tr>
<tr>
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<td>NCoA</td>
<td>nuclear receptor co-activator</td>
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<td>nuclear receptor co-repressor</td>
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</tr>
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</tr>
<tr>
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</tr>
<tr>
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</tr>
<tr>
<td>Abbreviation</td>
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</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCP-2</td>
<td>Purkinje cell protein-2</td>
</tr>
<tr>
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</tr>
<tr>
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</tr>
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</tr>
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</tr>
<tr>
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</tr>
<tr>
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</tr>
<tr>
<td>PTU</td>
<td>propylthiouracil</td>
</tr>
<tr>
<td>RAR</td>
<td>retinoic acid receptor</td>
</tr>
<tr>
<td>RAP46</td>
<td>receptor-associated protein, 46 kDa</td>
</tr>
<tr>
<td>RC3</td>
<td>rat cortex clone 3</td>
</tr>
<tr>
<td>RER</td>
<td>rough endoplasmic reticulum</td>
</tr>
<tr>
<td>Rf</td>
<td>migration relative to dye front</td>
</tr>
<tr>
<td>RIP140</td>
<td>receptor interacting protein, 140 kDa</td>
</tr>
<tr>
<td>rRNA</td>
<td>ribosomal RNA</td>
</tr>
<tr>
<td>RXR</td>
<td>retinoid X receptor</td>
</tr>
<tr>
<td>RT</td>
<td>reverse transcription</td>
</tr>
<tr>
<td>RTx</td>
<td>radiothyroidectomised</td>
</tr>
<tr>
<td>rT3</td>
<td>3,3',5'-triiodothyronine (reverse triiodothyronine)</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error mean</td>
</tr>
<tr>
<td>SMRT</td>
<td>silencing mediator for retinoid and thyroid hormone receptor</td>
</tr>
<tr>
<td>SRC</td>
<td>steroid receptor co-activator</td>
</tr>
<tr>
<td>Srg1</td>
<td>synaptotagmin related gene-1</td>
</tr>
<tr>
<td>SSC</td>
<td>saline sodium citrate buffer</td>
</tr>
<tr>
<td>SSPE</td>
<td>saline sodium phosphate EDTA buffer</td>
</tr>
<tr>
<td>Syt</td>
<td>synaptotagmin</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris-borate-EDTA buffer</td>
</tr>
<tr>
<td>TBP</td>
<td>TATA binding protein</td>
</tr>
<tr>
<td>TBG</td>
<td>thyroid binding globulin</td>
</tr>
<tr>
<td>TE</td>
<td>Tris-EDTA buffer</td>
</tr>
<tr>
<td>Tetrac</td>
<td>tetra-iodothyroacetic acid</td>
</tr>
<tr>
<td>TFIIB</td>
<td>transcription factor IIB</td>
</tr>
<tr>
<td>TH</td>
<td>thyroid hormone</td>
</tr>
<tr>
<td>Tm</td>
<td>melting temperature</td>
</tr>
<tr>
<td>TR</td>
<td>nuclear T3 receptor</td>
</tr>
<tr>
<td>TRH</td>
<td>thyrotrophin releasing hormone</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------------------------------------------------</td>
</tr>
<tr>
<td>TRE</td>
<td>thyroid hormone response element</td>
</tr>
<tr>
<td>Triac</td>
<td>tri-iodothyroacetic acid</td>
</tr>
<tr>
<td>trkA</td>
<td>tyrosine kinase receptor-A</td>
</tr>
<tr>
<td>trkB</td>
<td>tyrosine kinase receptor-B</td>
</tr>
<tr>
<td>trkC</td>
<td>tyrosine kinase receptor-C</td>
</tr>
<tr>
<td>Trip1</td>
<td>thyroid hormone receptor interacting protein 1</td>
</tr>
<tr>
<td>TSH</td>
<td>thyroid stimulating hormone</td>
</tr>
<tr>
<td>TTR</td>
<td>transthyretin</td>
</tr>
<tr>
<td>Tx</td>
<td>thyroidectomised</td>
</tr>
<tr>
<td>TyrH</td>
<td>tyrosine hydroxylase</td>
</tr>
<tr>
<td>3,5-T2</td>
<td>3,5-diiodothyronine</td>
</tr>
<tr>
<td>T3</td>
<td>3,5,3'-triiodothyronine</td>
</tr>
<tr>
<td>T3NB</td>
<td>T3 nuclei binding buffer</td>
</tr>
<tr>
<td>T4</td>
<td>thyroxine</td>
</tr>
<tr>
<td>VDR</td>
<td>vitamin D receptor</td>
</tr>
<tr>
<td>v-erbA</td>
<td>avian erythroblastosis virus oncogene</td>
</tr>
<tr>
<td>wg</td>
<td>weeks gestation</td>
</tr>
</tbody>
</table>
ACKNOWLEDGMENTS

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CHAPTER 1.

INTRODUCTION
1.1 Historical review

The only established biological role for iodine is as a constituent of thyroid hormones (TH). When iodine is lacking in the diet, a number of pathologies including goitre and cretinism result—the exact pathology being determined by the timing and extent of the deficiency—and these are collectively known as iodine deficiency disorders (IDD). The relationship between iodine intake and goitre, however, has only relatively recently (in the past eighty years) been accepted—despite the long and mostly separate histories of the thyroid, iodine and goitre.

The first recorded mention of the thyroid gland is thought to have been in ancient Greece (circa 200 AD), where Galen thought that it may function as a mucous secreting gland for lubricating the pharynx and larynx (1). However this is disputed by Merke (2) who argued that the anatomical descriptions attributed to Galen actually refer to the tonsils, and that the thyroid was not anatomically described until the 1500s by Leonardo da Vinci (2). The organ was formally named “the thyroid gland” by Wharton in 1656, due to its proximity in the larynx to the thyroid cartilage (so named because of its distinctive shape—thryoid derives from the Greek word for “oblong shield”) (3). Wharton thought that the thyroid served a purely aesthetic role, giving the neck a rounded shape (3). It was later noted, however that particular conditions were always associated with an alteration in the size of the thyroid, leading to the hypothesis that the thyroid was important for day to day maintenance of body function (cited in (1)).

Elemental iodine was discovered by Bernard Courtois in 1811 during the manufacture of saltpetre from burnt seaweed (4). In 1820, Coindet deduced that the active ingredient of burnt sponge (a commonly used remedy for goitre at the time) was iodine and successfully treated goitrous patients with potassium iodide and tincture of iodide (4). Another chemist, Boussingault, also recognised iodine deficiency as a cause of goitre in 1825. Unfortunately, these views were largely ignored by the medical profession and it was not until the 1850s that Adolphe Chatin, following his studies of measuring environmental iodine levels, again proposed that iodine deficiency was linked with goitre. This theory was once more received with scepticism due to problems with iodine overdoses in patients, and did not become generally accepted until the studies of Marine, 1909-17 and von Fellenberg in 1926 (2).
Meanwhile, the 1870s onwards saw rapid developments in determining the role of the thyroid gland. The relationship between atrophy of the thyroid and cretinism was first recognised by Fagge in 1871 (5). This was followed by Gull in 1874, who described several cases of adult-onset cretinism (6). The connection between the thyroid gland and these disorders was confirmed in 1883, when Kocher and the Reverdin brothers performed thyroidectomies on goitrous patients. They noticed an initial cosmetic improvement but the patients' health soon deteriorated with symptoms resembling cretinism and myxoedema (2,5). Following on from Fagge's work, Ord chaired a committee of the Clinical Society in London to investigate myxoedema. The report, published in 1888, concluded that myxoedema was a result of atrophy of the thyroid gland and essentially the same disease as cretinism; the factor(s) behind the damage remained unknown (7). Initial success in treating myxoedema by grafting a lobe of sheep thyroid by Bettencourt and Serrano in 1890 led to the treatment of hypothyroidism, first by injection, and later by oral administration of thyroid extract (8,9). These successes, together with the established remedies of burnt sponge and iodine, led Kocher in 1895 to speculate that the thyroid contained iodine (5). This was confirmed by Baumann in the same year when he discovered that the thyroid was capable of incorporating iodine into organic compounds (1,2).

A major advance in the clinical classification of cretinism came in 1908 (10) when McCarrison divided the disorder into two groups, myxoedematous and neurological, depending on the symptoms. This classification is still used today, although there is considerable debate whether two distinct groups actually exist (see section 1.7.1.2). In 1912, Gudernastch fed thyroid extract to tadpoles to demonstrate the role of TH in maturation (11). In 1914 a major breakthrough came with the successful isolation and crystallization of thyroxine (T4) by Kendall (12,13), and in 1927, Harrington and Berger synthesised T4 and elucidated its chemical composition (13). It was not until 1952 however, that Gross and Pitt-Rivers discovered 3,5,3'-triiodothyronine (T3) and demonstrated it to be more potent than T4 (14).

Research then centred on defining the effects and mechanisms of action of TH. It had been speculated since the late 1960s that TH acted via the nucleus (15) and this was confirmed with the demonstration of high affinity nuclear T3 receptors (16). Until recently, the nature of these receptors was largely unknown; although their binding parameters could be determined, attempts to purify the receptors led to loss of affinity. With the development of molecular biological techniques in the 1980s two groups independently discovered candidate genes coding for the nuclear T3 receptor in human (17) and avian (18) cDNA libraries, the former on chromosome 3 and the latter having a human homologue on chromosome 17. Both were related forms of the c-erbA proto-oncogene, the cellular
homologue of the viral v-erbA gene which had kindled researchers' interest because of its resemblance to steroid hormone receptor genes (19,20). It thus became evident that the nuclear T3 receptors (TR) belonged to a superfamily of ligand-activated transcription factors (section 1.6).

Despite the many advances in our knowledge that have occurred during nearly a century of research into TH action there are still several controversies - can maternal TH cross the placenta and thereby influence early fetal development? Does TH affect the adult brain? Are all the effects of TH mediated by nuclear receptors or are there extranuclear sites of action? This thesis is concerned with the first question, and in particular will focus upon the possible role of maternal TH in fetal brain development.

1.2 Development of the thyroid

The basic course of events in the development of the thyroid gland is virtually the same in all mammals studied (1), although the rate of maturation of the thyroid gland tends to vary greatly between species. For example, the thyroid follicles start to form in humans at 11-12 weeks gestation (wg) and in rats at 16 days gestation (dg) (21).

The thyroid gland originates from endodermal tissue as an invagination in the floor of the pharynx known as the foramen caecum. This can be first seen in the human at 16-17 dg (22) and in the rat at 10 dg (1). Proliferation of the original cells, augmented by movement of pharyngeal epithelial cells, results in downward growth and evolution into a flask-like vesicle with a narrow canal known as the thyroglossal duct. By 13-14 dg in rats, and 45-50 dg in humans, the vesicle has bifurcated into two lateral lobes and arrived at its final location in front of the upper trachea. The vesicle, now consisting of two compact clusters of epithelial cells, loses contact with the pharyngeal cavity as the thyroglossal duct solidifies and atrophies. Blood vessels infiltrate the vesicle, leading to a high degree of vascularisation in the developed gland. Finally the vesicle fuses with the ultimobranchial body, which supplies the parafollicular and calcitonin-secreting C cells (1,23).

Follicle formation is a crucial stage in thyroid development as iodine organification and TH synthesis cannot occur until they are fully formed (1). Folliculogenesis starts just after fusion with the ultimobranchial body. Junctional complexes form in the cell membranes between neighbouring cells leading to the formation of narrow clefts which develop into follicular lumens (24). Coincident with this is the polarization of the follicle cells (section 1.3). Iodination and TH synthesis soon follows—at 17.5 dg in rats (1), whereas in humans the timing is more contentious. In vitro studies of developing thyroid explants
suggest TH synthesis starts at around 10.5 wg, however, *in vivo* studies indicate that TH synthesis may begin later, at any time between 15-24 wg (25). Accurate determination of fetal TH synthesis *in vivo* is complicated by the presence of maternally transferred TH.

1.3 Thyroid hormone synthesis

The primary function of the thyroid gland is to concentrate inorganic iodide and use it for TH synthesis. The thyroid gland also produces and secretes another hormone, calcitonin, from the parafollicular cells but this will not be considered here as it is involved in calcium homeostasis, and as such is outside the scope of this thesis. The functional unit of the thyroid gland, the follicle, is roughly spherical in shape, ranging from 0.02 to 1.0 mm in diameter (23). There are generally 20 to 50 follicles in each lobe of the gland, each consisting of a single layer of epithelial cells surrounding the lumen (Fig. 1.1). The epithelial cells are highly polarised in that the apical cell membrane (facing the lumen) has many microvilli and pseudopods, whereas the basal cell membrane is relatively smooth. The polarization is further reflected by differences in functional components between the two membranes and the distribution of the organelles within the cell. For example, nuclei and rough endoplasmic reticulum (RER) tend to be situated in the basal or central area of the cell, while the Golgi apparatus and secretory vesicles are found in the apical area (26). This highly organised arrangement is important for the coordination of the many processes that occur during TH synthesis, as described below.

1.3.1 Iodide accumulation

Situated on the basal membrane are efficient active transport mechanisms capable of importing iodide into the follicles against chemical and electrical gradients. Not surprisingly, studies with uncouplers of oxidative phosphorylation have shown the mechanism to be energy-dependent (27). Iodide accumulation also requires extracellular Na\(^+\) and K\(^+\) ions (28), and the enzyme Na\(^+\),K\(^+\)-ATPase has been demonstrated on the basal cell membrane (29). Recently the cDNA encoding a Na\(^+\)/I\(^-\) symporter has been cloned from rat, and subsequently human, thyroid (30,31). Sequence analysis suggests that the symporter is composed of a membrane protein with 12 putative transmembrane domains, sharing 24.6% homology with the glucose/Na\(^+\) co-transporter (30,32). The cellular interior is kept negatively charged with respect to the lumen, allowing intracellular iodide to diffuse rapidly across the apical cell membrane (33).
Figure 1.1 Structural composition of a follicle, showing distribution of organelles within a representative epithelial cell
1.3.2 Iodination of thyroglobulin

Thyroglobulin is synthesised in the RER and transported, via the Golgi apparatus, to the apical membrane where it is secreted into the follicular lumen—the site of iodination (reviewed in (26,34)). The iodination of thyroglobulin is an oxidative reaction—requiring an electron acceptor, hydrogen peroxide, which is generated by a NADPH oxidase complex located in the apical membrane (26,34). In the presence of hydrogen peroxide, thyroperoxidase catalyses the iodination of tyrosyl residues in thyroglobulin (35). This process results in the formation of mono- and di-iodotyrosyls which are coupled by ether linkage to form T3 and T4 (see Fig. 1.2 for chemical structures) (26,34). The exact mechanisms of iodination and coupling are not yet known but they occur concurrently (see (35) for the current theory). Thyroid stimulating hormone (TSH) exerts a powerful stimulatory effect—enhancing iodination by two mechanisms. Firstly, by stimulating exocytosis of vesicles at the apical membrane, TSH raises levels of non-iodinated thyroglobulin, thyroperoxidase and NADPH oxidase (26). The second effect, increased hydrogen peroxide release, occurs at high TSH concentrations (> 10 mU/ml) and is Ca²⁺-dependent (34).

1.3.3 Thyroid hormone release and secretion

Iodinated thyroglobulin is taken up from the lumen into the follicle cells by endocytosis. The endosomes then fuse with lysosomes, containing proteases and glycolytic hydrolases (34), resulting in degradation of thyroglobulin to T3, T4 and mono- and di-iodotyrosines (MIT and DIT respectively). Under normal conditions, preferential proteolysis results in 3-fold higher production of T4 than T3 (34). Furthermore, serum T3 has a shorter half-life than T4 (1 and 7 days respectively), so that the proportion of circulating T4:T3 increases to 50-fold. In hypothyroid conditions however, TSH-stimulated 5'-deiodination of T4 in the thyroid results in a higher proportion of T3 being secreted (section 1.5.4). MIT and DIT are deiodinated in follicle cells by microsomal iodotyrosine dehalogenase and the iodide is recycled. The mechanism by which TH are transported across the basal membrane, into the bloodstream is not yet known, but may occur through passive diffusion (34).

1.3.4 Regulation of thyroid function: the hypothalamic-pituitary axis

In the adult, TH synthesis is largely under the control of TSH, a pituitary glycoprotein. TSH enhances iodination and also has widespread effects on thyrocyte metabolism (reviewed in (11)). Following the binding of TSH to its cell-surface receptor, rapid
increases in thyroidal glucose oxidation, and the synthesis of lipid, RNA and protein (particularly thyroglobulin) are observed (36). These effects are mediated via a cAMP second messenger system (37). TSH synthesis in the pituitary is stimulated by a hypothalamic hormone, thyrotrophin releasing hormone (TRH) and inhibited by TH. Furthermore TRH synthesis is also inhibited by TH. TH regulation of both TSH and TRH synthesis is primarily at the level of transcription (38), although post-transcriptional modification of TSHβ mRNA has also been demonstrated (39).

Development of this regulatory axis occurs prenatally in humans but largely postnatally in rats (21,25). TRH can be first detected in human fetal hypothalamus at 9 wg, but TSH cannot be detected in the pituitary until 12 wg. At around 18-22 wg however, there is a surge in pituitary and serum TSH levels, and a corresponding rise in fetal serum TH levels—suggesting that TSH-stimulated TH synthesis has developed by this time. Furthermore, TH levels continue to rise as TSH and TRH levels decline, indicating feedback regulation is also in place. In cases of congenital hypothyroidism however, TSH levels are not elevated in response to the low TH levels, suggesting the feedback mechanism has not yet fully matured (21,25). In the rat, TSH can be detected from 18 dg and drug induced fetal hypothyroidism results in increased TSH levels. Nevertheless, TH levels appear unresponsive to TSH until after birth when TRH, TSH and TH levels all rise correspondingly (21).

1.4 Thyroid hormone transport and cellular uptake

In humans, 99.97% of T4 and 99.7% of T3 in the bloodstream is bound reversibly to three plasma proteins, thyroid binding globulin (TBG), transthyretin (TTR) and albumin (23). The relevant characteristics of the TH binding proteins are summarised in Table 1.1.

Although TBG is present at the lowest concentration, it possesses the highest affinity for T3 and T4 and is therefore the most physiologically important protein in terms of serum TH binding. It is interesting to note that although TBG only binds TH, TTR also binds retinol binding protein (and consequently retinol) and albumin can bind many other compounds (41).
Table 1.1 Plasma concentration and binding characteristics of the TH binding proteins in humans

<table>
<thead>
<tr>
<th></th>
<th>TBG</th>
<th>TTR</th>
<th>Albumin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma concentration (mg/dl)</td>
<td>1-2</td>
<td>12-24</td>
<td>3000-4500</td>
</tr>
<tr>
<td>Ka for T4 (M⁻¹)</td>
<td>1 x 10¹⁰</td>
<td>7 x 10⁵ - 7 x 10⁷</td>
<td>5 x 10⁴ - 7 x 10⁶</td>
</tr>
<tr>
<td>Ka for T3 (M⁻¹)</td>
<td>5 x 10⁸</td>
<td>6 x 10⁵ - 1 x 10⁷</td>
<td>5 x 10³ - 1 x 10⁵</td>
</tr>
<tr>
<td>% T4 bound</td>
<td>60-80</td>
<td>10-20</td>
<td>5-15</td>
</tr>
<tr>
<td>% T3 bound</td>
<td>50-70</td>
<td>≤1</td>
<td>30-50</td>
</tr>
</tbody>
</table>

Affinity constants (Ka) were determined at pH 7.4, 37 °C. The range of affinities is due to co-operativity between multiple TH binding sites. Adapted from (23,40)

Under normal circumstances none of the binding proteins, even TBG, are saturated. This gives the homeostatic mechanism considerable flexibility in that moderate changes in total TH levels do not significantly alter free TH levels. A similar situation exists with respect to the steroid hormones and their serum binding proteins, and has thus given rise to the free hormone hypothesis which suggests that only free hormone is available to target tissues—the hormone binding proteins acting as buffers, to mop up excess hormone or release it in times of deficit (42). There is some evidence that increases in binding protein levels affect the tissue distribution of hormone (43). Other possible functions of serum TH-binding proteins include increasing the biological half-life, by restricting access to degradative enzymes, and prevention of loss by excretion from the renal system.

Although the free hormone hypothesis is generally well accepted, the functions of binding proteins can also be served by intracellular TH binding sites (44). Indeed, it has been estimated that 37% of the extrathyroidal T4 pool is intracellular (45). Experiments with perfused liver have shown that in the absence of binding proteins, T4 is quickly extracted from serum by the periportal cells (44). It has been proposed therefore, that serum TH binding proteins ensure even distribution of TH within tissues (44).
None of the proposed functions adequately explain why, in the case of TH, three different hormone binding proteins exist. TBG is the only serum binding protein that exclusively binds TH, could it therefore have a specific TH-related function not ascribed to TTR or albumin? It is well known that TBG levels increase two to three-fold during pregnancy (due to decreased metabolism and increased hepatic synthesis) with a concomitant rise in TH levels (particularly T4) (46,47). It was originally suggested that the rise in TBG prevented maternal TH transfer across the placenta, thus protecting the fetus from maternal TH exposure (48). In contrast, it is now thought that maternal TH plays a role in fetal brain development (49-52) and that TBG specifically transports maternal TH to the feto-placental unit in humans, thereby facilitating transfer to the fetus (53). Placental TH transfer is considered in detail in section 1.7.1.4.

1.4.1 Thyroid hormone cellular uptake

Thyroid hormones are lipophilic and should therefore be capable of diffusing through cell membranes unaided, however there is evidence of cell membrane carrier-mediated uptake systems for T3 and T4 in a range of tissues (41,54-56), including the brain. The developing brain is a major target for TH (section 1.7), but there are two barriers hindering its entry: the blood-brain barrier (consisting of the endothelial lining of the brain capillaries) and the cerebrospinal fluid-brain barrier (consisting of the epithelium lining the vesicular surface of the choroid plexus) (57). Radioactive T4 is rapidly taken up into the brain after injection of an intravascular bolus (57), and free T3 and T4 concentrations in the cerebrospinal fluid (CSF) are several-fold higher than in serum (58). Carrier-mediated transport seems to be a plausible mechanism and recent studies suggest that TTR, synthesised in the choroid plexus, mediates the passage of TH from choroid plexus into the brain (59,60). It seems probable that TH also transverses the blood-brain barrier via endothelial cell surface receptors similar to those found in other cell types (see below for details) (57,61-63). There is some debate regarding the relative importance of these two barriers in TH uptake into the brain (see (63,64)) and studies using radiolabelled TH suggest that the blood-brain barrier route accounts for the majority of TH uptake into the CNS (62,65). Other radiolabelled-TH experiments however, suggest that the choroid plexus is an important site for T4, but not T3, uptake into the CSF (64)—in agreement with studies indicating that the brain is dependant on local deiodination of T4 for its T3 supply (section 1.5.2). Furthermore, TTR is expressed in the rat concomitantly with neural tube formation, long before the blood-brain barrier develops (59,66). Despite this, mice with a null mutation of the TTR gene are normal except for decreased serum TH and retinol levels (67). This casts doubt on the importance of TTR in TH transport into the brain, at least in mice, but further studies are needed to resolve this issue.
TH uptake systems have also been demonstrated in neuronal and glial cell cultures (56,68) although there are considerable discrepancies between studies. One report characterised an uptake system in neurons with a similar Km (310-400 nM) for T3 and T4, mediated either by two distinct carriers or a single carrier with separate binding sites (56). In contrast, an earlier study using a mouse neuroblastoma cell line reported Km values of 2.83 and 6.07 nM for T3 and T4 uptake, respectively, which was inhibited by amino acids (69). Another study, using rat brain synaptosomes, suggested that only T3 uptake is carrier-mediated via a Na⁺ co-transporter system (consisting of high affinity/low density and low affinity/high density components), whereas T4 enters by diffusion (70). In astroglia, the transport system is stereospecific and may be similarly linked to a Na⁺/H⁺ exchanger (68,71). T3 uptake in synaptosomes is inhibited by desmethylimipramine—a selective inhibitor of presynaptic noradrenaline uptake (72,73). This is noteworthy because there are reports in other tissues of cell surface T3 binding sites interacting with adrenergic agonists and antagonists (74-76). Despite the differences outlined above, it is clear that TH is almost certainly actively taken up into brain tissues. TH transport in the brain does not end at the cell membrane however, there is evidence that TH is directed away from the initial site of uptake via anterograde axonal transport (77) and synaptosomes have a higher TH concentration than anywhere else in the brain (78), which may be related to putative extranuclear TH actions (section 1.6.6).

1.5 Thyroid hormone metabolism

The extent and type of TH metabolism within a tissue or cell determines the local effect of TH. There are several pathways by which TH are metabolised (Fig. 1.2), with the most physiologically important being deiodination. This involves the removal of an iodine atom from either the tyrosyl or phenol rings, and accounts for 80% of metabolised T4 (23). Furthermore, metabolism of T4 via outer ring deiodination contributes 70-80% of T3 production in the body (the rest being synthesised in the thyroid) (79). There are at least three deiodinases and their properties are summarised in Table 1.2.

5'- (or outer ring) deiodinases removes iodine atoms from the 5'- (or the chemically equivalent 3'-) position on the phenolic ring whereas the 5- (or inner ring) deiodinase removes iodine atoms from the 5- or 3- position on the tyrosyl ring (Fig. 1.2).
Table 1.2 Properties of the thyroid hormone deiodinases

<table>
<thead>
<tr>
<th>Property</th>
<th>Type I 5'-deiodinase</th>
<th>Type II 5'-deiodinase</th>
<th>5-deiodinase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrate preference</td>
<td>rT3 &gt;&gt; T4 &gt;&gt; T3</td>
<td>T4 &gt; rT3 &gt;&gt; T3</td>
<td>T3 ≥ T4</td>
</tr>
<tr>
<td>Inhibition by:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>thiouracils</td>
<td>strong</td>
<td>weak</td>
<td>weak</td>
</tr>
<tr>
<td>iopanate</td>
<td>strong</td>
<td>strong</td>
<td>strong</td>
</tr>
<tr>
<td>iodoacetates</td>
<td>strong</td>
<td>moderate/weak</td>
<td>unknown</td>
</tr>
<tr>
<td>Response to:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hypothyroidism</td>
<td>increased activity</td>
<td>decreased activity</td>
<td>increased activity</td>
</tr>
<tr>
<td>hyperthyroidism</td>
<td>decreased activity</td>
<td>increased activity</td>
<td>decreased activity</td>
</tr>
</tbody>
</table>

Adapted from (79,80)

1.5.1 Type I 5'-deiodinase

Type I 5'-deiodinase (5'D-I) is highly abundant and widely distributed, with the liver, kidney and thyroid having the highest levels. 5'D-I differs from type II 5'-deiodinase (5'D-II) in that it is strongly inhibited by thiouracils, and 3,3',5'-triiodothyronine (reverse T3; rT3) is the preferred substrate over T4. It has been known for some time that 5'D-I is a selenoprotein (81), and selenocysteine may be an essential active site residue (82).

5'D-I has restricted distribution within organs, e.g. in the kidney it is only found in the proximal convoluted tubule and the outer portion of the renal cortex; in the liver it is found in the hepatocyte (83); while in the brain, activity is confined to glial cells (80,84). At the subcellular level, 5'D-I is a membrane bound protein (82). 5'D-I activity in fetal rat brain has not been directly estimated, but is probably present, albeit at lower levels than 5'D-II (85). Neonatal 5'D-I levels are generally very low, but show a gradual increase in activity throughout postnatal development, reaching adult levels in most brain regions. In the brain stem however, 5'D-I activity peaks at 30 postnatal days (pnd) whereas cerebellar activity peaks at 11-12 pnd (86). Depending on the substrate, 5'D-I can serve either as an activating (converting T4 to T3) or inactivating (converting T3 to di-iodothyronine) enzyme. In addition, under alkaline conditions or when TH has been sulphated (section 1.5.5), 5'D-I can also deiodinate the inner tyrosyl ring, thereby converting T4 to rT3 (i.e. 5-deiodinase).
Figure 1.2 Thyroid hormone metabolic pathways. For the sake of simplicity, nondeiodinative pathways are shown for thyroxine only.
activity) (79,87). Its main physiological role is thought to be the production of peripheral T3 (88), however since its preferred substrate is rT3 it is also responsible for the clearance of rT3 from the circulation and tissues (79,84).

1.5.2 Type II 5'-deiodinase

The placenta and brown adipose tissue have the highest levels of 5'D-II, with lower levels being found in the brain and pituitary. It has been reported that 5'D-II is located mainly in neurons (84), though others have demonstrated this enzyme in astroglial cell cultures (89-91). Indeed, it has recently been proposed that 5'D-II is expressed predominantly in astrocytes, where T4 is taken up, deiodinated to T3 and released for neuronal uptake (92). At the subcellular level, 5'D-II is a microsomal protein (88). Until recently it was thought that 5'D-II did not contain selenocysteine, however sequencing of rat and human 5'D-II cDNA have shown that they code for selenoproteins (93).

5'D-II displays a higher affinity for T4 than rT3 and its main physiological role is thought to be the generation of T3 from T4. It is therefore primarily an activating enzyme. In connection with this, there is evidence that 5'D-II regulates TH action in the developing brain; 5'D-II activity is expressed earlier than 5'D-I (79) and has been demonstrated in 17 dg rat fetal brain (85,94). There is a 4-fold increase in 5'D-II activity between 17 and 22 dg with the main increase occurring in the latter stages of pregnancy (19-22 dg) (85). After birth, 5'D-II activity declines to low levels by 4-5 pnd, increases steadily to term levels by 12 pnd, then gradually declines—remaining low in the adult (86,94). Both the fetal and neonatal peaks in 5'D-II activity occur during important periods of brain development, namely neuroblast and glial cell proliferation, respectively (94). Additionally, the fetal brain is wholly dependant on the local deiodination of T4 for its T3 supply (95,96). Thus 5'D-II plays a pivotal role in TH action in fetal brain (94,96).

1.5.3 5-Deiodinase

5-Deiodinase (5-D) has been demonstrated in the CNS, placenta, skin and pituitary (97). Like 5'D-II, it was thought that 5-D was not a selenoprotein but recent studies have indicated otherwise (93,98-100). 5-D activity is found glial cells (84) and may also be present in neurons (101). The enzyme plays an inactivating role, converting T4 to rT3 and T3 to di-iodothyronine. It may therefore serve to regulate T3 action and prevent excess tissue levels of T3 (101).
Placental 5-D has been implicated as a barrier to maternal TH transfer to the fetus (99,102). Human placental 5-D activity is 200 times higher than 5'D-II activity throughout gestation, and total 5-D activity increases with gestation, although activity decreases over time when expressed as a function of DNA or protein (103,104). Koopdonk-Kool et al. also suggest that human placental 5-D activity is negatively regulated by maternal serum T3 but not T4, and this is supported by a study in the rat showing placental 5-D to be less responsive to T4 than brain 5-D (105). However earlier studies showed no effect of thyroidectomy in rat (106), or hyperthyroidism in humans (104) on placental 5-D activity. It is difficult to envisage how maternal TH can cross the placenta without being deiodinated by the high levels of placental 5-D. A recent study however, has found that placenta is not the only tissue where 5-D activity during development appears to be higher than the other deiodinases (107). The authors suggest that in vitro 5-D activity determined under optimal conditions may not be representative of in vivo activity. It has been suggested that in humans, placental 5'D-II activity in the first trimester converts transferred T4 to T3 in the fetus, whereas during the second and third trimesters of pregnancy, higher 5-D activity is responsible for the provision of iodine to the fetus (53,108). Although this remains to be proved, amniotic and exocoelomic fluids contain iodothyronine metabolites (particularly rT3) generated from placental 5-D activity (109).

In contrast to the other deiodinases, brain 5-D activity is 3-6 fold higher in the fetus than in the adult (110,111). It has been detected at 14 dg, and by 19 dg activity has increased 1.5-fold (111). After birth, activity increases sharply during the first few days of postnatal life, peaking at 2-3 days, thereafter there is a decrease to adult levels by 20 pnd (86,110). This general pattern varies with brain region and assay substrate, for example cortical levels are low at birth and rise slowly, peaking at 20 or 30 pnd depending on whether T3 or T4 is the assay substrate (86).

1.5.4 Regulation of deiodinases

Deiodinases respond in unison to changes in circulating TH levels (Table 1.2), in order to keep both circulating and tissue T3 levels within narrow limits. Thus in hypothyroid rats, liver and kidney 5'D-I activity decreases to reduce catabolism of circulating TH. Thyroidal 5'D-I activity however, is under TSH control (112) and increases during hypothyroidism in order to elevate the proportion of T3 secreted. In the brain, 5'D-II plays a major role in maintaining TH homeostasis, indeed the fetal brain is more resistant than other tissues to decreased circulating TH levels. Although part of this response may involve increased brain T4 uptake, a major factor is the rapid increase in 5'D-II activity that occurs in this tissue (85,94,96). This is also true in neonatal brain albeit in a region-specific manner, with 5'D-
II activity increasing in the cerebral cortex, but not cerebellum, in response to hypothyroidism (113). The increase in brain 5'D-II activity occurs independently of protein synthesis and transcription, suggesting activation of pre-existing enzyme (114,115). Studying 5'D-II inactivation by TH analogues, it has been found that T4 and rT3 are more potent than T3 (116,117), which is of interest since these TH-analogues are traditionally considered to have no direct physiological role. A proposed mechanism of action is that T4/rT3 promote actin cytoskeleton polymerisation resulting in internalisation and inactivation of microsomal membrane-bound 5'D-II (84). Presumably, low TH levels reverse this process and allow more 5'D-II to reach the microsomal membrane. In contrast to liver and kidney, adult rat brain 5'D-I is insensitive to changes in TH levels (82,118), however whether this is also so in the developing rat brain is unknown. In fetal and adult brain 5-D activity is upregulated by increased TH levels and downregulated by decreased TH levels (110,119). This response occurs via a multistep protein synthetic pathway and is therefore not so rapid as for 5'D-II (120).

Factors other than TH levels also influence deiodinase activity including; cAMP, phorbol esters, fibroblast growth factor (89-91,121), protein disulphide isomerase activity (122) and selenium deficiency (79). Selenium deficiency results in a lowering of 5'D-I activity in all tissues except the thyroid (123,124), which seems to conserve selenium in times of deficit (82). In extreme selenium deficiency however, thyroidal 5'D-I activity is reduced leading to a reduction of circulating T3. Indeed, selenium regulates 5'D-I mRNA at both the transcriptional and post-transcriptional level (82). The effect of selenium deficiency on the other deiodinases is more complex, 5'D-II activity being reduced while 5-D activity remains relatively unaffected (82,123). Selenium deficiency may exacerbate the effects of iodine deficiency via the deiodinases (88). Selenium deficiency in adult rats decreased cortical 5'D-II activity and T3 levels in most brain regions, but increased or did not affect T4 levels (125). In contrast, combined iodine/selenium deficiency increased cortical 5'D-II activity and decreased T3 and T4 levels in most regions and was very similar in effect to iodine deficiency alone (125). This suggests that the effect of selenium deficiency in brain is secondary to iodine deficiency, particularly with regards to deiodinase activities and TH levels. There are other problems however, associated with selenium deficiency which may impact upon brain development and these are discussed in section 1.7.1.2.

1.5.5 Non-deiodinative thyroid hormone metabolism

Of the other pathways of TH metabolism, the most physiologically important is glucuronidation (Fig. 1.2). This increases the water solubility of TH, resulting in secretion of glucuronide conjugates in bile, and up to 30% of T4 may be metabolised in this way.
Actual bodily loss of T4 through bile excretion is only 15% however, since de-glucuronidation occurs, enabling reabsorption of T4 through the large intestine (126). Another important metabolic pathway is sulphation (Fig. 1.2) which produces biologically inactive analogues. Higher levels of sulphated TH have been found in fetal serum, amniotic fluid and cord blood than in maternal serum (127), indicating that sulphation may play an important role in TH homeostasis in early development (79, 128). This may be linked to the ability of 5\(^\prime\)-D-I to preferentially deiodinate TH sulphates (particularly T3-sulphate) at the 5\(^\prime\)-position of the tyrosyl ring, thus allowing 5\(^\prime\)-D-I to metabolise TH in a manner normally restricted to the 5-D (87). 5\(^\prime\)-D-I activity is low in fetal brain however, and T3-sulphate is poorly metabolised via this pathway (129). T3-sulphate may therefore be a source of T3, with sulphatases in tissues (notably cerebral cortex) producing active T3 when needed (128). Other pathways of TH metabolism include oxidative deamination (Fig. 1.2) which accounts for under 2% of T3 metabolism and leads to the formation of triac (or tetrac in the case of T4), and ether link cleavage (Fig. 1.2) to form DITs and MITs (from T4 and T3 respectively) as well as reactive iodine and oxygen radicals. This reaction may play a role in the immune response against bacterial infection, but is of minor importance in terms of TH metabolism (79).

1.6 Thyroid hormone action

It is generally thought that the primary mechanism of TH action is mediated via nuclear receptors (TRs) (130), however there is evidence to indicate extranuclear actions as well (section 1.6.6). TRs are members of a superfamily which includes receptors for vitamin D (VDR), 9-cis-retinoic acid (retinoid X receptors; RXR), retinoic acid (RAR) and steroid hormones, as well as orphan receptors—for which no ligands have been identified. Orphan receptors apart, members of the superfamily can be divided into two classes; A (e.g. steroid hormone receptors) and B (e.g. TR, RXR and RAR), depending on certain characteristics. For example, unliganded class A receptors exist in the cytosol complexed with heat shock proteins. Binding of ligand (steroid hormone) induces dissociation from the complex, allowing liganded receptor to enter the nucleus and bind to specific regions of DNA known as hormone response elements (HREs) (131). The HRE usually lies upstream of the gene to be regulated and binding of the hormone-receptor complex to the HRE induces a conformational change in the DNA structure (132) that activates transcription by recruitment of the basal transcription complex (see section 1.6.3.1). In contrast, class B receptors such as TRs, tend to be bound to their HREs even in the absence of ligand. Nevertheless, crystallographic studies of ligand-bound TR indicate that T3 binding produces the active receptor conformation (133), which can then induce transcription in a similar manner to the steroid hormone-receptor-HRE complex (134).
1.6.1 Superfamily receptor structure

All superfamily receptors have the same basic structure which can be subdivided into functional domains (Fig. 1.3). At the N-terminus of the receptor is the A/B domain which varies considerably in size and composition between members. This domain may determine target gene specificity for receptor isoforms which recognise the same HRE. The A/B domain also contains a sequence known as the AF1 region which exhibits ligand-independent transactivation *i.e.* it mediates transcription or repression (135) *via* interaction with components of the core transcription machinery (131).

The C domain is responsible for DNA binding, and as such is highly conserved throughout the family members. The functional elements of the domain are two zinc fingers, so called because of their distinctive shape formed by basic amino acids and four cysteine residues tetrahedrally coordinated with a zinc atom (136). DNA binding specificity is determined by several amino acids in the first zinc finger, known as the P box. All class B receptors have identical P boxes, binding preferentially to HREs containing the sequence AGGTCA (known as a half site), whereas Class A receptors have a slightly different P box which binds preferentially to half sites of sequence TGTTCT (131,137). The half site is so called because the receptors generally bind as dimers. The spacing and orientation of the two half sites of the HRE determine which receptors will bind and also influence receptor homo/heterodimerisation, as discussed in section 1.6.3.

The D domain is a small, variable region, also known as the hinge region due to its structural flexibility. Its function seems to be related to the conformational changes that occur in the receptor upon ligand binding. It is highly variable between receptor species (*i.e.* between TRs and RXRs) but is conserved between receptor isoforms (*i.e.* between TRβ1 and TRβ2) (138). The E domain is large and variable and comprises the ligand binding site. This domain is also implicated in dimerisation and DNA binding (138,139), and contains the AF2 region, which is required for ligand-dependant transactivation *via* interaction with co-activators/co-repressors (section 1.6.3.1) (140-142).

1.6.2 Thyroid hormone receptor isoforms

TRs exist in several isoforms (Fig. 1.3), categorised into two classes, termed α or β, depending on their chromosomal localisation. The TRα isoforms derive from a gene located on human chromosome 3 (143) and the TRβ isoforms from a gene on human chromosome 17 (17). Differential mRNA splicing produces at least three full length TRα
Figure 1.3 Schematic diagrams showing comparison of rat thyroid hormone receptor isoforms. Numbers inside boxes indicate percentage homology in amino acid sequences compared with Trα1, shaded regions indicate areas of no or little homology (identical shading indicates identical homology). Numbers outside boxes refer to the amino acid sequence.
isoforms (TRα1-3), two truncated TRα products (144) and two TRβ isoforms (TRβ1-2) (145,146). TRα1 is the only TRα isoform that binds T3, since it contains a critical portion of the E domain absent from TRα2 and α3 (145,147,148).

The function of the full length non-T3 binding isoforms is unknown but it has been suggested, on the basis of transient transfection studies, that they serve as dominant negative regulators of TRα1 and TRβ1 function, via competition at the level of the TR response element (TRE) (149). Indeed it has been suggested that the high levels of TRα2 expression seen in adult brain and spleen may account for the supposed TH-insensitivity of these tissues (in terms of O2 consumption). However there is much evidence to suggest the adult brain is TH-sensitive and the developing brain is unquestionably so, despite a high TRα2 abundance. Furthermore, TRα2 does not bind to DNA as avidly as TRα1, nor does it heterodimerise with TRα1 or other receptor superfamily members (e.g. RAR and RXR), at least not on DNA (it weakly dimersises with TRα1 in solution). (148). It is therefore difficult to see how TRα2 could have a dominant negative effect on transcription. Further studies of TRα2 function however, have shown that its poor DNA affinity is due to the phosphorylation of serine residues on its C-terminus (150). Dephosphorylation improves the interaction between TRα2 and DNA, and may therefore regulate its dominant negative activity (150). Additionally, TRα2 may exert its dominant negative effect independent of TRE-binding (151), possibly by titration of other factors such as co-activators (section 1.6.3.1). Indeed, recent evidence has pointed to a dual mechanism of action, at low levels of TRα2 inhibition occurs via TRE binding, whereas at higher TRα2 levels inhibition becomes TRE-independent (152).

A related gene product, derived from the noncoding strand of the TRα gene, is Rev-erbAα. The coding strand at the 3' end of this gene is complimentary to a TRα2 specific exon. Treatment of cultured cells with protein synthesis inhibitors increases Rev-erbAα mRNA expression, and the ratio of TRα1:α2 mRNA levels (153). The mechanism of action is unknown, but the effect is post-transcriptional, possibly due to Rev-erbAα mRNA hybridising to the TRα2 specific portion of the TRα gene transcript, thereby inhibiting TRα2 message splicing in favour of TRα1 (153). This mechanism may therefore function as a modulator of TRα2 expression—exerting a positive effect on TH action (153). Rev-erbAα binds T3 very weakly (154), nevertheless in vitro studies have shown that it can bind DNA as a monomer on a novel HRE, consisting of a 5 base pair AT-rich sequence adjacent to a TRE halfsite, or as a homodimer (but not heterodimer) on direct repeat TREs (section 1.6.3). In both cases Rev-ErbAα binding stimulated transcription
(154,155), however the level of stimulation was low (relative to TRs) and the functional significance of these results remains to be determined.

The truncated TRα isoforms derive from an internal promoter in intron 7 of the c-erbAα gene which produces shortened forms of TRα1 and α2 (termed TRΔα1 and TRΔα2 respectively) (144). These transcripts are expressed in chicken, mouse and human tissues, including brain, and their protein products repress transcriptional activity. TRΔα1 specifically inhibits TRα1, TRβ1 and RAR-activated transcription, whereas the repressive action of TRΔα2 is non-specific. Repression is thought to occur by prevention of coactivator binding (144) (section 1.6.3.1).

In contrast to the TRα isoforms, both TRβ isoforms bind T3—and differ only in the A/B domain (146). This difference arises from an additional upstream promoter which induces transcription of an alternative, larger, N-terminal exon to produce the TRβ2 isoform mRNA (156).

There is some question regarding the relevance of multiple T3-binding isoforms, particularly when they have similar affinities for T3; structural variations may hold some clues. All three T3-binding isoforms vary considerably in their A/B domains (Fig. 1.3). There is some indication that differences in the N-termini of TRβ isoforms allow TRβ2 to form more stable homodimers on palindromic response elements (section 1.6.3) (157). TRα isoforms also differ from TRβ isoforms in the second zinc finger of the DNA binding domain (156). These differences may influence their TRE preference, or their ability to stimulate transcription. Indeed, TRα1 homodimers exhibit two-fold higher maximal transcriptional activation than TRβ1 for several TH-responsive genes in vitro (158,159), this difference being independent of the TRE sequence despite the fact that the TRES determined overall transcriptional potency (159). There is some evidence suggesting T3 differentially regulates TR transcription (160-163), albeit not in adult rat brain (162,164). T3 upregulates TRβ1 mRNA expression in astrocytic cultures without any effect on TRα1 or α2 mRNA expression (165). Nuclear T3-binding activity did not increase however, casting doubt on the physiological significance of this result. Several studies indicate that TR isoforms may have specific roles during development (section 1.6.4), and there is evidence for an RXR-related protein in rat brain which heterodimerises specifically to TRβ1, and is only present during perinatal development (166).
1.6.3 Mechanisms of thyroid hormone receptor action

The mechanism of action of TRs has been intensively studied in the past decade, and as more details have been uncovered, the picture has become increasingly complex. TR can bind to TRE as a monomer, homodimer or heterodimer (usually, but not exclusively, with RXR). Furthermore, the TRE half sites may take the form of palindromes (PALS), inverted palindromes (IPs) or direct repeats (DRs) (167). TREs are distinguished from other Class B receptor HREs by the nucleotide spacing of the half-sites, however there are numerous half-site arrangements capable of binding several Class B receptors, TR included (Table 1.3). There is considerable potential therefore for interplay and competition between Class B receptors for these sites.

Table 1.3 Known half-site combinations for TR homo/heterodimers

<table>
<thead>
<tr>
<th>Homo/Heterodimer</th>
<th>Half-site orientation</th>
<th>Half-site spacing</th>
</tr>
</thead>
<tbody>
<tr>
<td>TR-TR</td>
<td>DR</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>PAL</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>IP</td>
<td>4-6</td>
</tr>
<tr>
<td>RXR-TR</td>
<td>DR</td>
<td>4 or 1-2</td>
</tr>
<tr>
<td></td>
<td>PAL</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>IP</td>
<td>5-6</td>
</tr>
<tr>
<td>TR-RAR</td>
<td>DR</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>PAL</td>
<td>0</td>
</tr>
<tr>
<td>TR-VDR</td>
<td>DR</td>
<td>3-4</td>
</tr>
<tr>
<td></td>
<td>IP</td>
<td>7</td>
</tr>
</tbody>
</table>

Adapted from (138,168-170).

Factors governing these interactions are not yet fully known, although TR binds with higher affinity to TREs when heterodimerised to RXR (138). Nevertheless, TR homodimers can form, especially in the absence of T3 when they tend to bind to DRs and IPs to repress basal transcription (section 1.6.3.1). On DRs, T3 causes dissociation of TR homodimers, thereby promoting TR-RXR heterodimerisation (170-172), whereas, 9-cis-retinoic acid generally promotes RXR homodimerisation (138,168). Furthermore, only T3 is able to bind to TR-RXR heterodimers on DRs, 9-cis-retinoic acid cannot (173). In contrast, both ligands are usually required for efficient transcriptional activation by heterodimers on IPs (168,174).
The commonly held notion of TREs consisting solely of two hexamer half-sites has been challenged recently by studies showing that TRα1 exhibits optimal binding as a monomer to an octamer TRE (175,176). This TRE is able to induce transcription as efficiently as the naturally occurring rat growth hormone TRE. When arranged as DRs with varying spacing, both TR homodimers and TR-RXR heterodimers can bind to the response elements and activate transcription regardless of nucleotide spacing. In contrast VDR and RAR homo/heterodimers (with RXR) exhibited no binding at all on these HREs (176). The authors proposed that the first two 5' bases of the sequence (TA in the case of TRs) determined which receptor species (i.e. RXR, RAR and VDR) will bind the octamers optimally (175). These studies also cast doubt on the role of RXR as universal mediator in receptor action as TR-RXR heterodimers were no more effective than TR mono/homodimers at binding or activating transcription (176).

1.6.3.1 TR-mediated activation and repression of transcription

For positively regulated genes (e.g. malic enzyme and rat growth hormone (177,178)), transcription is enhanced by T3. There is some evidence for direct interaction between TR and components of the transcriptional complex, particularly TATA binding protein (TBP) and TFIIB (131,135,142). Although TRs are essential for T3-dependant transcriptional activation, several other proteins (termed co-activators) are necessary for efficient transcriptional activation.

Trip1 (thyroid hormone receptor interacting protein) is a co-activator which is thought to interact with the TR AF1 transactivation domain, and TBP (179). Another co-activator, CREB binding protein (CBP), interacts with the TR AF2 domain and the RNA polymerase complex (141,180,181). CBP also promotes transcription through its intrinsic histone acetyltransferase activity (182,183), which destabilises the nucleosome, facilitating access of the transcriptional complex to the target gene. CBP can bind nuclear receptors on its own or via association with another family of co-activator proteins, the steroid receptor co-activators (SRC; also known as nuclear receptor co-activators or NCoA-1) (141,180,181,184,185). Other proteins thought to have TR co-activating functions include RIP140 (receptor interacting protein, 140 kDa) and RAP46 (receptor-associated protein, 46 kDa), however their modes of action are not fully understood (142). There is also evidence that TRs can stimulate gene expression without binding to TREs, furthermore this effect is not influenced by T3. The mechanism of action is unknown but may involve titration of transcriptional inhibitors or interaction with the transcriptional machinery (186,187).
Unliganded TRs tend to form homodimers and silence basal transcription on positively regulated genes. This silencing activity is mediated via co-repressors, which deacetylate histones—thereby preventing access of the transcriptional complex to the TRE. For example, NCoR (nuclear receptor co-repressor) associates with two other proteins, also known to be co-repressors (mSin3; mammalian suppressor interacting gene-3 and mRPD3; mammalian reduced potassium dependency gene-3), to inhibit TH-mediated transcription (188,189). Although mRPD3 alone has histone deacetylase activity, NCoR is required for binding to the E domain of unliganded TRs, and mSin3 links the other two components, thus all three components are required for effective transcriptional repression (188). SMRT (silencing mediator for retinoid and thyroid hormone receptors) is another co-repressor that binds to unliganded TRs (and RXRs/RARs) to silence basal transcription (190). Whether SMRT functions via association with mSin3 and mRPD3 is unknown, however as SMRT shares 48% homology with NCoR a similar mechanism of action is likely. In addition, the E domain of unliganded TRs can bind directly with TFIIB to repress basal transcription in vitro (135,140), and this is likely to be reinforced in vivo with co-repressors such as NCoR acting as bridging proteins between unliganded receptors and components of the transcriptional complex. Unliganded TRα1 homo/heterodimers also repress basal transcription of RAR palindromic response elements as well as TREs, thereby affecting retinoic acid-mediated responses (156,191,192). The concept of unliganded TR action has important implications for the effects of maternal hypothyroxinemia on early fetal brain development (section 1.8).

Negatively regulated genes (e.g. TRH and the TSH β- and α-subunits (146,163,177)) show repressed transcription in the presence of T3. The position and sequence of the TRE is important in determining whether transcription is positively or negatively regulated (163,193). Negative TREs tend to be close to the TATA box, suggesting an inhibitory mechanism involving steric interference of the basal transcription complex (146,170,194). Curiously, co-repressors have been shown to enhance transcription of these genes, although the mechanism of action is currently unknown (195,196).

1.6.3.2 Orphan receptors

Orphan receptors are capable, at least in vitro, of modulating TH action—the roles of TRα2 and Rev-ErbAα in this respect have already been mentioned. Another orphan receptor, COUP-TF (chicken ovalbumin upstream promoter transcription factor), represses TR-, RAR- and VDR-mediated transcription (197). COUP-TF does not bind to these receptors, but directly competes for the RXR heterodimer partner, thereby preventing formation of active TR-RXR etc. heterodimers. Furthermore COUP-TF also competes
with these receptors at the HRE level, and suppress basal transcriptional activity in a similar fashion to unliganded TRs (197). Which of these modes of action, if any, is dominant in vivo is unclear, but probably depends on the relative levels of receptors involved.

1.6.3.3 Phosphorylation of thyroid hormone receptors

Phosphorylation may affect TR action at many levels. Binding studies with salt-extracted TRs originally provided evidence that their affinity for T3 increases under phosphorylating conditions (198), and the non T3-binding TRα2 isoform also appears to be influenced by phosphorylation (section 1.6.2). More recently, several studies have focused on human TRβ1 (hTRβ1), which can be phosphorylated at multiple serine and threonine residues (199-202). Phosphorylated hTRβ1 exhibits increased transcriptional activation and DNA binding, but not T3-binding (199). There is inconsistent data regarding TR phosphorylation and dimerisation. Bhat et al. found that phosphorylation of hTRβ1 promoted heterodimerisation (200), whereas another study reported that phosphorylation increased hTRβ1 homodimer binding to DNA (201). A further role for phosphorylation may be to increase hTRβ1 stability but this effect was only seen in COS-1 cell line, and not in GH3 or neuro-2a cells (202), therefore the physiological relevance is hard to determine. Studies with TRα1 have been largely limited to the chicken receptor which can be phosphorylated by cAMP-dependent protein kinase, caesin kinase II and protein kinase C (203,204). Importantly, the serine residue phosphorylated by caesin kinase II is conserved in rat and human TRα1 and α2 (203). The functional significance remains to be determined, but mutation of this serine residue to alanine does not affect the affinity of TRs for ligand (203). More recently, inhibition of protein phosphatases in CV-1 cells, transiently transfected with either rat TRα1 or TRβ1 resulted in increased transcriptional activity, particularly by TRα1 (205). However, effects of phosphorylation on auxiliary receptor proteins and/or co-activators could not be ruled out (205).

1.6.4 TRs and brain development

T3 binding studies with salt-extracted receptor preparations initially showed that TRs could first be detected in whole rat fetuses at 13 dg and in the brain at 14 dg (206,207). TR levels in brain increased nearly 3-fold by 17 dg, then remained constant until after birth (207). In humans, TRs were detected from at least 10 wg (earliest age studied), and receptor number increased 10-fold by 16 wg (208,209). More recent work using molecular biological and immunocytochemical techniques has shed some light on the developmental profiles of the TR isoforms. A study of rat brain TR mRNA ontogeny by Northern hybridisation analysis
found that TRα1 mRNA expression correlated strongly with TRα2, the mRNA levels of the latter being an order of magnitude greater (210). Concentrations of both isoforms doubled between 19 dg and birth, and again by 4 pnd. From 10 pnd, levels declined to adult values—which were slightly higher than those at 19 dg. Levels of TRβ1 mRNA were less than 10% those of TRα1 at 19 dg, but increased 40-fold to reach adult levels by 10 pnd (210). Other studies have reported similar findings (164,211,212), although there are inconsistencies regarding the magnitude of TRα1 increase and the prenatal ontogeny of TRα2 mRNA, with evidence for (211) and against (164,212) co-ordinate expression with TRα1. Rev-erbAα mRNA is not detectable in brain, at least by RNase protection assay, until after 5 pnd (212).

In situ hybridisation, though only semi-quantitative, has been used to determine regional expression of TR mRNA isoforms during development (213,214). At 11.5 dg (the earliest age studied) TRα1 and α2 mRNA are expressed in the neural tube, and by 12.5 dg in all five major subdivisions of the brain (214). TRβ1 mRNA is also detectable at 12.5 dg, but only within ventral areas of hind brain and diencephalon (214). Both TRα1 and α2 mRNAs are widely expressed (with TRα2 mRNA levels generally higher) at subsequent stages of development and reach maximal levels by the end of the first postnatal week, before declining to adult values. TRβ1 mRNA was only expressed in areas where TRα isoforms were already present and, during fetal life, moderate to high levels are seen only in caudate putamen and hippocampus. TRβ1 levels increase markedly during the first postnatal week however, and peak one to two weeks later than TRα1 (213,214). TRβ2 mRNA expression is first detectable at 13.5 dg, but only in the pituitary. Low levels of expression can also be seen in striatum and hippocampus near term, and in neocortex postnatally (161,214). During postnatal development, several regional differences in levels of isoform expression become apparent. For example, TRβ1 mRNA is most abundant in layers 2-3 of the frontal cortex whereas TRα1 mRNA expression is greatest in layers 1-2 and 5-6 (213).

Accumulating evidence suggests that the various TR isoforms may be subject to differential translational/post-translational control since mRNA levels often show little correspondence to the amount of TR protein present, as measured by Western blotting or binding assays (210,211,215-217), however the existence of extranuclear TR-related proteins should also be considered (section 1.6.5). This difference is most marked in the case of TRβ2 whose mRNA expression in the adult rat appears to be mainly restricted to the pituitary (161), whereas immunocytochemical studies have demonstrated that TRβ2 protein is widely expressed throughout adult rat brain (218) and other tissues (217). It is important therefore...
to assess TR protein levels before conclusions can be made regarding possible functions/roles. T3-binding following immunoprecipitation indicates that in 19 dg fetal brain, TRα1 accounts for over 90% of the nuclear T3-binding activity whereas TRβ1 T3-binding activity is negligible (217). This is in contrast to a previous report which suggested that TRβ1 may account for up to 5% of nuclear T3-binding (215). Nevertheless the consensus is that at 19 dg TRα1 is more likely to be involved in mediating TH action in fetal brain than TRβ1. In general agreement with these results, a semi-quantitative immunohistochemical study showed TRα1 to be the predominant TRα isoform from 14 - 17 dg, however TRβ1 was also detectable, albeit at low to moderate levels, at 14 dg. TRβ1 was up-regulated between 17 and 21 dg, but remained fairly stable thereafter, up to 3 postnatal weeks. In contrast to mRNA studies, the temporal pattern of TRα2 protein expression was similar to that of TRβ1 rather than TRα1, however it should be noted that the TRα1 levels were not directly determined, but inferred from TRα2 and TRα-common antibody levels (219).

1.6.4.1 Cellular localisation

The cellular localisation of TRs is still uncertain, with some contradictory results still to be resolved. In vivo nuclear T3-binding studies in adult rats indicate TRs exist in both neurons and glia—although neuronal TRs are more numerous (220,221). During postnatal development however, the concentration of TRs in glial nuclei is nearly two-fold higher than that in neuronal nuclei at 5 pnd, and equivalent to neurons at 20 pnd (222). A developmentally regulated decline in oligodendroglial TR expression during postnatal development is supported by an immunocytochemical study in rat corpus callosum (223). TRs could only be detected in immature oligodendrocytes, suggesting a transient requirement for these receptors during myelogenesis (223). Another immunocytochemical study detected TRα1 (inferred from TRα2 and α-common staining), TRα2 and TRβ1 protein in neurons and glia (219).

Nuclear T3-binding using primary cell cultures suggested that TRs were present only in neurons (224). Nuclear T3-binding has, however, also been detected in cultured astrocytes, albeit at lower levels (165,225). Leonard et al. demonstrated TRα1 and TRβ1 (mRNA and protein) but not TRα2 in neuronal nuclei; and TRα2 (mRNA and protein), TRα1 (mRNA only) but not TRβ1 in astroglial nuclei (226). TR immunoreactivity has been demonstrated in secondary cultures of oligodendrocytic subtypes using a non-specific TR monoclonal antibody (223). As with the in vivo study, TR expression declined with time in culture and was most evident in immature oligodendrocytes (223). Other studies
looking at both TR mRNA and protein however, have shown that oligodendrocytic progenitor cells contain only TRα isoforms, whereas mature oligodendrocytes contain both TRα isoforms and TRβ1 (227-229). The presence of type 2 astrocytes in these studies may account for these discrepancies as they contain all four TR isoforms (in the form of TRα1, α2 and β1 mRNA and TRβ2 protein) (230).

Lebel and coworkers have demonstrated TRα1, α2 and β1 mRNAs in astroglial cultures from neonatal rat brain. No changes in mRNA levels were seen with time in culture, however TR protein levels were not determined. TRβ1 protein was detected in cultured astrocytes, but immunoreactivity was localised to perinuclear and cytoplasmic regions (165). In contrast, Carlson et al. could not detect any TR isoform protein using immunohistochemistry in type 1 astrocytes cultured from adult brain (229), although in a later study they demonstrated TRβ2 mRNA and protein (230). This study is also noteworthy because the investigation distinguished between type 1 (glial fibrillary acidic protein-positive; GFAP+ve, found in vivo and in vitro) and type 2 (GFAP-ve, only found in vitro) astrocytes (230). Up to 95% of the astrocytes Lebel et al. had studied were GFAP+ve, however (165) indicating that the different ages of the brains used for culture may have accounted for the differences in TR expression.

1.6.4.2 Isoform specific roles in development

Analysis by in situ hybridisation of TR isoform mRNA expression in different cortical layers in fetal rats has suggested that TRα1 is expressed most strongly in cells undergoing differentiation, whereas TRβ1 is expressed most strongly in proliferating cells. (214). A role for TRβ1 in modulating postnatal TH action in brain has been also proposed, based on the ontogeny of TR isoform mRNA and tissue T3 levels (210,231). Indeed, cerebellar Purkinje cells have been cited as a model of TRβ1 action in this respect (231) and TRβ1 seems to influence myelogenesis (see section 1.7.3). Furthermore, a novel nuclear protein (probably related to the RXR family) is transiently expressed during perinatal brain development and only heterodimerises to TRβ1 on the myelin basic protein TRE (166).

In contrast, in vitro studies have implicated TRα1 in cell proliferation. In chick optic lobe neuronal cell cultures, antisense oligonucleotides complementary to the TRα gene blocked neuroblast proliferation whereas oligonucleotides complementary to the TRβ sequence did not (232). Further evidence that TRα1 has a role in differentiation/proliferation comes from studies of rat PC12 pheochromocytoma cells and E18 immortalised brain neuroblasts, stably expressing TRα1 (233). Unliganded TRα1 inhibits nerve growth factor (NGF)
dependent neuronal differentiation—as a result NGF induces proliferation. In the presence
of T3 the cells differentiate and require NGF for survival (233). In contrast, over
expression of human TRβ1, but not rat TRα1, induced differentiation and prevented
proliferation of neuroblastoma cells in a T3-dependent manner (234), although no effects
were seen in the absence of T3. Interpretation of these in vitro studies is difficult however,
due to the uncertainties involved in extrapolating from immortalised cell lines (with non-
physiological TR levels) to the brain. Nevertheless, these studies, together with the
different spatial and temporal expression in brain, suggest distinct functions for the various
TR isoforms. TRα1 tends to predominate early in development, when cell proliferation is
active, whereas TRβ1 (and possibly TRβ2) is up-regulated concomitant with terminal
differentiation. This relationship has been demonstrated in neuronal (235), oligodendroglial
(227,228) and astrocytic (165) cultures. Their exact roles however remain unclear.

1.6.5 Thyroid hormone receptor knockouts

The recent development of mice carrying null mutations of one or more of the genes
encoding the TRs has provided a new perspective on the roles of these receptors (236). In
mice in which both TRα1 and TRα2 were not expressed, severe and progressive
hypothyroidism developed by 14 pnd and they died before 5 weeks (237). Body weight
was retarded, as was small intestine and bone growth, but no obvious abnormalities were
seen in the brain. T3 injection rescued these mice, returning T3 levels, and bone and
intestinal growth to normal, suggesting normal pituitary-thyroid axis function. Body
weight did not catch-up however and life span was still shortened (237). The rapid onset of
postnatal hypothyroidism suggests that TRα isoforms are responsible for upregulating TH
production, probably via TSH, during this period. In contrast, when just TRα1
expression was inactivated, mice showed normal growth and development but exhibited
bradycardia, reduced body temperature and mild hypothyroidism (238). Thus TRα2 seems
to have an important role in mouse development.

TRβ knockout mice show similar symptoms to humans suffering from resistance to thyroid
hormone (163). Mice were deaf and showed a blunted response to TSH but brain
development appeared normal and, unlike the combined TRα1/2 knockout, the life span
appeared normal.

The absence of crippling defects in brain development in these knockout mice may seem
puzzling, particularly when compared to the effects of fetal/neonatal TH deficits (section
1.7). One explanation is that the early effects of TH are mediated predominantly through
extranuclear binding sites (section 1.6.6), although the presence of nuclear TRs during fetal development argue against this. A more likely explanation is related to the repressive activities of the unliganded receptors. Thus, in TH-deficient brain, gene transcription is repressed by the unliganded receptors, whereas in the knockout mice, the lack of receptors per se will not repress transcription.

1.6.6 Extranuclear TH binding sites

Although it is accepted that TH effects are mediated by nuclear receptors there is considerable evidence for extranuclear binding sites in a wide variety of tissues (reviewed in (76)). The brain in particular has a number of potential extranuclear TH binding sites for which a variety of physiological functions have been proposed. TH binding sites in neuronal, glial and synaptosomal membranes have already been discussed with regards to their role in TH uptake (see section 1.4.1). Synaptosomal TH binding sites may have additional roles—amino acid and 2-deoxyglucose uptake into nerve endings of hypothyroid (but not euthyroid) mouse cerebro-cortical slices are T3-regulated (239), as is 2-deoxyglucose uptake into hypothyroid (but again, not euthyroid) adult rat synaptosomes (240). In euthyroid and hypothyroid adult rat synaptosomes, T3 augments the fast phase of depolarisation-induced Ca\(^{2+}\) uptake (241), and thereby, Ca\(^{2+}\)-dependent GABA (\(\gamma\)-amino butyric acid) release (242). T3 concentrated in pre-synaptic nerve terminals is itself released during depolarisation (78). Furthermore, the presence of synaptic T3 processing systems, provide evidence for a role for synaptic T3 in neurotransmission and the maintenance of plasticity in adult brain (78,241-243). Possibly related to this are observations that adult hypothyroidism increases the abundance of the inhibitory G-protein \(\alpha\) subunit (244), thereby increasing adenylate cyclase inhibition (245). Indeed, a G-protein-linked TH-binding site has been recently reported in chick embryos (246). The functional significance of these finding are, as yet, unclear but since many neurotransmitter receptors are coupled with G-proteins, TH may regulate synaptic transmission through these sites.

1.6.6.1 Cytosolic TH binding sites

The existence of cytosolic T3 binding proteins (CTBPs) have been known for over two decades, but they remain poorly characterised. Initial studies in postnatal rat brain described the existence of a single class of low affinity (\(K_a = 0.07-5.0 \times 10^8 \text{ M}^{-1}\)) cytosolic T3 (247-250) and T4 (251) binding sites which were expressed in a region-specific manner and developmentally regulated, although many inconsistencies were apparent between studies (252). It was thought the binding sites served either as a reservoir of TRs (247-249) or to regulate the cytosolic free TH concentration—analogous to serum TH binding proteins.
Other studies of cytosolic iodothyronine binding in cell culture models have suggested that the CTBPs are predominantly neuronal (253,254) with high and low affinity sites which bind T4 and T3 (the preferred ligand). Low levels of cytosolic binding were also present in glial cell subcultures (253,254).

More recently, a high affinity ($K_a = 1.56 \times 10^9 \text{ M}^{-1}$) 58 kDa CTBP has been purified from adult rat cerebral cortex (255) which may be identical to an extensively characterised 58 kDa kidney CTBP (256-258). Indeed, the ontogeny of this CTBP has also been reported in brain where cytosolic T3 binding activity ($K_a \approx 1.1 \times 10^9 \text{ M}^{-1}$) was detectable at 17 dg (the earliest age point studied) (259). The number of sites peaked around the time of birth in cerebrum and cerebellum, before declining concomitant with the main phase of glial proliferation. The function of the 58 kDa brain CTBP is unknown, however NADPH seems to regulate the number, but not affinity of CTBP sites (258-260). In kidney at least, NADPH-activated CTBP appears to retain T3 within the cytosol, whereas the NADP-activated form may enhance T3 delivery to the nucleus (257,260), and/or mitochondria (258). The physiological relevance of these observations is however questionable, since the intracellular NADPH concentration is such that nuclear translocation of T3 should be impossible (255). It may be that other compounds, such as thioredoxin are involved in the regulation of CTBP activity (255).

Similarly, a CTBP has been demonstrated in the JEG-3 and COS-1 cell lines which also modulates the availability of cytoplasmic T3 to the nucleus (261). This protein has been identified as a subunit (also 58 kDa) of the M2 subtype of tetrameric pyruvate kinase. Only the monomeric form binds T3 and tetramer-monomer interconversion is regulated by the cellular glucose level via fructose 1,6-bisphosphate. Thus, as cellular glucose levels decrease, CTBP activity increases. Furthermore in the absence of glucose, TRβ1 transcriptional activity decreased, suggesting that in times of energy deprivation non-essential protein synthesis is restricted (261). It remains to be determined however whether this CTBP is expressed in brain.

1.6.6.2 Mitochondrial TH binding sites

Mitochondria have long been viewed as a possible site of extranuclear TH action, since the effect of TH on oxygen consumption occurs too promptly for it to be mediated by changes in gene expression (262,263). There are several reports of high affinity, low capacity T3 binding sites ($ca. 150 \text{ kDa in size, } K_a \approx 10^{11} \text{ M}^{-1}$) located mainly in the inner mitochondrial membrane (the site of oxidative phosphorylation) (264-266). Curiously,
these sites are not seen in brains from rats older than 12 pnd (265). Furthermore, direct effects of T3 upon oxygen consumption in various tissues, including brain, only occur at supra-physiological doses of TH (262,267) and/or in preparations from hypothyroid rats (262-264,268). The physiological significance of such effects therefore remain controversial (reviewed in (269)).

A recent study has indicated that TH influences phospholipid composition and membrane fluidity of adult rat brain mitochondria, however the physiological consequences remain to be determined (270). An in vitro study using liver homogenate reported that 3,5-diiodothyronine (3,5-T2) binds to mitochondrial sites with higher affinity than T3 (271). 3,5-T2 increased mitochondrial respiration, however reliable binding data could not be obtained from isolated mitochondria—suggesting the involvement of cytoplasmic factors in the process (271). Also in rat liver mitochondria, two groups have independently demonstrated the presence of TR-related proteins using immunochemical approaches (272,273). The earlier study reported the presence of TRα- and TRβ-related proteins (48 and 55 kDa, respectively) in the mitochondria, with a strong signal for the latter protein. No TR mRNA could be detected in the mitochondria, suggesting that the TRs are transported from the nucleus to the mitochondrion (272). The other group detected only a TRα1-related protein (at 43 kDa) and an unidentified 28 kDa protein (273). The TRα1-related protein could not be detected in mitochondria from adult brain—possibly related to the lack of mitochondrial T3-binding in this tissue—but developing brain was not studied (273). Inconsistencies in the isoform identity of these proteins between the reports may have been due to the different antibodies used.

The TRα1-related protein bound to TREs, as well as to a sequence within the mitochondrial promoter, suggesting a possible role as a regulator of mitochondrial gene transcription (273). It is of interest, therefore, that the expression of certain mitochondrial-encoded genes in brain are TH-responsive (274,275). Combined feto-maternal hypothyroidism resulted in decreased steady state levels of brain 16S rRNA at 19 dg. Furthermore, reductions in 12S rRNA and cytochrome c oxidase subunit III (COX-III) mRNA levels were seen immediately after birth and at 15 pnd, respectively. T3 replacement therapy normalised mitochondrial gene expression but only 48 hours after administration. The mRNA levels of the COX subunits encoded by the nucleus (types IV and VIc) were also decreased postnatally, but normalised by 30 pnd, even though COX-III expression remained depressed. As a result, COX enzyme activity was reduced, at least at 15 pnd. Mitochondrial morphology was also disturbed, possibly as a result of the observed changes in gene expression, with a decreases in transmembrane potential and inner membrane cristae organisation (276). A further study has identified another mitochondrial gene,
NADH dehydrogenase subunit 3, as being TH-sensitive in postnatal brain (but not in the adult) (275). Postnatal hypothyroidism depressed the steady state mRNA levels in cerebral cortex and hippocampus but not striatum.

1.6.6.3 TH extranuclear action and protein disulphide isomerase

T3 has also been reported to bind to a membrane-associated protein, subsequently identified as protein disulphide isomerase (PDI) (277-279). This enzyme is associated with the RER and has multiple functions, including the post-translational modification of secretory proteins (280). It was initially thought that PDI may be related to 5'D-II (281), however this was subsequently shown to be incorrect (88). In glial cells, most PDI is associated with RER, though in the absence of TH ca. 25% is found in the cytosol. T4 induces relocation of a 55-kDA subunit of PDI from the cytosol to the F-actin microfilaments (282). The functional significance of this effect is not known but may be related to the proposed role of T4 in regulating 5'D-II activity and TH-dependant actin polymerisation (282). The effectiveness of T3 in inducing this effect, however was not determined—if this is part of the regulation of 5'D-II, then T3 should be at least 100-fold less effective than T4.

1.7 Thyroid hormone and brain development

The classical notion of TH function is that of an age-dependant dual role i.e., modulating fetal/postnatal brain development, and regulating cellular metabolism in all adult tissues except in the brain, spleen and testis (283,284). The adult brain was traditionally considered insensitive since TH had no effect on oxygen consumption, or activities of malic enzyme and \(\alpha\)-glycerophosphate dehydrogenase (285). These criteria may not be relevant to the brain however, as no change in these parameters is seen in neonatal brain [Schapiro, 1966 #144; Schwartz, 1978 #143], despite the acknowledged role of TH in this organ.

The importance of TH for brain development is unquestionable and has been demonstrated in numerous clinical studies of iodine deficiency and congenital hypothyroidism in human offspring, and in studies using rat models of congenital hypothyroidism (reviewed in 286-289). There is however considerable debate regarding the period during which brain development is TH-sensitive.
1.7.1 Studies in humans

1.7.1.1 Iodine deficiency - prevalence and prophylaxis

Iodine deficiency is a primary cause of goitre and hypothyroidism. Nearly one third of the world's population (1.5 billion people) are at risk from IDDs and 12% exhibit goitre of varying severity (290). The problem is most severe in developing countries such as China and the Republic of Guinea (291, 292), however iodine deficiency is also prevalent in many areas of Europe (290, 293). Despite a growing awareness of this problem, there is considerable controversy regarding the most effective and safe remedial action. Iodine prophylaxis by distribution of iodised salt or bread is relatively inexpensive but rather non-specific. For instance, even after 1-2 years of salt iodination, mild iodine deficiency was still prevalent in pregnant women in one iodine deficient endemia (294). Indeed, poorly supervised iodine replacement can produce pockets of iodine excess and the attendant problems of Wolff-Chaikoff syndrome in which thyroglobulin iodination is inhibited (295), and iodine-induced thyrotoxicosis. A sudden influx of iodine to a hypothyroid patient can also induce necrosis of the thyroid gland—especially when selenium is also deficient (296). Although these problems do not occur with T4 replacement therapy, cost and logistics prevent the widespread use of this option in developing countries. Thus iodine is the treatment of choice but it must be tightly controlled to ensure the right amount is going to the right people. The problem is not wholly overt, since subclinical iodine deficiency may only become apparent during pregnancy, when the increased demand for iodine can induce goitre—with serious consequences for the offspring (46, 297)

1.7.1.2 Cretinism

The effects of iodine deficiency on brain development are manifested in their most extreme form as cretinism. Cretinism has been classified into two related forms, neurological and myxoedematous. Neurological cretinism is the more common form and presents as spastic diplegia, clonus, deaf-mutism and severe mental retardation. Myxoedematous cretinism results in gait disorders, impaired motor coordination, partial deafness, speech defects and reduced IQ (4). Although it was originally thought that they represented distinct syndromes, it is generally accepted that they are two extremes of a continuous spectrum. There are several factors that determine whether the form of cretinism will tend towards neurological or myxoedematous, although the precise aetiology is unknown. Neurological cretinism is thought to result from maternal TH deficit during early fetal development (4, 298-300). Iodine/TH replacement therapy given after the second half of pregnancy fails
to correct the damage—indeed, neurological cretins have normal thyroid function (4,298,299,301). Myxoedematous cretinism, on the other hand, seems to arise from iodine deficiency and thus hypothyroidism, during later development. Thus myxoedematous cretins have less severe mental retardation and are overtly hypothyroid—the thyroid gland commonly being atrophied (4,302,303). Thus myxoedematous cretinism shares many pathological features with congenital hypothyroidism (section 1.7.1.4) and TH/iodine replacement therapy produces some improvement (302). It has been suggested that many myxoedematous cretins display a similar degree of mental retardation to neurologic cretins, thus myxoedematous cretinism simply results from an extension of hypothyroidism from the early fetal period into postnatal life (300). It is difficult however, to reconcile the conclusions of this study with the seminal studies discussed above, and it cannot be ruled out that the myxoedematous cretins studied actually had combined neurological/myxoedematous cretinism.

Selenium deficiency has been implicated in the pathogenesis of myxoedematous cretinism (304). In many areas of joint iodine and selenium deficiency, myxoedematous cretinism is more prevalent than the neurological form (303,304). Selenium deficiency impairs hydrogen peroxide catabolism, since the activity of the selenoprotein, glutathione peroxidase, is reduced. High levels of hydrogen peroxide within the thyroid gland may cause necrosis and atrophy (303). Alternatively, thyroid autoimmune disease may be responsible for the atrophy, since IgG antibodies which inhibit TSH-induced DNA synthesis have been found in 86% of patients with myxoedematous cretinism, but were absent in euthyroid controls (305). Furthermore, the serum concentration of these antibodies correlated with the degree of thyroid gland atrophy (305). The involvement of thyroid autoimmune disease would also explain why myxoedematous cretins are apparently spared the effects of hypothyroidism until later in development. The factors inducing the presence of these antibodies in myxoedematous cretins, however are not known (305).

1.7.1.3 Maternal thyroid hormone and fetal brain development

The studies regarding the aetiology of neurological cretinism discussed above, gave the first indications that TH was important for early fetal brain development. In the light of the then prevalent belief that the placenta was impermeable to maternal TH, the results were interpreted as suggesting a role for elemental iodine in early fetal brain development (299). No evidence to support such a role could be found however, and further studies demonstrated a direct correlation between maternal T4 levels during pregnancy and parameters of cognitive/motor function in the offspring (306). More recently, Xue-Yi et.al. confirmed that only iodine replacement therapy during the first and second trimesters
protected fetal brain development in an iodine deficient endemia (307). Iodine replacement
given in the last trimester improved brain growth but not neurological outcome.
Furthermore, schoolchildren in an area of Italy with mild iodine deficiency born prior to an
iodine prophylaxis program, showed no signs of deficient cognitive performance but
exhibited slower reaction times compared to controls—suggestive of blunted motor
performance (308).

Thyroid hypofunction during pregnancy is not limited to the iodine deficient endemias. It
has been estimated that thyroid deficiency exists in 0.3 - 3% of iodine sufficient pregnant
women (309,310), and 20% of pregnant hypothyroid women are asymptomatic (47). In
clinical studies of these cases, there is a clear correlation between maternal
hypothyroxinemia and impaired cognitive and motor performance in offspring when
adequate TH replacement therapy was not administered during pregnancy (309,311). In
contrast, another study found no evidence of deficient IQ in children born to
hypothyroxinemic mothers (treated with T4 supplementation from between 13 - 28 wg)
(312). The reason for this conflicting data is unknown, however the study was limited in
size and scope.

1.7.1.4 Placental transfer of thyroid hormone

Controversy over whether maternal TH can cross the placenta and reach the fetus has raged
since the 1950s. Until the late 1980s the general consensus was that TH did not cross the
placenta in either rat or man (21). Many of these studies were flawed however, either
investigating late in gestation—when TH transfer is minimal, or focusing on T3 (e.g. see
(313)) whose transfer is minor compared to T4. In addition, techniques for estimating TH
levels were relatively insensitive compared with those used today.

Partly as a result of Pharoahs studies, Ekins postulated the hypothesis that maternal TH is
necessary for fetal brain development, and furthermore, that TBG plays a vital role in the
delivery of TH (T4 in particular) to the placenta (53). This proposal was based largely on
mathematical modelling of the behaviour of free and protein-bound TH in the
microvasculature (108). These models indicate that an increase in hormone binding protein
concentration increases the concentration of free hormone at the capillary wall, thus
encouraging rather than preventing hormone release (108). In addition, binding proteins
capable of transporting more than one hormone can enhance the delivery of one hormone
relative to others in certain tissues, if the binding characteristics of the individual hormone
binding site vary. Thus in the case of TBG, T4 delivery may be increased relative to T3
when TBG levels rise (such as during pregnancy) (108). The placenta, with its unique
vascular anatomy is a possible site at which TBG enhances T4 delivery (53). This theory has been criticised for considering only unidirectional hormone flux into tissues (44). The contention being that intracellular metabolism of the hormone is much slower than movement either into or out of the cell, thus at steady-state hormone influx into the cell will be almost equalled by hormone efflux. Nevertheless, TH transfer from mother to fetus has been demonstrated in humans (51), and both T3 and T4 are detectable in human embryonic limbs at 6-8 wg and brain at 9 wg (314).

Very little is known regarding the mechanism and regulation of placental TH transfer. There are two barriers to such transfer; the first is mechanical—placental membranes being relatively impermeable to iodothyronines; the second is that high levels of 5-D activity are expressed in placenta (102) (see section 1.5.3). Human placental cell membranes exhibit two T3 binding sites, one of low affinity, high capacity (Kd = 18.5 μM; Bmax ~ 2.2 pmol/mg protein) and the other high affinity but low capacity (Kd = 2.0 nM; Bmax ~ 320 fmol/mg protein) (315). It is not known if they play any role in placental transfer of TH, although they could conceivably play a part in overcoming the mechanical barrier.

1.7.1.5 Congenital hypothyroidism

Additional evidence supporting TH placental transfer has come from studies of congenitally hypothyroid children (51). Congenital hypothyroidism affects 1 in 4000 live births and one of the common characteristics of this disease is that newborn infants appear asymptomatic at birth (316). Indeed if sufficient TH-replacement therapy is initiated by the second postnatal week, little or no permanent brain damage (as measured by IQ tests) is sustained (317,318). It was originally thought that this indicated that TH did not affect development until the postnatal period, however placental transfer of TH to congenitally hypothyroid fetuses is indicated by the presence of T4 in cord serum (51). Furthermore, in cases of untreated congenital hypothyroidism there is no evidence of the hearing loss and pyramidal/extrapyramidal defects associated with severe neurological cretinism, suggesting that maternal TH transfer provides at least partial protection from the prenatal effects of TH-deprivation in the congenitally hypothyroid fetus (317). Indeed, the higher incidences of cerebral palsy and mental retardation seen in premature infants may be related to the transient perinatal hypothyroxinemia consequent upon the premature loss of the maternal TH contribution, although secondary metabolic effects arising from premature birth cannot be ruled out (319-321).

Analysis of maternal TH function during early pregnancy has provided further, albeit circumstantial, evidence for a fetal requirement for maternal TH in humans. Serum TH
levels rise concomitant with the increase in TBG, occasionally to the point of transient hyperthyroidism (46), even though TSH levels fall in the same period. This suggests the existence of an alternative thyroid simulator, and human chorionic gonadotrophin (hCG) is considered the most likely candidate (322-324). Serum levels of this placental glycoprotein rise during the first trimester in correspondence with increasing TH and decreasing TSH levels (322). Furthermore hCG is structurally related to TSH, containing an identical α-subunit, and exists in various isoforms—some of which exhibit considerable thyrotrophic activity. Thyrotrophic activity is partly determined by the degree of glycosylation and acidity of the isoform and there is a higher proportion of acidic hCG isoforms in the first trimester compared with the second (322,323). It has been proposed therefore that hCG takes over the thyrotrophic role of TSH during early pregnancy, to ensure the increased serum TH level required by the fetus (322,323). This remains to be proved, but seems to be a plausible hypothesis.

1.7.2 Congenital hypothyroidism models and brain development

There is considerable evidence that significant transfer of maternal TH across the placenta occurs in a variety of mammals, including humans and rats (49-51,109,325-327). The long held erroneous assumption that TH did not cross the placenta has however meant that the effects of TH on brain development have largely been investigated in the perinatal period, using rat models in which hypothyroidism is induced in the late fetal/neonatal period. During this period (17 dg to birth) in the rat, cerebral neurogenesis and migration is virtually complete; neuronal differentiation, axonal outgrowth, dendritic ontogeny, synaptogenesis, gliogenesis and cerebellar neurogenesis are just commencing and myelinogenesis does not occur until 10 pnd (52,287,328).

Early studies with this model revealed gross morphological abnormalities, predominantly in the cerebellum but also in earlier developing brain regions, such as the forebrain. Cell number and size were reduced—indicating alterations in the rate of cell migration, proliferation and terminal differentiation (reviewed in (287,288,329)). Generally, the proliferative phase was extended, particularly in the extragranular layer of the cerebellum, and cell migration was impaired—probably as a result of delayed differentiation (329). Other studies revealed severe hypoplasia of the neuropil, particularly in the Purkinje cells of the cerebellum (287,329), indicative of a reduction in the length and number of dendrites and axons, and impaired interneuronal connectivity and function. Synaptogenesis was also delayed as a result of decreased interneuronal connectivity and this is reflected by changes in many aspects of neurotransmitter function, including neurotransmitter levels.
metabolic enzyme activities (330-336) and receptor number and affinity (337-341).

The morphological effects of postnatal TH deficiency are reflected by other biochemical alterations, from reductions in DNA, RNA and protein synthesis, to more specific effects on markers of neuronal terminal differentiation such as succinic acid dehydrogenase and glutamic acid dehydrogenase activities (52,288). Levels of sulphated glycosaminoglycans (extracellular matrix components with possible roles in cell differentiation) and gangliosides are also reduced in the cerebellum of postnatal rats after feto-matemal hypothyroidism (342,343). These changes however, are thought to be symptom of the general delay in cerebellar development, rather than a specific effect of TH on sulphated glycosaminoglycan expression (342,343). It has been suggested that changes in the cytoskeleton underlie many of the neuronal morphological abnormalities (289), since TH regulates microtubule assembly and stability, as well as the expression of two microtubule-associated proteins (MAP2 and tau) (52,288,329,344). In addition, the transition between immature and mature tau isoforms is delayed in neonatal hypothyroidism (289,344). Steady-state levels of tau and MAP2 mRNA are however, unaffected (345) so the mode of regulation remains unknown. More recently, β- and γ-actin mRNA levels were shown to be TH-regulated (346). T3 directly increased their rates of transcription of these two cytoskeletal proteins, with β-actin showing a greater response than γ-actin. There is a lack of correlation between the rates of transcription and steady state mRNA levels however, which suggests post-transcriptional regulation may occur (346). β- and γ-actin protein expression is upregulated in the normal rat concomitant with the the main phase of synaptogenesis (346), suggesting a role for these cytoskeletal proteins in this process. Decreased levels arising from neonatal hypothyroidism may therefore partly account for the defects in synaptogenesis mentioned above.

Neurotransmission is severely affected by the retardation in myelinogenesis evident in congenital hypothyroid rat models (329). Myelin synthetic enzymes activities are reduced and myelin synthesis is delayed, albeit transiently. Furthermore, several genes encoding myelin-associated proteins exhibit decreased expression in oligodendrocytes from postnatal hypothyroid rats (345,347). Of these genes, myelin basic protein (MBP) and myelin-associated glycoprotein (MAG) have been extensively studied. The promoter region of the MBP gene contains a TRE (348,349) and this, together with the presence TR isoforms (particularly TRβ1) in oligodendrocytes during myelination, suggests that MBP transcription is directly regulated by T3 (223,227,229). Indeed, TRβ1 elicits a stronger response from the MBP TRE than TRα1 (349). T3 may also influence MBP mRNA stability however (350,351). Postnatal hypothyroidism also delays MAG mRNA and protein expression (352), particularly in cerebral cortex and hippocampus. The lack of T3-
induced increase in MAG mRNA transcription in nuclear run-on assays indicates that TH acts post-transcriptionally, possibly via regulation of mRNA stability (352). This conclusion should be viewed with caution however, since in oligodendrocyte cultures, T3 increases mRNA levels of MAG and two other myelin-associated genes, 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase) and proteolipid protein (PLP) (351); MAG and CNPase mRNA were affected via direct transcriptional mechanisms whereas PLP mRNA was affected via post-transcriptional regulation—probably increased mRNA stability (350,351). Together these studies indicate that TH influences myelin gene expression at multiple levels.

More recently, a comprehensive study of all four major myelin protein genes has shown that neonatal hypothyroidism results in a transient decrease in their mRNA levels—all to a similar degree (353). Regional variation was evident however, with rostral regions such as cortex and striatum more severely affected, in terms of duration and degree of deficit, than caudal regions. Myelination spreads in a caudal to rostral manner, thus it is the regions that undergo myelination later that are affected most. As T3 can potentially affect the myelin genes by several different mechanisms, it is surprising that the study shows such a uniform response to neonatal hypothyroidism. TH may act therefore mainly in an indirect manner, by promoting the differentiation of oligodendrocytes which in turn express the myelin genes (353). This also explains the transient nature of the reduction in mRNA expression; oligodendrocytes continue to differentiate, albeit at a slower rate, even at subnormal T3 levels. The other mechanisms of TH action such as alteration of gene transcription and mRNA stability are thought to have supplementary roles.

Astrocytes are also sensitive to TH during development (sections 1.6.4.1 and 1.7.3), and as indicated by studies showing abnormal astrogial differentiation in hypothyroid postnatal rats (354). It has been suggested that, like neurons, these effects are primarily due to cytoskeletal deficiencies, arising from a lack of TH (355). Postnatal hypothyroidism results in decreased GFAP expression in the cerebellum and hippocampus (355) and neonatal TH supplementation induces premature appearance of GFAP-immunoreactive astrocytes in basal forebrain and hippocampus (356). GFAP is required in astrocytes for process outgrowth and morphogenesis (355), thus the effects of TH on astrocytic development may be due to a reduction in GFAP expression. Other astroglial markers however, such as vimentin, S100 protein and glutamine synthetase are also TH-sensitive, and may therefore also be involved (355-358).

The consequence of the structural and biochemical compromise seen in developing hypothyroid rats is abnormal behaviour. Neonatal hypothyroid rats have impaired learning
ability, motor coordination and exploratory performance (52). Nevertheless despite our knowledge of TH action on gene transcription, it is not yet clear how the lack of TH results in the functional deficits, with the possible exception of myelin synthesis—even here much remains to be elucidated. Several candidate genes have been proposed which may be primary targets for transcriptional regulation by T3 (reviewed in (274,275,289,359,360)). One such candidate is RC3 (rat cortex clone 3 or neurogranin) since combined maternal/fetal/neonatal hypothyroidism reduces RC3 mRNA and protein expression from 5 pnd onwards in a region-dependent manner, with the striatum being most severely affected (345). The disturbances in RC3 expression are most prominent after 10 pnd (360). Despite intensive study however, a TRE has not been found in the flanking sequence of the RC3 gene, although retinoic acid and steroid HREs were identified (361). RC3 is a protein kinase C substrate, localised in dendritic spines and cell bodies in postnatal forebrain neurons, and implicated in postsynaptic calcium signalling and long-term potentiation (360). It is possible therefore that the decreased RC3 levels in hypothyroid postnatal animals may be related to their decreased cognitive performance.

Expression of immediate early gene NGFI-A (NGF-induced gene A) mRNA and protein are both T3-sensitive in neonatal rat brain, levels being reduced in an age- and region-dependent manner in hypothyroid neonates (362,363). A sequence upstream of the promoter region in the mouse gene may represent a TRE (363) but this awaits confirmation. NGFI-A is also induced by a variety of growth factors (particularly NGF) (364,365), which may themselves be TH-sensitive. The putative function of NGFI-A as a transcriptional activator in the regulation of cell proliferation, differentiation and synaptic plasticity (366-368) suggests that its altered expression in congenital hypothyroid models may, at least partly, underlie some of the effects discussed. Postnatal hypothyroidism also increases the steady state level of c-jun mRNA by an uncharacterised mechanism (345).

Several neurotrophins and, in some cases, their receptors show altered gene expression in rat brain as a consequence of neonatal hypo/hyperthyroidism. For example, TH transiently increased NGF mRNA levels in cultured postnatal cerebellar neuroblasts (369), and fetal/postnatal hypothyroidism reduced NGF mRNA levels in cortex and hippocampus, but not striatum (370). Curiously, the latter study showed that mRNA levels of the high affinity NGF receptor (trkA) were reduced in striatum, while low affinity NGF receptor (p75NGFR) mRNA was elevated in cerebellum (370). T3 replacement therapy normalised NGF mRNA levels in the hippocampus, but not cortex. Neurotrophin-3 (NT-3) mRNA levels were also affected by fetal/postnatal hypothyroidism, being increased in most regions except cerebellum, but gene expression of brain-derived neurotrophic factor, and tyrosine
kinase, trkB and trkC, receptors were unaffected (370). Other studies utilising perinatally induced hypothyroidism also describe increases in mRNA and protein levels of p75NGFR in postnatal rat cerebellum, while hyperthyroidism was associated with a transient decrease in p75NGFR levels (347). NGF protein levels were unaffected by hypothyroidism, but were transiently increased by fetal/postnatal hyperthyroidism (347). Unlike the other studies, neonatal hypothyroidism has also been seen to decrease NT-3 mRNA levels in cerebellum while T3 administration increased NT-3 gene expression in vivo and in vitro. The authors proposed a novel interaction between the granule neurone and the developing Purkinje cell, whereby T3 induces NT-3 in the granule cells, which in turn promotes Purkinje cell differentiation via interaction with trkC receptors. (371). The reasons for the discrepancies between the studies outlined above are unknown—as is the exact mechanism of TH-modulation. Nevertheless, neurotrophins are vital for normal brain development, and their regulation by TH may be important for normal development. For example, NGF has been shown to induce apoptosis in mature oligodendrocytes via the p75NGFR (372)—indicating another possible mechanism of TH regulation of myelination.

In agreement with the known TH sensitivity of cerebellar development, the accumulation of Purkinje cell protein-2 (PCP-2) mRNA, a Purkinje cell-specific gene is delayed in rat cerebellum by postnatal hypothyroidism (373). The promoter region of the PCP-2 gene contains TREs, suggesting direct transcriptional regulation (373). The function of PCP-2 is unknown but it is expressed coincident with Purkinje cell differentiation. Subtractive hybridisation followed by PCR amplification has revealed two further TH-responsive genes, expressed in the rat cerebellum at 10-15 pnd. These genes, synaptotagmin-related gene 1 (Srg1) and the rat homologue of the mouse hairless gene (hr), are rapidly induced by TH, suggesting direct transcriptional regulation, although the precise mechanism is uncharacterised (374). Srg1 is only expressed in the brain, whereas hr is also expressed in skin—but in a TH-independent manner. The functions of these gene products are not known, although Srg1 may be involved neurotransmitter release (374). Synaptotagmins so far studied (syt I and syt III) are not TH-sensitive however this may not be true of the other synaptotagmins (374). It is feasible that Srg1 expression may be disturbed by TH deficits, thus perturbing synaptogenesis. The hr protein contains a putative zinc finger domain, thus disturbance in its expression may alter the transcriptional regulation of multiple genes (374).

Other genes apparently under TH control include the myelin and mitochondrial genes discussed previously (sections 1.6.6.2 and 1.7.2). None of the genes so far identified have been shown to be capable of producing the the wide range of compromise seen in congenital hypothyroidism models. Few of the genes examined appear to have upstream
TREs and thus many changes in gene expression may simply reflect, for example, cell loss
or abnormal cellular differentiation. In other cases, post-transcriptional mechanisms, such
as altered mRNA stability, may be important.

1.7.3 Cell culture studies

In vitro studies of the effects of TH on rat neural cell cultures have provided many insights
into TH action. T3 induces stem cells to become lineage-restricted oligodendrocyte
progenitors, blocking their proliferation and enhancing differentiation into oligodendrocytes
(375-377). Indeed, earlier studies showed that T3-induced morphological changes in
oligodendrocytes, increasing process length and neurite number (228). Furthermore, T3
continues to promote maturation in postmitotic oligodendrocytes, i.e. after differentiation
is complete, by a distinct (and as yet unknown) mechanism to that of
antiproliferation/differentiation (377). The differentiating effects of T3 are not confined to
oligodendrocytes, but occur in glia in general (375,376). In astroglial cell cultures, T3
transforms flat cells into process bearing ones and increases GFAP mRNA expression
along with the secretion of several uncharacterised proteins (378,379). The effect of T3 on
astrocytic cultures varies with the site of origin and age of the astrocytes prior to culture.
For example, astrocytes cultured from newborn cerebellum are insensitive to T3 whereas
they are T3-responsive at 19 dg and 10 pnd; mesencephalic astrocytes, in contrast, are
affected at all ages studied (379). A more detailed study has identified two TH-sensitive
stages in astrocyte development (380). Lack of T3 delays the first stage, a change in
morphology from radial glia to flat polygonal, and prevents the second—differentiation into
mature process-bearing cells. Both stages are correlated with changes in the rate of actin
synthesis, which increases during transition to the first stage and declines prior to the
second stage. Thus, in hypothyroid astrocytic cultures, the initial increase in actin synthesis
is delayed, but a high rate is maintained thereafter (380). Other effects of TH on
cytoskeletal development have been studied in cell culture models, for example
physiological levels of T3 stimulated tubulin synthesis (independent of total protein
synthesis) in cultured rat fetal/neonatal whole brain. This effect was age-dependant, and
was not seen in cultures from rats 2 pnd and older (381).

T3 increases neuronal protein synthesis in general, as shown by leucine uptake and
incorporation into membrane-bound and cytosolic proteins (382). The effect of TH on
neurotransmitter turnover has also been extensively studied with neuronal cell culture
models. Several studies have demonstrated that T3 induces acetylcholinesterase (AChE)
and choline acetyltransferase (ChAT) activities, alone (383-385) or in concert with NGF
(386-388) or estrogen (389). T3 also induces muscarinic cholinergic receptor expression
and has morphological effects, increasing perikarya size, neurite outgrowth and branching (384,385,387). Several other growth/neurotrophic factors induce similar morphological changes but do not affect AChE and ChAT activities (387). Thus the T3-mediated induction of these enzyme activities is via a mechanism distinct from that causing the morphological effects. Similar findings have been reported for T3-induced tyrosine hydroxylase and monoamine oxidase activities in two neuroblastoma cell lines (391). A recent study using neuro-2a cells over-expressing TRβ1 implicated a cytosolic serine/threonine protein kinase pathway in the T3-dependent stabilisation of AChE mRNA (359), leading to an increase in AChE activity (234). It is not known however, if the other enzymes are regulated in a similar manner. As with astrocytes, the morphological effects of TH vary with the origin and age of neurons being cultured. In cultures of fetal mouse mesencephalic dopaminergic neurons, T3 increased perikarya size, but not neurite outgrowth or dopamine uptake (392). In contrast, fetal mouse hypothalamic cultured neurons exhibit increases in perikarya size, neurite outgrowth and dopamine uptake in response to T3 (393).

1.7.4 Maternal hypothyroxinemia models

In addition to the studies in humans, various biochemical studies in a number of mammals (particularly rats) indicate that maternal T4 is able to reach the fetus in biologically significant amounts in early pregnancy, before the onset of fetal thyroid function (49,50,325,326). TH is taken up and metabolised as early as 9-10 dg in whole rat fetuses, when their only possible source of TH is maternal (49). More specifically, T3 and T4 are detectable in rat fetal brain at 13 dg (326). There is also considerable evidence to suggest that systems are active in fetal brain to utilise the maternal TH. The role of 5′D-II in maintaining fetal brain T3 concentrations in the rat has been discussed (section 1.5.4), as has the expression of TRs in fetal brain (section 1.6.4). In order to investigate the role of maternal TH in fetal brain, rat models have been developed which exhibit deficient maternal thyroid function, without affecting fetal thyroid function. In the model used here rat dams (made hypothyroxinemic through partial thyroidectomy; Tx) were mated with normal males and the brain biochemistry of their offspring was compared with age-matched offspring from normal dams (section 2.1.2).

1.7.4.1 Adult progeny

Using this model, several biochemical changes in the brains of partial Tx dam adult progeny (7 months old) have been demonstrated, in comparison with controls (394-398). The changes are generally region-specific; usually (but not always) the cerebellum is
unaffected, consistent with the TH deficit occurring prior to the main phase of cerebellar
development (which occurs postnatally). Gross biochemical parameters such as protein and
DNA concentrations are reduced in midbrain, cerebral cortex and medulla (DNA only),
whilst the protein:DNA ratio is affected in every region studied. Leucine uptake is reduced
in cerebral cortex preparations \textit{in vitro} but not \textit{in vivo} (399). Lysosomal enzyme function
is impaired—particularly those activities with preferential neuronal localisation, \textit{e.g.} \(\beta\)-
galactosidase and aryl sulphatase (400) have reduced activities in all brain regions except
cerebellum (398). AChE activity (present in neurons and glia) is also reduced in most brain
regions, however specific neuronal markers are more selectively affected. For example,
ChAT activity (a specific cholinergic neuronal marker) is only increased in subcortex
whereas GABAergic and glutamatergic neuronal markers are unaffected (401).

Glial markers such as N-acetylgalactosaminidase (general glia marker) (402) and glutamine
synthetase (astrocytic marker) are unaffected. Several oligodenrogial-associated enzymes
(5’ nucleotidase, CNPase and oleate esterase) are however affected in a complex manner
(396, 401). These altered enzyme activities, together with the observed reductions in \(\beta\)-
galactosidase and aryl sulphatase activities, and deficits in brain galactocerebroside and
galactocerebrosido sulphate content, are strongly indicative of compromised myelination in
adult Tx dam progeny (398). Thus although myelination occurs largely postnatally in the
rat, it appears that the prenatal insult permanently affects oligodendrocyte function, perhaps
\textit{via} disruption of oligodendroglial progenitor cell development. Another group, using a
different rat dam model (consisting of a low iodine diet or methimazole administration)
found that the 21 dg progeny of Tx dams had a lower proportion of mature radial glial cell
fibres in the CA1 region of the hippocampus, although the number of immature glial cell
fibres was unchanged (403).

Adult Tx dam progeny also exhibit increased TR numbers in brain (as measured by nuclear
T3-binding assays) (397). The effect is region-specific, being seen only in cerebellum and
paleocortex. The adult progeny were euthyroid so it is not a homeostatic response to
reduced T3 levels, as has been postulated to explain the upregulation of TRs seen in
neonatal hypothyroid rat brain (404). Rather, it may indicate permanent disruption of the
TH regulatory/effectector systems.

Younger experimental progeny (2-3 months old) have been utilised in behavioural studies
that reinforce the biochemical data by indicating impairment of brain function (405, 406).
These tests show that Tx dam progeny are more cautious in their exploratory behaviour.
Sex differences are also apparent, female Tx dam progeny exhibit reduced locomotor
activity in an open field relative to controls, while males do not. When a novel object is introduced however, only male Tx dam progeny exhibit deficient locomotor behaviour. In addition, Tx dam progeny of both sexes show impaired learning behaviour as demonstrated in a food reward paradigm (406). Behavioural compromise has also been observed in adult progeny of radiothyroidectomised (RTx) dams. Male and female RTx dam progeny appear hyperactive and perform poorly in a Lashley alley maze compared with controls—indicative of impaired memory and learning abilities (407).

1.7.4.2 Pre- and postnatal progeny

In the rat model of maternal hypothyroxinemia, the fetal brain TH deficit is thought to be greatest just prior to the onset of fetal thyroid function, at 17.5 dg. Studies therefore usually include 15 or 16 dg as the initial age point. Progeny are then studied throughout late fetal and postnatal development to determine those effects that are corrected by fetal TH synthesis, and those that are irreversible. At 15 dg, Tx dam fetuses have lower body and brain weights relative to controls (408), but these parameters normalise by 19 dg and remain so through to adulthood (396,397,408). Tx dam fetuses also show decreased brain DNA content and protein:DNA ratios, indicative of reduced cell number and size, respectively; however cell density (as determined by DNA concentration) was increased (408). These parameters were all normal throughout postnatal development, at least from 5 to 14 days—in contrast to the 7 month Tx dam progeny. Other groups, using more severely thyroidectomised rat dam models (409-411), have demonstrated similar changes in fetal brain DNA content, DNA concentration and protein:DNA ratio during early development, along with body and brain weight reductions during late fetal life (407,409,411,412). The effects however, tend to be more prolonged—persisting well into the postnatal period (407,409,412). For example, transient reductions occur in whole brain galactolipid concentrations at term, and in ganglioside accumulation at 5 pnd, in RTx dams (410). The increased persistence of changes is probably due to the more severe degree of maternal hypothyroidism induced in these models. Overall the above results from the various models are indicative of delayed neuronal maturation in progeny.

Total protein concentrations in whole fetal brain and postnatal brain regions are unaffected in the partial Tx dam model (408), although isolated differences in cytosolic and membrane glycoprotein fractions have been observed postnatally (395,398). These findings are in contrast with data in 7-month-old progeny, which also exhibit widespread abnormalities in various enzyme activities not detected at earlier stages. These inconsistencies may be due to differences in the degree of maternal hypothyroxinemia induced, or they could indicate degenerative changes in the brain. Longitudinal studies are required to confirm this
possibility. Other groups also report slightly different results, Morreale de Escobar et al. observed protein concentrations to be normal at 20 dg but increased at 21 dg in experimental progeny (411), whereas Porterfield and Hendrich observed deficits in brain protein concentration at 22 dg and at 1, 5 and 60 pnd (52,407,409).

Discrepant results between the various models may partly be accounted for by the different methods used to induce hypothyroxinemia/hypothyroidism. In the model of Porterfield and Hendrich (409), dams were fed an iodine deficient diet for a week, then radiothyroidectomised by administration of 300 μCi $^{131}$I. The RTx dams were then placed on T4 replacement therapy for 2 weeks which was usually withdrawn the day after mating. Although RTx dams show similar circulating TH levels to those seen in the partial Tx model throughout pregnancy, the sudden withdrawal of T4 replacement during early pregnancy may disturb the dams metabolism. Morreale de Escobar et. al. surgically thyroidectomise rat dams prior to $^{131}$I administration (100 μCi $^{131}$I) and are only mated when serum T4 and T3 levels are below detection levels (411). Thus maternal metabolic compromise is likely in both models and may explain the more chronic changes in progeny brain protein and DNA levels observed. Porterfield et. al. reported numerous metabolic changes in mothers and pre/postnatal progeny, including growth hormone levels and homeostasis (412) and this may explain the disturbances in progeny thyroid function in this model (407). Indeed, severely hypothyroid dams are unable to accumulate fat stores during the first half of gestation, and consequently, their fetuses suffer from reduced nutrient supply during the second half of gestation and suckling (413,414). The partial Tx model used in this thesis attempts to minimise these effects by inducing hypothyroxinemia rather than hypothyroidism. Indeed, previous studies have shown that postnatal cerebellar growth and biochemical functions are mostly normal, arguing against metabolic compromise in the dam and, although body and brain weights are impaired during early fetal life, they become normal by 19 dg and remain so at term (408). Placental dysfunction is another possible confounding factor, however, there is no evidence of changes in placental weight or gross indices of placental cellular development, *e.g.* ornithine decarboxylase (ODC) activity (which is affected in fetal brain) (408). Changes in placental glucose transporter 1 (GLUT1) protein occur, but only near term (415) and, rat placenta shows only low levels of expression of TR mRNAs and nuclear T3-binding suggesting this organ is largely TH-insensitive (416). These observations indicate that placental dysfunction is also unlikely to contribute significantly to fetal brain damage in the partial Tx dam model.

Developing Tx dam progeny also show a range of altered brain biochemistry. ODC is the rate-limiting polyamine synthetic enzyme, and is known to be a highly sensitive marker for normal early brain development. Changes in ODC activity occur as a consequence of a
wide range of insults (417), including perinatal thyroid dysfunction (418-420). Maternal hypothyroxinemia results in reduced ODC activity in fetal brain at 15 dg, normal activity at 19 dg, but elevated activity at 22 dg. In the early postnatal period ODC activity is transiently reduced in all brain regions except cerebellum, but normal in all regions by 14 pnd (408). It is not known whether the changes in ODC activity merely reflect changes in the TH-deprived fetal brain, or whether the effects of TH are, at least partly, mediated by ODC and polyamines. The latter possibility is feasible as polyamines regulate gene expression through modulation of histone acetylation and phosphorylation, and ODC itself may be a RNA polymerase I initiation factor. Polyamines also have poorly defined roles in axonogenesis and synaptogenesis (417).

GLUT1 is also expressed at high levels in rapidly developing tissues and after trophic stimulation (421). Its expression is also deficient in Tx dam fetal brain at 16 dg, when Western blotting of fetal brain microsome preparations with a GLUT1-specific antibody reveals a large deficit in a subtype of this protein (415). Although GLUT1 is predominantly localised to the blood-brain barrier at this stage of development (422), this reduction is unlikely to reflect a general compromise in blood-brain barrier function, since another marker, alkaline phosphatase activity, appears normal (423).

Signal transduction systems in fetal brain are also affected in a selective manner by maternal hypothyroxinemia (424). Protein kinase C specific activity is increased in Tx dam progeny at 15 and 19 dg but normal by 22 dg. In contrast, protein kinase A was unaffected, while Ca^{2+}-ATPase activity is decreased at 15 and 19 dg, but increased at 22 dg (424). Whole brain neutral and alkaline components of calmodulin-dependent phosphatase (calcineurin) activity are markedly reduced in newborn Tx dam progeny (398), whereas by 2 months only the cerebellum is affected, exhibiting increases in the activities of the neutral and acidic components (395). Thus, although postnatal cell acquisition (408) and most other parameters appear normal in cerebellum from Tx dam progeny, other aspects of its development may be dependent on maternal thyroid status (395,398).

Together, these studies strongly suggest that maternal TH is necessary for normal brain development. In Tx dam models, brain development is affected not only before the onset of fetal TH synthesis but also in the late fetal/postnatal period, culminating in irreversible brain dysfunction. In addition, the fact that many of the observed changes show varying degrees of age and region-dependency suggests that TH has selective, rather than generalised effects on early brain development.
1.8 Rationale

Despite evidence implicating maternal TH as a regulator of early brain development, the prenatal brain remains a relatively neglected tissue for studying TH action. When considering the effects of maternal hypothyroxinemia, the period of brain development before the onset of fetal thyroid function is of particular importance. This period (corresponding to 0-17 dg in the rat and 0-12 wg in humans; see section 1.2) encompasses the beginning of cerebral and brain stem neurogenesis and also some neuronal migration (328). Only developmentally labile neurons undergoing at the time of TH insult are vulnerable (425), therefore it would be expected that neurological compromise would be limited to phylogenetically older regions, and previous studies with the Tx dam animal model have shown this to be largely so (see section 1.7.4). Some effects are however, irreversible and even processes that do not begin until after birth can be affected (e.g. myelination). These chronic effects arising from a transient insult can be explained by the role of TH as a biological clock during development (288). Brain development is highly coordinated and requires the correct integration of the different regions of the brain, which develop at different rates, to establish communications within and between brain regions and also with the peripheral nervous system. This extremely complex process is influenced directly and indirectly by signals from many factors, other than TH (e.g. growth hormones, various developmental signals and neurotrophins), and little is known about the effects and interplay between these factors. Furthermore, the CNS is heterogenous, with groups of neurons occupying the same region but differing biochemically, morphologically and functionally. Changes in the developmental environment often result in very different responses in the various neuronal populations (426). Thus a derangement in synchronisation during early neurogenesis may have a catastrophic effect on the rest of brain development and the functional performance of the fully developed brain may be sub-optimal (i.e. the features of cretinism). In order to investigate the immediate effects of an early TH deficit in fetal brain and their reversibility, progeny were studied before and after the onset of fetal TH synthesis.

Neurotransmitters are probably the most directly relevant TH responsive system in the developing brain, and a vast body of evidence indicates that thyroid status can affect CNS neurotransmitter function—not only during rat postnatal development in vivo (330,331,333,336-340) but also in rat neural cell culture models (section 1.7.3). Furthermore, previous work has identified region-specific changes in brain AChE and ChAT activities in Tx dam adult progeny (427), together with behavioural abnormalities, including deficits in motor function, cognition and learning behaviour (405). It is possible
therefore that TH regulates neurotransmitter function during fetal development. Several studies suggest that certain neurotransmitters (and their metabolic enzymes) play a neurotrophic role in the early stages of brain ontogenesis (428-430). Certainly, many neurotransmitters, their metabolic enzymes and their receptors have been demonstrated in the brain long before the onset of electrical activity (426,431-434). Disruption of neurotransmitter systems by TH deficits in the early fetal brain could therefore have wide ranging effects beyond synaptic transmission. In order to confirm this and investigate the effects in more detail, a Tx dam model was used to study the effects of maternal hypothyroxinemia on the ontogeny of cholinergic and catecholaminergic neurotransmitter metabolic enzymes in the brains of pre- and postnatal progeny. This was supplemented by a study on muscarinic cholinergic, β-adrenergic and D2 dopaminergic receptor binding during postnatal development to characterise the chronic effects.

As discussed in section 6.1, the nucleus appears to be the primary site of TH action. It is important therefore to establish whether TH status can affect TR ontogeny. The human TRβ1 gene has two TREs in its promoter region which can be activated by both TRα1 and TRβ1, in the presence of T3, to enhance transcription (435). Indeed, T3 induction of TRβ1 expression has been demonstrated in cultured astrocytes (165). Neonatal hypothyroidism has been shown to increase the number of T3 receptors in the brain, although the affinity was unaffected (404). Detrimental effects arising from a fetal TH deficit have also been demonstrated, as adult progeny of Tx dams exhibit an increased number of TR binding sites in the cerebellum and paleocortex (397). Furthermore, regardless of any direct effects of a TH deficit on TR function, unliganded TRs may repress basal transcription of TH and retinoic acid responsive genes (section 1.6.3.1). Maternal hypothyroxinemia may lead to an increase in unliganded TR concentration in fetal brain (unless they are downregulated) and the increase in transcriptional inhibition may be responsible for the asynchronous development observed in Tx dam progeny. It is therefore important to assess the levels of TRs in Tx and N dam progeny in fetal brain at the protein and mRNA level. Thus receptor binding assays using whole nuclei and extracted receptor preparations were utilised to determine the number and affinities of functional TRs, while Northern hybridisation analysis and reverse transcription polymerase chain reaction were used to characterise the mRNA levels of the three main TR isoforms (i.e. TRα1, TRα2 and TRβ1).

Together, these studies should provide important insights into the role and mechanism(s) of action of maternal TH in fetal brain development. Furthermore, as well as providing some answers, this study will hopefully act as a basis for further research.
CHAPTER 2.
MATERIALS
AND
METHODS
2.1 General

2.1.1 Materials

General laboratory reagents were purchased from Merck-BDH Ltd. (Hertfordshire, U.K.) and fine chemicals from Sigma Chemical Co. (Dorset, U.K.), unless specified otherwise. The radiochemicals, D,L-3,4-[alanine-1-14C]dihydroxyphenylalanine, L-[1-14C]tyrosine, [acetyl-3H]acetyl coenzyme A, L-[benzilic-4,4'-3H(N)]quinuclidinyl benzilate and [benzene ring-3H]spiperone were purchased from NEN Life Science Products Ltd. (Hounslow, U.K.); acetyl[methyl-3H]choline and L-[propyl-2,3-3H]dihydrolaprenolol were obtained from Amersham-Pharmacia Biotech Ltd. (Bucks, U.K.); [125I]T3 from BM Browne Ltd (Reading, U.K.) and [32P]labelled isotopes from ICN Biomedicals Ltd. (Oxon, U.K.). Amersham-Pharmacia Biotech Ltd. was also the source for Hybond N nylon membranes, NICK columns, Hyperfilm MP, Biomax MS film and the Megaprime and 5' end labelling kits. SOC medium, LB (Luria-Bertani) broth and agar, and all reagents and enzymes used in the reverse transcription-polymerase chain reaction studies (section 2.6.7) were obtained from Gibco Life Technologies Ltd (Paisley, U.K.). Dowex (1-X-8, Cl- form, 200-400 mesh) was obtained from Bio-Rad Laboratories Ltd. (Herts., U.K.). Sprague-Dawley rats were obtained from Charles River Ltd (Margate, Kent, U.K.) and bred in the local animal house facilities.

2.1.2 Animal model

Proven rat dam breeders were partially thyroidectomised (parathyroid-spared) by surgery. Two weeks after surgery, blood was collected from the tail veins of the thyroidectomised rat dams (Tx) and from age-matched normal rat dams (N), for serum TH and TSH determination (section 2.1.4). Tx dams were mated with normal males when circulating T4 levels were < 25 nM. The control group constituted N dams, also mated with normal males. All animals were maintained at 22 °C on a cycle of 14 h light : 10 h darkness with free access to an iodine replete diet. The drinking water of the Tx dams was supplemented with calcium lactate (0.1% w/v). When pregnancy was allowed to continue to term, N and Tx dam litter sizes were standardised to seven pups on the day of birth. Occasionally, Tx dams had litters with fewer than seven pups, and in these cases N dam litters were reduced to the same number. N or Tx dam litters with fewer than three pups were not used.
2.1.3 Sample preparation

Pregnant dams were stunned and killed by cervical dislocation at 14, 16, 19 and 21 dg, and a blood sample obtained via cardiac puncture for serum TH determination. Conceptuses were removed to ice, and fetuses and placentae were separated and weighed. Placentae were kept for protein and DNA determination (sections 2.1.5 and 2.1.6). Fetal brains were dissected, cleansed of meninges and blood vessels, and weighed. Postnatal progeny were weighed and killed (either by decapitation or cervical dislocation depending on size) on the day of birth and at 10, 20 and 30 pnd; brains were dissected and cleansed as for fetal brains. From 10 pnd onwards, brains were dissected into four gross anatomical regions—cerebral cortex, cerebellum, brain stem (comprising the pons and medulla) and subcortex.

For protein, DNA and enzyme assays, tissues were homogenised in 9 vol. of ice-cold 0.32 M sucrose, aliquoted and stored at -20 °C. For membrane receptor binding assays, tissues (20 and 30 pnd brain regions only) were homogenised in 9 vol. of ice-cold 50 mM Tris-HCl buffer (pH 7.4) and immediately used to prepare crude membrane fractions (section 2.3). For T3-binding assays, whole or extracted nuclei were prepared from fresh tissue (16 to 21 dg brains only) as detailed in section 2.4. For RNA extraction (section 2.6), brains (16 to 21 dg only) were frozen on dry ice and stored at -70 °C.

2.1.4 Determination of serum thyroid hormones and TSH

Blood was allowed to clot and serum was obtained by centrifugation (2500 g for 10 min; 4 °C). Serum Total T4 and T3 were determined using commercial radioimmunoassay kits (North East Thames Radioimmunoassay Service, London, U.K.), according to the suppliers protocol. Briefly, 25-50 μl serum was incubated with a primary antibody (anti-T3 or -T4), tracer (125I[T3] or 125I[T4]; 20000 cpm), 200 μg 8-anilino-1-naphthalene sulphonic acid (to displace thyroid hormone from the serum binding proteins) and a secondary antibody (directed against the primary antibody). Antibody complexes were precipitated with 4% (w/v) polyethylene glycol 6000 and centrifuged (1000 g for 30 min; 4 °C). The supernatant was decanted and the tracer remaining in the pellet determined by γ-spectrometry (Nuclear Enterprises NE1600). The T3/T4 concentration was calculated by the use of standards (0 - 160 nM for T4 and 0 - 12 nM for T3). Non-specific binding (NSB) was determined by omitting primary antibody, and quality controls (BM Browne Ltd.) were assayed in parallel. The detection limits of these assays were 3.19 nM for total T4 and 0.29 nM for total T3.
Serum TSH level was also determined using a commercial radioimmunoassay kit (Biocode Biotechnology, Liege, Belgium). Serum, 0.1 ml was mixed with anti-rat TSH antibody and incubated overnight at ambient temperature. $^{125}\text{I}[\text{TSH}]$ (48000 cpm) was added and the solution incubated at 37 °C for 3 h. A polyethylene glycol/secondary antibody mix was then added, incubated for a further 30 min (at ambient temperature) and centrifuged (1500 g for 30 min). Supernatant was aspirated and the $^{125}\text{I}[\text{TSH}]$ in tubes determined by γ-spectrometry. TSH level was calculated using standards (0 - 20 ng/ml) and NSB was determined in tubes without primary antibody. Internal quality controls were also assayed in parallel.

2.1.5 Protein determination

Two different methods were used for protein estimation; the Folin-Lowry method and the Bradford method. The former was used for estimation of the protein content of tissue homogenates, whereas the latter method was used for membrane fractions, or as indicated in the text.

2.1.5.1 Folin-Lowry method

This method works on the principle that Cu$^{3+}$ is reduced to Cu$^{+}$ in the presence of protein at alkaline pH (436). The Cu$^{+}$ is chelated and forms a blue coloured complex with Folin-Ciocalteau's phenol reagent enhancing the colour formation. 3 ml alkaline copper sulphate solution (0.04% w/v sodium carbonate, 0.5% w/v copper sulphate and 1% w/v sodium-potassium tartrate in 0.02 M sodium hydroxide) was added to 1 ml sample, standards (0 - 150 μg bovine serum albumin; Pierce & Warriner Ltd, Chester, U.K.) or water (reagent blank). After mixing, tubes were allowed to stand at room temperature for 15 min. Folin-Ciocalteau's phenol reagent (0.3 ml; diluted 1 in 2 with water) was added and the tube contents were incubated at room temperature for 40 min. The absorbance at 500 nm was determined in a Pye-Unicam spectrophotometer. Samples and standards were assayed in triplicate.

2.1.5.2 Bradford method

For convenience, the dye binding method of Bradford (437) was used to determine protein in crude membrane fractions (section 2.3.1) and partially purified hog kidney dihydroxyphenylalanine decarboxylase (section 2.2.4.1). This method relies on the quantitative binding of Coomassie Plus Brilliant Blue G250 reagent (Pierce & Warriner Ltd) to protein. To 0.5 ml sample (diluted 1 in 5000), standards (0 - 6 μg bovine serum
albumin) or water (reagent blank), 0.5 ml Coomassie Plus reagent was added. Absorbance was immediately read at 595 nm in a Pye-Unicam spectrophotometer. Samples and standards were assayed in triplicate.

2.1.6 DNA determination

DNA was estimated using the fluorimetric method of Labarca and Paigen (438). Samples were diluted in phosphate buffered saline (PBS; 0.05 M NaH₂PO₄·2H₂O, 2 M NaCl and 2 mM EDTA; pH 7.4) then to 0.1 ml of each sample, 3 ml bis-benzimide (1 μg/ml in PBS) was added. After incubation in the dark at room temperature for 30 min, fluorescence was measured in a Perkin Elmer fluorimeter (excitation wavelength of 356 nm; emission wavelength of 458 nm). Calf thymus DNA standards (0 - 25 μg) were treated in the same way as samples and both were assayed in triplicate.

2.2 Enzyme assays

For each enzyme assay preliminary experiments were performed to determine suitable conditions for a linear reaction. Samples and blanks were assayed in triplicate.

2.2.1 Acetylcholinesterase (EC 3.1.1.7)

Acetylcholinesterase (AChE) degrades acetylcholine thereby terminating synaptic activation (439). It is present throughout the brain and other tissues, in both cholinergic and noncholinergic neurons. AChE activity was measured using the method of Ellman (440). In a cuvette, 10 μl of homogenate was mixed with 3 ml assay buffer (0.1 M sodium phosphate buffer; pH 8.0) and either 30 μl of water, or 1 mM eserine (for blanks). Eserine is a specific inhibitor of acetylcholinesterase and therefore allows breakdown of substrate by non-specific cholinesterases (such as butylylcholinesterase) to be accounted for. The mixture was incubated at room temperature for 5 min before the addition of 100 μl reagent (0.01 M 5,5’-dithiobis-(2-nitrobenzoic acid) and 18 mM sodium bicarbonate in 0.1 M sodium phosphate buffer; pH 7.0) and 75 mM acetylthiocholine iodide. The increase in absorbance at 412 nm over time was measured by spectrophotometry.

2.2.2 Choline acetyltransferase (EC 2.3.1.6)

Choline acetyltransferase (ChAT) catalyses acetylcholine synthesis. Choline transport into neurons is the limiting factor in acetylcholine synthesis, however, rather than ChAT activity (439). Unlike AChE, ChAT serves as a specific marker of cholinergic neurons. ChAT
activity was measured using the radiochemical method of Fonnum (441,442). In glass vials
on ice, 95 µl of homogenate was mixed with 5 µl 10% (w/v) Triton X-100. After 30 min,
0.5 ml assay mix (0.3 M NaCl, 20 mM EDTA, 8 mM choline bromide, 0.1 mM eserine
and 0.2 mM [3H]acetyl coenzyme A in 50 mM sodium phosphate buffer; pH 7.4) was
added, and the solution incubated for 15 min at 37 °C. 5 ml ice-cold sodium phosphate
buffer (10 mM; pH 7.4) was added to terminate the reaction. [3H]Acetylcholine was
separated from unreacted [3H]acetyl coenzyme A by the addition of 2 ml acetonitrile
(containing 10 mg sodium tetraphenylboron) followed by 10 ml scintillant (0.05% w/v
diphenyloxazole and 0.02% w/v 1,4-bis-4-methyl-5-phenyloxazole-2-yl benzene in
toluene). The vials were gently mixed facilitating the extraction of [3H]acetylcholine into
the upper phase (containing the scintillant). Phases were allowed to separate and vials
counted in a scintillation counter (LKB-Wallac Minibeta). Blanks consisted of 95 µl of
0.32 M sucrose and 5 µl 10% (v/v) Triton X-100 instead of homogenate, and were taken
throughout the entire procedure. Counting efficiency was determined using a known
amount of [3H]acetylcholine in the same two-phase scintillant-buffer mix as the samples.

2.2.3 Dihydroxyphenylalanine (DOPA) decarboxylase (EC 4.1.1.28)

DOPA decarboxylase (DDC) is an enzyme in the monoaminergic neurotransmitter pathway
which catalyses the synthesis of dopamine from DOPA. DDC can also decarboxylate 5-
hydroxytryptophan and therefore also plays a role in serotonin synthesis (443). DDC
activity was measured using the method of Okuno and Fujisawa (444). 100 µl Homogenate
(or 0.32 M sucrose for blanks) and 400 µl of assay mix (1 mM [alanine-1-14C]DOPA D,L-
3,4, 70 µM pyridoxal phosphate, 1 mM 2-mercaptoethanol, 0.1 mM EDTA and 0.01% v/v
Triton X-100 in 0.1 M sodium phosphate buffer; pH 7.0) were mixed on ice in glass vials.
A microfuge tube containing a filter paper wick, moistened with 0.2 ml potassium
hydroxide (30% w/v) was added to each vial. The vials were sealed with a Suba cap and
incubated at 37 °C for 30 min with vigorous shaking. Reactions were terminated by the
injection of 0.4 ml perchloric acid (8% v/v). This also facilitated the release of [14C]CO2,
produced by the action of the enzyme, which was collected by the alkaline wicks in the
microfuge tubes during 1 h of vigorous shaking. The contents of the microfuge tubes were
transferred to scintillation vials containing 4 ml Picoflour scintillant (Canberra-Packard Ltd,
Berks, U.K.) and counted in the scintillation counter. Counting efficiency was determined
using a known amount of assay mix in 4 ml scintillant.
2.2.4 Tyrosine hydroxylase (EC 1.14.16.2)

Tyrosine hydroxylase (TyrH) is the initial, and rate-limiting, enzyme in the monoaminergic neurotransmitter synthetic pathway. The assay was based on the method of Waymire (445) which utilises DDC, purified from hog kidney (which has no detectable endogenous TyrH activity), to release \([^{14}\text{C}]\text{CO}_2\) from DOPA produced by the action of TyrH on tyrosine.

2.2.4.1 Partial purification of DDC from hog kidney

DDC was partially purified from frozen hog kidney (Advanced Protein Products Ltd, West Midlands, U.K.) as described previously (445), all procedures being performed at 4 °C. The cortex was dissected from the medulla and homogenised in 4 vol. sucrose (0.32 M) using a Waring blender at 3/4 speed for two 1 min periods. The homogenate was centrifuged (6000 g for 15 min) and the supernatant was further centrifuged (27000 g for 1 h). Saturated ammonium sulphate solution (pH 8.0) was slowly added to the supernatant with constant mixing to produce 37% (v/v) saturation. Once achieved, this was stirred for 30 min before centrifugation (27000 g for 30 min). The supernatant was collected and additional saturated ammonium sulphate solution was added to produce 55% (v/v) saturation. This was stirred for 30 min then centrifuged (27000 g for 30 min). The resulting pellet containing partially purified DDC was resuspended in 0.5 ml 0.01 M sodium phosphate buffer (pH 7.0), containing 20% (v/v) glycerol and stored at -70 °C.

It is vital that the DDC activity does not become rate limiting in the TyrH assay, therefore the DDC activity of each preparation was determined. The protein concentration of each fraction was determined by the Bradford method (section 2.1.5.2) and its DDC activity was assayed with increasing amounts of protein under the conditions of the TyrH assay (section 2.2.4.2). The amount of protein which did not further increase DDC activity, was used in subsequent TyrH assays.

2.2.4.2 Assay procedure

The TyrH assay was performed as follows, 100 μl homogenate (or 0.32 M sucrose for blanks) and 400 μl assay mix (80 μM L-[1-\(^{14}\text{C}\)]tyrosine, 10 μM pyridoxal phosphate, 40 mM 2-mercaptoethanol, 2 mM 6,7-dimethyl-5,6,7,8-tetrahydropterine hydrochloride, 1 mM ferrous sulphate, 1 mM sodium phosphate, partially purified hog kidney DDC and 0.1% v/v Triton X-100 in 0.2 M sodium acetate buffer; pH 6.1) were mixed on ice in glass vials. They were then treated as described for the DDC assay (section 2.2.3).
2.2.5 Monoamine oxidase (EC 1.4.3.4)

Monoamine oxidase (MAO) is one of the main degradative enzymes of monoaminergic neurotransmitters, capable of deactivating dopamine, noradrenaline, adrenaline and serotonin. It has widespread distribution throughout the brain and other tissues. MAO exists in two isoforms, A and B, which can be distinguished by substrate preference and specific inhibitors (446). Total MAO activity was assayed using the fluorimetric method of Krajl (447). Homogenate (20 µl) was mixed with kynuramine dihydrobromide (0.1 mg) and sodium phosphate buffer (0.083 M; pH 7.4) to a final volume of 3 ml. The tubes were incubated at 37 °C for 30 min and the reaction was terminated by the addition of 2 ml trichloroacetic acid (10% w/v). Tubes were centrifuged (1000 g for 15 min; 4 °C) to remove precipitated protein, and 1 ml of supernatant was added to 2 ml 1 M NaOH in a quartz cuvette. Fluorescence was measured with an excitation wavelength of 315 nm and emission wavelength of 380 nm. Standards (0 - 25 nmoles 4-hydroxyquinolene instead of homogenate) and blanks (20 µl water instead of homogenate) were assayed in parallel.

In addition to total MAO, the activities of the A and B isoforms were assayed in brain at 16 and 19 dg. The same assay procedure was employed, but with the addition of specific inhibitors for each isoform, namely clorgyline (MAO-A inhibitor) and R-(−)-deprenyl hydrochloride (MAO-B inhibitor; ICN Biomedicals Ltd.). Preliminary experiments were performed, using brain homogenate from 19 dg N dam progeny, to determine the optimal concentrations for each inhibitor (section 4.1.2.3).

2.3 Neurotransmitter receptor binding studies

2.3.1 Crude membrane preparation

Tissue homogenates (section 2.1.3) were centrifuged (104000 g for 60 min; 4 °C). The pellet was washed in ice-cold Tris-HCl buffer (50 mM; pH 7.4), by resuspension/centrifugation, resuspended in the same buffer and assayed for protein concentration using the Bradford method (section 2.1.5.2). Crude membrane preparations were aliquoted and stored at -20 °C.

2.3.2 β-adrenergic receptor binding assay

The method was based on that of Smith et al. (338). Membrane fractions (150 to 300 µg protein) were incubated with increasing concentrations (0.25, 0.5, 1.0, 2.0, 4.0 and 8.0 nM) of [³H]dihydroalprenolol (DHA; 39 Ci/mmol) in assay buffer (50mM Tris-HCl; pH
7.8), in a total volume of 0.25 ml, for 30 min at 25 °C. NSB was determined in parallel
tubes containing 10 μM propranolol (Cambridge Research Biochemicals Ltd, Cambridge,
U.K.). The reaction was terminated by the addition of 3 ml ice-cold assay buffer and
membrane-bound [³H]DHA was separated from free by immediate filtration through GF/B
glass fibre filters (Whatman Int., Kent, U.K.) on a vacuum manifold (Millipore Ltd.,
Herts, U.K.). Filters were washed twice with 5 ml ice-cold assay buffer, air-dried then
prepared for liquid scintillation counting. Counting efficiency was determined by counting
a known amount of [³H]DHA on a filter in 10 ml scintillant. Samples were assayed in
duplicate at each DHA concentration, with NSB being determined in singlicate.

2.3.3 Muscarinic cholinergic receptor binding assay

Muscarinic receptor binding was determined using the method of Patel et al. (337).
Membrane fractions (75 - 150 μg protein) were incubated with increasing concentrations
(0.1, 0.3, 0.75, 1.5 and 3.0 nM) of [³H]quinuclidinyl benzilate (44.9 Ci/mmol), in 50 mM
tris-HCl (pH 7.4), in a total volume of 0.25 ml, for 60 min at 25 °C. NSB was determined
in parallel tubes containing 100 μM oxotremorine. The assay was terminated and processed
as described in section 2.3.2.

2.3.4 Dopaminergic D2 receptor binding assav

Using the method of Atterwill (448), membrane fractions (150 - 300 μg protein) were
incubated with increasing concentrations (0.1, 0.25, 0.5, 1.0, 2.5 and 5.0 nM) of
[³H]spiperone (17.7 Ci/mmol), in 50 mM Tris-HCl (pH 6.9), in a total volume of 0.25 ml,
for 30 min at 37 °C. NSB was determined in parallel tubes containing 1 μM (+)-butaclamol
HCl (Semat Technical Ltd, Herts, U.K.). The assay was terminated and processed as
described in section 2.3.2.

2.4 Nuclear T3-binding studies

2.4.1 Nuclei isolation

The method used was a modification of the procedure used for adult brain (221,449). Fetal
brains were soaked in 9 vol. ice cold 10 mM MgCl₂ in 0.25 M sucrose for 10 min. Tissue
was then minced and soaked again in the same solution for a further 10 min before
homogenisation. Homogenates were filtered through nylon mesh (110 μm pore size)
followed by steel gauze (40 μm pore size), and centrifuged (100 g for 10 min; 4 °C). The
crude nuclear pellet was resuspended in 10 vol. 2.0 M sucrose and centrifuged (104000 g
for 40 min; 4 °C). The pellet was washed once in ice-cold 10 mM MgCl₂/0.25 M sucrose by resuspension/centrifugation (100 g for 10 min; 4 °C) before final resuspension in 5 ml T3 nuclei binding buffer (T3NB; 1 mM dithiothreitol, 3 mM MgCl₂ and 0.25 M sucrose in 20 mM Tris-HCl buffer; pH 7.4). All procedures were performed at 4 °C. Fractions from each stage were kept for protein (Folin-Lowry method) and DNA determination (sections 2.1.5.1 and 2.1.6, respectively).

2.4.1.1 Receptor extraction

In a separate set of experiments, crude receptor extracts were obtained, using a published procedure (450). Nuclei were isolated as detailed above and the nuclear suspension was adjusted to a DNA concentration of 4 mg/ml T3NB. Nuclei were centrifuged (100 g for 10 min; 4 °C) and resuspended in the same volume of extraction buffer (0.4 M KCl, 1 mM MgCl₂ and 1 mM dithiothreitol in 10 mM Tris-HCl; pH 7.4). After vigorous mixing and incubation at 4 °C for 45 min, DNA was pelleted by centrifugation (50000 g for 30 min; 4 °C) and the supernatant was used in the T3 binding assay.

2.4.2 Binding assay: whole nuclei

The binding assay was based on the procedure of Gullo et al. (449). In 0.5 ml microfuge tubes, 160 μl nuclear suspension (ca. 40 μg DNA) were mixed on ice with 0.1 nM [125I]X3 (specific activity >1200 μCi/μg) and unlabelled T3 to give a range of concentrations (0.1, 0.2, 0.5, 1.0, 2.5, 5.0 and 10.0 nM) in a final volume of 200 μl. NSB was determined in parallel tubes containing a large excess of by unlabelled T3 (1 μM). Samples were incubated at 37 °C for 30 min and the reaction was terminated the addition of 200 μl ice-cold Triton X-100 (2% v/v) in T3NB. After mixing, tubes were incubated on ice for 15 min and centrifuged (12000 g for 3 min; 4 °C). The supernatant (free fraction) was removed by aspiration and the pellet (bound fraction) was washed with 400 μl Triton X-100 in T3NB (1% v/v) by resuspension and centrifugation. After removal of supernatant, the bottom of the microfuge tube containing the pellet was cut off and counted by γ-spectrometry. Counting efficiency was determined using a known amount of [125I]T3.

2.4.3 Binding assay: salt-extracted receptors

The assay was performed as detailed above, except after incubation, the reaction was terminated by the addition of 200 μl of a suspension of Dowex (160 mg/ml) in assay buffer
(0.3 M KCl, 1 mM MgCl$_2$ and 2 mM dithiothreitol in 20 mM Tris-HCl; pH 8.5). The tubes were incubated for 5 min at 4° C, with three mixes during the incubation. After centrifugation (10000 g for 10 min; 4 °C), 200 μl supernatant (bound fraction) was removed and counted by γ-spectrometry.

2.5 Receptor binding analysis

Data from the receptor binding studies outlined in sections 2.3 and 2.4 were analysed by nonlinear regression using a computer program, Prism 2.0 (Graphpad Software, CA, USA) to determine the affinity (Kd) and receptor number (Bmax). Ligand receptor binding data has traditionally been processed using “Scatchard” analysis which involves transforming the data by dividing bound over free. This not only violates one of the assumptions of linear regression, by altering the relationship between the parameters being plotted, but also distorts the experimental error. Nonlinear regression does not require data transformation, but is difficult to perform without the aid of a computer. It is based on an iterative procedure in which the variables in the equation of the line are adjusted to minimise the sum of squares. In the case of an assumed one-site saturation binding curve, the equation is as follows:

\[
Y = \frac{B_{\text{max}}(X)}{K_d + X}
\]

where X is the initial ligand concentration, Y is the amount of ligand specifically bound and Kd and Bmax are the variables to be determined.

2.6 Expression of TR isoform mRNA in fetal brain

Analysis of RNA presents a major methodological problem in that RNA is highly susceptible to degradation by RNases, which are abundantly present in tissues and the laboratory environment. Therefore precautions were taken to ensure a nuclease-free environment. To this end most solutions were pre-treated with 0.1% (v/v) diethyl pyrocarbonate (DEPC) overnight, then autoclaved to sterilise the solution and remove residual DEPC. Amine-containing compounds such as Tris react with DEPC, therefore these solutions were prepared in DEPC-treated water and autoclaved. Solutions containing heat-sensitive compounds such as 3-(N-morpholino) propane sulphonic acid (MOPS), were prepared in DEPC-treated water and filter-sterilised. Disposable plasticware was either purchased sterilised or, where possible, autoclaved and glassware was baked at 180 °C overnight. All chemicals used were of “Molecular Biology Grade” i.e. certified as
nuclease free by suppliers.

Certain solutions were used in several procedures and their compositions are detailed in Table 2.1

**Table 2.1 Composition of commonly used buffers and solutions**

<table>
<thead>
<tr>
<th>Solution</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 x Saline Sodium Citrate (SSC) buffer</td>
<td>3.0 M NaCl in 0.3 M sodium citrate buffer; pH 7.0</td>
</tr>
<tr>
<td>20 x Saline Sodium Phosphate EDTA (SSPE) buffer</td>
<td>2.98 M NaCl and 0.02 M EDTA in 0.2 M sodium phosphate buffer; pH 7.4</td>
</tr>
<tr>
<td>10 x MOPS-acetate-EDTA (MAE) buffer</td>
<td>0.8 M sodium acetate and 0.05 M EDTA in 0.5 M MOPS buffer; pH 7.5</td>
</tr>
<tr>
<td>Tris-EDTA (TE) buffer</td>
<td>1 mM EDTA in 10 mM Tris-HCl; pH 8.0</td>
</tr>
<tr>
<td>5 x Tris-Borate-EDTA (TBE) buffer</td>
<td>10 mM EDTA in 0.45 M Tris-Borate; pH 8.3</td>
</tr>
<tr>
<td>100 x Denhardt’s solution (Anachem, Beds, U.K.)</td>
<td>2% (w/v) Ficoll, 2% (w/v) polyvinylpyrrolidone and 2% (w/v) BSA</td>
</tr>
<tr>
<td>DNA loading buffer</td>
<td>0.25% (w/v) bromophenol blue and 15% (w/v) Ficoll</td>
</tr>
<tr>
<td>RNA loading buffer</td>
<td>0.4% (w/v) bromophenol blue and 1 mM EDTA in 50% (v/v) glycerol</td>
</tr>
</tbody>
</table>
2.6.1 Agarose gel electrophoresis

Agarose gel electrophoresis is commonly used to separate RNA or DNA species according to their molecular weight. It can also be used to determine the purity or concentration of a DNA or RNA preparation. Electrophoresed nucleic acids were visualised by either including ethidium bromide within the sample or the gel, or by staining the gel with ethidium bromide (0.5 μg/ml) after electrophoresis.

2.6.1.1 RNA electrophoresis

RNA was electrophoresed using a minigel (8 x 10.6 x 0.5 cm) to assess its integrity after extraction (section 2.6.2), or on a maxigel (20 x 20 x 0.5 cm) as a preliminary step to Northern blotting (section 2.6.3). For minigel electrophoresis, 4 μl (6 μg) RNA was added to 16 μl sample buffer (8% v/v formaldehyde, 65% v/v formamide and 0.02 μg/μl ethidium bromide in 0.6 x MAE), heated at 55 °C for 15 min to denature the RNA, then quenched on ice. To each sample, 2 μl RNA loading buffer was added and 20 μl was loaded on a 1% (w/v) agarose minigel (containing 1x MAE buffer and 6.6% v/v formaldehyde). The gel was run at 80 - 100 V, using 1x MAE as the running buffer, until the dye front had migrated at least 5 cm. The 18 and 28S ribosomal RNA species were visualised using a UV transilluminator and photographed. Only those samples which displayed the expected 2:1 ratio between the 28S and 18S rRNA bands were used in subsequent experiments.

For maxigel electrophoresis, 12 μl (20 μg) RNA, was mixed with 48 μl sample buffer (same constituents as used for the minigel, but without ethidium bromide). Samples were denatured as before, followed by the addition of 6 μl RNA loading buffer. 60 μl sample was loaded on a 1% (w/v) agarose maxigel (same constituents as in the minigel) and run at 70 - 100 V, again with 1x MAE as the running buffer, until the dye front had travelled 8 cm. In addition to the samples, 7 μg 0.24-9.5 kb RNA ladder (Gibco Life Technologies Ltd) was electrophoresed.

2.6.1.2 DNA electrophoresis

For DNA electrophoresis, agarose was dissolved in 0.5 x TBE, which was also used as the running buffer. The amount of DNA loaded, sample buffer constituents and % agarose used varied according to the particular application and are detailed in the text where necessary.
2.6.2 RNA isolation and standardisation

Total RNA was extracted from fetal brains using the method of Chomcynski and Saachi (451). Briefly, tissue was homogenised with 9 vol. denaturing solution (solution D; 4 M guanidium thiocyanate, 0.5 % w/v sarcosyl and 0.1 M 2-mercaptoethanol in 25 mM sodium citrate; pH 7.0). To this was added 0.1 vol. 2 M sodium acetate (pH 4.0), 1 vol. citrate buffer-saturated phenol (pH 4.3) and 0.2 vol. chloroform:isoamyl alcohol (49:1 mixture), the solution being vigorously shaken between each addition. Tubes were stood on ice for 15 min, then centrifuged (10000 \( g \) for 20 min; 4 °C) and the aqueous phase was removed to fresh tubes. An equal volume of isopropanol was added and the solution was left at -20 °C for at least one hour to precipitate the RNA. After centrifugation (as above), the RNA pellet was dissolved in 0.3 ml solution D and transferred to a microfuge tube. The RNA was re-precipitated with isopropanol and pelleted by centrifugation (15000 g for 10 min; 4 °C). The pellet was washed twice in 1 ml ethanol (75% v/v) by resuspension/centrifugation and left to air-dry for 15 min. Pellets were dissolved in a suitable volume (0.25 µl/mg wet weight tissue extracted) sodium dodecyl sulphate (SDS; 0.5% w/v) and stored at -20 °C. RNA concentration was determined by spectrophotometry (section 2.6.2.1) and RNA integrity by minigel electrophoresis (section 2.6.1.1) before subsequent use.

2.6.2.1 Estimation of RNA concentration

Samples were diluted 1 in 500 with DEPC-treated water and the absorbance at 260 and 280 nm was measured using a Pye Unicam spectrophotometer. The RNA concentration was calculated based on the assumption that a RNA solution of 40 µg/ml has an OD of 1 at 260 nM (452). The \( A_{260}/A_{280} \) ratio indicates the degree of protein contamination. A ratio of 1.7 - 2.0 indicated a reasonably pure preparation whereas a ratio of less than 1.7 indicated significant protein contamination thus the RNA was re-extracted with phenol-chloroform-isoamyl alcohol and isopropanol-precipitated as described previously.

2.6.3 Northern blotting

After RNA samples had been electrophoresed on a maxigel (section 2.6.1.1), the marker lane was removed, stained with ethidium bromide, visualised by UV transillumination and photographed. The distance travelled by the RNA marker bands relative to the dye front (Rf value) were measured and plotted against the log of their size in kb, allowing the later determination of size of any bands detected by autoradiography. The electrophoresed sample RNA was blotted onto nylon membrane (Hybond-N; Amersham-Pharmacia
Biotech) by overnight capillary transfer, using 20 x SSC as the transfer buffer (452). The gel was stained with ethidium bromide to confirm complete transfer of RNA, and RNA was covalently bound to the nylon membrane by UV irradiation (Spectrolinker XL-1000 UV crosslinker).

2.6.4 Preparation of cDNA probes

Probes for the TR isoforms were derived from Bluescript KS+ plasmids containing the rat TR cDNA coding sequences (Fig. 2.1). TRα1 and TRα2 cDNA (160) were gifts from Professor Chin (Howard Hughes Medical Institute, Boston, MA, USA), and TRβ1 cDNA was donated by Dr Koenig (University of Michigan Medical Centre, Michigan, MI, USA) (453).

2.6.4.1 Transformation and culture

Competent Escherichia coli, strain HB101 (Promega Corp, Southampton, U.K.), were transformed with 10 ng plasmid by heat shock (50 s at 42 °C, followed by immediate placement on ice for 2 min). 900 μL SOC medium was added and the cells were cultured for 1 hour at 37 °C with shaking at 225 rpm. A loopful of cells were streaked onto LB agar plates, containing 50 μg/ml ampicillin. As well as the TR cDNA, the plasmids also contain the gene for ampicillin resistance (Fig. 2.1A), allowing the selection of transformed cells. After overnight culture at 37 °C, several single colonies were removed from the plate, each being suspended in 10 ml LB broth, containing 0.1% w/v glucose and 25 μg/ml ampicillin. An aliquot (1 ml) of suspension was used to inoculate 500 ml LB broth, which was then incubated overnight at 37 °C with shaking as before. The remaining suspension was stored in 15% (v/v) glycerol at -70 °C.

2.6.4.2 Plasmid purification

Cells were harvested by centrifugation (10000 g for 5 min) and plasmids were isolated using a commercial kit (Qiagen Plasmid Maxi Kit; Qiagen Ltd, Surrey, U.K.). The pellet was resuspended in 10 ml buffer P1 (10 mM EDTA and 100 μg/ml RNase A in 50 mM Tris-HCl; pH 8.0) followed by addition of 10 ml buffer P2 (1% w/v SDS in 200 mM NaOH) to lyse the cells. After 5 min incubation and gentle mixing, 10 ml buffer P3 (3.0 M potassium acetate; pH 5.5) was added the solution was centrifuged (20000 g for 30 min; 4 °C). The supernatant was removed and re-centrifuged (20000 g for 15 min; 4 °C) to ensure complete removal of particulate matter. The resulting supernatant was applied to an equilibrated Qiagen-Tip anion exchange column, washed twice with 30 ml buffer QC (15%
Figure 2.1  A) Schematic diagram of Bluescript KS+ plasmid (MCS; multiple cloning site). B) Detail of the TR isoform cDNA inserts within the MCS.
v/v ethanol and 1.0 M NaCl in 50 mM MOPS; pH 7.0), and the plasmid was eluted with 15 ml buffer QF (15% v/v ethanol and 1.5 M NaCl in 50 mM Tris-HCl; pH 8.5). The plasmid was precipitated with 0.7 vol. isopropanol and centrifuged (15000 g for 30 min; 4 °C). After washing with 75% (v/v) ethanol, the plasmid was dissolved in 200 µl sterile water. An aliquot was diluted 10-fold and 2 µl of this was added to 8 µl TE and 2 µl DNA loading buffer. After mixing, 10 µl was electrophoresed on a 1% w/v agarose minigel (section 2.6.1.2). Varying amounts of a 1 kb ladder, of known DNA concentration (Stratagene Ltd, Cambridge, U.K.), were run with the sample to provide approximate quantitation and to confirm the expected size of the plasmid.

2.6.4.3 Plasmid digestion

Plasmids were digested with restriction enzymes by overnight incubation at 37 °C to excise the cDNA. TRα1 and TRα2 inserts were excised with EcoR1, although in the case of TRα2, the internal EcoR1 site meant that the insert was cleaved into two portions—the fragment of interest being the 600 bp TRα2-specific portion at the 3' end (Fig. 2.1B). The TRβ1 cDNA was excised with Xho1 and Pst1 (Fig. 2.1B). A TR common probe was also produced from the TRα1 cDNA (corresponding to nucleotides 964-1554). This probe has been shown to detect all three TR isoforms (226) and was produced by StuI digestion (Fig. 2.1B). Digestion products were separated by electrophoresis on a preparative 1% (w/v) low melting point agarose (Promega Corp.) minigel, with a single 54 x 1 x 4 mm well allowing the whole sample (100 µl of the digest added to 20 µl DNA loading buffer) to be loaded. After electrophoresis, the gel was stained with ethidium bromide and the fragment of interest cut from the gel under UV translumination. A commercial kit (Hybaid Recovery Purification Kit II; Hybaid) was used for purification of the cDNA fragment. Briefly, portions of the gel (300 mg) were melted at 55 °C and applied to a silica gel matrix column with 400 µl binding buffer in a spin filter tube. The column was washed twice then the cDNA insert was eluted in 25 µl elution buffer. An aliquot was used for quantitation on a gel as before, the rest being stored at -20 °C until required.

2.6.5 Probe labelling

cDNA probes were synthesised from template cDNA, incorporating [32P]dCTP using Klenow DNA polymerase, as described by Feinberg and Vogelstein (454). The reagents, radiolabel apart, were supplied in a commercial kit (Megaprime DNA labelling system; Amersham-Pharmacia Biotech) and the manufacturers protocol was followed. Template (25 ng cDNA) was added to random nonomer primers solution, denatured by boiling for 5
min, then incubated at 65 °C for 5 min to allow primers to anneal. After cooling, buffer (containing dATP, dGTP and dTTP) was added, followed by 2 U Klenow enzyme and 17 pmol [\(^{32}\)P]dCTP (specific activity; 3000 Ci/mmol). The reaction mix was incubated for 1 h at 37 °C. Unincorporated radioactivity was separated from the radiolabelled probe by gel chromatography, using Sephadex G25 (NICK column; Amersham-Pharmacia Biotech) and TE buffer as eluate. Eluate fractions were collected and 1 - 5 µl aliquots were added to 5 ml scintillant for counting. The labelled (and unlabelled) probe, being excluded from the sephadex matrix, is eluted before the free [\(^{32}\)P]dCTP. The fractions containing the first peak of eluted radioactivity were pooled therefore and added to hybridisation solution (section 2.6.6). The specific activity of the probe, calculated from the counts obtained via the column, was always > 1 x 10^9 cpm/µg cDNA.

For 18S hybridisation and confirmation of reverse transcription polymerase chain reaction products (section ), oligonucleotide probes were used. 18S was used as a control to account for RNA loading differences, and utilised an oligonucleotide probe complementary to bases 292-321 of the published rat 18S sequence (CCACTGAGATCTATTGGAGCCGGCTAGGCG; synthesised by Amersham-Pharmacia Biotech) (455,456). Oligonucleotides were labelled using a 5'-end labelling kit (Amersham-Pharmacia Biotech), in which T4 polynucleotide kinase transfers the terminal phosphate from [\(^{32}\)P]γ-ATP (ICN Biomedicals) to the 5'-terminal phosphate of the oligonucleotide. Briefly, 5 pmol oligonucleotide probe was added to 1 x phosphorylation buffer, 5.5 pmol [\(^{32}\)P]γ-ATP (specific activity <4000 Ci/mmol) and 5 U T4 polynucleotide kinase. After incubation for 30 min at 37 °C, the reaction mixture was heated to 70 °C for 5 min to deactivate the enzyme and the probe was purified on a Sephadex G25 column as described above.

2.6.6 Hybridisation

For hybridisation with cDNA probes, nylon membranes were pre-wetted in 2 x SSC then placed individually in bottles with 5 ml prehybridisation solution (5x SSC, 5x Denhardt’s solution, 50% v/v formamide, 0.5% w/v SDS and 50 µg/ml salmon sperm DNA) and incubated for 4 hours at 42 °C with constant rotation in a hybridisation oven (Hybaid). The solution was changed after the first hour. Membranes were then hybridised overnight at 42 °C with rotation in fresh prehybridisation solution, containing labelled probe, at 5 ml solution per bottle.

For hybridisation with oligonucleotide probes, formamide was not included in the prehyb/hybridisation solution, and the hybridisation temperature, which is dependent on the melting temperature (Tm) and therefore the GC content of the oligonucleotide, was
determined by the following formula:

\[ T_m (°C) = 81.5 + 0.41(\% \text{ probe GC content}) - \% \text{ mismatched bases} - \frac{675}{\text{probe length}} \]

Thus, for the 18S oligonucleotide the Tm was 83.6 °C and the prehyb/hybridisation was performed at 15 °C below this temperature, 68.6 °C. The prehybridisation/hybridisation solutions consisted of 6 x SSPE, 5 x Denhardt’s solution, and 50 µg/ml Salmon sperm DNA.

2.6.6.1 Washing

Hybridisation solution was decanted and the membranes were removed and washed according to a set protocol of increasing stringency. For cDNA hybridisations, the washing protocol was:

- Once with 2 x SSC/0.1% (w/v) SDS (50 ml per wash) at 60 °C for 10 min
- Twice with 1 x SSC/0.1% (w/v) SDS at 60 °C for 10 min
- Twice with 0.1 x SSC/0.1% (w/v) SDS at 60 °C for 10 min

After hybridisation with oligonucleotide probes, membranes were washed according to the following protocol:

- Twice with 6 x SSPE at room temperature for 10 min.
- Twice with 6 x SSPE/0.1% (w/v) SDS at hybridisation temperature for 10 min.
- Once with 6 x SSPE/0.1% (w/v) SDS at 5 °C below the Tm for 2 min.

The optimal washing conditions were determined in preliminary experiments.

2.6.6.2 Autoradiography

Washed membranes were wrapped in clingfilm and exposed at -70 °C to autoradiographic film in light-proof cassettes with intensifying screens. Generally, Hyperfilm-MP (Amersham-Pharmacia Biotech) was used and this was pre-flashed to bring the signal to within the linear range. For very faint signals Kodak BioMax MS film (Amersham-Pharmacia Biotech) was used, without pre-flashing, since this film is approximately 4-fold more light sensitive. Exposure times therefore varied with signal strength and are given in the text (section 5.3.1).
2.6.6.3 Image analysis

Autoradiographic images were digitised using a digital camera (Kodak DC40), with an optical density (OD) step-tablet (0 - 3 OD units; Kodak) included for calibration. Digitised images were subjected to densitometric analysis using the computer program NIH Image 1.61 (public domain program developed at the National Institutes of Health, USA and available on the internet at http://rsb.info.nih.gov/nih-image/). Using the calibration curve obtained from the step-tablet, the program is able to calculate the mean area OD of the bands of interest, which is proportional to the amount of radioactivity, and therefore mRNA content in those bands. The size of the bands (in kb) was also calculated, using the calibration curve generated by the RNA markers (section 2.6.3).

2.6.7 Reverse transcription and polymerase chain reaction

2.6.7.1 Reverse transcription

SDS inhibits reverse transcription, therefore RNA was re-precipitated by the addition of 0.1 vol. 3 M sodium acetate (pH 5.2) and 1 vol isopropanol and overnight incubation at -20 °C. Centrifugation and ethanol washing of the RNA pellet was performed as detailed previously (section 2.6.2), then the RNA was resuspended in nuclease-free water and purity and concentration were confirmed (sections 2.6.2.1 and 2.6.2.2). 2 μg of RNA were taken for reverse transcription, with residual genomic DNA being removed by incubation with 1 U DNase I, 1 x DNase buffer (2 mM MgCl$_2$ and 50 mM KCl in 20 mM Tris-HCl; pH 8.4) and 40 U RNasin (20 μl final volume) for 15 min at 25 °C. The reaction was terminated by adding 2 μl 25 mM EDTA (pH 8.0) and heating to 70 °C for 10 min. The mixture was divided into two equal aliquots, to which random hexamers were added (final concentration of 50 ng/μl) and heated to 70 °C for 10 min, then chilled on ice. Both aliquots had the following components of the reverse transcription reaction added to final concentrations of 1x first strand buffer (37.5 mM KCl and 1.5 mM MgCl$_2$ in 25 mM Tris-HCl; pH 8.3), 5 mM dithiothreitol and 500 μM dNTP mix (19 μl final volume. Then 200 U Superscript II RNase H$^+$ reverse transcriptase was added to one aliquot (RT$^+$; reverse transcribed sample), while the other aliquot (RT$^-$; negative control) received the same volume (1 μl) of water. Both aliquots were incubated at 42 °C for 50 min before being heated to 70 °C for 15 min to inactivate the enzyme, and stored at -20 °C until required.

89
2.6.7.2 Primer design

Designing primers for specific amplification of a desired product is a crucial step in polymerase chain reaction (PCR). Primers should satisfy several criteria, achieving a balance between high specificity and efficiency (457,458). Specificity can be obtained primarily by designing primers which exactly match the target sequence, though primer length is also important—the longer the primer the more specifically it will bind. For greater PCR efficiency however, primers should be as short as possible to enable them to quickly anneal to the DNA and form an effective template. An effective compromise between specificity and efficiency is achieved with oligos of 18-24 nucleotides (457). For efficient annealing, primers should have melting temperatures (Tm) of between 56 and 62°C, Tm being influenced by primer length and GC content, which ideally should be close to 50% (457). Primer pairs should have similar Tms to avoid mispriming if the lower Tm is used, or loss of signal at the higher Tm. Mispriming can also arise from the formation of primer dimers with either the same primer or its pair, this is particularly critical with the 3’ end. Product length should also be considered, amplification of a long sequence (> 1 kb) is unlikely to occur efficiently. Nevertheless, a product should be long enough to allow the generation of specific internal oligo probes to confirm its identity, products of between 250-750 bp are therefore preferred (457).

A computer program, Oligo 5.0 (National Biosciences Inc., MN, USA), was used to help choose suitable primers since it automatically rejects primers which do not fulfil the criteria. Complete cDNA sequences for each TR isoform were obtained from the Thyroid Hormone Receptor Resource internet site [http://xanadu.mgh.harvard.edu/receptor/trrfront.html]. Upstream and downstream primers were located on different exons, preventing possible interference from signals arising from residual genomic DNA. Furthermore, the identical sequences of the c-erbAα and c-erbAβ gene splice variants were exploited to produce common primers (Fig. 2.2). Thus, TRα1 and TRα2 share a common upper primer, and TRβ1 and TRβ2 share a common lower primer— in each case the other primer was complementary to an isoform specific sequence (Table 2.2). The TRα-common upper primer was upstream of the transcription start-site of the recently described truncated α1 and α2 receptors (144), therefore the amplified products can only be derived from full length TRα mRNAs. Additionally, the position of the TRα2 lower primer was chosen to allow differentiation between TRα2 and α3 splice variants according to product length (Fig. 2.2). 18S primers were also designed and used to control for the efficiency of the reverse transcription between samples. Primers were synthesised by Genosys Biotechnologies (Cambridge, U.K.) and details are given in Fig. 2.2 and Table. 2.2.
**Figure 2.2** Schematic representation of the PCR primer positions on the TR isoforms. Shading represent the main receptor domains (A/B domain, DNA binding domain and ligand binding domain from left to right, respectively) and identical shading indicates identical homology in those regions. Numbers represent bases, from the transcription start site onwards.
Table 2.2 RT-PCR primer pairs

<table>
<thead>
<tr>
<th>Product</th>
<th>Sequence (5' - 3')</th>
<th>Expected product length (bp)</th>
<th>Annealing temp(°C)</th>
</tr>
</thead>
</table>
| TRalpha1 | Upper primer CACCCCGGCCATCACC  
           Lower primer TGGGGCACTCGACTTTCATGT | 515 | 60 |
| TRalpha2 | Upper primer CACCCCGGCCATCACC  
           Lower primer ACTTCCCGCTTCACCAAACTG | 610* | 60 |
| TRbeta1 | Upper primer CAAGCGCCCAGACTTTC  
           Lower primer TTCCCCATTCAAGGTTAGAGT | 758 | 60 |
| TRbeta2 | Upper primer AAGCCTTTTCCTCAAGTGC  
           Lower primer TTCCCCATTCAAGGTTAGAGT | 766 | 56 |
| 18S     | Upper primer GTCCCCCAACTTCTTAGAG  
           Lower primer CACCTACGGAACCTTGTTAG | 413 | 53 |

*for the TRα3 variant, the expected product size will be 493 base pairs

2.6.7.3 Polymerase chain reaction

To minimise mispriming, “hot start” PCR was employed using “Platinum” Taq DNA polymerase which is inactive at low temperature due to an antibody binding at the active site. At 94 °C, the antibody denatures and the enzyme becomes active. PCR reactions consisted of: 0.5 μl of template (RT+/- or water), 1 x PCR buffer (50 mM KCl in 20 mM Tris-HCl; pH 8.4), 1 - 4 mM MgCl₂ (depending on optimisation; Table 2.3), 200 μM dNTP, 0.1 μM each primer and 50 mU/μl “Platinum” Taq DNA polymerase in a total volume of 25 μl. PCR was performed in a Perkin Elmer GeneAmp 9600 thermal cycler for the required number of cycles.

After PCR, an aliquot (10 μl) of each reaction was mixed with 2 μl DNA loading buffer and electrophoresed, along with a 100 bp marker (Gibco BRL) on a 2% (w/v) agarose gel (section 2.6.1.2), then stained with ethidium bromide (5 min staining followed by 1 h destaining). The 100 bp marker was used to confirm that the PCR products were of the expected size. Stained gels were photographed with UV transillumination using a digital
camera and an ethidium bromide filter. Digitised images were converted to 256 point greyscale, inverted and analysed by NIH image to determine band intensity.

To ensure maximal sensitivity and linearity of response, PCR conditions were optimised for each primer pair, using a template mix produced by pooling aliquots of all RT+ reactions; RT- reactions were similarly pooled for the negative control. Both RT+ and RT- template mixes were diluted 1:2 with water. Cycle number was first varied, to determine a suitable degree of amplification such that the product was detectable by ethidium bromide staining and within the linear range.

The 18S PCR reaction was first optimised using the standard conditions of 2 mM MgCl₂ and an annealing temperature of 52 °C (determined by the length and GC composition of the 18S primers). PCR was performed for 16, 18, 20, 22 and 24 cycles. A single band of the expected size was first seen after 18 cycles and a linear increase in signal strength was seen up to 24 cycles. The optimal MgCl₂ concentration was then determined by performing the PCR for 22 cycles at 1-4 mM MgCl₂. 1 mM seemed to be optimal with product amplification reduced by ~50% at greater than 3 mM MgCl₂. Thus 1 mM MgCl₂ was used in subsequent 18S PCR reactions. The final step was PCR (22 cycles; 1 mM MgCl₂) with serial dilutions of template concentration, starting with 1:2 and going down to a 1:16 dilution. A linear response was evident throughout the range of dilutions (Fig. 2.3).

TRα1 and TRα2/3 were optimised in parallel, due to the common upper primer. Initially, PCR was performed at 2 mM MgCl₂ and with an annealing temperature of 60 °C for 22, 26, 30, 34 and 38 cycles. For TRα1, a single band of the expected size was detectable from 30 cycles onwards whereas TRα2/3 PCR produced two bands. The main band, corresponding to the expected size for TRα2 was first seen at 26 cycles, with a fainter second band (corresponding to the expected TRα3 product size) apparent at 30 cycles. All three products increased linearly up to 38 cycles. Using 34 cycles of amplification and 1-4 mM MgCl₂, TRα1 and TRα3 signal strengths peaked at 1.5 mM MgCl₂ before declining while TRα2 peaked at 2.0 mM MgCl₂. There was not however much difference in signal strength between the two MgCl₂ concentrations therefore 2.0 mM MgCl₂ was subsequently used. Finally, using 2.0 mM MgCl₂ and 34 cycles of amplification, the effect of serial template dilutions was assessed (Fig. 2.4). Only TRα3 gave a linear response over the full range of dilutions; TRα1 and TRα2 signals were nonlinear when the template dilution was 1:2. Therefore, to ensure linearity TRα1 PCR was run for 30 cycles and TRα2/3 PCR for 28 cycles.
**Figure 2.3** Effect of template concentration on 18S PCR. 

**A)** Representative ethidium bromide stained gel. Lane A: 100 bp DNA ladder; Lanes B-E: serial template dilutions from 1 in 2 to 1 in 16, 18S PCR product size is given in bold. 

**B)** Plot using data from digitised images analysed with NIH image, each point represents the mean ± SEM of 2 replicates.
**Figure 2.4** Effect of template concentration on TRα isoform PCR. A) Representative ethidium bromide stained gel. Lanes E & J: 100 bp DNA ladder; Lanes A & F: 1 in 16 template dilution; Lanes B & G: 1 in 8 dilution; Lanes C & H: 1 in 4 dilution; Lanes D & I: 1 in 2 dilution, PCR product sizes are given in bold. B) Plot of TRα1 (circles), TRα2 (squares) and TRα3 (triangles) from digitised images analysed with NIH image, each point represents the mean ± SEM of 2 replicates.
TRβ1 and TRβ2 were also optimised parallel. Using 2.0 mM MgCl₂ and an annealing temperature of 56 °C, PCR was performed at 24, 28, 32, 36, 40 and 44 cycles. TRβ2 was undetectable after 44 cycles, which was not surprising as its presence is highly localised and in very small quantities in the fetal brain. No further optimisation was attempted for TRβ2. For TRα1, a single band of the expected size was visible after 32 cycles, its intensity increasing linearly up to 44 cycles, although some mispriming was evident after 40 cycles. The effect of varying MgCl₂ concentration was determined using 34 cycles. Signal strength peaked at 2.0 mM MgCl₂ and this was subsequently used. Template dilution experiments were initially hindered by mispriming, which was remedied by increasing the annealing temperature to 60 °C without loss of signal. TRβ1 PCR performed for 34 cycles at 60 °C with 2 mM MgCl₂, showed a linear response throughout the range of template dilutions used (Fig. 2.5). Optimised conditions for all PCR reactions are summarised in Table 2.3.

### Table 2.3 Optimised PCR conditions

<table>
<thead>
<tr>
<th>Amplicon</th>
<th>[MgCl₂] (mM)</th>
<th>Optimal annealing temperature (°C)</th>
<th>Template dilution</th>
<th>Cycle number</th>
</tr>
</thead>
<tbody>
<tr>
<td>IBS</td>
<td>1</td>
<td>52</td>
<td>1:2, 1:4 &amp; 1:8</td>
<td>20</td>
</tr>
<tr>
<td>TRα1</td>
<td>2</td>
<td>60</td>
<td>1:2, 1:4 &amp; 1:8</td>
<td>30</td>
</tr>
<tr>
<td>TRα2/3</td>
<td>2</td>
<td>60</td>
<td>1:2, 1:4 &amp; 1:8</td>
<td>28</td>
</tr>
<tr>
<td>TRβ1</td>
<td>2</td>
<td>60</td>
<td>1:2, 1:4 &amp; 1:8</td>
<td>34</td>
</tr>
</tbody>
</table>

As further confirmation of the identity of PCR products, Southern blotting was used to transfer DNA from the agarose gel onto Hybond N membranes, which were then probed with specific oligonucleotides. Prior to blotting, the DNA was denatured by soaking the gels in 0.5 M NaOH/1.5 M NaCl for 30 min then neutralised in 1.5 M Tris-HCl; pH 7.4/1.5 M NaCl for 30 min. DNA was transferred onto nylon membranes using the same set-up as for Northern blotting (section 2.6.3).
Figure 2.5 Effect of template concentration on TRβ1 PCR. A) Representative ethidium bromide stained gel. Lane A: 100 bp DNA ladder; Lanes B-D: serial template dilutions from 1 in 2 to 1 in 8, TRβ1 PCR product size is given in bold. B) Plot using data from digitised images analysed with NIH image, each point represents the mean ± SEM of 2 replicates.
2.6.1.4 Oligonucleotide probe design and hybridisation

Specific oligonucleotide probes were designed using Oligo 5, for 18S and each of the TR isoforms - including a common TRα2/α3 probe and a specific TRα2 probe (Table 2.4). These were synthesised by Genosys Biotechnologies Ltd. The labelling, hybridisation and washing procedures were performed as detailed in sections 2.6.5 and 2.6.6, and films were analysed as detailed in section 2.6.5.1.

Table 2.4 RT-PCR product oligonucleotide probes

<table>
<thead>
<tr>
<th>Product</th>
<th>Probe sequence</th>
<th>Hybridisation temp</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRα1</td>
<td>GGAAGCGGCTGGCGTGGCAGG</td>
<td>65.6 °C</td>
</tr>
<tr>
<td>TRα2</td>
<td>GGACGCCAGTGACCCCGCAGG</td>
<td>67.6 °C</td>
</tr>
<tr>
<td>TRα2/3</td>
<td>GCTCAAGCTGCCGGACCCTGCAG</td>
<td>63.7 °C</td>
</tr>
<tr>
<td>TRβ1</td>
<td>CCGTGGCTTGTCCCGGCACCA</td>
<td>61.7 °C</td>
</tr>
<tr>
<td>18S</td>
<td>TAAGTGCAGGCGTCGAGCTGCATAGTTA</td>
<td>61.6 °C</td>
</tr>
</tbody>
</table>

2.7 Statistical analysis

In most cases, statistical significance was assessed by two-way analysis of variance (ANOVA) with post-hoc analysis by Fisher’s protected least significant difference (PLSD) test where required. Prior to analysis, data were tested to ensure that the criteria for two-way ANOVA were satisfied. Firstly, the data was subjected to Bartlett’s test for homogeneity of variance, using the equation for unequal sample sizes (459). If the data passed this test, it was tested for normality. Residuals were determined, arranged in numerical order and split into four or more groups, based on the normal distribution. Chi-squared analysis was used to determine whether the number of residuals in each group differed significantly from the expected. When data failed to satisfy either of these tests it was transformed, either logarithmically or squared, and re-tested. Where two-way ANOVA was not appropriate, data was analysed by Student’s t-test, as indicated in text.
CHAPTER 3.

THE INFLUENCE OF MATERNAL HYPOTHYROXINEMIA ON FETO-PLACENTAL AND NEONATAL GROWTH
3.1 Animal model

The animal model used in this study is designed to enable investigation of the effects of maternal TH deprivation on fetal development. Fetal thyroid function should therefore be unaffected, indeed work with similar, albeit more severe, rat dam hypothyroid models indicates that fetal brain TH levels normalise soon after the onset of fetal thyroid function (411). The rat dams in this model are only partially thyroidectomised, the remaining thyroid gland responds by increasing T3 secretion compared to T4. Thus maternal hypothyroxinemia, rather hypothyroidism, is induced, which is analogous to the maternal hypothyroxinemia seen in humans in both iodine deficient and sufficient endemias. Although fetal thyroid function is thought to be normal, other potential confounding factors may occur when maternal TH function is disrupted. In severely hypothyroid dams, metabolic compromise occurs which impinges on fetal development (413,414). Similarly there is a risk that placental function is disturbed with detrimental effects upon the fetus. To avoid, or at least minimise, these confounding factors the degree of hypothyroxinemia induced in these studies is mild compared with that in other models (section 1.7.4).

Before analysing the results of maternal hypothyroxinemia on brain development therefore, it is important to ascertain whether the model satisfies the criteria of reduced maternal T4 without acquiring the confounding factors detailed above. One way of achieving this is to monitor the maternal serum TH and TSH levels throughout pregnancy and also examine fetal and neonatal parameters of growth and development—particularly brain, body and placental weights, and protein and DNA concentrations.

3.2 Maternal thyroid function

Maternal serum total T3, total T4 and TSH levels were all affected by treatment (Table 3.1 & Figs. 3.1 & 3.2). In addition, T4 levels exhibited age-dependency whereas TSH showed age-treatment interaction. In normal dams, serum T3 increased 1.5-fold from 14 to 16 dg, but showed no further change by 21 dg (Table 3.1). Partial thyroidectomy did not significantly change serum T3 levels at 14 dg, but they were reduced to 58-70% of control levels at the other ages.

T4 levels in N dams declined by 47% between 16 and 21 dg, with the largest fall (by 36%) occurring between 16 and 19 dg (Table 3.1). In contrast, Tx dam serum T4 levels decreased by 21% between 19 and 21 dg. Thus, while Tx dam T4 levels were ca. 35% of controls at 14 and 16 dg, they were 52% of control values at 19 and 21 dg.
Table 3.1 Maternal serum total 3,5,3'-triiodothyronine (T3) and thyroxine (T4) levels in normal (N) and partially thyroidectomised (Tx) rat dam pregnancies

A)

<table>
<thead>
<tr>
<th>Gestational stage</th>
<th>Dam status</th>
<th>T3 (nM)</th>
<th>T4 (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>14 dg N</td>
<td>0.89 ± 0.06 (7)</td>
<td>39.69 ± 1.70 (7)</td>
<td></td>
</tr>
<tr>
<td>14 dg Tx</td>
<td>0.60 ± 0.14 (5)</td>
<td>13.71 ± 1.69** (5)</td>
<td></td>
</tr>
<tr>
<td>16 dg N</td>
<td>1.31 ± 0.08 (34)</td>
<td>43.48 ± 1.60 (40)</td>
<td></td>
</tr>
<tr>
<td>16 dg Tx</td>
<td>0.76 ± 0.09** (29)</td>
<td>13.76 ± 0.92** (32)</td>
<td></td>
</tr>
<tr>
<td>19 dg N</td>
<td>1.27 ± 0.09 (28)</td>
<td>27.64 ± 1.27 (36)</td>
<td></td>
</tr>
<tr>
<td>19 dg Tx</td>
<td>0.89 ± 0.08* (27)</td>
<td>14.67 ± 0.83** (31)</td>
<td></td>
</tr>
<tr>
<td>21 dg N</td>
<td>1.39 ± 0.12 (14)</td>
<td>22.94 ± 1.27 (25)</td>
<td></td>
</tr>
<tr>
<td>21 dg Tx</td>
<td>0.88 ± 0.13* (18)</td>
<td>11.65 ± 0.74** (22)</td>
<td></td>
</tr>
</tbody>
</table>

Values are mean ± SEM; n given in parentheses

B) Statistical analysis, NS - No significant difference

<table>
<thead>
<tr>
<th>2-way ANOVA</th>
<th>T3</th>
<th>T4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>NS</td>
<td>P &lt; 0.0005</td>
</tr>
<tr>
<td>Treatment</td>
<td>P &lt; 0.0005</td>
<td>P &lt; 0.0005</td>
</tr>
<tr>
<td>Age-treatment interaction</td>
<td>NS</td>
<td>P &lt; 0.0005</td>
</tr>
</tbody>
</table>

Fisher’s PLSD

I. Treatment-related (Tx vs. N dam)
*P < 0.005 and **P < 0.0005

II. Age-related

<table>
<thead>
<tr>
<th>T3</th>
<th>T4</th>
</tr>
</thead>
<tbody>
<tr>
<td>N dams</td>
<td>Tx dams</td>
</tr>
<tr>
<td>16 vs. 14 dg</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>19 vs. 16 dg</td>
<td>NS</td>
</tr>
<tr>
<td>19 vs. 21 dg</td>
<td>NS</td>
</tr>
</tbody>
</table>
A) Gestational profile

Figure 3.1 The influence of maternal hypothyroxinemia on serum thyroid stimulating hormone (TSH) in dams throughout pregnancy. A) Gestational profile - each point represents the mean ± SEM of normal (N; open circles) or partially thyroidectomised (Tx; closed circles) dams, n is given in parentheses. B) Statistical analysis - NS; no significant difference.
Figure 3.2 Serum TSH concentration versus total T4 for normal (N; open symbols) and partially thyroidectomised (Tx; closed symbols) rat dams at 16 (squares), 19 (triangles) and 21 dg (circles).
In N dams, serum TSH levels (Fig. 3.1) did not change significantly between 16 and 19 dg although they tended to be higher at the latter age. Indeed by 21 dg, levels had fallen by 72% relative to 19 dg, although they were not significantly different to the 16 dg levels. In Tx dams, TSH levels were consistently higher than in controls, being 7-fold higher at 16 dg, 4-fold at 19 dg and 13-fold by 21 dg. Serum TSH levels did not change significantly with age in the Tx dams, although there was a tendency for levels to rise from 16 to 21 dg. It is interesting to note that when TSH levels are plotted against T4, there is little, or no, correlation between the two parameters in either group, yet TSH is always higher in Tx dams even when T4 levels are similar. TSH concentration is a more sensitive indicator of partial thyroidectomy, if not thyroid dysfunction, than T4.

3.3 Litter number

The number of progeny in each litter was strongly dependent on treatment and, to a lesser extent, on age (Fig. 3.3). In controls, litter number was constant between 14 and 19 dg, then decreased by 13% between 19 and 21 dg. The number of viable pups at birth was unchanged from the number of fetuses at 21 dg. In contrast, Tx dam litter size dropped by 34% between 21 dg and birth, but was unchanged prior to 21 dg. In addition, litter number was reduced in Tx dams at 16 dg (by 18%), and 19 dg (by 24%), relative to controls. Tx dam litter number normalised by 21 dg—due to the ontogenic decrease seen in N dams, but by birth, the average number of viable pups was lower (by 34%) than controls.

3.4 Gross parameters of growth in prenatal progeny

3.4.1 Fetal body and brain weights

As expected, body and brain weights were strongly dependent on age (Fig. 3.4 & Table 3.2). In addition, body weight was also treatment-dependent. Body weight increased 32-fold between 14 and 21 dg in N dam progeny and 29-fold in Tx dam progeny (Fig. 3.4). Between 16 and 21 dg however, body weight was consistently (7-8%) lower in Tx dam progeny relative to controls. Brain weight increased between 6.5 to 7-fold in N and Tx dam progeny between 14 and 21 dg, with no significant differences apparent between the treatment groups at any age (Table 3.2).
A) Ontogenic profile

<table>
<thead>
<tr>
<th>Age</th>
<th>N dam progeny</th>
<th>Tx dam progeny</th>
</tr>
</thead>
<tbody>
<tr>
<td>14 vs. 16 dg</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>19 vs. 16 dg</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>21 vs. 19 dg</td>
<td>*P &lt; 0.05</td>
<td>NS</td>
</tr>
<tr>
<td>birth vs. 21 dg</td>
<td>NS</td>
<td>*P &lt; 0.001</td>
</tr>
</tbody>
</table>

B) Statistical analysis

2-way ANOVA
- Age: *P < 0.005
- Treatment: **P < 0.0005
- Age-treatment interaction: NS

Fisher's PLSD

I. Treatment-related (Tx vs. N dam progeny)
*P < 0.005 and **P < 0.0005

II. Age-related

Figure 3.3 The influence of maternal hypothyroxinemia on the number of progeny per litter. A) Ontogenic profile - each bar represents the mean ± SEM of normal (N; open bars) or partially thyroidectomised (Tx; closed bars) dam litters, n is indicated in the bars. B) Statistical analysis - NS; no significant difference.
**A) Ontogenic profile**

![Bar chart showing ontogenic profile of body weight from 14 days gestation (dg) to 30 postnatal (pnd)].

**B) Statistical analysis**

<table>
<thead>
<tr>
<th>2-way ANOVA</th>
<th>Prenatal</th>
<th>Postnatal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>$P &lt; 0.0005$</td>
<td>$P &lt; 0.0005$</td>
</tr>
<tr>
<td>Treatment</td>
<td>$P &lt; 0.0005$</td>
<td>NS</td>
</tr>
<tr>
<td>Age-treatment interaction</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

**Fisher's PLSD**

*I. Treatment-related* (Tx vs. N dam progeny)

*P* < 0.01 and **P* < 0.001

**II. Age-related**

<table>
<thead>
<tr>
<th>Comparison</th>
<th>N dam progeny</th>
<th>Tx dam progeny</th>
</tr>
</thead>
<tbody>
<tr>
<td>16 vs. 14 dg</td>
<td>$P &lt; 0.0005$</td>
<td>$P &lt; 0.0005$</td>
</tr>
<tr>
<td>19 vs. 16 dg</td>
<td>$P &lt; 0.0005$</td>
<td>$P &lt; 0.0005$</td>
</tr>
<tr>
<td>21 vs. 19 dg</td>
<td>$P &lt; 0.0005$</td>
<td>$P &lt; 0.0005$</td>
</tr>
<tr>
<td>birth vs. 21 dg</td>
<td>$P &lt; 0.0005$</td>
<td>$P &lt; 0.0005$</td>
</tr>
<tr>
<td>10 pnd vs. birth</td>
<td>$P &lt; 0.0005$</td>
<td>$P &lt; 0.0005$</td>
</tr>
<tr>
<td>20 vs. 10 pnd</td>
<td>$P &lt; 0.0005$</td>
<td>$P &lt; 0.0005$</td>
</tr>
<tr>
<td>30 vs. 20 pnd</td>
<td>$P &lt; 0.0005$</td>
<td>$P &lt; 0.0005$</td>
</tr>
</tbody>
</table>

**Figure 3.4** The influence of maternal hypothyroxinemia on progeny body weight. *A*) Ontogenic profile - each bar represents the mean ± SEM of normal (N; open bars) or partially thyroidectomised (Tx; closed bars) dam progeny, n is indicated in the bars. *B*) Statistical analysis - NS; no significant difference.
Table 3.2 Fetal brain weight, protein and DNA levels in normal (N) and partially thyroidectomised (Tx) dam progeny

<table>
<thead>
<tr>
<th>Age (dg)</th>
<th>Dam status</th>
<th>Fetal brain weight (mg)</th>
<th>Fetal brain protein concentration (mg/g wet weight)</th>
<th>Fetal brain protein content (mg)</th>
<th>Fetal brain DNA concentration (mg/g wet weight)</th>
<th>Fetal brain DNA content (mg)</th>
<th>Fetal brain protein:DNA ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>N</td>
<td>27.3 ± 1.3 (7)</td>
<td>41.64 ± 1.67 (7)</td>
<td>1.13 ± 0.06 (7)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Tx</td>
<td>26.0 ± 0.5 (7)</td>
<td>38.62 ± 1.53 (5)</td>
<td>1.00 ± 0.02 (5)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>16</td>
<td>N</td>
<td>47.3 ± 1.9 (38)</td>
<td>60.62 ± 1.80 (15)</td>
<td>3.08 ± 0.18 (15)</td>
<td>8.79 ± 1.14 (6)</td>
<td>0.47 ± 0.07 (6)</td>
<td>7.67 ± 1.05 (6)</td>
</tr>
<tr>
<td></td>
<td>Tx</td>
<td>48.4 ± 1.9 (30)</td>
<td>62.14 ± 4.65 (10)</td>
<td>2.83 ± 0.23 (10)</td>
<td>9.51 ± 0.71 (6)</td>
<td>0.43 ± 0.03 (6)</td>
<td>7.67 ± 0.79 (6)</td>
</tr>
<tr>
<td>19</td>
<td>N</td>
<td>127.8 ± 2.0 (37)</td>
<td>63.34 ± 2.24 (12)</td>
<td>8.36 ± 0.34 (12)</td>
<td>9.52 ± 0.49 (4)</td>
<td>1.34 ± 0.11 (4)</td>
<td>7.41 ± 0.67 (4)</td>
</tr>
<tr>
<td></td>
<td>Tx</td>
<td>121.7 ± 2.2 (27)</td>
<td>67.08 ± 2.32 (13)</td>
<td>8.08 ± 0.29 (13)</td>
<td>9.47 ± 0.79 (4)</td>
<td>1.13 ± 0.18 (4)</td>
<td>7.95 ± 0.71 (4)</td>
</tr>
<tr>
<td>21</td>
<td>N</td>
<td>177.2 ± 2.8 (25)</td>
<td>72.72 ± 4.85 (9)</td>
<td>12.61 ± 0.78 (9)</td>
<td>6.98 ± 0.61 (7)</td>
<td>1.20 ± 0.10 (7)</td>
<td>11.71 ± 1.08 (7)</td>
</tr>
<tr>
<td></td>
<td>Tx</td>
<td>179.9 ± 5.7 (20)</td>
<td>68.69 ± 2.86 (8)</td>
<td>12.49 ± 0.78 (8)</td>
<td>6.91 ± 1.31 (4)</td>
<td>1.17 ± 0.22 (4)</td>
<td>11.91 ± 2.66 (4)</td>
</tr>
</tbody>
</table>

2-way ANOVA

<table>
<thead>
<tr>
<th>Age</th>
<th>Treatment</th>
<th>Age-treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P</td>
<td>P</td>
</tr>
<tr>
<td></td>
<td>&lt; 0.0005</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>&lt; 0.0005</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>&lt; 0.0005</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>&lt; 0.0005</td>
<td>&lt; 0.0005</td>
</tr>
<tr>
<td></td>
<td>&lt; 0.0005</td>
<td>&lt; 0.0005</td>
</tr>
<tr>
<td></td>
<td>&lt; 0.0005</td>
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<td></td>
<td>&lt; 0.0005</td>
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<tr>
<td></td>
<td>&lt; 0.0005</td>
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<tr>
<td></td>
<td>&lt; 0.0005</td>
<td>&lt; 0.0005</td>
</tr>
<tr>
<td></td>
<td>&lt; 0.0005</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>&lt; 0.0005</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>&lt; 0.0005</td>
<td>NS</td>
</tr>
</tbody>
</table>

Fisher’s PLSD

I. Treatment-related
NS at all ages and parameters

II. Age-related
N dam progeny

| 16 vs. 14 dg | P         | < 0.0005 |
| 19 vs. 16 dg | P         | < 0.0005 |
| 21 vs. 19 dg | P         | < 0.0005 |

Tx dam progeny

| 16 vs. 14 dg | P         | < 0.0005 |
| 19 vs. 16 dg | P         | < 0.0005 |
| 21 vs. 19 dg | P         | < 0.0005 |

Values are mean ± SEM, n is given in parentheses; NS - no significant difference, ND - not determined
Body weights increased at a greater rate than brain weight, thus brain:body weight ratios also showed age-dependency; decreasing by almost 80% from 14 to 21 dg in N and Tx dam progeny (Fig. 3.5). A treatment effect was also apparent, with the ratio being 11% higher in the experimental group relative to control at 16 dg, but no other age. This was of course due primarily to the depression in Tx dam progeny body weight at this age.

3.4.2 Fetal brain protein

Protein concentration in fetal brain was age-dependant (Table 3.2). Post-hoc analysis indicated however that the age effect was limited to between 14 and 16 dg with an increase in protein concentration of ca. 1.6-fold in both groups. No treatment effects were evident. Protein content also exhibited age-dependency, increasing 11 to 12-fold between 14 and 21 dg in both groups, and no significant treatment effect was found at any age for this parameter.

3.4.3 Fetal brain DNA and protein:DNA ratio

DNA concentration is a measure of cell density. In fetal brain this parameter was age-dependent, though no significant differences were noted between any ages by post-hoc analysis (Table 3.2). No treatment-related effects were found. DNA content per brain can be used as an estimate of cell number. This parameter was age-dependent in both groups, due to a ca. 2.9-fold increase between 16 and 19 dg (Table 3.2). No further change occurred by 21 dg and no treatment effects were apparent.

The protein:DNA ratio gives an indication of cell size and this parameter was also age-dependent in fetal brain (Table 3.2). In N dam progeny, the ratio increased 1.6-fold between 19 and 21 dg. This ratio in Tx dam progeny also increased 1.6-fold by 21 dg, but only significantly so, when compared with 16 dg. No significant treatment effect was evident.

3.5 Gross parameters of placental growth

3.5.1 Placental weight

Placental weight was age-dependent but unaffected by treatment (Table 3.3). In N dam pregnancies, placental weight increased 1.9-fold from 16 to 21 dg. Tx dam placental weight also increased, but only between 16 and 19 dg (by 1.6-fold).
A) Ontogenic profile

B) Statistical analysis

<table>
<thead>
<tr>
<th>2-way ANOVA</th>
<th>Prenatal</th>
<th>Postnatal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>$P &lt; 0.0005$</td>
<td>$P &lt; 0.0005$</td>
</tr>
<tr>
<td>Treatment</td>
<td>$P &lt; 0.05$</td>
<td>NS</td>
</tr>
<tr>
<td>Age-treatment interaction</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

Fisher's PLSD

I. Treatment-related (Tx vs. N dam progeny)

* $P < 0.01$

II. Age-related

| 16 vs. 14 dg                | $P < 0.0005$ | $P < 0.0005$ |
| 19 vs. 16 dg                | $P < 0.0005$ | $P < 0.0005$ |
| 21 vs. 19 dg                | $P < 0.0005$ | $P < 0.0005$ |
| birth vs. 21 dg             | NS          | NS         |
| 10 pnd vs. birth            | NS          | NS         |
| 20 vs. 10 pnd               | $P < 0.005$  | $P < 0.0005$ |
| 30 vs. 20 pnd               | $P < 0.0005$ | $P < 0.0005$ |

Figure 3.5 The influence of maternal hypothyroxinemia on progeny brain/body weight ratio. A) Ontogenic profile - each bar represents the mean ± SEM of normal (N; open bars) or partially thyroidectomised (Tx; closed bars) dam progeny, n is indicated in the bars. B) Statistical analysis - NS; no significant difference.
Table 3.3 Placental growth and development in normal (N) and partially thyroidectomised (Tx) dams

<table>
<thead>
<tr>
<th>Age (dg)</th>
<th>Dam status</th>
<th>Placental weight (mg)</th>
<th>Placental protein concentration (mg/g wet weight)</th>
<th>Placental protein content (mg)</th>
<th>Placental DNA concentration (mg/g wet weight)</th>
<th>Placental DNA content (mg)</th>
<th>Placental protein:DNA ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>N</td>
<td>314 ± 22 (9)</td>
<td>100.32 ± 6.73 (6)</td>
<td>30.45 ± 5.88 (6)</td>
<td>3.98 ± 0.39 (5)</td>
<td>1.25 ± 0.20 (5)</td>
<td>25.93 ± 2.76 (5)</td>
</tr>
<tr>
<td></td>
<td>Tx</td>
<td>304 ± 20 (9)</td>
<td>102.40 ± 4.80 (5)</td>
<td>27.74 ± 1.75 (5)</td>
<td>4.59 ± 0.27 (5)</td>
<td>1.24 ± 0.10 (5)</td>
<td>22.80 ± 2.42 (5)</td>
</tr>
<tr>
<td>19</td>
<td>N</td>
<td>488 ± 9 (13)</td>
<td>115.93 ± 5.29 (5)</td>
<td>57.98 ± 1.67 (5)</td>
<td>3.11 ± 0.29 (5)</td>
<td>1.55 ± 0.12 (5)</td>
<td>38.66 ± 4.09 (5)</td>
</tr>
<tr>
<td></td>
<td>Tx</td>
<td>484 ± 36 (5)</td>
<td>106.56 ± 8.57 (4)</td>
<td>48.37 ± 5.29* (4)</td>
<td>4.02 ± 0.53 (4)</td>
<td>1.84 ± 0.30 (4)</td>
<td>27.01 ± 1.52* (4)</td>
</tr>
<tr>
<td>21</td>
<td>N</td>
<td>583 ± 22 (5)</td>
<td>125.91 ± 6.13 (5)</td>
<td>75.80 ± 4.81 (5)</td>
<td>3.23 ± 0.28 (5)</td>
<td>1.95 ± 0.15 (5)</td>
<td>39.57 ± 2.73 (5)</td>
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<tr>
<td></td>
<td>Tx</td>
<td>545 ± 32 (7)</td>
<td>130.85 ± 10.44 (5)</td>
<td>67.11 ± 4.87 (5)</td>
<td>2.77 ± 0.10 (5)</td>
<td>1.44 ± 0.14 (5)</td>
<td>47.27 ± 3.00 (5)</td>
</tr>
</tbody>
</table>

2-way ANOVA

<table>
<thead>
<tr>
<th>Age</th>
<th>P</th>
<th>Treatment</th>
<th>P</th>
<th>Age-treatment</th>
<th>P</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>&lt; 0.0005</td>
<td>NS</td>
<td>&lt; 0.05</td>
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<td>NS</td>
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<td>&lt; 0.005</td>
<td>NS</td>
<td>&lt; 0.05</td>
<td>NS</td>
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<td>&lt; 0.05</td>
<td>NS</td>
<td>&lt; 0.005</td>
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</tr>
</tbody>
</table>

Fisher's PLSD

I. Treatment-related

*P < 0.05 Tx vs. N dam progeny

II. Age-related

N dam progeny

<table>
<thead>
<tr>
<th>19 vs. 16 dg</th>
<th>P</th>
<th>NS</th>
<th>&lt; 0.0005</th>
<th>NS</th>
<th>&lt; 0.005</th>
</tr>
</thead>
<tbody>
<tr>
<td>21 vs. 19 dg</td>
<td>P &lt; 0.01</td>
<td>NS</td>
<td>&lt; 0.005</td>
<td>NS</td>
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</tbody>
</table>

Tx dam progeny

<table>
<thead>
<tr>
<th>19 vs. 16 dg</th>
<th>P &lt; 0.0005</th>
<th>NS</th>
<th>&lt; 0.0005</th>
<th>NS</th>
<th>NS</th>
</tr>
</thead>
<tbody>
<tr>
<td>21 vs. 19 dg</td>
<td>P &lt; 0.05</td>
<td>&lt; 0.05</td>
<td>&lt; 0.05</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values are mean ± SEM, n is given in parentheses; NS - no significant difference
3.5.2 Placental protein

Protein concentration in placenta was also dependent on age (Table 3.3). In controls there was a tendency for protein concentration to increase with gestation, but no age point differed significantly from any other. Tx dam placental protein concentration showed a stronger age effect in that there was a significant 1.3-fold increase between 19 and 21 dg. Placental protein concentration was not significantly different between the normal and experimental groups however.

Placental protein content was treatment and age-dependent (Table 3.3). In controls, protein content increased 2.1-fold from 16 to 21 dg. Protein content also increased, 2.6-fold, in Tx dam progeny but there was a tendency for this parameter to be reduced compared to the age-matched control, although only at 19 dg was the difference (83% of control value) significant by post-hoc analysis.

3.5.3 Placental DNA and protein:DNA ratio

Placental DNA concentration was age-dependent (Table 3.3), and approached treatment-dependency ($P = 0.0954$). No age effects were apparent in N dam placentae by post-hoc analysis, whereas Tx dam placental DNA concentration decreased by 21% between 19 and 21 dg. In addition, there was a tendency for Tx dam placental DNA concentration to be higher than controls at 16 and 19 dg, and lower than controls at 21 dg—none of these differences were significant by post-hoc analysis however. Placental DNA content was also age-dependent; in controls this parameter increased 1.6-fold between 16 and 21 dg (Table 3.3). In Tx dam placentae, the increase (1.5-fold) in DNA content was limited to between 16 and 19 dg. No significant treatment-related differences were apparent however.

Placental protein:DNA ratio was age-dependent and also exhibited age-treatment interaction (Table 3.3). In controls, this ratio increased 1.5-fold between 16 and 19 dg with no further change by 21 dg. This increase appeared to be delayed in Tx dam progeny, occurring between 19 and 21 dg (by 1.7-fold). As a result, at 19 dg the ratio in Tx dam progeny was 30% lower than in controls. The ratio had increased to normal levels by 21 dg however.
3.6 Gross parameters of growth in postnatal progeny

3.6.1 Postnatal body weights

Postnatal body weight was age-dependent (Fig. 3.4), and also approached treatment-dependency ($P = 0.0562$). In both N and Tx dam progeny, body weight increased between 15 to 16-fold from the day of birth to 30 pnd. No significant treatment-related differences were apparent at any age, although there was a tendency for body weights to be lower in Tx dam progeny on the day of birth—following on from the reduced body weight in Tx dam progeny at 21 dg (Fig. 3.4). Brain:body weight ratio was age-dependent, continuing the fetal trend to decrease with age. In contrast to fetal development, however no treatment effects were apparent in this parameter.

3.6.2 Postnatal brain region weights

The weight of all four regions showed strong age-dependency (Table 3.4). Cerebral cortex weight increased in both groups by between 1.6 to 1.9-fold from 10 to 30 pnd. No treatment effects were found at any age. Cerebellar weight increased in both groups by between 2.1 to 2.3-fold from 10 to 20 pnd, but no further significant increase occurred by 30 pnd. No treatment effects were seen at any age.

Brain stem weight increased 2-fold in N dam progeny between 10 and 30 pnd (Table 3.4). Tx dam progeny also exhibited a 2-fold increase in brain stem weight, but only between 10 and 20 pnd. Nevertheless, no significant treatment-related differences were found at any age. Subcortical weight in N dam progeny increased 1.3-fold between 10 and 20 pnd, but then did not change significantly by 30 pnd (Table 3.4). In Tx dam progeny subcortical weight similarly increased 1.4-fold between 10 and 20 pnd with a further 1.2-fold increase by 30 pnd. As a result, subcortical weight was 14% higher in Tx dam progeny at 30 pnd relative to controls. The relevance of this observation is, however, questionable since no treatment or age-treatment effects were found by 2-way ANOVA.

3.6.3 Postnatal brain region protein

Protein concentration was age-dependent in all brain regions studied, but varied little between regions (Table 3.5). In N and Tx dam progeny, cerebral cortical protein concentration increased 1.4-fold from 10 to 20 pnd. There was a tendency for a further increase by 30 pnd, although this was only significant in Tx dam progeny. Values were however, very similar in both control and experimental groups and there was no significant
Table 3.4 Postnatal brain region weights in normal (N) and partially thyroidectomised (Tx) dam progeny

A)

<table>
<thead>
<tr>
<th>Region</th>
<th>Age (pnd)</th>
<th>Dam status</th>
<th>n</th>
<th>Brain weight (mg)</th>
</tr>
</thead>
<tbody>
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<td>7</td>
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<tr>
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<td></td>
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<td>17</td>
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</tr>
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<td>Tx</td>
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<td>836 ± 21</td>
</tr>
<tr>
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<td>N</td>
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</tr>
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</tr>
<tr>
<td></td>
<td></td>
<td>Tx</td>
<td>14</td>
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</tr>
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<td></td>
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</table>

Values are mean ± SEM

B) Statistical analysis, NS - No significant difference

<table>
<thead>
<tr>
<th>2-way ANOVA</th>
<th>Cerebral cortex</th>
<th>Cerebellum</th>
<th>Brain stem</th>
<th>Subcortex</th>
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<td>( P &lt; 0.0005 )</td>
<td>( P &lt; 0.0005 )</td>
<td>( P &lt; 0.0005 )</td>
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<td>NS</td>
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</tr>
<tr>
<td>Age-treatment interaction</td>
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<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

Fisher's PLSD

I. Treatment-related

*\( P < 0.05 \) Tx vs. N dam progeny

II. Age-related

<table>
<thead>
<tr>
<th>N dam progeny</th>
<th>Cerebral cortex</th>
<th>Cerebellum</th>
<th>Brain stem</th>
<th>Subcortex</th>
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<tbody>
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<td>( P &lt; 0.0005 )</td>
<td>( P &lt; 0.0005 )</td>
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<td>( P &lt; 0.01 )</td>
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<td>Tx dam progeny</td>
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<tr>
<td>20 vs. 10 pnd</td>
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<td>( P &lt; 0.0005 )</td>
<td>( P &lt; 0.0005 )</td>
<td>( P &lt; 0.0005 )</td>
</tr>
<tr>
<td>30 vs. 20 pnd</td>
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<td>NS</td>
<td>( P &lt; 0.05 )</td>
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</tbody>
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Table 3.5 Protein concentration in postnatal normal (N) and partially thyroidectomised (Tx) dam progeny brain regions

<table>
<thead>
<tr>
<th>Region</th>
<th>Age (pnd)</th>
<th>Dam status</th>
<th>n</th>
<th>Protein concentration (mg/g wt weight)</th>
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</thead>
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<td></td>
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<td>Tx</td>
<td>7</td>
<td>109.64 ± 5.98</td>
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<td>30</td>
<td>N</td>
<td>7</td>
<td>121.73 ± 3.93</td>
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<td>Tx</td>
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<td>N</td>
<td>7</td>
<td>73.64 ± 3.04</td>
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<td>Tx</td>
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<td>N</td>
<td>7</td>
<td>73.14 ± 4.18</td>
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<td>Tx</td>
<td>9</td>
<td>106.75 ± 5.89</td>
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</table>

Values are mean ± SEM

B) Statistical analysis, NS - No significant difference

<table>
<thead>
<tr>
<th>2-way ANOVA</th>
<th>Cerebral cortex</th>
<th>Cerebellum</th>
<th>Brain stem</th>
<th>Subcortex</th>
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<tbody>
<tr>
<td>Age</td>
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<td>P &lt; 0.0005</td>
<td>P &lt; 0.0005</td>
<td>P &lt; 0.0005</td>
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<tr>
<td>Treatment</td>
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<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Age-treatment interaction</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

Fisher’s PLSD

I. Treatment-related
NS at all ages and regions

II. Age-related

<table>
<thead>
<tr>
<th>N dam progeny</th>
<th>Cerebral cortex</th>
<th>Cerebellum</th>
<th>Brain stem</th>
<th>Subcortex</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 vs. 10 pnd</td>
<td>P &lt; 0.005</td>
<td>P &lt; 0.05</td>
<td>P &lt; 0.0005</td>
<td>P &lt; 0.005</td>
</tr>
<tr>
<td>30 vs. 20 pnd</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Tx dam progeny</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.05</td>
<td>P &lt; 0.005</td>
<td>P &lt; 0.05</td>
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<td>20 vs. 10 pnd</td>
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<td>NS</td>
<td>NS</td>
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<tr>
<td>30 vs. 20 pnd</td>
<td>P &lt; 0.05</td>
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</tbody>
</table>
treatment effect. Cerebellar protein concentration increased in N and Tx dam progeny by between 1.2 and 1.4-fold from 10 to 20 pnd but showed no further change by 30 pnd. No significant treatment-related differences were observed. Brain stem protein concentration increased by 1.4-fold in N and Tx dam progeny from 10 to 20 pnd, with no further change by 30 pnd, and no differences between the treatment groups. Similarly, protein concentration in subcortex increased in both groups between 10 and 20 pnd by 1.3-fold. No further change was seen at 30 pnd and no differences were observed between groups.

Brain region protein content exhibited similar, albeit larger, age-related changes to protein concentration (Table 3.6). No treatment-related effects were noted for any region or age. In cerebral cortex, protein content increased 2.7-fold between 10 and 30 pnd. In the other regions, ontogenic increases occurred between 10 and 20 pnd. In cerebellum and brain stem, protein content increased by between 2.6 and 3.0-fold during this period whereas subcortical protein content increased by 1.9-fold.

3.6.4 Postnatal brain region DNA

Age-related changes in DNA concentration and content were less uniform in the different brain regions than for protein. In cerebral cortex, DNA concentration was age-dependent, decreasing by 19-24% from 10 to 20 pnd in N and Tx dam progeny (Table 3.7). No treatment-related differences were apparent at any age. Cerebellar DNA concentration was greater than in the other brain regions and showed age-dependent changes. Post-hoc analysis however indicated this age effect was only seen in N dam progeny, whose DNA concentration decreased by 36% between 20 and 30 pnd. Tx dam progeny DNA concentration did not change with age in this region and was reduced by 24% relative to controls at 20 pnd, but not at the other ages. The absence of an overall treatment or age-treatment effect by 2-way ANOVA casts doubt on the relevance of this difference however. Brain stem DNA concentration did not change significantly with age or treatment, although a trend for DNA concentration to decrease with age was visible. Subcortical DNA concentration was age-dependent, with a tendency to decrease between 10 and 20 pnd. No age was shown to be significantly different by post-hoc analysis however, and no treatment effects were apparent.

Cerebral cortical DNA content was age-dependent but post-hoc analysis did not highlight any particular age point as being different from any other. No treatment effects were observed (Table 3.8). DNA content in cerebellum was more strongly age-dependent, increasing in N and Tx dam progeny by between 2 to 2.5-fold from 10 to 20 pnd. No
Table 3.6 Protein content in postnatal normal (N) and partially thyroidectomised (Tx) dam progeny brain regions

<table>
<thead>
<tr>
<th>Region</th>
<th>Age (pnd)</th>
<th>Dam status</th>
<th>n</th>
<th>Protein content (mg)</th>
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<tr>
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<td>N</td>
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<td>37.94 ± 3.47</td>
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<td>5</td>
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<td>20</td>
<td>N</td>
<td>8</td>
<td>85.68 ± 5.34</td>
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<td>Cerebellum</td>
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<td>N</td>
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<td>5.19 ± 0.66</td>
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Values are mean ± SEM

B) Statistical analysis, NS - No significant difference

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<th>Cerebellum</th>
<th>Brain stem</th>
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Fisher's PLSD

I. Treatment-related
NS at all ages and regions

II. Age-related

<table>
<thead>
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<th>N dam progeny</th>
<th>Cerebral cortex</th>
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<td></td>
<td></td>
</tr>
<tr>
<td>20 vs. 10 pnd</td>
<td>P &lt; 0.0005</td>
<td>P &lt; 0.0005</td>
<td>P &lt; 0.0005</td>
<td>P &lt; 0.005</td>
</tr>
<tr>
<td>30 vs. 20 pnd</td>
<td>P &lt; 0.05</td>
<td>NS</td>
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<td>NS</td>
</tr>
</tbody>
</table>
Table 3.7 DNA concentration in postnatal normal (N) and partially thyroidectomised (Tx) dam progeny brain regions

<table>
<thead>
<tr>
<th>Region</th>
<th>Age (pnd)</th>
<th>Dam status</th>
<th>n</th>
<th>DNA concentration (mg/g wt weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cerebral cortex</td>
<td>10</td>
<td>N</td>
<td>5</td>
<td>3.51 ± 0.11</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>N</td>
<td>5</td>
<td>2.85 ± 0.10</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>N</td>
<td>6</td>
<td>2.62 ± 0.18</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>10</td>
<td>N</td>
<td>5</td>
<td>13.65 ± 0.73</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>N</td>
<td>5</td>
<td>15.53 ± 0.48</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>N</td>
<td>6</td>
<td>10.00 ± 0.98</td>
</tr>
<tr>
<td>Brain stem</td>
<td>10</td>
<td>N</td>
<td>5</td>
<td>3.97 ± 0.22</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>N</td>
<td>5</td>
<td>3.52 ± 0.36</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>N</td>
<td>6</td>
<td>2.93 ± 0.54</td>
</tr>
<tr>
<td>Subcortex</td>
<td>10</td>
<td>N</td>
<td>5</td>
<td>3.31 ± 0.12</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>N</td>
<td>5</td>
<td>2.50 ± 0.17</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>N</td>
<td>6</td>
<td>2.40 ± 0.24</td>
</tr>
</tbody>
</table>

Values are mean ± SEM

B) Statistical analysis, NS - No significant difference

<table>
<thead>
<tr>
<th>2-way ANOVA</th>
<th>Cerebral cortex</th>
<th>Cerebellum</th>
<th>Brain stem</th>
<th>Subcortex</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>P &lt; 0.0005</td>
<td>P &lt; 0.05</td>
<td>NS</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>Treatment</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Age-treatment interaction</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

Fisher’s PLSD

I. Treatment-related

*P < 0.05 Tx vs. N dam progeny

II. Age-related

N dam progeny

| 20 vs. 10 pnd | P < 0.05 | NS | NS | NS |
| 30 vs. 20 pnd | NS       | P < 0.005 | NS | NS |

Tx dam progeny

| 20 vs. 10 pnd | P < 0.05 | NS | NS | NS |
| 30 vs. 20 pnd | NS       | NS | NS | NS |
Table 3.8 DNA content in postnatal normal (N) and partially thyroidectomised (Tx) dam progeny brain regions

<table>
<thead>
<tr>
<th>Region</th>
<th>Age (pnd)</th>
<th>Dam status</th>
<th>DNA content (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cerebral cortex</td>
<td>10</td>
<td>N</td>
<td>1.64 ± 0.10</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>N</td>
<td>2.11 ± 0.14</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>N</td>
<td>2.15 ± 0.20</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>Tx</td>
<td>1.62 ± 0.16</td>
</tr>
<tr>
<td></td>
<td>Tx</td>
<td>N</td>
<td>2.01 ± 0.25</td>
</tr>
<tr>
<td></td>
<td>Tx</td>
<td>N</td>
<td>2.06 ± 0.14</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>10</td>
<td>N</td>
<td>0.99 ± 0.16</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>N</td>
<td>2.48 ± 0.13</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>N</td>
<td>1.82 ± 0.36</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>Tx</td>
<td>0.99 ± 0.14</td>
</tr>
<tr>
<td></td>
<td>Tx</td>
<td>N</td>
<td>2.03 ± 0.39</td>
</tr>
<tr>
<td></td>
<td>Tx</td>
<td>N</td>
<td>2.04 ± 0.30</td>
</tr>
<tr>
<td>Brain stem</td>
<td>10</td>
<td>N</td>
<td>0.41 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>N</td>
<td>0.62 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>N</td>
<td>0.57 ± 0.10</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>Tx</td>
<td>0.55 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>Tx</td>
<td>N</td>
<td>0.50 ± 0.03</td>
</tr>
<tr>
<td>Subcortex</td>
<td>10</td>
<td>N</td>
<td>0.41 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>N</td>
<td>0.52 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>N</td>
<td>0.48 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>Tx</td>
<td>0.41 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>Tx</td>
<td>N</td>
<td>0.54 ± 0.07</td>
</tr>
</tbody>
</table>

Values are mean ± SEM; n = 5

B) Statistical analysis, NS - No significant difference

<table>
<thead>
<tr>
<th>2-way ANOVA</th>
<th>Cerebral cortex</th>
<th>Cerebellum</th>
<th>Brain stem</th>
<th>Subcortex</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>P &lt; 0.05</td>
<td>P &lt; 0.0005</td>
<td>P &lt; 0.005</td>
<td>NS</td>
</tr>
<tr>
<td>Treatment</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Age-treatment interaction</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

Fisher’s PLSD

I. Treatment-related

NS at all ages and regions

II. Age-related

<table>
<thead>
<tr>
<th>N dam progeny</th>
<th>Cerebral cortex</th>
<th>Cerebellum</th>
<th>Brain stem</th>
<th>Subcortex</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 vs. 10 pnd</td>
<td>NS</td>
<td>P &lt; 0.001</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>30 vs. 20 pnd</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Tx dam progeny</th>
<th>Cerebral cortex</th>
<th>Cerebellum</th>
<th>Brain stem</th>
<th>Subcortex</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 vs. 10 pnd</td>
<td>NS</td>
<td>P &lt; 0.05</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>30 vs. 20 pnd</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>
further change was seen by 30 pnd and there was no treatment effect. Brain stem DNA content also appeared age-dependent, however post-hoc analysis indicated that only Tx dam progeny showed any significant change—DNA content increasing 1.8-fold between 10 and 20 pnd. N dam progeny showed the same trend however, thus no treatment effects were apparent. DNA content in subcortex did not change significantly with age or treatment between 10 and 30 pnd.

3.6.5 Postnatal brain region protein:DNA ratio

Protein:DNA ratio showed age-dependency in all four regions with little or no indication of treatment effects (Table 3.9). In cerebral cortex this ratio increased by between 1.6 to 1.8-fold in N and Tx dam progeny between 10 and 20 pnd, with no further change up to 30 pnd. Protein:DNA ratio in N dam progeny cerebellum showed no significant change until 20 pnd, the ratio increasing 1.7-fold by 30 pnd. In contrast, there was an initial 1.5-fold rise in protein:DNA ratio between 10 and 20 pnd in Tx dam progeny which then remained unchanged up to 30 pnd. As a result, post-hoc analysis indicated that the protein:DNA ratio in Tx dam progeny cerebellum was 41% higher at 20 pnd relative to controls, but normal at the other ages. Again, the lack of any overall treatment effect by 2-way ANOVA suggests that this result probably arose by chance. Brain stem protein:DNA ratio increased 1.6-fold from 10 to 20 pnd in N dam progeny with a further, albeit insignificant, 1.3-fold increase by 30 pnd. The ratio similarly increased in Tx dam progeny brain stem, rising 2.1-fold between 10 and 30 pnd; no treatment effects were apparent. Subcortical protein:DNA ratio showed a 1.6-fold increase in N dam progeny between 10 and 20 pnd, no further increase occurred by 30 pnd. Minor, but insignificant, differences in the Tx dam progeny ratios meant that a 1.6-fold increase was seen between 10 and 30 pnd, but there was no indication of treatment-related differences.

3.7. Summary of results

As expected in this model, T4 was more severely depressed than T3, indicating that the dams were hypothyroxinemic rather than hypothyroid. The consistently high TSH levels in Tx dams indicated that the pituitary feedback response was normal, and also that this response was not able to compensate for the decreased synthetic capacity of the thyroid. Partial compensation was seen however, in that T4 levels in Tx dams did not fall so steeply during pregnancy as in N dams. Nevertheless, as serum T3 levels were reduced in the Tx dams, some degree of tissue hypothyroidism was probably present.
Table 3.9 Protein:DNA ratios in postnatal normal (N) and partially thyroidectomised (Tx) dam progeny brain regions

<table>
<thead>
<tr>
<th>Region</th>
<th>Age (pnd)</th>
<th>Dam status</th>
<th>Protein:DNA ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cerebral cortex</td>
<td>10</td>
<td>N</td>
<td>24.08 ± 2.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tx</td>
<td>23.33 ± 1.18</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>N</td>
<td>38.22 ± 1.65</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tx</td>
<td>42.48 ± 6.60</td>
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<td></td>
<td>30</td>
<td>N</td>
<td>47.28 ± 4.81</td>
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<tr>
<td></td>
<td></td>
<td>Tx</td>
<td>49.08 ± 5.71</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>10</td>
<td>N</td>
<td>5.59 ± 0.28</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tx</td>
<td>5.79 ± 0.43</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>N</td>
<td>6.01 ± 0.39</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tx</td>
<td>8.49 ± 1.25*</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>N</td>
<td>10.00 ± 1.56</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tx</td>
<td>9.56 ± 0.47</td>
</tr>
<tr>
<td>Brain stem</td>
<td>10</td>
<td>N</td>
<td>19.52 ± 1.43</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tx</td>
<td>21.20 ± 1.38</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>N</td>
<td>30.58 ± 1.67</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tx</td>
<td>32.15 ± 7.74</td>
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<tr>
<td></td>
<td>30</td>
<td>N</td>
<td>40.25 ± 8.99</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tx</td>
<td>43.48 ± 7.46</td>
</tr>
<tr>
<td>Subcortex</td>
<td>10</td>
<td>N</td>
<td>23.63 ± 1.50</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tx</td>
<td>26.19 ± 3.02</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>N</td>
<td>38.26 ± 2.43</td>
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<td></td>
<td></td>
<td>Tx</td>
<td>38.56 ± 6.99</td>
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<td></td>
<td>30</td>
<td>N</td>
<td>39.08 ± 5.54</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tx</td>
<td>41.31 ± 3.73</td>
</tr>
</tbody>
</table>

Values are mean ± SEM; n = 5

B) Statistical analysis, NS - No significant difference

<table>
<thead>
<tr>
<th>2-way ANOVA</th>
<th>Cerebral cortex</th>
<th>Cerebellum</th>
<th>Brain stem</th>
<th>Subcortex</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>P &lt; 0.0005</td>
<td>P &lt; 0.0005</td>
<td>P &lt; 0.0005</td>
<td>P &lt; 0.005</td>
</tr>
<tr>
<td>Treatment</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Age-treatment interaction</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

Fisher's PLSD

I. Treatment-related

*P < 0.05 Tx vs. N dam progeny

II. Age-related

<table>
<thead>
<tr>
<th>N dam progeny</th>
<th>Cerebral cortex</th>
<th>Cerebellum</th>
<th>Brain stem</th>
<th>Subcortex</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 vs. 10 pnd</td>
<td>P &lt; 0.005</td>
<td>NS</td>
<td>P &lt; 0.05</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>30 vs. 20 pnd</td>
<td>NS</td>
<td>P &lt; 0.005</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Tx dam progeny</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 vs. 10 pnd</td>
<td>P &lt; 0.0005</td>
<td>P &lt; 0.05</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>30 vs. 20 pnd</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

120
Body weight was reduced in Tx dam fetuses from 16 to 21 dg. This parameter had largely normalised by the postnatal stages of development, although a trend for lower body weight could still be seen at birth in Tx dam progeny. Brain weight in contrast, was not affected pre- or postnatally in Tx dam progeny, neither were brain protein or DNA levels. These results indicate the presence of a mechanism sparing the brain from the effects of maternal hypothyroidism such as that proposed by Morreale de Escobar et al. (96), involving increased 5′D-II activity (section 1.5.4). Fetal litter size was significantly reduced in Tx dams at 16 and 19 dg, and there was a tendency for lower litter sizes at 14 dg (the lack of significance at this age was probably due to the smaller number of litters studied). This decrease in Tx dam litter size is expected as maternal hypothyroidism inhibits ovulation (460). No such effect is seen at 21 dg, however, due to the decrease in control litter size between 19 and 21 dg, for which no simple explanation exists. A further decrease is seen in viable pups from Tx dams on the day of birth. This could be due to a number of reasons, including difficulties during parturition, late-stage metabolic compromise and decreased maternal inclination. In addition, Tx dams may cannibalise pups they see as unfit—which may also explain the normalisation of body weight at this age (i.e. the runts of the litter are removed). Decreased litter size in Tx dam progeny may also provide another explanation for why serum T4 levels do not fall as rapidly as controls. The decrease in serum total T4 levels in normal dams as gestation progresses has been previously reported (94,408), and may occur due to increased placental demand for TH as a source of iodine for the fetal thyroid. Thus the fall in circulating maternal T4 levels is steepest between 16 and 19 dg, the period during which the fetal thyroid becomes active. The reduced litter size in Tx dams equates to reduced placental T4 demand, hence the maternal T4 level drops less rapidly in Tx dams, conserving the limited maternal T4 pool.

Certain aspects of placental development were disturbed in Tx dams; protein content and protein:DNA ratio (cell size) were reduced at 19 dg, although protein concentration, DNA content (cell number) and DNA concentration (cell density) appeared normal. The placental compromise was largely limited to 19 dg, and did not seem to affect fetal development (body weight being compromised from 16 dg). Late stage placental dysfunction in Tx dams may partly explain the decrease in the number of viable progeny per litter that occurs between 21 dg and birth. Placental dysfunction may have arisen as a result of maternal metabolic compromise, alternatively, the observed changes may be due to a direct effect of TH deficiency on placental development.

In summary, the use of a partially thyroidectomised rat dam model results in reduced Tx dam serum T4 levels, but does not appear to induce severe metabolic compromise in the
dams despite serum T3 levels being depressed. Hence all parameters of brain growth were normal in pre- and postnatal Tx dam progeny, and body weight normalised after birth. Of particular significance is the normal development of the cerebellum, a region which develops largely postnatally and is therefore particularly sensitive to maternal nutritional deficiencies during the suckling period. Similarly, placental abnormalities were only seen at 19 dg and did not appear to impinge upon fetal development, as judged by the absence of late fetal abnormalities.
CHAPTER 4.

THE EFFECT OF MATERNAL HYPOTHYROXINEMIA ON CHOLINERGIC AND CATECHOLAMINERGIC NEUROTRANSMITTER SYSTEMS IN DEVELOPING BRAIN
4.1 Neurotransmitter metabolic enzyme activities

Numerous studies using rat models of congenital hypothyroidism have shown a relationship between postnatal thyroid hormone status and neurotransmitter system development in brain (section 1.7.2). Many of the enzymes associated with cholinergic and monoaminergic neurotransmitter metabolism have been extensively studied. Although the results tend to vary greatly between studies—being dependent on the exact nature of the animal model and assay method used, enzyme activities are generally reduced in hypothyroid animals. Similar findings for these enzymes have also been reported in neuronal cell culture models, indicating they may be directly regulated by TH. Previous studies have shown that adult progeny of partially thyroidectomised rat dams also exhibit changes in brain neurotransmitter enzymes, namely acetylcholinesterase and choline acetyltransferase (397). It is possible therefore that these, and other, neurotransmitter metabolic enzymes are TH-sensitive in fetal brain. If so, this could provide important insights into the effects of maternal hypothyroxinemia on fetal brain development, since considerable evidence suggests that various neurotransmitters (including acetylcholine, noradrenaline and dopamine) have neurotrophic roles in the early stages of brain ontogeny (428,430,461). Certainly, many neurotransmitters and their receptors and metabolic enzymes have been demonstrated in the brain long before the onset of electrical activity (431,433,434). Disruption of neurotransmitter ontogeny at this crucial time could therefore result in a catastrophic scenario, whereby brain development is affected not only by the primary effects of a TH deficit, but also via secondary effects, arising from perturbation of neurotransmitter systems.

In initial experiments, fetal whole brain was studied at 16, 19 and 21 dg, i.e. both before and after the onset of fetal TH synthesis at 17.5 dg. This was in order to determine whether brain neurotransmitter ontogeny is dependent on maternal TH before fetal thyroid function is established and whether any disturbances that arise are mitigated by fetal thyroid secretion. Earlier work has suggested that the consequences of maternal hypothyroxinemia (in terms of disturbed brain biochemistry) can persist into postnatal development and even adulthood (427), therefore progeny were studied up to 30 pnd. Postnatal brains were dissected into four gross anatomical regions since the effects arising from maternal hypothyroxinemia can be highly localised. Results are expressed as specific activities and, since brain protein concentrations were unaffected by maternal hypothyroxinemia (sections 3.4.2 and 3.6.3), any treatment-related changes in enzyme activity represent selective effects on those enzymes, rather than protein levels in general.
4.1.1 *Cholinergic metabolic enzymes*

4.1.1.1 *Choline acetyltransferase (ChAT) activity*

The specific activity of the acetylcholine synthetic enzyme, ChAT (439) in fetal brain was dependent on age; age-treatment interaction was also evident (Fig. 4.1). In normal progeny, ChAT activity increased 1.5-fold from 16 to 19 dg, but showed no further change through to 21 dg (Fig. 4.1). In fetal brain from Tx dam pregnancies, ChAT ontogeny was disturbed in an age-dependent manner; activities appeared reduced at 16 dg and more markedly so at 19 dg, but elevated at 21 dg relative to controls. Post-hoc analysis however revealed no significant difference between N and Tx dam progeny at any individual age, though it confirmed an increase in ChAT activity between 19 and 21 dg in Tx dam fetal brain that was absent in normal pregnancy. Thus, the main effect of maternal hypothyroxinemia on prenatal ChAT activity is to extend the period of enzyme induction.

Postnatal brain regions varied considerably in both their levels of ChAT activity and the developmental profiles thereof, although activity in all regions was dependent on age but not treatment (Fig. 4.2). In both N and Tx groups, ChAT activity in cerebral cortex, brain stem and subcortex increased by between 2- and 4-fold from 10 to 20 pnd, with no change thereafter (Fig. 4.2). In contrast, cerebellar ChAT activity declined ca. 50% from 10 to 20 pnd, with no further change by 30 pnd. As ChAT is a specific marker of cholinergic neurons, it appears that their development is largely complete by 20 pnd. In contrast to prenatal development, no differences in postnatal brain ChAT activity were apparent in Tx dam progeny relative to controls.

4.1.1.2 *Acetylcholinesterase (AChE) activity*

Fetal brain AChE specific activity was dependent on age; control and experimental progeny exhibited linear increases (by between 3.2 and 3.6-fold) in activity from 16 to 21 dg. No treatment-related differences were apparent (Fig. 4.3).

During postnatal development, cerebral cortical, brain stem and subcortical AChE activities were age-dependent (Fig. 4.4). No region showed treatment-related effects by 2-way ANOVA, however AChE activity in cerebral cortex was close to being significantly different \( (P = 0.075) \) between N and Tx groups, and post-hoc analysis also indicated that maternal hypothyroxinemia may influence AChE activity in this region (Fig. 4.4). In N dam progeny, cerebral cortical AChE activity increased 1.8-fold from 10 to 20 pnd and then a further 1.3-fold to 30 pnd. At 10 pnd, activity was 38% higher in Tx dam progeny.
A) **Ontogenic profile**

![Graph showing specific activity of choline acetyltransferase across different ages for normal (open circles) and thyroidectomised (closed circles) dam progeny.](image)

**Age** (days gestation)

**Specific activity** (nmol acetyl-CoA/h/mg protein)

10 1.5 2.0 2.5 3.0 3.5

15 16 17 18 19 20 21 22

B) **Statistical analysis**

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>2-way ANOVA</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Age</strong></td>
<td><em>P &lt; 0.0005</em></td>
<td></td>
</tr>
<tr>
<td><strong>Treatment</strong></td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td><strong>Age-treatment interaction</strong></td>
<td><em>P &lt; 0.05</em></td>
<td></td>
</tr>
</tbody>
</table>

**Fisher's PLSD**

**I. Treatment-related** (Tx vs. N dam progeny):

NS at any age

**II. Age-related**:  

<table>
<thead>
<tr>
<th>Age</th>
<th>N dam progeny</th>
<th>Tx dam progeny</th>
</tr>
</thead>
<tbody>
<tr>
<td>19 vs. 16 dg</td>
<td><em>P &lt; 0.0005</em></td>
<td><em>P &lt; 0.001</em></td>
</tr>
<tr>
<td>21 vs. 19 dg</td>
<td>NS</td>
<td><em>P &lt; 0.01</em></td>
</tr>
</tbody>
</table>

**Figure 4.1** The influence of maternal hypothyroxinemia on the prenatal ontogeny of choline acetyltransferase specific activity in whole brain. **A)** Ontogenic profile - each point represents the mean ± SEM of normal (N; open circles) or thyroidectomised (Tx; closed circles) dam progeny, n is given in parentheses. **B)** Statistical analysis - NS; no significant difference.
A) **Ontogenic profile**

![Ontogenic profile diagram]

B) **Statistical analysis**

<table>
<thead>
<tr>
<th></th>
<th>Cerebral cortex</th>
<th>Cerebellum</th>
<th>Brain stem</th>
<th>Subcortex</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>$P &lt; 0.0005$</td>
<td>$P &lt; 0.0005$</td>
<td>$P &lt; 0.0005$</td>
<td>$P &lt; 0.0005$</td>
</tr>
<tr>
<td>Treatment</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Age-treatment interaction</td>
<td>NS</td>
<td>NS</td>
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<td>NS</td>
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</table>

**Fisher's PLSD**

<table>
<thead>
<tr>
<th></th>
<th>Cerebral cortex</th>
<th>Cerebellum</th>
<th>Brain stem</th>
<th>Subcortex</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age-related</td>
<td>$P &lt; 0.0005$</td>
<td>$P &lt; 0.0005$</td>
<td>$P &lt; 0.0005$</td>
<td>$P &lt; 0.0005$</td>
</tr>
<tr>
<td>N dam 20 vs. 10 pnd</td>
<td>$P &lt; 0.0005$</td>
<td>$P &lt; 0.0005$</td>
<td>$P &lt; 0.0005$</td>
<td>$P &lt; 0.0005$</td>
</tr>
<tr>
<td>Tx dam 20 vs. 10 pnd</td>
<td>$P &lt; 0.0005$</td>
<td>$P &lt; 0.0005$</td>
<td>$P &lt; 0.0005$</td>
<td>$P &lt; 0.0005$</td>
</tr>
</tbody>
</table>

**Figure 4.2** The influence of maternal hypothyroxinemia on the ontogeny of choline acetyltransferase specific activity in postnatal brain regions. A) Ontogenic profile - values are mean ± SEM of normal (N; open bars) or thyroidectomised (Tx; closed bars) dam progeny, n is indicated in the bars. B) Statistical analysis - NS; no significant difference.
A) Ontogenic profile

![Graph showing ontogenic profile of acetylcholinesterase specific activity.](image)

B) Statistical analysis

<table>
<thead>
<tr>
<th>2-way ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
</tr>
<tr>
<td>Treatment</td>
</tr>
<tr>
<td>Age-treatment interaction</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Fisher's PLSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age-related</td>
</tr>
<tr>
<td>N dam progeny</td>
</tr>
<tr>
<td>19 vs. 16 dg $P &lt; 0.0005$</td>
</tr>
<tr>
<td>21 vs. 19 dg $P &lt; 0.005$</td>
</tr>
</tbody>
</table>

Figure 4.3 The influence of maternal hypothyroxinemia on the prenatal ontogeny of acetylcholinesterase specific activity in whole brain. A) Ontogenic profile - each point represents the mean $\pm$ SEM of normal (N; open circles) or thyroidectomised (Tx; closed circles) dam progeny. B) Statistical analysis - NS; no significant difference.
A) Ontogenic profile

![Graph showing ontogenic profile with specific activity in brain regions (cerebral cortex, cerebellum, brain stem, and subcortex) across different ages (10, 20, 30 days)].

B) Statistical analysis

<table>
<thead>
<tr>
<th>2-way ANOVA</th>
<th>Cerebral cortex</th>
<th>Cerebellum</th>
<th>Brain stem</th>
<th>Subcortex</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>$P &lt; 0.0005$</td>
<td>NS</td>
<td>$P &lt; 0.05$</td>
<td>$P &lt; 0.001$</td>
</tr>
<tr>
<td>Treatment</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Age-treatment interaction</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

Fisher's PLSD

I. Treatment-related

* $P < 0.05$ Tx vs. N dam progeny

II. Age-related

<table>
<thead>
<tr>
<th></th>
<th>Cerebral cortex</th>
<th>Cerebellum</th>
<th>Brain stem</th>
<th>Subcortex</th>
</tr>
</thead>
<tbody>
<tr>
<td>N dam 20 vs. 10 pnd</td>
<td>$P &lt; 0.0005$</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>N dam 30 vs. 10 pnd</td>
<td>$P &lt; 0.0005$</td>
<td>NS</td>
<td>NS</td>
<td>$P &lt; 0.05$</td>
</tr>
<tr>
<td>N dam 30 vs. 20 pnd</td>
<td>$P &lt; 0.05$</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Tx dam 20 vs. 10 pnd</td>
<td>$P &lt; 0.0005$</td>
<td>NS</td>
<td>NS</td>
<td>$P &lt; 0.005$</td>
</tr>
</tbody>
</table>

**Figure 4.4** The influence of maternal hypothyroxinemia on the ontogeny of acetylcholinesterase specific activity in postnatal brain regions. A) Ontogenic profile - values are mean ± SEM of normal (N; open bars) or thyroidectomised (Tx; closed bars) dam progeny. B) Statistical analysis - NS; no significant difference.
relative to controls, but not at any other age. Furthermore, no age-related increase was apparent between 20 and 30 pnd in Tx dam progeny. Without a significant 2-way ANOVA however, these results must be interpreted with caution, particularly since no treatment effects were observed prenatally. Cerebellar AChE activity was found to be unaffected by age or treatment throughout the postnatal period. In the brain stem, AChE activity was age-dependent, but activities at 10 and 30 pnd were equivalent and no particular point was judged different from any other by post-hoc analysis, in terms of age or treatment. In subcortex, activity in controls increased linearly 1.5-fold from 10 to 30 pnd, whereas in Tx dam progeny an increase was only observed between 10 and 20 pnd, but no significant treatment effects were apparent.

4.1.2 Catecholaminergic metabolic enzymes

4.1.2.1 Tyrosine hydroxylase (TyrH) activity

Fetal brain tyrosine hydroxylase (TyrH) activity showed age-, but not treatment-dependency. Thus, in both N and Tx dam progeny, TyrH activity increased linearly by between 1.4 and 1.8-fold from 16 to 21 dg (Fig. 4.5). There was however evidence of disrupted ontogeny in certain postnatal Tx dam progeny brain regions (Fig. 4.6), namely cerebral cortex and subcortex. Indeed, cerebral cortical activity exhibited an overall treatment effect. In controls, activity increased 1.5-fold between 10 and 30 pnd, whereas in experimental progeny TyrH activity was 46% higher than in controls at 10 pnd but showed no further change with age, resulting in normal activities at subsequent ages. Cerebellar TyrH activity was ca. 10-fold lower than in other regions and dependent on age alone with no indication of treatment effects. Activity declined ca. 50% by 20 pnd, with no further change apparent at 30 pnd (Fig. 4.6). A similar age-dependent effect was seen in brain stem (albeit at consistently higher activity), though again maternal hypothyroxinemia had no influence on TyrH activity in this region (Fig. 4.6). Subcortical TyrH activity appeared to be dependent on age alone, however the treatment effect approached significance ($P = 0.09$) and post-hoc analysis indicated Tx dam progeny had a distinct TyrH ontogeny in this region. In controls, activity decreased by 30% from 10 to 30 pnd, whereas in Tx dam progeny, no decrease was seen until after 20 pnd. As a result, TyrH activity in Tx dam progeny subcortex was 32% higher at 20 pnd relative to controls. Activity subsequently decreased (by 40%) to normal levels by 30 pnd (Fig. 4.6).
**A) Ontogenic profile**

![Graph showing specific activity (nmol DOPA/h/mg protein) vs. age (days gestation).](image)

**B) Statistical analysis**

<table>
<thead>
<tr>
<th>2-way ANOVA</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td><em>P</em> &lt; 0.0005</td>
</tr>
<tr>
<td>Treatment</td>
<td>NS</td>
</tr>
<tr>
<td>Age-treatment interaction</td>
<td>NS</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Fisher's PLSD</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Age-related</td>
<td></td>
</tr>
<tr>
<td>N dam progeny</td>
<td>Tx dam progeny</td>
</tr>
<tr>
<td>19 vs. 16 dg</td>
<td>NS</td>
</tr>
<tr>
<td>21 vs. 16 dg</td>
<td><em>P</em> &lt; 0.01</td>
</tr>
<tr>
<td></td>
<td><em>P</em> &lt; 0.0005</td>
</tr>
</tbody>
</table>

**Figure 4.5** The influence of maternal hypothyroxinemia on the prenatal ontogeny of tyrosine hydroxylase specific activity in whole brain. **A)** Ontogenic profile - each point represents the mean ± SEM of normal (N; open circles) or thyroidectomised (Tx; closed circles) dam progeny, n is given in parentheses. **B)** Statistical analysis - NS; no significant difference.
**A) Ontogenic profile**

![Graphs showing specific activity of tyrosine hydroxylase in postnatal brain regions.](image)

**B) Statistical analysis**

<table>
<thead>
<tr>
<th>2-way ANOVA</th>
<th>Cerebral cortex</th>
<th>Cerebellum</th>
<th>Brain stem</th>
<th>Subcortex</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>NS</td>
<td>*P &lt; 0.0005</td>
<td>*P &lt; 0.0005</td>
<td>*P &lt; 0.005</td>
</tr>
<tr>
<td>Treatment</td>
<td>*P &lt; 0.05</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Age-treatment interaction</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

Fisher's PLSD

I. Treatment-related

*P < 0.05 Tx vs. N dam

II. Age-related

<table>
<thead>
<tr>
<th>N dam 20 vs. 10 pnd</th>
<th>Cerebral cortex</th>
<th>Cerebellum</th>
<th>Brain stem</th>
<th>Subcortex</th>
</tr>
</thead>
<tbody>
<tr>
<td>NS</td>
<td>*P &lt; 0.05</td>
<td>*P &lt; 0.01</td>
<td>*P &lt; 0.005</td>
<td>*P &lt; 0.05</td>
</tr>
</tbody>
</table>

**Figure 4.6** The influence of maternal hypothyroxinemia on the ontogeny of tyrosine hydroxylase specific activity in postnatal brain regions. A) Ontogenic profile - values are mean ± SEM of normal (N; open bars) or thyroidectomised (Tx; closed bars) dam progeny, n is indicated in the bars. B) Statistical analysis - NS; no significant difference.
4.1.2.2 DOPA decarboxylase (DDC) activity

The ontogeny of DDC activity in fetal brain was rather different to the neurotransmitter metabolic enzymes discussed previously, being subject to age-treatment interaction but not age or treatment per se (Fig. 4.7). This was due to the marked difference in DDC ontogeny between the two groups. In N dam progeny, activity was age-dependent, declining by 43% between 16 and 21 dg (Fig. 4.7). In Tx dam progeny in contrast, no change in activity was apparent and, as a result, DDC activity in Tx dam progeny was 58% higher relative to controls by 21 dg.

The higher DDC activity in Tx dam progeny fetal brain appeared to persist in certain postnatal brain regions. In cerebral cortex, treatment and age effects were apparent due to what may be a delay in the developmental profile of DDC activity in this region (Fig. 4.8). In controls, cortical DDC activity declined by 38% from 10 to 30 pnd whereas activity in Tx dam progeny did not decrease until after 20 pnd. As a result, DDC activity at 20 pnd was 61% higher in experimental progeny compared with controls, but normal by 30 pnd (Fig. 4.8). It should be noted that this effect was strikingly similar to that seen for subcortical TyrH activity (Fig. 4.6). Cerebellar DDC activity was age- but not treatment-dependent. This age effect seemed to be limited to the N dam progeny however, in which DDC activity declined by 62% between 10 and 20 pnd, then increased 1.7-fold by 30 pnd (Fig. 4.8). In brain stem there were clear treatment- and age-related effects. In both groups, activity approximately halved between 10 and 30 pnd, however Tx dam progeny exhibited higher DDC activities at 20 (by 30%) and 30 pnd (by 45%), relative to controls (Fig. 4.8). Subcortical DDC activity was insensitive to age and treatment (Fig. 4.8).

4.1.2.3 Monoamine oxidase (MAO) activity

Fetal brain MAO activity was affected by age and treatment (Fig. 4.9). Activity in controls increased 1.7-fold between 16 and 19 dg and remained stable to 21 dg (Fig. 4.9). MAO activity in experimental progeny also increased between 16 and 19 dg, however when compared with controls, the activities at 16 and 19 dg were reduced, by 20 and 24% respectively (Fig. 4.9). By 21 dg brain MAO activity in Tx dam progeny had normalised. It should be noted that the profiles of MAO activity in both groups were strikingly similar to those of ChAT (Fig. 4.1), albeit MAO activity was more severely affected by maternal hypothyroxinemia.
**B) Statistical analysis**

<table>
<thead>
<tr>
<th>ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-way ANOVA</td>
</tr>
</tbody>
</table>
| Age                          | NS  
| Treatment                    | NS  
| Age-treatment interaction    | $P < 0.05$  

Fisher's PLSD

1. Treatment-related

* $P < 0.01$ Tx vs. N

2. Age-related

| N dam progeny | Tx dam progeny  
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>21 vs. 16 dg</td>
<td>$P &lt; 0.005$</td>
</tr>
</tbody>
</table>

**Figure 4.7.** The influence of maternal hypothyroxinemia on the prenatal ontogeny of DOPA decarboxylase specific activity in whole brain. A) Ontogenic profile - each point represents the mean ± SEM of normal (N; open circles) or thyroidectomised (Tx; closed circles) dam progeny, n is given in parentheses. B) Statistical analysis - NS; no significant difference.
A) Ontogenic profile

![Ontogenic profile](image)

B) Statistical analysis

<table>
<thead>
<tr>
<th>2-way ANOVA</th>
<th>Cerebral cortex</th>
<th>Cerebellum</th>
<th>Brain stem</th>
<th>Subcortex</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>$P &lt; 0.01$</td>
<td>$P &lt; 0.005$</td>
<td>$P &lt; 0.0005$</td>
<td>NS</td>
</tr>
<tr>
<td>Treatment</td>
<td>$P &lt; 0.01$</td>
<td>NS</td>
<td>$P &lt; 0.001$</td>
<td>NS</td>
</tr>
<tr>
<td>Age-treatment interaction</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

**Fisher's PLSD**

I. Treatment-related

* $P < 0.05$ and ** $P < 0.005$ Tx vs. N dam progeny

II. Age-related

<table>
<thead>
<tr>
<th></th>
<th>Cerebral cortex</th>
<th>Cerebellum</th>
<th>Brain stem</th>
<th>Subcortex</th>
</tr>
</thead>
<tbody>
<tr>
<td>N dam 20 vs. 10 pnd</td>
<td>NS</td>
<td>$P &lt; 0.0005$</td>
<td>$P &lt; 0.01$</td>
<td>NS</td>
</tr>
<tr>
<td>N dam 30 vs. 10 pnd</td>
<td>$P &lt; 0.05$</td>
<td>$P &lt; 0.0005$</td>
<td>$P &lt; 0.01$</td>
<td>NS</td>
</tr>
<tr>
<td>N dam 30 vs. 20 pnd</td>
<td>NS</td>
<td>$P &lt; 0.05$</td>
<td>$P &lt; 0.01$</td>
<td>NS</td>
</tr>
<tr>
<td>Tx dam 20 vs. 10 pnd</td>
<td>NS</td>
<td>NS</td>
<td>$P &lt; 0.05$</td>
<td>NS</td>
</tr>
<tr>
<td>Tx dam 30 vs. 20 pnd</td>
<td>$P &lt; 0.005$</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
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</tbody>
</table>

Figure 4.8 The influence of maternal hypothyroxinemia on the ontogeny of DOPA decarboxylase specific activity in postnatal brain regions. A) Ontogenic profile - values are mean ± SEM of normal (N; open bars) or thyroidectomised (Tx; closed bars) dam progeny, n is indicated in the bars. B) Statistical analysis - NS; no significant difference.
**Figure 4.9** The influence of maternal hypothyroxinemia on the prenatal ontogeny of monoamine oxidase specific activity in whole brain.  

A) Ontogenic profile - each point represents the mean ± SEM of normal (N; open circles) or thyroidectomised (Tx; closed circles) dam progeny, n is given in parentheses.  

B) Statistical analysis - NS; no significant difference.
Since MAO exists as two isoforms (A and B), the effect of maternal hypothyroxinemia on the activity of each isoform at 16 and 19 dg (when treatment effects are most apparent) was explored. Initially, a range of concentrations of the MAO isoform-specific inhibitors, clorgyline (MAO-A specific) and deprenyl (MAO-B specific) were used in the conventional assay with homogenate from 19 dg normal brain to determine the optimal inhibitory concentrations for further study. For clorgyline, MAO activity was not inhibited until a concentration of $1 \times 10^{-9}$ M was used, after which the degree of inhibition increased sharply up to $1 \times 10^{-8}$ M at which activity was reduced by ca. 70% (Fig. 4.10). Further increases in clorgyline concentration, up to $1 \times 10^{-6}$ M, produced little change in MAO activity, the residual activity being due to isoform MAO-B. A further decrease in MAO activity is seen at $5 \times 10^{-5}$ M clorgyline and this represents inhibition of both isoforms. Deprenyl-induced inhibition of MAO activity is evident from $1 \times 10^{-8}$ M onwards. Between this concentration and $1 \times 10^{-6}$ M MAO activity (approximately 90% of controls) does not decrease further, and is likely to represent MAO-A activity (Fig. 4.10). Further increases in deprenyl concentration sharply decreases MAO activity and near total inhibition occurs at $1 \times 10^{-4}$ M. Some overlap is evident, deprenyl inhibition suggests the ratio of MAO-A:MAO-B was 9:1, whereas clorgyline inhibition suggests a ratio of 7:3 (Fig. 4.10). Nevertheless, for both inhibitors a concentration of $1 \times 10^{-7}$ M was within their respective plateaus, where inhibition was judged specific and was therefore used in subsequent assays.

At 16 dg, isoform A predominated—accounting for 70 - 75% of total MAO activity (Table 4.1). In contrast to the results for total MAO, neither isoform appeared to be affected by maternal hypothyroxinemia at this age (although the number of litters studied were small). It should be noted that different samples were used from those in the original study, however the degree of maternal hypothyroxinemia was similar, ruling this out as a reason for the observed discrepancy. Indeed, at 19 dg a treatment effect was observed for MAO-A activity, which was reduced by 16% in Tx dam progeny, relative to controls. In contrast the MAO-B isoform was unaffected (Table 4.1). MAO-A was still the predominant isoform contributing 74-77% of the total activity in both groups. An age-related effect was also observed, but only for MAO-A activity in N dam progeny, which increased 1.3-fold from 16 to 19 dg.

During postnatal development MAO activities increased slightly with age in most regions (Fig. 4.11). In cerebral cortex, activity was age- but not treatment-dependent, increasing 1.5-fold from 10 to 20 pnd in both groups (Fig. 4.11). Cerebellar MAO activity was also age- but not treatment-dependent. In contrast, brain stem MAO activity did not alter with age, but a treatment effect was evident due to increased activities in Tx dam progeny (Fig.
Figure 4.10 Effects of increasing deprenyl (circles) and clorgyline (triangles) concentrations on brain monoamine oxidase activity in 19 dg N dam progeny. Each point represents the mean ± SEM of two experiments.
Table 4.1 Activity of monoamine oxidase (MAO) isoforms A and B in normal (N) and partially thyroidectomised (Tx) dam fetal brain at 16 and 19 days gestation (dg)

<table>
<thead>
<tr>
<th>Progeny (age &amp; dam thyroid status)</th>
<th>n</th>
<th>MAO-A activity (nmol 4-hydroquinolene/hour/mg protein)</th>
<th>MAO-B activity (nmol 4-hydroquinolene/hour/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16 dg N</td>
<td>3</td>
<td>12.49 ± 1.05</td>
<td>4.20 ± 0.42^</td>
</tr>
<tr>
<td>16 dg TX</td>
<td>4</td>
<td>11.55 ± 0.56</td>
<td>4.50 ± 0.28^</td>
</tr>
<tr>
<td>19 dg N</td>
<td>5</td>
<td>15.80 ± 0.65|$|$</td>
<td>4.68 ± 0.15^</td>
</tr>
<tr>
<td>19 dg Tx</td>
<td>5</td>
<td>13.34 ± 1.42*</td>
<td>4.59 ± 0.31^</td>
</tr>
</tbody>
</table>

Values are mean ± SEM. 2-way ANOVA indicates treatment and treatment-isoform interaction effects for MAO-A activity at 19 dg (both \( P < 0.05 \)). Isoform effects are apparent at both ages (\( P < 0.0005 \)). *\( P < 0.005 \) for Tx vs. N, \( \|P < 0.05 \) for 19 vs. 16 dg and ^\( P < 0.0005 \) for MAO-A vs. MAO-B by Fishers PLSD.

4.11). This was most marked (20% increase) at 10 and 20 pnd, being only 7% higher at 30 pnd. No statistically significantly difference between N and Tx dam progeny could however be discerned at age by post-hoc analysis. Subcortical MAO activity displayed a similar developmental profile to cerebral cortex and cerebellum, being age- but not treatment-dependent. Thus, in N and Tx dam progeny subcortical activity increased 1.4-fold between 20 and 30 pnd (Fig. 4.11).

4.1.3 Maternal hypothyroxinemia and metabolic enzyme activities at 14 dg

It is thought that in this model, the effects of maternal hypothyroxinemia on brain development are greatest prior to the onset of fetal thyroid function, since fetal brain TH levels normalise shortly after this period (section 1.7.4.2). Therefore the effect of maternal hypothyroxinemia was investigated at 14 dg (when TR proteins can first be detected in brain), on those enzymes shown to have altered fetal ontogeny (i.e. ChAT, DDC and MAO). Surprisingly, no significant treatment-related differences were observed for any of the enzymes (Table 4.2). In terms of their ontogeny, all three enzymes in N and Tx dam progeny appeared to have lower activities at 14 dg compared with 16 dg—although this was not verified statistically. Particularly notable is DDC activity, which increased by 6 to 8-fold between 14 and 16 dg (Table 4.2 and Fig. 4.7). Indeed, this rapid increase may
A) Ontogenic profile

![Graphs showing ontogenic profile of different brain regions](image)

B) Statistical analysis

<table>
<thead>
<tr>
<th>2-way ANOVA</th>
<th>Cerebral cortex</th>
<th>Cerebellum</th>
<th>Brain stem</th>
<th>Subcortex</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>$P &lt; 0.0005$</td>
<td>$P &lt; 0.0005$</td>
<td>NS</td>
<td>$P &lt; 0.0005$</td>
</tr>
<tr>
<td>Treatment</td>
<td>NS</td>
<td>NS</td>
<td>$P &lt; 0.05$</td>
<td>NS</td>
</tr>
<tr>
<td>Age-treatment interaction</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

Fisher’s PLSD

I. Treatment-related
NS at any age

II. Age-related

<table>
<thead>
<tr>
<th></th>
<th>Cerebral cortex</th>
<th>Cerebellum</th>
<th>Brain stem</th>
<th>Subcortex</th>
</tr>
</thead>
<tbody>
<tr>
<td>N dam 20 vs. 10 pnd</td>
<td>$P &lt; 0.01$</td>
<td>$P &lt; 0.05$</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>N dam 30 vs. 20 pnd</td>
<td>$P &lt; 0.05$</td>
<td>$P &lt; 0.001$</td>
<td>NS</td>
<td>$P &lt; 0.01$</td>
</tr>
<tr>
<td>Tx dam 20 vs. 10 pnd</td>
<td>$P &lt; 0.05$</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Tx dam 30 vs. 20 pnd</td>
<td>NS</td>
<td>$P &lt; 0.0005$</td>
<td>NS</td>
<td>$P &lt; 0.005$</td>
</tr>
</tbody>
</table>

Figure 4.11 The influence of maternal hypothyroxinemia on the ontogeny of monoamine oxidase specific activity in postnatal brain regions. A) Ontogenic profile - values are mean ± SEM of normal (N; open bars) or thyroidectomised (Tx; closed bars) dam progeny, n is indicated in the bars. B) Statistical analysis - NS; no significant difference.
account for the variable data for this enzyme at 14 dg, i.e. small variations in age may produce relatively large variations in DDC activity. All three enzymes showed a tendency to be lower in Tx dam progeny, however as they stand these results suggest that the enzymes concerned become TH-sensitive after 14 dg.

Table 4.2 Whole brain DOPA decarboxylase (DDC), monoamine oxidase (MAO) and choline acetyltransferase (ChAT) specific activities in 14 days gestation normal (N) and partially thyroidectomised (Tx) dam progeny.

<table>
<thead>
<tr>
<th>Dam status</th>
<th>DDC (nmol DOPA/hour/mg protein)</th>
<th>MAO (nmol 4-hydroxyquinolene/hour/mg protein)</th>
<th>ChAT (nmol acetylCoA/hour/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>0.958 ± 0.223 (7)</td>
<td>9.963 ± 0.701 (5)</td>
<td>0.702 ± 0.053 (7)</td>
</tr>
<tr>
<td>Tx</td>
<td>0.694 ± 0.112 (6)</td>
<td>9.068 ± 0.354 (4)</td>
<td>0.635 ± 0.054 (6)</td>
</tr>
</tbody>
</table>

Values are mean ± SEM, n is given in parentheses. No significant differences were observed as determined by Student’s t-test.

4.2 Neurotransmitter receptor binding studies

Having shown disturbances in monoaminergic, and to a lesser extent, cholinergic neurotransmitter metabolic enzyme activities in postnatal brain regions, neurotransmitter receptor binding was investigated. As with the enzymes, previous studies with late fetal/maternal hypothyroid models have shown changes in neurotransmitter receptor number (337-339,462). Postnatal brain regions at 20 and 30 pnd were studied to assess the long term effects of maternal hypothyroxinemia on neurotransmitter receptor ontogeny. Although the data is displayed in the form of Scatchard plots, Kd and Bmax were calculated by non-linear regression (section 2.5). NSB varied between the ligands. In muscarinic cholinergic receptor binding studies, NSB accounted for < 10% total binding. For β-adrenergic and D2 dopaminergic receptor binding assays however, NSB tended to be higher, accounting for 20% of total binding at the lower ligand concentrations and up to 75% at the higher ligand concentrations. The increased NSB was not due to greater binding of the ligands to filter paper/crude membrane fractions. Rather, it was a reflection of the lower specific binding these ligands exhibited, relative to quinuclidinyl benzilate, and was also illustrated by the lower number of β-adrenergic and dopaminergic receptors detected compared with muscarinic receptors.
4.2.1 Muscarinic cholinergic receptor binding

Both receptor number and affinity showed wide regional variation (Table 4.3, Fig. 4.12 & Fig. 4.13). Cerebral cortex exhibited a high number of receptors with low affinity and these parameters were not affected by age or treatment.

In contrast, cerebellar binding consisted of relatively few high affinity receptors. Receptor number was again unaffected by age and treatment, however receptor affinity age-related, tending to decrease from 20 to 30 pnd. This decline was not however significant in post-hoc analysis (Table 4.3).

Brain stem had intermediate receptor number and affinity (Table 4.3) and like cerebral cortex, was unaffected by age or treatment. Subcortical receptor binding was age-dependent but not affected by treatment. At 20 pnd receptor number and affinity were similar to that in brain stem, however, by 30 pnd receptor number had increased and affinity decreased to levels approaching those in cerebral cortex.

4.2.2 β-Adrenergic receptor binding

β-adrenergic receptor number was not affected by age or treatment in cerebral cortex. Affinity was age-dependent—increasing slightly, ca. 1.3-fold, from 20 to 30 pnd in both groups (Table 4.4, Fig. 4.14 & Fig. 4.15).

The situation in cerebellum was more complex, with receptor number being affected by age and treatment, while affinity exhibited age-treatment interaction. Receptor number in both groups increased ca. 1.5-fold from 20 to 30 pnd, though the number of receptors were consistently lower in Tx dam progeny (by 46% and 35% at 20 and 30 pnd respectively) than in controls (Table 4.4). Conversely, affinity did not alter with age in controls but increased in Tx dam progeny. Thus, receptor affinity was 38% lower in Tx dam progeny relative to controls at 20 pnd, but normal by 30 pnd.

In brain stem, receptor number was treatment-dependent, and also sensitive to age-treatment interaction. N dam progeny exhibited a 42% decline in receptor number between 20 and 30 pnd which was absent in Tx dam progeny. Furthermore, the latter group had 54% fewer receptors at 20 pnd—consequently, receptor number had normalised by 30 pnd. Affinity was not affected by treatment at any age.
Table 4.3 Muscarinic cholinergic receptor number (Bmax) and affinity (Kd) in normal (N) and thyroidectomised (Tx) dam progeny brain regions at 20 and 30 postnatal days (pnd)

<table>
<thead>
<tr>
<th>Region</th>
<th>Age (pnd)</th>
<th>Dam status</th>
<th>n</th>
<th>Bmax (pmol/mg protein)</th>
<th>Kd (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cerebral cortex</td>
<td>20</td>
<td>N</td>
<td>4</td>
<td>4.126 ± 0.410</td>
<td>2.375 ± 0.244</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tx</td>
<td>4</td>
<td>4.253 ± 0.361</td>
<td>2.123 ± 0.185</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>N</td>
<td>4</td>
<td>4.348 ± 0.757</td>
<td>2.256 ± 0.417</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tx</td>
<td>3</td>
<td>2.724 ± 0.666</td>
<td>1.352 ± 0.165</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>20</td>
<td>N</td>
<td>5</td>
<td>0.500 ± 0.078</td>
<td>0.241 ± 0.024</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tx</td>
<td>5</td>
<td>0.305 ± 0.034</td>
<td>0.307 ± 0.032</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>N</td>
<td>3</td>
<td>0.365 ± 0.051</td>
<td>0.372 ± 0.022</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tx</td>
<td>3</td>
<td>0.413 ± 0.041</td>
<td>0.356 ± 0.067</td>
</tr>
<tr>
<td>Brain stem</td>
<td>20</td>
<td>N</td>
<td>6</td>
<td>1.588 ± 0.197</td>
<td>0.615 ± 0.059</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tx</td>
<td>6</td>
<td>1.245 ± 0.173</td>
<td>0.595 ± 0.033</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>N</td>
<td>5</td>
<td>1.556 ± 0.253</td>
<td>0.684 ± 0.078</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tx</td>
<td>5</td>
<td>1.172 ± 0.138</td>
<td>0.610 ± 0.057</td>
</tr>
<tr>
<td>Subcortex</td>
<td>20</td>
<td>N</td>
<td>5</td>
<td>1.620 ± 0.132</td>
<td>0.641 ± 0.062</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tx</td>
<td>6</td>
<td>1.737 ± 0.241</td>
<td>0.895 ± 0.113</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>N</td>
<td>5</td>
<td>3.066 ± 0.345</td>
<td>1.163 ± 0.171</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tx</td>
<td>5</td>
<td>2.588 ± 0.364</td>
<td>1.381 ± 0.132</td>
</tr>
</tbody>
</table>

Values are mean ± SEM.

B) Bmax statistical analysis, NS - no significant difference

<table>
<thead>
<tr>
<th>2-way ANOVA</th>
<th>Cerebral cortex</th>
<th>Cerebellum</th>
<th>Brain stem</th>
<th>Subcortex</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>Treatment</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Age-treatment interaction</td>
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<td>NS</td>
<td>NS</td>
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</table>

Fisher’s PLSD
I. Treatment-related
NS at either age

II. Age-related
<table>
<thead>
<tr>
<th>Cerebral cortex</th>
<th>Cerebellum</th>
<th>Brain stem</th>
<th>Subcortex</th>
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<tbody>
<tr>
<td>N dam</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Tx dam</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

C) Kd statistical analysis, NS - no significant difference

<table>
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<th>2-way ANOVA</th>
<th>Cerebral cortex</th>
<th>Cerebellum</th>
<th>Brain stem</th>
<th>Subcortex</th>
</tr>
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<tbody>
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<td>NS</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>Treatment</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Age-treatment interaction</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

Fisher’s PLSD
I. Treatment-related
NS at either age

II. Age-related
<table>
<thead>
<tr>
<th>Cerebral cortex</th>
<th>Cerebellum</th>
<th>Brain stem</th>
<th>Subcortex</th>
</tr>
</thead>
<tbody>
<tr>
<td>N dam</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Tx dam</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>
Figure 4.12 Scatchard plots of cholinergic muscarinic membrane binding in normal (open circles) and partially thyoidectomised (closed circles) dam progeny brain regions at 20 pnd. The scatchard plots were only used to display the data, binding parameters were calculated by non-linear regression.
Figure 4.13 Scatchard plots of cholinergic muscarinic membrane binding in normal (open circles) and partially thyroidecтомised (closed circles) dam progeny brain regions at 30 pnd. The scatchard plots were only used to display the data, binding parameters were calculated by non-linear regression.
Table 4.4 β-adrenergic receptor number (Bmax) and affinity (Kd) in normal (N) and thyroidectomised (Tx) dam progeny brain regions at 20 and 30 postnatal days (pnd)

A) Table

<table>
<thead>
<tr>
<th>Region</th>
<th>Age (pnd)</th>
<th>Dam status</th>
<th>n</th>
<th>Bmax (fmol/mg protein)</th>
<th>Kd (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cerebral cortex</td>
<td>20</td>
<td>N</td>
<td>4</td>
<td>142 ± 21</td>
<td>1.648 ± 0.137</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tx</td>
<td>5</td>
<td>113 ± 10</td>
<td>1.720 ± 0.088</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>N</td>
<td>4</td>
<td>148 ± 20</td>
<td>1.395 ± 0.082</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tx</td>
<td>4</td>
<td>109 ± 28</td>
<td>1.261 ± 0.225</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>20</td>
<td>N</td>
<td>5</td>
<td>74 ± 7</td>
<td>0.962 ± 0.104</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tx</td>
<td>4</td>
<td>40 ± 4*</td>
<td>1.558 ± 0.217**</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>N</td>
<td>5</td>
<td>108 ± 15</td>
<td>1.161 ± 0.074</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tx</td>
<td>4</td>
<td>70 ± 11*</td>
<td>0.947 ± 0.068</td>
</tr>
<tr>
<td>Brain stem</td>
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<td>N</td>
<td>4</td>
<td>116 ± 12</td>
<td>1.652 ± 0.141</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tx</td>
<td>4</td>
<td>53 ± 7***</td>
<td>1.783 ± 0.299</td>
</tr>
<tr>
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<td>30</td>
<td>N</td>
<td>4</td>
<td>68 ± 10</td>
<td>1.358 ± 0.202</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tx</td>
<td>3</td>
<td>60 ± 9</td>
<td>1.852 ± 0.092</td>
</tr>
<tr>
<td>Subcortex</td>
<td>20</td>
<td>N</td>
<td>5</td>
<td>100 ± 12</td>
<td>1.549 ± 0.140</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tx</td>
<td>4</td>
<td>55 ± 2**</td>
<td>1.221 ± 0.159</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>N</td>
<td>5</td>
<td>116 ± 9</td>
<td>1.115 ± 0.110</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tx</td>
<td>5</td>
<td>83 ± 8</td>
<td>1.006 ± 0.130</td>
</tr>
</tbody>
</table>

Values are mean ± SEM.

B) Bmax statistical analysis, NS - no significant difference

<table>
<thead>
<tr>
<th>2-way ANOVA</th>
<th>Cerebral cortex</th>
<th>Cerebellum</th>
<th>Brain stem</th>
<th>Subcortex</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>NS</td>
<td>P &lt; 0.01</td>
<td>NS</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>Treatment</td>
<td>NS</td>
<td>P &lt; 0.005</td>
<td>P &lt; 0.005</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>Age-treatment interaction</td>
<td>NS</td>
<td>NS</td>
<td>P &lt; 0.05</td>
<td>NS</td>
</tr>
</tbody>
</table>

Fisher's PLSD

I. Treatment-related

*P < 0.05, **P < 0.001 and ***P < 0.005 Tx vs. N dam progeny

II. Age-related

<table>
<thead>
<tr>
<th>N dam</th>
<th>Cerebral cortex</th>
<th>Cerebellum</th>
<th>Brain stem</th>
<th>Subcortex</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NS</td>
<td>P &lt; 0.05</td>
<td>P &lt; 0.005</td>
<td>P &lt; 0.05</td>
</tr>
</tbody>
</table>

| Tx dam      | NS              | P < 0.005  | NS         | NS        |

C) Kd statistical analysis, NS - no significant difference

<table>
<thead>
<tr>
<th>2-way ANOVA</th>
<th>Cerebral cortex</th>
<th>Cerebellum</th>
<th>Brain stem</th>
<th>Subcortex</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>P &lt; 0.05</td>
<td>NS</td>
<td>NS</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>Treatment</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Age-treatment interaction</td>
<td>NS</td>
<td>P &lt; 0.005</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

Fisher's PLSD

I. Treatment-related

**P < 0.005 Tx vs. N dam progeny

II. Age-related

<table>
<thead>
<tr>
<th>N dam</th>
<th>Cerebral cortex</th>
<th>Cerebellum</th>
<th>Brain stem</th>
<th>Subcortex</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

| Tx dam      | NS              | P < 0.005  | NS         | NS        |
Figure 4.14 Scatchard plots of β-adrenergic membrane binding in normal (open circles) and partially thyroidectomised (closed circles) dam progeny brain regions at 20 pnd. The scatchard plots were only used to display the data, binding parameters were calculated by non-linear regression.
Figure 4.15 Scatchard plots of β-adrenergic membrane binding in normal (open circles) and partially thryoidectomised (closed circles) dam progeny brain regions at 30 pnd. The scatchard plots were only used to display the data, binding parameters were calculated by non-linear regression.
Receptor number in the subcortex, like the previous two regions, exhibited treatment-dependency. This parameter was also age-dependent, increasing by 1.2- and 1.5-fold in N and Tx dam progeny respectively between 20 and 30 pnd. At 20 pnd, Tx dam progeny had 45% fewer receptors than controls, but no difference existed at 30 pnd due to the larger ontogenic increase in Tx dam progeny. Affinity was age-dependent, tending to increase slightly between 20 and 30 pnd, however this increase was not significant by post-hoc analysis in either group.

4.2.3 D2 Dopaminergic receptor binding

In cerebral cortex, D2 receptor number was subject to age-treatment interaction, while affinity was treatment-dependent (Table 4.5 and Fig. 4.16). In controls there was a 1.8-fold increase in receptor number with age, whereas no change was seen in Tx dam progeny. At neither age point, however was the difference between groups significant by post hoc analysis. Affinity did not change with age in either group but was 20-40% higher in Tx dam progeny relative to controls.

D2 receptor binding in cerebellum could not be accurately determined in either group due to the low number of receptors present (463-466). In brain stem, receptor number was neither age- nor treatment-dependent, however affinity was treatment-dependent and exhibited age-treatment interaction (Table 4.5). Thus in N dam progeny, affinity increased 2.2-fold between 20 and 30 pnd, whereas in Tx dam progeny affinity was initially 231% higher than in controls at 20 pnd, but then decreased by 55% at 30 pnd, such that there was no significant difference between the two groups. Subcortical receptor number showed age-dependency, tending to increase with age, but without significant differences by post-hoc analysis. Affinity was insensitive to age and treatment.

4.3 Summary of results

The presence of measurable activities for neurotransmitter metabolic enzymes at the earliest time point studied, 14 dg, lends support to the theory that the neurotransmitters may play a significant role in neural development before the onset of synaptogenesis (429,461). Published data regarding pre- and postnatal ontogeny of these enzymes is incomplete, but most studies indicate similar trends to those observed here, with specific activities of the same order of magnitude (331,333,334,467-471). The exception is fetal brain DDC activity (468-472), which has been reported to increase 4-6 fold from 16 to 19 dg, whereas in this study an earlier increase between 14 to 16 dg was noted, followed by a decrease between
Table 4.5 D2 dopaminergic receptor number (Bmax) and affinity (Kd) in normal (N) and thyroidectomised (Tx) dam progeny brain regions at 20 and 30 postnatal days (pnd)

A)  

<table>
<thead>
<tr>
<th>Region</th>
<th>Age (pnd)</th>
<th>Dam status</th>
<th>n</th>
<th>Bmax (fmol/mg protein)</th>
<th>Kd (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cerebral cortex</td>
<td>20</td>
<td>N</td>
<td>4</td>
<td>175 ± 5</td>
<td>0.589 ± 0.098</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tx</td>
<td>4</td>
<td>271 ± 38</td>
<td>0.945 ± 0.145</td>
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<td>1.182 ± 0.199</td>
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<tr>
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<td>0.554 ± 0.145</td>
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Values are mean ± SEM.

B) Bmax statistical analysis, NS - no significant difference

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<th>Subcortex</th>
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<td>Age-treatment interaction</td>
<td>P &lt; 0.05</td>
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<td>NS</td>
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</table>

Fisher's PLSD
I. Treatment-related
NS at either age

II. Age-related

| N dam       | P < 0.0005 | NS         | NS        |
| Tx dam      | NS         | NS         | NS        |

C) Kd statistical analysis, NS - no significant difference

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<th>Brain stem</th>
<th>Subcortex</th>
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<tbody>
<tr>
<td>Age</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Treatment</td>
<td>P &lt; 0.05</td>
<td>P &lt; 0.05</td>
<td>NS</td>
</tr>
<tr>
<td>Age-treatment interaction</td>
<td>NS</td>
<td>P &lt; 0.0005</td>
<td>NS</td>
</tr>
</tbody>
</table>

Fisher's PLSD
I. Treatment-related
*P < 0.001 Tx vs. N dam progeny

II. Age-related

| N dam       | NS         | P < 0.005  | NS        |
| Tx dam      | NS         | P < 0.005  | NS        |

150
Figure 4.16 Scatchard plots of D2 dopaminergic membrane binding in normal (open circles) and partially thyroidectomised (closed circles) dam progeny brain regions at 20 (left hand plots) and 30 (right hand plots) pnd. The scatchard plots were only used to display data, binding parameters were calculated by non-linear regression.
16 and 19 dg. Different methods of tissue preparation and enzyme assay were employed and may explain the discrepancy.

Maternal hypothyroxinemia disrupted the ontogeny of several neurotransmitter enzymes. In the fetal brain, MAO, ChAT and DDC all exhibited disturbed developmental profiles in Tx dam progeny, however these changes occurred after 14 dg. MAO and ChAT showed similar developmental profiles in N and Tx dam fetal brain, the increase in activities from 16 dg being less pronounced in Tx dam progeny compared to controls—with MAO activity being more severely affected. Further study showed that the TH-sensitivity was MAO isoform-specific, MAO-A being affected at 19 dg while MAO-B was not. DDC ontogeny was markedly different as was the response to maternal hypothyroxinemia, since activity remained unchanged from 16 to 21 dg in Tx dam fetal brain, whereas it declined during the same period in controls. In postnatal brain, age- and region-specific differences were apparent between N and Tx dam progeny, TyrH, DDC, MAO and, to a lesser extent, AChE activities being increased in tx dam progeny relative to controls. Affected regions were mainly those which undergo significant neurogenesis during fetal life, whereas enzyme activities were unaffected in cerebellum, which develops largely postnatally in the rat. Although no region was consistently affected, cerebral cortex appeared the most severely disrupted region with regard to the number of different enzymes affected. In terms of chronic effects however, brain stem was the only region which exhibited a change at 30 pnd (in DDC activity).

Generally, the binding kinetics displayed by the ligands in this study agree with those in the literature (337-339,462,463), although there is variation, particularly for Kd. This is likely to be due to methodological differences in tissue preparation and buffer used (473,474).

Muscarinic cholinergic receptors were unaffected by maternal hypothyroxinemia, but showed distinct regional variation in receptor number and affinity. Indeed, these parameters seemed inversely correlated such that regions with the highest receptor number, such as cerebral cortex and subcortex, had the lowest affinities, whereas cerebellum exhibited low numbers of high affinity receptors. This pattern is suggestive of co-operativity between ligand and receptor, as previously reported in a study using quinuclidinyl benzilate to investigate muscarinic cholinergic binding in rat brain (475).

In contrast, β-adrenergic receptor number varied considerably between N and Tx dam progeny in several brain regions—particularly at 20 pnd. To a lesser extent, affinity was also affected in a region- and age-specific manner. The cerebellum was most severely
affected, the receptor number remaining depressed at 30 pnd and affinity perturbed at 20 pnd. Dopaminergic receptor affinity was more consistently affected than receptor number by maternal hypothyroxinemia, Tx dam progeny tending to have higher affinity receptors than controls. In contrast to the other neurotransmitter receptors studied, no binding could be detected in cerebellum.

The ligands used in this study are relatively non-specific, for example both spiperone and dihydroalprenolol are thought to also bind to serotonergic receptors (476-479). It is possible therefore that changes in receptor affinity between age, region and treatment are actually indicative of changes in receptor subtype populations rather than affinity per se. It has also been suggested however, that receptor affinity may differ due to the local membrane microenvironment (480). Non-linear regression analysis was unable to define multiple binding sites with the data available, and confirmation would require a considerably larger number of data points, and an extension of the range of ligand concentrations studied.

Curiously, changes in β-adrenergic receptor binding occurred in the cerebellum whereas enzyme activities were unaffected. This may indicate that enzyme and receptors activities are modulated by different mechanisms. Alternatively, the disturbed neurotransmitter pathway may originate in a TH-sensitive region and terminate in the cerebellum, such as the noradrenergic pathway that arises from the locus ceruleus. In common with the metabolic enzyme activities however, the monoaminergic system (β-adrenergic receptor binding in particular) was more severely affected by maternal hypothyroxinemia than the cholinergic system. This would suggest that the monoaminergic neurotransmitter system as a whole is more susceptible to maternal hypothyroxinemia than the cholinergic system, however, since this system comprises a range of neurotransmitters (e.g. dopamine, noradrenaline and serotonin) further work is required to define which monoamines are affected.
CHAPTER 5.

THE EFFECT OF MATERNAL HYPOTHYROXINEMIA ON THYROID HORMONE NUCLEAR RECEPTORS IN DEVELOPING BRAIN
5.1 Thyroid hormone nuclear receptor expression in fetal rat brain

The ontogeny of TRs in developing normal rat brain has been characterised at the mRNA and protein level (section 1.6). *In situ* hybridisation studies (213,214) indicate that mRNAs encoding TRα isoforms are expressed from 11.5 dg onwards, TRα2 being predominant. TRβ1 expression is first seen at 12.5 dg at lower levels than, and confined to, areas of TRα1 mRNA expression. TRβ2 mRNA expression is first seen at 13.5 dg in pituitary, with low levels also seen in striatum, hippocampus and neocortex near term. More quantitative data utilising Northern hybridisation analysis has unfortunately focused mainly on the postnatal period, with only one prenatal time point (usually 19 dg) being investigated. Such studies show that TRα2 mRNA is the most abundant isoform in fetal brain, followed by TRα1 then TRβ1 (210-212). Thereafter, studies show either no change (212), or a steep increase in TRα2 mRNA expression between 19 dg and birth (210,211). TRα1 mRNA expression increases during this period but there is uncertainty regarding the magnitude of increase. TRβ1 mRNA has only been detected by Northern hybridisation at 19 dg in one study, which indicated a large increase in mRNA expression after this time (210).

Protein levels do not consistently correspond to mRNA levels. Measurable T3 receptor-binding is apparent at 14 dg, increasing approximately 3-fold by 17 dg and then (unlike mRNA), remaining stable until term (207). More recent studies using isoform-specific antibodies indicate that the majority of T3-binding TR protein expressed during fetal development is TRα1, which accounts for over 90% of binding at 19 dg (215).

Data regarding the effects of TH deficiency on brain TRs during fetal development are scarce. A single study has investigated TRβ1 mRNA ontogeny in connection with materno-fetal hypothyroidism and found no effect (210). Another study reported that materno-fetal hypothyroidism tended to increase neonatal levels of TRα1 mRNA, with TRα2 being less consistently affected (481). In terms of nuclear T3-binding, neonatal hypothyroidism increases the number of T3 receptors, although affinity is unaffected (404). Detrimental effects arising from an earlier insult have also been demonstrated, as adult progeny of Tx dams exhibit an increased number of TR binding sites in the cerebellum and palaeocortex relative to control progeny (397). Another study however, using materno-fetal hypothyroidism, failed to show any effect on T3 receptor binding in nuclear extracts from fetal brains (207).
5.2 Triiodothyronine nuclear receptor binding in fetal brain

5.2.1 Whole nuclei studies

Receptor number was dependent on age and treatment, with interaction between the two parameters (Table 5.1). In N dam progeny, receptor number increased 1.5-fold from 16 to 19 dg, then remained stable up to 21 dg (Table 5.1 and Fig. 5.1). Receptor number was elevated by 64% in Tx dam progeny at 16 dg, but normal at 19 dg due to the absence in experimental progeny, of the ontogenic increase seen in controls between these ages. There was however, an ontogenic increase in receptor number of 1.9-fold between 19 and 21 dg in Tx dam progeny, resulting in 53% higher T3-binding at 21 dg compared with controls.

Receptor affinity was age-dependent, but unaffected by treatment (Table 5.1). In N dam progeny, affinity increased by 43% from 16 to 19 dg, with no further change by 21 dg. A similar ontogenic pattern was exhibited by Tx dam progeny.

5.2.2 Salt-extracted receptor studies

The changes in the Tx dam fetal brain whole nuclei receptor number seen here are in contrast to those reported for salt-extracted receptor binding in a materno-fetal hypothyroid model (207). It is known however that chromatin-associated factors influence TR binding parameters (482) therefore T3-binding in salt-extracted receptors was also studied.

In agreement with the literature (207), the number of extracted receptors was not significantly affected by age or treatment (Table 5.2 & Fig. 5.2). Extracted receptor number was higher than in whole nuclei except in extracts from 21 dg Tx dam progeny (Table 5.2 & Fig. 5.2). Indeed, the differences in receptor number in whole nuclei and extracted receptor preparations declines with age, but only in Tx dam progeny do the estimates converge.

Extracted receptor affinity was age- but not treatment-dependent. In N and Tx dam progeny, affinity decreased 20-40% between 16 and 19 dg, then increased 70-80% by 21 dg. At 19 and 21 dg, extracted receptor affinity was lower than that found in whole nuclei (Table 5.1 & Fig. 5.1).
Table 5.1 Nuclear T3-binding in whole nuclei from normal (N) and partially thyroidectomised (Tx) dam fetal brains

A) Kinetic parameters

<table>
<thead>
<tr>
<th>Age (dg)</th>
<th>Dam</th>
<th>n</th>
<th>Bmax (fmol/mg DNA)</th>
<th>Kd (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>N</td>
<td>5</td>
<td>105.16 ± 6.12</td>
<td>1.907 ± 0.231</td>
</tr>
<tr>
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<td>Tx</td>
<td>5</td>
<td>178.14 ± 23.54**</td>
<td>1.887 ± 0.232</td>
</tr>
<tr>
<td>19</td>
<td>N</td>
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<td>158.74 ± 6.32</td>
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</tr>
<tr>
<td></td>
<td>Tx</td>
<td>6</td>
<td>163.12 ± 18.39</td>
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</tr>
<tr>
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<td>N</td>
<td>5</td>
<td>199.62 ± 16.26</td>
<td>1.073 ± 0.102</td>
</tr>
<tr>
<td></td>
<td>Tx</td>
<td>6</td>
<td>309.53 ± 33.94*</td>
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Values are mean ± SEM.

B) Statistical analysis, NS - no significant difference

<table>
<thead>
<tr>
<th>2-way ANOVA</th>
<th>Bmax</th>
<th>Kd</th>
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<td>Age</td>
<td>$P &lt; 0.0005$</td>
<td>$P &lt; 0.0005$</td>
</tr>
<tr>
<td>Treatment</td>
<td>$P &lt; 0.005$</td>
<td>NS</td>
</tr>
<tr>
<td>Age-treatment interaction</td>
<td>$P &lt; 0.05$</td>
<td>NS</td>
</tr>
</tbody>
</table>

Fisher's PLSD

I. Treatment-related

* $P < 0.01$, and ** $P < 0.005$ Tx vs. N dam progeny

II. Age-related

<table>
<thead>
<tr>
<th>Bmax</th>
<th>Kd</th>
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<td>N dam</td>
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<tr>
<td>19 vs. 16 dg</td>
<td>$P &lt; 0.01$</td>
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<tr>
<td>21 vs. 19 dg</td>
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<tr>
<td>Tx dam</td>
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<tr>
<td>19 vs. 16 dg</td>
<td>NS</td>
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<tr>
<td>21 vs. 19 dg</td>
<td>$P &lt; 0.0005$</td>
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</tbody>
</table>
Figure 5.1 Scatchard plots of T3-receptor binding in whole nuclei from normal (open circles) and partially thyroidectomised (closed circles) dam fetal brain. Scatchard plots were used to display the data only—binding characteristics were determined by non-linear regression.
Table 5.2  T3-binding in extracted receptor preparations from normal (N) and partially thyroidectomised (Tx) dam fetal brains

A) Kinetic parameters

<table>
<thead>
<tr>
<th>Age (dg)</th>
<th>Dam</th>
<th>n</th>
<th>Bmax (fmol/mg DNA)</th>
<th>Kd (nM)</th>
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<tr>
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<td>256.70 ± 31.72</td>
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<td>271.49 ± 20.28</td>
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<td>274.72 ± 27.67</td>
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<td>291.72 ± 27.97</td>
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Values are mean ± SEM

B) Statistical analysis, NS - no significant difference

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<tr>
<td>Treatment</td>
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<td>NS</td>
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<tr>
<td>Age-treatment interaction</td>
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<td>NS</td>
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Fisher’s PLSD

I. Treatment-related

NS at any age

II. Age-related

<table>
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<th>Bmax</th>
<th>Kd</th>
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</tr>
<tr>
<td>21 vs. 19 dg</td>
<td>NS</td>
<td>P &lt; 0.0005</td>
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</table>

<table>
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<th>Bmax</th>
<th>Kd</th>
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<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>21 vs. 19 dg</td>
<td>NS</td>
<td>P &lt; 0.005</td>
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Figure 5.2 Scatchard plots of T3-receptor binding in extracted receptor preparations from normal (open circles) and partially thyroidectomised (closed circles) dam fetal brain. Scatchard plots were used to display the data only—binding characteristics were determined by non-linear regression.
5.3 Thyroid hormone receptor isoform mRNA ontogeny

The binding studies indicate that the differences in whole nuclei receptor number between N and Tx dam progeny, may be due to chromatin-associated factors rather than differences in the number of receptor proteins per se. This does not however rule out an effect of maternal hypothyroxinemia on TR mRNA levels. Furthermore, it is not possible to study the non-T3 binding isoform, TRα2, with binding assays, therefore TR isoform mRNA levels were studied in N and Tx dam fetal brain.

5.3.1 Northern hybridisation studies

Blots were first probed with the TR common probe (section 2.6.4). After washing, membranes were exposed to Hyperfilm, initially for 18 h (Fig. 5.3). Although the TRα2 mRNA band at 2.6 kb was clearly visible, the TRα1 signal (consisting of the characteristic doublet at 5.5 and 6.1 kb) was very faint and TRβ1 could not be seen at all. A longer exposure (3 weeks) neither revealed TRβ1 nor produced a stronger TRα1 signal relative to background.

In an attempt to improve the strength of the TRα1 signal, the membranes were stripped and re-probed with the TRα1 specific cDNA probe. An initial exposure for 3 nights on Hyperfilm (Fig. 5.4) produced a similar result to the TR common probe, with non-specific binding to the TRα2 mRNA clearly visible, while TRα1 mRNA binding was not enhanced. It should be noted that non-specific binding of TRα1 probes to TRα2 mRNA is not unusual and has been previously reported (211). Membranes were then exposed to the more sensitive Biomax film, however even after 40 h exposure, TRα1 signals were not greatly enhanced relative to background. The TRα2 specific probe was not used, since the TRα common probe had already effectively detected TRα2 mRNA levels.

Stripping and re-probing with TRβ1 cDNA was unsuccessful, with no signal being detected after overnight exposure to Biomax film (data not shown). This result, in view of the TRα1 signal strength, was not particularly surprising as fetal brain TRβ1 mRNA is thought to be present in much lower levels than TRα1. Failure to detect TRβ1 was not due to the probe being defective, since it successfully detected TRβ1 at 6.5 kb in several adult rat tissues (Fig. 5.5). After stripping once more, membranes were finally probed with an 18S rRNA oligonucleotide probe. A strong signal was apparent after 5 h exposure to hyperfilm (Fig. 5.3), indicating that RNA degradation was unlikely to be the reason for the
Figure 5.3 Ontogeny of TRα isoform mRNAs in normal (N) and partially thyroidectomised (Tx) dam fetal brain by Northern hybridisation. A) A representative autoradiograph of TRα1 and TRα2 mRNA in fetal brain using a TR common cDNA probe. Membranes were exposed for 18 h to Hyperfilm. B) The same autoradiograph after stripping and re-probing with the 18S oligonucleotide. Membranes were exposed to Hyperfilm for 5 h. C) TRα2 mRNA ontogeny relative to 18S in N (open bars) and Tx (closed bars) dam fetal brain. Values are mean ± SEM, n is indicated in the bars. 2-Way ANOVA indicated age ($P < 0.0005$) and treatment ($P < 0.05$) effects. In addition, $P < 0.0005$ for 19 vs. 16 dg in both groups by Fishers PLSD.
**Figure 5.4** Northern hybridisation of TRα1 cDNA probe to total RNA from normal (N) and partially thyroidectomised (Tx) dam progeny fetal brain, after 18 h exposure to hyperfilm.
Figure 5.5. Northern hybridisation of TRβ1 cDNA probe to total RNA from normal adult liver (Lane A), kidney (Lane B) and brain (Lane C), after 3 nights exposure to Biomax film.
failure to detect TRα1 (at quantifiable levels) or TRβ1. Rather, it is likely that the levels of TRα1 and β1 mRNA were too low for reliable detection by this method. Levels of TRα2 mRNA were however detectable and, after normalisation by 18S, the ontogeny of this species in N and Tx dam fetal brains was calculated (Fig. 5.3).

The expression of TRα2 mRNA was age- and treatment-dependant (Fig. 5.3). In N and Tx dam progeny, levels increased between 16 and 19 dg by 3.4 to 3.8-fold. A treatment effect was apparent by 2-way ANOVA largely due to an overall trend for TRα2 mRNA levels to be increased in Tx dam progeny—though expression at no single age was judged to be significantly different by Fishers PLSD.

5.3.2 Semi-quantitative RT-PCR

The results of the Northern hybridisation studies indicated that a more sensitive method was required to investigate TRα1 and TRβ1 mRNA ontogeny. Analysis by RT-PCR was therefore used. RT samples from N and Tx dam fetal brains at 16, 19 and 21 dg were used in PCR reactions with each primer set under optimised conditions (Table 2.3). PCR results from the TR isoforms were expressed relative to 18S PCR amplification of the same RT samples (Fig. 5.6). TRα1 was age- but not treatment-dependent, with both groups showing an increase (of between 2- to 2.6-fold) from 16 to 21 dg. TRα2 expression was similarly age- but not treatment-dependent, although there was some evidence of differential TRα2 ontogeny between N and Tx dam progeny. In N dam progeny, TRα2 mRNA levels increased 2.4-fold between 16 and 21 dg, whereas in Tx dam progeny the increase (of 1.9-fold) was limited to between 16 and 19 dg. TRα3 mRNA levels showed age-dependency but also tended towards significant treatment-dependency ($P = 0.0553$); mRNA levels increased 2.8-fold in controls, and 3.9-fold in Tx dam progeny, with a trend for higher levels at 19 and 21 dg in the latter group. The TRα2 signal seen by Northern hybridisation analysis comprises of both TRα2 and TRα3 species since they migrate at the same rate, therefore the PCR signals were combined (Fig. 5.7). As expected, age-dependency remained; the TRα3 component being too small to have any effect on overall treatment-dependency. Both groups showed ontogenic profiles essentially the same as for TRα2 alone. TRβ1 mRNA, like the other TR isoforms, showed age-dependency, albeit to a lesser degree with levels increasing 1.4-fold in both groups. No treatment-related effects were observed (Fig. 5.6). The TRα2:TRα3 ratio did not alter significantly with age or treatment (Fig. 5.7). Similarly the TRα1:TRα2 and TRα1:TRβ1 ratios were not age- or treatment-dependant, suggesting they are coordinately expressed during the period of study (Fig. 5.7).
Figure 5.6 RT-PCR of TR isoforms in normal (open circles) and partially thyroidectomised (closed circles) dam fetal brains. Each point represents mean ± SEM of 3 separate experiments. 2-Way ANOVA indicates age-effects ($P < 0.0005$) for TRα1, TRα2, TRα3 and TRβ1 but no treatment or age-treatment interactions. $\dagger$ $P < 0.05$ for N dam fetal brain; $\wedge P < 0.05$ and $\wedge\wedge P < 0.01$ for Tx dam fetal brain, higher vs. lower age by Fishers PLSD.
Figure 5.7 TR isoform expression ratios/combination in normal (open circles) and partially thyroidectomised (closed circles) dam fetal brains. Each point represents mean ± SEM of 3 separate RT-PCR experiments. 2-Way ANOVA indicates an age-effect ($P < 0.0005$) for TRα2+TRα3 but no treatment or age-treatment interactions. ^ $P < 0.05$ and ^^ $P < 0.005$ for Tx dam progeny, higher vs. lower age by Fishers PLSD.
5.3.3 Southern hybridisation of PCR products

To confirm the identity of the PCR products amplified, Southern hybridisation analysis with specific oligonucleotide probes was employed (section 2.6.7.4 & Table 2.4). For 18S and TRα1 PCR product characterisation, a complete set of samples from all three template dilutions were blotted and probed (Fig. 5.8). For the other isoforms, samples were taken from a single set of dilutions and probed as this gave a cleaner picture. After washing under standard conditions, it was found that the TRα2 specific probe also bound to the TRα3 PCR product. Washing at higher stringency (0.5 x SSC/0.1 % SDS at 78 °C) removed the TRα3-bound probe while retaining the TRα2 signal. The cross-hybridisation was subsequently found to be due to 40% homology between the TRα2 specific probe and the TRα3 product sequence. Otherwise, the probes confirmed the identities of the products as expected (Fig. 5.8).

5.4 Summary of results

5.4.1 T3-receptor binding assays

Fetal brain T3-receptor binding varied depending on whether whole nuclei or extracted receptors were used. In general, receptor number was increased and affinity decreased in extracted receptor preparations relative to whole nuclei at the same age. Extraction of nuclear receptors therefore not only alters their binding properties, but also seems to reveal hidden sites/receptors. In whole nuclei, receptor number increased with age between 16 and 19 dg, whereas extracted receptor number did not change. The latter result is in general agreement with Perez-Castillo et.al. (207), who found that after an initial 3-fold increase in receptor number between 14 dg and 17 dg, levels were static until birth. Affinity was age-dependant in whole nuclei and extracted receptors, albeit in different ways. Extraction of receptors also abolished any treatment effects observed between N and Tx dam progeny, indicating that these differences may have been due to chromatin-associated factors. Such factors cannot however account for the differences in extracted-receptor affinity at different stages of gestation and it is possible that the receptors are directly modified during development, possibly by phosphorylation (198).
**Figure 5.8** Southern hybridisation of specific oligonucleotide probes to RT-PCR products from normal (N) and partially thyroidectomised (Tx) dam fetal brains.
5.4.2 TR isoform mRNA expression

The inability to reliably detect TRα1 and TRβ1 mRNA by Northern hybridisation was not too surprising, given the low levels of expression of these isoforms during fetal development. TRα2 transcripts were however detected by Northern hybridisation and RT-PCR analysis results and, given the relatively high margins of error in both methods, the results of both studies are in reasonably good agreement. Both indicate that TRα2 mRNA levels increase between 16 and 21 dg to a similar degree (i.e. 3.4- to 3.8-fold vs. 2.1- to 2.4 fold increase). Although only the Northern analysis indicated a significant treatment effect, a similar trend is seen in the RT-PCR data, especially with regard to TRα3, which would be a component of the signal seen on the autoradiographs after Northern hybridisation. Despite this however combining the RT-PCR data for TRα2 and TRα3 did not produce a significant difference—probably due to the low number of litters used in the study.

As measured by RT-PCR, TRα1 and TRβ1 showed ontogenic increases in mRNA expression—in agreement with published studies (210,211). Relative to T3-binding in whole nuclei, the magnitude of ontogenic increase was greater—this discrepancy between mRNA and protein levels is not unusual for TRs (210,211,217). In addition, no treatment-related effects were found in mRNA expression, suggesting that the differences observed in T3-binding were due to post-transcriptional modification. Indeed, this was also indicated by the abolishment of treatment effects in extracted receptor studies. The effect on TRα2/3 however, may be transcriptional but it is not known whether these changes in mRNA expression result in altered TRα2 protein levels.
CHAPTER 6.

DISCUSSION
The effects of TH on neonatal brain development in the rat have been intensively studied in models of congenital hypothyroidism and several genes have been shown to be TH-responsive during this period (289). In the past decade, it has become apparent that the early fetal brain, in both rats and humans, is also dependent on TH for normal development. Thus, the period of TH-dependency exists prior to the onset of fetal TH synthesis (occurring at 17.5 dg in rats and between 10-24 wg in humans) such that maternally derived TH, T4 in particular, is required. Indeed, transfer of T4 from mother to fetus has been demonstrated in both rats and humans (49,51). TH deprivation as early as the first trimester in humans results in irreversible neurological defects, the degree of deficit being related to the extent of maternal hypothyroxinemia (306,311). Studies in rat models of maternal hypothyroxinemia have indicated that experimental progeny also exhibit region-specific disturbances in brain biochemistry and behavioural abnormalities (406). In comparison to the effects of congenital hypothyroidism however, this area has not been extensively studied and targets for TH in fetal brain remain to be elucidated.

Many of the actions of TH are thought to be mediated by their nuclear receptors, TRs. In recent years, several candidate genes have been proposed to be under TH control during neonatal rat brain development (section 1.7.2). Most of these genes however, are unlikely to be implicated during early development; either because they are not expressed prior to fetal TH synthesis (e.g. the myelin protein genes), or they are developmentally regulated with regards to their TH responsiveness. For example, PCP-2 gene expression is repressed by, as yet unidentified, proteins during fetal development and is therefore only TH responsive during postnatal and adult life (483); a similar mechanism of action is thought to prevent TH-regulation of RC3 gene transcription before 10 pnd. Indeed, there is still scepticism regarding the importance of TH during fetal development (484), despite evidence to the contrary—such as the presence of TH and TRs in fetal brain before the onset of fetal thyroid function and the irreversible neurological dysfunction seen in children of hypothyroxinemic mothers. This thesis therefore attempted to examine in further detail the role of maternal thyroid status during pregnancy in fetal brain development and to identify possible targets for TH during this period. Identification of such TH-regulated processes may be useful in developing therapies to combat the consequences of an \textit{in utero} TH deficit.

The actions of T3 appear to be mediated largely through TRs, it seemed logical therefore to assess the effect of maternal hypothyroxinemia on fetal brain TR ontogeny. There are several \textit{in vitro} and \textit{in vivo} studies suggesting that T3 influences TR expression. For example, T3 up-regulates TR\(\beta\)1 mRNA expression in cultured astrocytes, albeit without
effect on T3 nuclei binding levels (165). In addition, neonatal hypothyroidism increases T3-receptor binding in rat brain (404) and elevated TR number is also seen palaeocortex and cerebellum of adult Tx dam progeny (397).

Neurotransmitter systems are also likely targets for TH action in the fetal brain, particularly the cholinergic and catecholaminergic systems. Not only are they T3-sensitive in models of congenital hypothyroidism (section 1.7.2) and \textit{in vitro} (section 1.7.3), but changes in cholinergic metabolic enzymes have been observed in adult Tx dam progeny (section 1.7.4.1). Given the likely role of neurotransmitters and their metabolic enzymes as growth factors in early fetal brain, it is important to assess the extent to which their ontogeny is affected by maternal hypothyroxinemia.

6.1 Animal model

The effects of maternal hypothyroxinemia on parameters of growth and development in the current study are largely in agreement with previous studies using the same model (398,408). Some differences are apparent however, for example a previous study using this model indicated reduced brain weight at 15 dg and some alterations in brain protein and DNA (408). These differences may be dependent on the degree of hypothyroxinemia induced; T4 levels in the Tx dams in the earlier study were only 20-30% of controls throughout gestation (408), compared 30-50% in this study. Similarly, models of overt hypothyroidism exhibit prolonged changes in brain protein and DNA levels, as well as reduced brain and body weights (411). The range and duration of compromise in these parameters may therefore be correlated to the severity of hypothyroxinemia/hypothyroidism in the experimental dams. As maternal TH levels fall however, it becomes increasingly difficult to separate effects caused directly by the TH deficit on the fetus from those caused indirectly \textit{via} metabolic compromise in the dams (413,414). It is important therefore that such confounding factors are minimised, if not eliminated. To this end, rats were only partially thyroidectomised in this study, resulting in the aforementioned reductions in T4 levels; further, T3 levels were unaffected at 14 dg and reduced by only 30-40% at later stages of gestation. Thus, in terms of circulating TH levels, the Tx dams exhibit hypothyroxinemia rather than overt hypothyroidism. Given the high serum TSH levels in the Tx dams throughout pregnancy however, some degree of tissue hypothyroidism cannot be ruled out. Nevertheless, the results of the current study strongly suggest that maternal metabolic compromise did not play a major role in disrupting fetal brain development, since parameters of growth such as brain weight, protein and DNA were normal pre- and postnatally. Of particular relevance in this respect is the normal development of the cerebellum. This brain region is prone to nutritional deficiency and is therefore a sensitive
indicator of deficits arising from maternal compromise. Furthermore, body weight also normalised in postnatal Tx dam progeny.

Placental dysfunction is also unlikely to have impinged upon fetal development to any major degree. Previous studies using this model show that placental development is largely unaffected (408) and in the current study, most placental parameters, such as weight, were normal and those that were affected, i.e. protein content and protein:DNA ratio, were reduced only at 19 dg. One result that may be due to maternal or placental compromise is the reduction in the number of viable Tx dam progeny at birth. The Tx dams may have trouble giving birth, alternatively sickly pups may be cannibalised by the dam or they may die through maternal neglect. Whatever the reason, it should be emphasised that the surviving progeny exhibit normal parameters of growth, such as brain weight, and protein and DNA levels. Therefore the reduction in litter number at birth is unlikely to be a sign of fetal malnutrition.

Fetal brain TH levels were not determined in this model, however in a study utilising a slightly different model of maternal hypothyroxinemia (achieved by radiothyroidectomy), but with similar reductions in maternal serum TH levels, fetal brain TH was decreased by 50% (326). This is despite the operation of brain-sparing mechanisms such as increased 5'D-II activity (85). Nevertheless, some degree of brain-sparing was seen in the current study as fetal brain weights were normal even though fetal body weights were reduced. Fetal and postnatal brain protein and DNA levels were also unaffected. It was assumed that the onset of fetal thyroid function normalised Tx dam fetal brain TH levels. This assumption is supported by data from a maternal hypothyroid model in which fetal brain TH levels in the experimental group were normal relative to controls at 20 dg, despite serum TH levels in the dams being below the limit of detection (411).

6.2 Maternal hypothyroxinemia and thyroid hormone receptor ontogeny

Maternal hypothyroxinemia resulted in increased T3 receptor number in whole nuclei at 16 and 21 dg, but not at 19 dg; no effect was observed on receptor affinity at any age. An increase in receptor number has also been reported in neonatal hypothyroid rat studies (404,485). No differences in receptor number or affinity were seen between N and Tx dam fetal brains when receptors were salt-extracted—as noted in previous studies (207). Receptor number was generally increased and affinity decreased, in extracted receptor preparations compared with whole nuclei. It could be argued that the increased receptor number observed in whole nuclei from brains of Tx dam progeny at 16 dg was due to a lack of endogenous T3 compared with controls—hence more receptors are available to bind
exogenous T3 label. This would not however account for the increased receptor number seen at 21 dg, as brain TH levels are likely to have normalised at this age. In addition, the incubation time of 30 min used in the assay should have been sufficient to allow endogenous T3 to dissociate (486), not to mention the nuclei preparation time.

Of the two assay methods, the whole nuclei preparation is more physiologically relevant. Comparison of binding parameters from whole nuclei and extracted receptor preparations suggests that at earlier stages of gestation in particular, some binding sites are masked in whole nuclei. This masking process appears to be developmentally regulated—more sites becoming available as gestation progresses. The regulation of this process seems disturbed in Tx dam fetal brain, so that more binding sites are accessible to T3 at 16 and 21 dg. Little is known regarding the factors governing T3 access to receptors; with the advent of recombinant DNA techniques research has focused on regulation of transcription and TR-DNA binding (section 1.6.3.1). Earlier studies however, suggested that chromatin-associated factors modulated T3-TR binding (482) and it has been suggested that histone acetylation is one of the processes involved (485). Recent studies have focused on the role of histone acetylation in the activation of transcription (182,183), however alteration of chromatin structure by acetylation/deacetylation could also mediate T3 access to receptors. Thus maternal thyroid status may directly modulate the transcription of one or more of the regulatory proteins involved in determining chromatin structure.

An alternative explanation is that T3 transport through the nuclear membrane is enhanced by maternal hypothyroxinemia, thus during the whole nuclei assays more T3 crossed the nuclear membrane and bound to T3. Indeed, there is evidence for a stereospecific transport mechanism for T3 into the nucleus (487). This would be an adaptive response to the fetal brain TH deficit, allowing whatever T3 was available to enter the nucleus rather than stay in the cytoplasm. As T3 is normally preferentially localised in the nuclei however (487), it is questionable how effective this mechanism would be. Of the two explanations, the former can best account for the effects of maternal hypothyroxinemia, since the unmasking of additional receptors without extra T3 being present (as in 16 dg Tx dam fetal brain) may repress transcription via an increase in unliganded TR homodimer concentration (156,191,192). In either case, the results argue against a direct effect of T3 on the T3-binding receptors since no changes were seen in extracted receptor preparations.

The effects of maternal hypothyroxinemia on the mRNA levels of TR isoforms were less conclusive. There appeared to be no significant effect on the mRNA levels of the T3 binding isoforms—consistent with the results of the extracted receptor studies. Analysis of TRα2 mRNA using Northern hybridisation however indicated a general increase in steady-
state levels of this isoform between 16 and 21 dg as a result of maternal hypothyroxinemia. The RT-PCR data did not suggest a significant difference between N and Tx dam fetal brain, but the general pattern was similar. It has been reported that materno-fetal hypothyroidism increases neonatal TRα1 mRNA expression in most brain regions, whereas TRα2 and TRα3 mRNA levels are less consistently affected (481). The discrepancies between these findings and the current study are probably due to the different extent and timing of TH deficit.

Little is known regarding the role of TRα2 apart from *in vitro* studies suggesting it may play a dominant negative role in TR-mediated transcription (149,150,152); even less is known about the role of TRα3. Thus, it may be possible to speculate that increased TRα2 levels in Tx dam fetal brain would result in further inhibition of TR-mediated transcription. This assumes however that the increased mRNA levels would be reflected by increased protein levels and it is well established that TR mRNA and protein levels do not always correspond (211). Nevertheless, recent studies using TR-knockout mice have indicated that TRα2 may play a critical role during early development (section 1.6.6) and merits further investigation.

### 6.3 Maternal hypothyroxinemia and cholinergic and monoaminergic neurotransmitter systems

#### 6.3.1 Fetal brain neurotransmitter enzyme ontogeny

In Tx dam fetal brain, total MAO activity was reduced at 16 and 19 dg, relative to controls; a similar pattern was seen for ChAT—albeit the effects were not so pronounced. These results resemble those seen in neonatally hypothyroid rat and cell culture studies. Combined materno-fetal (488) and neonatal hypothyroidism (489) have both been shown to reduce MAO activity in brain. Furthermore, MAO-A activity is TH-responsive in neuroblastoma cell lines (391) and was reduced by maternal hypothyroxinemia in the current study, at least at 19 dg. ChAT activity is also reduced by postnatal hypothyroidism in rat (490,491) and is induced by T3 in neuronal cultures (383,384). It is feasible therefore that TH regulates MAO and ChAT activities through common mechanisms during fetal and postnatal development. In postnatal brain, TH is generally thought to act via TRs (section 1.6). In normal fetal brain, TR binding increases 1.5-fold from 16 to 19 dg, coincident with the increase in ChAT and MAO activity, whereas in Tx dam fetal brain TR binding is higher than controls at 16 dg before normalising at 19 dg—coincident with the reduced ChAT and MAO activities. This suggests a possible direct effect by T3 on these
enzymes, whereby reduced levels of T3 in 16 dg Tx dam fetal brain together with higher than normal levels of unliganded TRs leads to transcriptional repression of ChAT and MAO-A genes. Indeed, several potential TREs have been localised in the 5' flanking region of the human ChAT gene and T3 induced transcription of this gene in a fusion gene construct in neuronal cell lines (492). TR binding activity and, probably, TH levels normalise in Tx dam fetal brain at 19 dg (411) however, whereas MAO and ChAT activities remain disturbed at this age. Nevertheless, T3 is unlikely to be the only factor regulating these enzymes and other mediators may be responsible for the divergence between T3-TR binding and enzyme activity.

TyrH and AChE have also been shown to be affected by postnatal thyroid status (332,493), however in this study they were unaffected prenatally by maternal hypothyroxinemia. Examination of the literature suggests that a hierarchy of TH-responsiveness exists for the enzymes investigated in this study. Thus, TyrH activity was less TH-sensitive than ChAT to postnatal hypothyroidism (491) or MAO-A in neuroblastoma cells (391). In addition, AChE is less responsive to T3 treatment than ChAT in neuronal cultures (383,384). It is feasible therefore that prenatal changes in brain AChE and TyrH activities may occur in more severe cases of maternal thyroid dysfunction than those induced in this study. Similar hierarchical responses to increasing T3 doses have been demonstrated for several parameters in pituitary cell lines (494,495). The mechanisms behind the hierarchy of TH-responsiveness are unknown, but may be related to the differing ligand-receptor sensitivities displayed by TREs (169). Thus ChAT and MAO genes may contain TREs that have a relatively high sensitivity for T3-induced transactivation compared with those in the other, less TH-sensitive enzymes. Alternatively, different mechanisms of T3 action may be responsible, for example, it has been reported that T3 stabilises AChE mRNA stability in Neuro-2A cell culture (359). These indirect mechanisms may be less susceptible to a TH-deficit than the direct ones which perhaps mediate ChAT and MAO activities. This is of course highly speculative and further work is required such as the screening of the upstream sequences of the genes encoding these enzymes for TREs.

The prenatal ontogeny of DDC was also disturbed in Tx dam progeny, but only at 21 dg, when the enzyme activity was 58% higher than control brain. As fetal brain TH levels should have normalised by this age, this is unlikely to be a direct effect. Indeed, unlike the other enzymes in this study, postnatal dysthyroidism has little effect on DDC activity (496). The altered ontogeny of DDC activity suggests that a proportion of the neurons containing this enzyme do not undergo apoptosis, in contrast to controls. DDC is ubiquitously expressed however, and it is unlikely to have been catecholaminergic neurons that were
affected since TyrH did not show a similar pattern. It may be serotonergic neurons that were affected therefore, since DDC also plays a role in serotonin synthesis (443).

6.3.2 Postnatal neurotransmitter system dysfunction

Chronic effects arising from the original insult were evident in catecholaminergic metabolic enzymes and receptors. The cholinergic system, at least those aspects investigated, were unaffected postnatally despite the observed disruption to ChAT ontogeny prenatally. At variance with these results, earlier work on adult Tx dam progeny showed region-specific disturbances in brain ChAT and AChE activities (427). A possible explanation for this difference could be that the changes in adult progeny reflects covert disturbances which become apparent over time—perhaps indicating premature degeneration. Alternatively, the more severe maternal hypothyroxinemia induced in the study of adult progeny, where T4 levels were depressed to 10% of controls, may account for the long term disturbances in these parameters (427).

Postnatal disturbances in catecholaminergic enzymes were confined to brain regions in which neurogenesis occurs during early gestation (52). DDC activity was elevated in brain stem and cerebral cortex, indicative of long term or, in the case of brain stem, permanent compromise to monoaminergic neurons. Changes were also apparent for TyrH in cerebral cortex and MAO in brain stem. Fetal TyrH activity appeared unaffected by maternal hypothyroxinemia. The postnatal effect on TyrH may indicate that the fetal changes were highly localised and therefore effectively masked since whole brain was studied. As the region affected postnatally was cerebral cortex however, which comprises ca. 60% of total brain weight this is unlikely. Alternatively, TyrH may not have been affected directly by the fetal TH deficit but its later ontogeny became disturbed as a result of the desynchronisation in development. Cerebellum was unaffected possibly because this region develops largely after the onset of fetal TH synthesis and only those neurons exhibiting developmental lability at the time of insult are susceptible to a deficient TH environment (425).

All of the enzymes affected postnatally showed increased activities. The reason for this is unknown but, as proposed for the increased fetal DDC activity, it may reflect elevated numbers of neurons expressing these enzymes. Of the three enzymes studied, only TyrH is specifically localised in catecholaminergic neurons. The increased activity of TyrH in the cerebral cortex, together with DDC, is therefore suggestive of elevated numbers of catecholaminergic neurons in this region. Both DDC and MAO have multiple substrates and are widely expressed and it is possible that their increased activities reflect perturbation of other aspects of the monoaminergic system, such as serotonin metabolism for example.
Neurotransmitter receptor binding was also studied in postnatal animals to further investigate the chronic effects of maternal hypothyroxinemia. Like the enzymes, monoaminergic receptors exhibited region-specific altered postnatal ontogeny as a result of maternal hypothyroxinemia, whereas cholinergic muscarinic binding was unaffected. Thus, in terms of long term changes at least, the monoaminergic system is more susceptible to maternal hypothyroxinemia than the cholinergic system. β-Adrenergic receptor number was decreased in the cerebellum, brain stem and subcortex of Tx dam progeny relative to controls, whereas dopaminergic D2 receptor number tended to be lower in the cerebral cortex. Receptor affinity was less consistently affected, with β-adrenergic receptors exhibiting reduced affinity in cerebellum while dopaminergic D2 receptor affinity was increased in brain stem. The effects on receptor number at least, seem to be similar to those seen in congenital hypothyroid models. For example, β-adrenergic receptor number was decreased in cerebellum and forebrain as a result of postnatal hypothyroidism (338). Similarly, D2 dopaminergic receptor number, was decreased in striatum of neonatal hypothyroid rats (341). Neither study showed any effect on receptor affinity as a result of hypothyroidism, although hyperthyroidism decreased dopaminergic receptor affinity in the latter study. Neonatal hypothyroidism also affects cholinergic muscarinic receptor ontogeny, but only in the cerebellum, where receptor number was decreased at 21 pnd but increased at 35 pnd relative to controls (337). Interestingly, a similar trend was seen in the current study, the lack of statistical significance may be due to the different ages studied, or the degree of TH deficit. A more likely explanation however is the difference in timing of the insults i.e. the changes occurring in the congenital hypothyroid models arise directly from the altered TH environment whereas the changes observed in the current study occur when brain TH levels are normal and therefore reflect chronic compromise in these parameters due to the earlier fetal TH deficits.

As discussed previously (section 4.3), differences in receptor affinity as measured by these ligands are probably a result of changes in heterogenous receptor populations. Thus maternal hypothyroxinemia is unlikely to alter receptor affinity per se, rather it alters the proportion of different receptors as characterised by the changes in overall affinity. For example, in cerebellum, at least at 20 pnd, not only are there less β-adrenergic receptors in Tx dam progeny, but their affinity is also lower—suggesting that it is the relatively high affinity receptor population that is deficient. By 30 pnd, although receptor number is still deficient, affinity has increased to that in controls. Relative to 20 pnd however receptor number has increased in Tx and N dam progeny, it is possible therefore that the increase in receptor binding seen in Tx dam progeny comprises the higher affinity receptor population that was subnormal at 20 pnd. Regions where receptor number changes without affecting
affinity are probably indicative of relatively homogenous receptor populations. Changes in affinity without altered receptor number, such as those seen for dopaminergic D2 receptor binding in cerebral cortex, are harder to explain. There are studies however that suggest that the D2 receptor exists in high and low affinity states (497,498), therefore it is possible that in the example mentioned above the high affinity state predominates in Tx dam progeny cerebral cortex at this age.

Curiously, the cerebellum not only displayed altered β-adrenergic receptor binding but was also the only region where the changes (in receptor number) persisted to 30 pnd. These changes in cerebellum are puzzling given that no changes in metabolic enzyme activities were seen in this region. Indeed, the cerebellum develops largely postnatally and should therefore be relatively protected from the effects of maternal hypothyroidism. Nevertheless, alterations in TH levels during development can alter the neuronal pathways (337) and the changes may therefore arise from alterations in noradrenergic pathways which develop in more vulnerable areas, but terminate in the cerebellum, namely the axons arising from the locus ceruleus (443). This suggests that damage arising from an early insult not only persists after correction of TH brain levels but may also disrupt the development of regions not directly affected. Alternatively, the disparity between the effects of maternal hypothyroidism on neurotransmitter metabolic enzymes and receptors may be due to different underlying causes such as disturbances in other growth factors as detailed below.

6.2.3 Suggested mechanisms of thyroid hormone-induced changes in brain development

As discussed earlier, maternal TH status may directly affect the activity of MAO and ChAT in fetal brain, however the long term changes, in the monoaminergic system in particular, are indicative of more widespread disruption. Brain development is a highly synchronised process incorporating many different regulatory signals. Disruption of one of these signals, namely T3, may have knock-on effects such that other regulatory signals are also affected. This catastrophic scenario is likely to be the principle cause of the chronic changes seen in this study and there are several developmental mediators that may be affected either directly or indirectly by the TH deficit in the fetal brain.

There is increasing evidence that neurotransmitters have neurotrophic roles in early gestation (429,430) which are probably mediated through their receptors. A range of neurotransmitter receptors are expressed in rat fetal brain (432,466,499-507) which may be targets for maternal TH. Changes in neurotransmitter receptor binding are likely to arise either through direct effects on receptor gene expression, or as a result of altered
neurotransmitter levels. There is evidence to support both possibilities; α- and β-adrenergic receptor mRNA expression is disturbed in a tissue-specific manner by hypothyroidism in adult rats (508), although brain was not studied, and postnatal hypothyroidism has also been shown to alter neurotransmitter levels in rat brain (330,331). It remains to be determined whether maternal hypothyroxinemia induces similar effects, nevertheless it is feasible that altered neurotransmitter function prior to synaptogenesis disturbs neuronal differentiation, leading to chronic brain maldevelopment and possibly the observed postnatal effects.

The early TH deficit may also impact on other developmental signals, such as polyamines (417). Indeed, ornithine decarboxylase regulates polyamine biosynthesis and is sensitive to maternal hypothyroxinemia in fetal and postnatal rat brain (408). Furthermore, there is considerable interaction between polyamines and the noradrenergic system. Polyamines regulate the development of noradrenergic pathways (417), similarly noradrenergic input via β-adrenergic receptors is thought to be a major regulator of ODC activity, and T3 has been reported to be essential for the establishment of this regulatory mechanism (509).

Other possible factors include the neurotrophins themselves. In cell culture models NGF acts synergistically with T3 to induce ChAT activity (386,510,511). Furthermore several neurotrophins, including NGF, are TH-sensitive—at least in the rat neonate (section 1.7.2). Indeed, with the close association between neurotransmitters and neurotrophins it is possible that they are affected by maternal hypothyroxinemia—the questions being whether the effects are directly or indirectly induced by TH status, and whether they account for the observed chronic changes in postnatal brain.

The current study also indicates that mRNA transcription in general may be disrupted in Tx dam fetal brain by two possible mechanisms (section 6.2), the first being an increase in transcriptional silencing mediated by elevated numbers of unliganded TR homodimers. Alternatively, if the raised TRα2 mRNA expression in Tx dam progeny is reflected by increased TRα2 protein levels there would be higher level of dominant negative inhibition of TR-mediated transcription. Either of these mechanisms could disrupt normal brain development and result in the observed changes.

The effects seen in this study may also occur via an extranuclear T3-mediated mechanism, acting at the synapse where T3 is thought to influence synaptic transmission (section 1.6.6). There is also evidence for TH interaction with adrenergic agonists at the cell surface (section 1.4.2), which together with the recent finding of a G-protein-linked T3 binding
site (246), suggests that T3 can influence neurotransmitter function at many levels. In fact, it is probable that the effects noted in this study are due to a combination of these, and other, mechanisms and further is required to clarify which ones are the most important.

It is known that both the cholinergic and catecholaminergic systems in the CNS are involved in behavioural functions. For example the cholinergic neurons in the basal forebrain play a role in learning and memory (512) and disturbed catecholamine metabolism is implicated in the pathogenesis of depression (513). It is possible therefore that the wide range of behavioural compromise exhibited in Tx dam progeny (427) is, at least partly, associated with the changes in neurotransmitter metabolism seen in this study. It can be misleading to extrapolate animal model results to humans, particularly with respect to a process so complicated as brain development (for example see (514)). It is possible, however that similar disturbances to those seen in the current study may underlie the neurological dysfunction seen in children born to hypothyroxinemic mothers in iodine deficient and sufficient endemias.

6.4 Summary

This thesis provides further evidence for a role for maternal thyroid status in fetal brain development and identifies at least three candidates genes, the transcription of which may be directly affected by TH in the fetal brain, namely ChAT, MAO-A and TRα2. In addition, the observed changes may be exacerbated by the general increase in inhibition of TR- and retinoic acid-mediated transcription via increased formation of TR homodimers. It is proposed that the disturbed ontogeny of these proteins, amongst others, may form the basis of the region-specific biochemical and behavioural abnormalities identified in this model (427). Thus, the changes seen in this study may also reflect those that occur in TH-deficient human fetal brain during the first half of pregnancy.

6.5 Future work

The results in this thesis have opened up several potentially fruitful avenues of research. Firstly, it is imperative that fetal brain TH levels are measured before and after the onset of fetal thyroid function in order to confirm the degree of deficit and the age at which TH levels normalise in Tx dam progeny.

Further work is also required to clarify the mechanisms behind the observed changes. For example, are the increased enzyme activities due to increased enzyme synthesis or increased numbers of neurons expressing the enzymes? Fetal brain neurotransmitter enzyme mRNA
levels, MAO-A and ChAT in particular, should be investigated using this model to confirm or refute the possibility of direct TH-modulation prior to the onset of fetal TH synthesis. It is also important to assess if neurotransmitter levels are affected in Tx dam fetal brain and whether these changes persist after the onset of fetal thyroid synthesis. Western blotting and immunohistochemical studies could be employed to determine whether TRα2 protein levels are increased in Tx dam fetal brains, relative to controls. It would also be interesting to see if maternal hypothyroxinemia affected histone acetyltransferase activities.

Several findings in this study implicate the serotonergic system as a possible target for TH during fetal brain development and this should be investigated. The research could also be widened to include the enzymes involved in GABA and glutamate metabolism, as synaptic plasticity and long term potentiation are important processes in brain development and there is some evidence that aspects of these neurotransmitter systems are TH-responsive (242,337,515-517). The effect of maternal hypothyroxinemia on fetal neurotransmitter receptor ontogeny should also be studied, at protein and mRNA levels. Immunohistochemistry and in situ hybridisation using receptor isoform specific antibodies/probes could be employed to pinpoint regional effects. Neurotrophins—particularly NGF are also potential targets for maternally derived TH in fetal brain. Both protein and mRNA expression should be studied to give a clearer indication of the mechanism behind TH-mediated effects.
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200

202

203


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Introduction

The large amount of thyroid hormone pills taken by patients all over the world — in the United States alone it is an estimated amount of 18 million prescriptions per year — together with the fact that thyroid hormones regulate a great number of biochemical reactions in cellular functions like brain function, myoccardial contractility, metabolic rate, growth and many more, induced us to invite for a discussion of "Thyroxine Excess".

The basic idea was to discuss the consequences of exogenous thyroxine, that is, Excess Thyroxine Medication selectively used to treat malignant and benign goitre, psychiatric disorders and hypothyroidism (especially drug-induced) in pregnancy. Too conflicting and too trivial is experience with excess thyroxine medication in weight reduction and its discussion should therefore be kept for a later occasion.

Excess thyroxine medication is not a thing apart from hyperthyroidism and to some extent also not from hypothyroidism and, therefore, the whole field of thyroid physiology is alluded to when the authors discuss normal and disturbed transport, effects and regulation of thyroid hormones.

In the part orientated to more clinical topics the possible "side effects" occupied plenty of ground. Any disturbance of thyroid hormone homeostasis will impinge upon normal cell function and beneficial and adverse effects occur, and the whole spectrum of such effects is very carefully discussed in the papers.

We feel we have hit upon a good idea when inviting to a meeting on the topic of Excess Thyroxine Medication and we are very happy and thankful indeed that all these prominent scientists came. Papers and discussions have shown that "Thyroxine Excess" really is a matter of interest and we hope that the meeting and the publication in this issue of ACTA MEDICA AUSTRIACA will help to focus interest on it.

We would like to thank once more the authors for the great care they took in preparing for the conference and in preparing the manuscripts.

Finally, it has to be said that the meeting and the publication was only made possible by the generous sponsorship of SANABO, Vienna.

R. Höfer, M. Weissel, Wien
(Editors of this issue of the ACTA MEDICA AUSTRIACA)

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Transport of Thyroid Hormones to Target Tissues


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


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

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


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


Introduction

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

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

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

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

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

This model embodies several key ideas:

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

b) A control system is envisaged as existing within the mother ensuring a sufficiency of the T4 supply to the feto-placental unit. The main components of this
putative control system comprise TBG (whose elevated concentration during pregnancy is postulated as enhancing T4 release from the maternal thyroid and from the placenta governing T4 synthesis in the thyroid field, including the supposed that has previously been accepted in the maternal thyroid stimulator deriving from the placenta governing T4 synthesis per se by the maternal thyroid gland.

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

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

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

Speculation on the physiological role of the specific thyroid (and steroid) hormone binding proteins has also occasionally centred on the possibility that they are in some way implicated in reproduction, as exemplified by the notion that the rise in TBG "protects" the fetus from maternal TH (23). Such speculation has stemmed in part from the marked changes observed in pregnancy in these proteins' maternal serum concentrations.

Clearly the role of TBG and other binding proteins in TH transport merits consideration in the context of an examination of maternal, placental and fetal TH requirements throughout gestation. Coincidentally this issue is one which, because of its wider relevance to endocrinology and reproductive physiology, has attracted much recent interest and controversy. Discussion has particularly centred on the validity of the "free hormone hypothesis" of (thyroid) hormone transport and action.

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

The "free hormone hypothesis" of hormone delivery is widely accepted amongst endocrinologists. It enshrines the concept that, in the case of hormones which exist in blood (largely) in protein-bound form, the serum free hormone concentration as determined under equilibrium conditions in vitro constitutes the key determinant of hormone action, the protein-bound concentration being physiologically irrelevant. A corollary is that only hormone in the free state is able to traverse target tissue capillary walls and penetrate target cells. These concepts reflect the observation that, in circumstances in which serum protein-bound hormone levels differ significantly from normal (e.g., during pregnancy,
or as a consequence of genetic abnormality), overall endocrine status appears to correlate with the serum free concentration, not the bound. The thyroid/pituitary/hypothalamic feedback system provides the classic example of these propositions, the system appearing to operate in such a way as to maintain measured free TH concentrations at a (near) normal level in the face of wide differences in the concentrations (or compositions) of the TH binding proteins present in blood. Similar concepts are applicable in the case of the steroid hormones.

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

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

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

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

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

The experimental basis for this revised proposal centred on the discrepancy between Pardridge and Landaw’s observa-
predictions based on a revised hormone equation, described by these authors as the "modified Kety-Renkin-Crone" equation (26, 27), which takes the form:

\[ FE = l - e^{-\frac{k_{pl}}{1 + K[P]}} \]  
(1)

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

\( [P] \) = protein concentration,

\( K \) = affinity constant,

\( t \) = capillary transit time,

\( k_p \) = capillary wall permeation rate constant.

Partridge et al.'s more recent views depended crucially on the demonstration that increase in the protein content of the injected labelled-hormone bolus used in his organ-perfusion experiments failed to cause a reduction in tissue uptake of hormone of the magnitude predicted by Eqn. 1 (27). However, it is readily demonstrable that this simplified equation (which is essentially identical to one proposed in our critique of Partridge's earlier theoretical analysis [8]) depends on the assumption that all kinetic events occurring within, and adjacent to, the capillary in the course of unidirectional hormone efflux proceed at infinite speed, i.e., that the only constraint on the rate of hormone efflux is the rate constant governing capillary wall hormone permeation.

The invalidity of this proposition can readily be demonstrated by examination of a more correct efflux equation applicable to Partridge's experiments, assuming (for the sake of clarity) that the extracapillary free (labelled) hormone concentration remains essentially at zero as the tracer bolus transits organ capillaries, and that intracapillary free hormone mixing and diffusion are infinitely rapid (implying that the free hormone concentration across the entire capillary radius is uniform [7, 8]):

\[ FE = l - e^{-\frac{k_{pl}}{1 + K[P] + k_p/k_d}} \]  
(2)

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

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

Fig. 2. Unidirectional extraction of \([125I]T3\) by rat brain plotted versus arterial albumin concentration (data reproduced from Figure 5 [27]). Note the typically good fit of Equation 2 to the experimental data using the dissociation constant determined in vitro. When the term \( k_p/k_d \) is omitted from the efflux equation, a 10-fold higher dissociation constant is required to obtain a good fit to the observations (27).

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

\[ FE = l - e^{-\frac{k_{pl}}{1 + K[P] + k_p/k_d + \sum K_i [P]}} \]  
(3)

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

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

Kinetic model of hormone transport

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

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

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

Equilibrium in vitro. In short, our analysis reveals that serum binding proteins assist in sustaining a high free hormone concentration at the capillary wall in the face of hormone efflux, thus enhancing (not, as commonly thought, restricting) hormone efflux from the capillary. Moreover it is demonstrable that a rise in the serum concentration of a binding protein that carries two hormones (as does TBG) is potentially capable of specifically enhancing transport to certain tissues of one hormone relative to the other in consequence of differences in their binding characteristics. Thus, in the case of T4 and T3, a rise in TBG is likely to selectively increase T4 delivery to target tissues in which the rate of hormone efflux per unit area of capillary wall is high. This suggests that, although the free hormone concept may broadly apply throughout the body, binding proteins may indeed serve a subtle role in directing particular hormones to particular target organs in particular physiological circumstances (albeit for physicochemical reasons differing from those advanced by Pardridge et al.).

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

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

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

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

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

In summary, the reasons for the high protein-bound levels of T4 in mammalian blood, and the raised levels of TBG seen in human pregnancy continue to be matters of speculation and controversy. We have attempted to show that detailed analysis of the physicochemical effects of binding proteins on hormone transport kinetics suggests that TBG is capable of selectively transporting T4 to the feto-placental unit, and that this would constitute a biological advantage in conditions of iodine deficiency. If maintenance of an adequate supply of T4 to the fetus
Fig. 4. Calculated free T4 concentration contours across the capillary diameter for two different binding protein (albumin) concentrations, assuming the equilibrium concentration to be the same in each case. An increase in the binding protein concentration results in a higher free hormone concentration at the capillary wall, implying a greater hormone loss rate from the capillary.

were indeed TBG's basic physiological raison d'être, total absence of TBG in males would clearly be of little consequence. Even in the extremely rare cases of total TBG deficiency in females, the high levels of T4 seen in the generally iodine-replete populations of Europe and the US might obviate or obscure any adverse consequences on the offspring that absence of TBG might otherwise cause.

Maternal hypothyroxinemia and fetal brain development

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

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

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

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

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

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

Phosphate and calcium metabolism

A wide range of biochemical and metabolic functions ultimately depend upon the availability of an adequate free phosphate pool (inorganic phosphate). A host of phosphorylated substrates, along with a high energy input, are necessary to sustain the almost exponential increase in nucleic acid and protein synthesis characteristic of explosive phases of neural development. Derangement in phosphate metabolism may therefore critically impinge upon the normal development of the fetal brain. Indeed, we have found the brain inorganic phosphate (Pi) concentration to be drastically reduced at 15 days gestation, but elevated at 19 and 22 days gestation in the Tx dam fetus (1). Similar changes are also observed for lipid phosphate and protein phosphate concentrations, at least at 15 and 19 days gestation (1). Of two protein kinase (PK) activities examined, PKC is elevated (by 25%; $P < 0.05$) at 15 days gestation, diminished (by 25%; $P < 0.05$) at 19 days gestation, but normal at term, whereas PKA is normal at all stages of gestation investigated. The intrauterine TH environment also influences fetal brain phosphohydrolase activities in a selective manner: both Ca$^{2+}$-ATPase and Na$^+$, K$^+$-ATPase are adversely affected at 15, 19 and 22 days gestation, whereas acid and alkaline phosphatases are completely unaffected (1). The activity of brain Ca$^{2+}$-stimulated, calmodulin-dependant phosphatase (calcineurin) is also known to be severely compromised in newborn Tx dam progeny (38). These findings taken with those of others in endemic neurological cretins, showing calcification of the basal ganglia and prominence of the Sylvian fissure due to mild asymmetry of the temporal lobes (13), strongly suggest an important influence of the intrauterine TH environment on phosphate and calcium metabolism in the fetal/neonatal CNS, possibly resulting in gross structural dysgenesis.

Neurotransmitter turnover

Various degrees of neurobehavioural and cognitive dysfunction, such as di­plegia, gait disorders, deaf-mutism, learning disability and severe mental retardation are primary features of neurological and mixed type cretinism (31). A range of behavioural parameters (emotion, motor function, cognition and learning ability) are also compromised in adult prog-
eny of Tx dams (3, 14, 38). Such changes are strongly suggestive of disturbances in neurotransmitter function — indeed, an early study of adult progeny born to Tx dams has demonstrated brain region-specific compromise of monoamine oxidase, acetylcholinesterase and choline acetyltransferase (ChAT) activities (9, 38). We have now investigated in detail the influence of maternal thyroid function on the early development of cholinergic and catecholaminergic metabolic systems. Preliminary results indicate that in Tx dam progeny, ChAT activity is reduced (by 24%; P < 0.02) at 19 days gestation but increased (by 30%; P < 0.05) at 21 days gestation. Although unaffected during the prenatal period, DOPA decarboxylase activity is transiently up-regulated in cerebellum (by 44%; P < 0.02) at 30 postnatal days and brainstem (by 44%; P < 0.02) at 30 postnatal days. The intraterine TH environment therefore appears to exert both short term and long term influences on the development of neurotransmitter systems. Disruption of multiple neurotransmitter systems (epigenetic influence) (12), which catalyses the initial, rate-limiting step in polyamine synthesis, is present in the postnatal period; activity of TH homeostasis results in irreversible neurobehavioural compromise. Restoration of the maternal TH environment during pregnancy by T4 replacement therapy may therefore correct/prevent much of the neurological damage. Indeed, T4 therapy to pregnant women has recently been recommended by the FDA of the United States.

Polymine biosynthesis

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

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

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25. JAHRESTAGUNG
DER ÖSTERREICHISCHEN GESELLSCHAFT FÜR INNERE MEDIZIN,
Salzburg, 15.–17. September 1994


Wissenschaftliches Sekretariat und Organisation: Univ.-Doz. Dr. O. Traindl, Univ.-Klinik für Innere Medizin III – Nephrologie, A-1090 Wien, Währinger Gürtel 18–20, Tel.: (0222) 40 400 – 2195, 2138; Fax: (0222) 40 400 – 2194.
Perturbation of Thyroid Hormone Homeostasis in the Adult and Brain Function


Key-words: Hypothyroidism - hyperthyroidism - brain - neurotransmitters - metabolism - behaviour.

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

In summary, our observations, taken together with those in the literature, demonstrate that thyroid hormones play a direct critical role in a range of biochemical and metabolic functions in the adult CNS. It is postulated that these deficits may be the underlying causes of the psychobehavioural, cognitive and motor disorders that accompany adult-onset dysthyroid states.

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


Zusammenfassend zeigen unsere Beobachtungen in Zusammenhang mit den in der Literatur berichteten, daß die Schilddrüsenhormone eine direkte kritische Rolle in einer Reihe von biochemischen und metabolischen Funktionen des erwachsenen Zentralnervensystems spielen. Es wird daher die Forderung erhoben, daß diese durch Änderungen der Schilddrüsenhomonkonzentrationen ausgelösten Defizite der Zellfunktion die zugrundelie-
Thyroid disorders are amongst the most prevalent pathological conditions in the world. Recent estimates have suggested that upwards of one billion people worldwide live under varying degrees of iodine deficiency and are at risk from the iodine deficiency disorders (IDD). Although mainly prevalent in developing countries, large pockets of iodine deficiency also exist in parts of Europe, including regions of Greece, Spain, Portugal, Italy, Germany and Ireland (25). A recent survey in Munich, Germany suggests that as many as 20% of women of college-going age have at least grade one goitre, and the incidence of cretinism in this area has been shown to be 1 in 4000 of the population. Similarly, in iodine-deficient areas of Sicily, the incidence of goitre may be as high as 44%, with a sizeable proportion of the children suffering some degree of cognitive dysfunction (14%), although overt neurological cretinism is rare (114). In the developing countries, the incidence of endemic iodine deficiency-related goitres is much higher; upwards of 60% of women of child bearing age may have palpable goitres and the incidence of overt neurological cretinism in offspring may approach 5 to 7% of total live births per annum. The diagnostic features of these cretins are diplegia, clonus, strabismus, deaf-mutism and severe mental retardation. Much larger number of children in these endemias also suffer from less overt disease states such as gait disorder, impaired motor coordination, loss of cognitive faculties, diminished IQ, partial deafness, speech defects and compromised school performance.

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

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

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

Thyroid hormone homeostasis

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

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

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

The deiodinases are differentially distributed in the CNS (58) and, although primary cell culture studies indicate cell-specific localization as well, the data are somewhat contradictory. In serum-containing primary cultures, 5'D-I and 5'D-III are predominantly localized in glial cells, and 5'D-II is neuronal (68). This latter observation is consistent with the presence of 5'D-II in neuroblastoma cells (99) and its preferential localization in the nerve terminal plasma membrane fraction of cerebral cortex (69). In serum-free medium however, significant 5'D-II activity has been observed in glial cells (11, 14, 15), whereas 5'D-III may be present in both neurons (15) and glia (11, 19, 36). The discrepancies may in part be related to the composition of the culture medium and/or the differentiation state of the cells. In astroglia, for example, catecholamines, cyclic AMP, phorbol esters, fibroblast growth factors (FGF) and glucocorticoids all serve as inducers of 5'D-II (16, 17, 18, 65, 70), and many of these factors play a similar role in the regulation of 5'D-III (19).
In thyroidectomized rats, thyroid hormones are conserved in the brain, being detectable long after they have disappeared from the serum (83). Thyroidectomy is associated with a stimulation in the fractional rate of T3 formation from T4 in brain, whereas hyperthyroidism results in inhibition (31). Opposite changes occur in the liver, and this coordinated action of liver and brain permits intracerebral thyroid hormone levels to be kept within narrow limits despite wide fluctuations in circulating concentrations (31).

At the enzymic level, chronic hypothyroidism results in up-regulation of brain 5'D-II, less marked down-regulation of brain 5D-III and liver 5'D-I, but no change in brain 5'D-I (57, 58, 60, 62). Analysis of the time course of these changes has revealed a very rapid response of cerebrocortical 5'D-II to changes in thyroid status: a 3-fold increase in activity is observed at 24 h post thyroidectomy, and the administration of T3 to chronically hypothyroid rats normalizes enzyme activity within 4 h (67). In contrast, significant changes in cerebrocortical 5D-III and liver 5'D-I are not found until 5 days post thyroidectomy (67).

The observations that both T4 and rT3 are much more potent than T3 in inhibiting 5'D-II in vivo (56, 106) and in a variety of cell culture models (70, 99, 100), that inhibition occurs independently of transcription and protein synthesis (70, 71), and that the effect is due to increased inactivation of the enzyme rather than decreased synthesis (70, 71), indicate an extranuclear-mediated mechanism of action. These findings further suggest that T4 and rT3, which are thought to be prohormone and inactive metabolite, respectively, play a critical role in thyroid hormone homeostasis in the brain. According to the current model of Leonard and coworkers, T4 and other active analogues promote polymerization of the actin cytoskeleton, which in turn serves to stimulate internalization and inactivation of the enzyme (66). In contrast, analysis of the induction of 5D-III in cultured astrocytes by thyroid hormone analogues is consistent with a multistep, protein synthetic-dependent pathway (36). Whether FGF and/or protein kinase C-mediated phosphorylation play a role in this process is unknown.

### Thyroid hormone receptor systems

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

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

The anatomical distribution of the various transcripts within the adult brain has been studied by several groups (9, 10, 13, 78). These studies have demonstrated widespread distribution of the c-erbA α1 transcript, with the c-erbA β1 isoform exhibiting a more restricted pattern. Nevertheless, considerable overlap exists between these two functional isoforms. In contrast the c-erbB β2 transcript is largely, but not exclusively, localized to the pituitary. With respect to the transcripts encoding nonbinding variants, Rev-ErbAα is characterized by a unique and somewhat restricted distribution, whereas both c-erbBα2 and α3 exhibit almost identical patterns to that of c-erbAα1. Of the 3 c-erbBα-derived transcripts, α2 is the most and α3 is the least abundant species. In view of the observation that the TR-α2 protein can inhibit T3-induced changes in gene expression mediated by the TR-α1 and -β1 proteins (63), at least in transient transfection studies, it has been suggested that the supposed nonresponsiveness of the adult brain may be related to the very high levels of the α2 transcript (13).

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

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

Hypothyroidism, as a consequence of thyroidectomy or propylthiouracil (PTU) treatment, results in a significant increase in nuclear T3 binding capacity (28, 47, 112) and a decrease in the affinity constant (28, 47). Induction of hyperthyroidism by T3 administration has also been reported to result in a slight increase in receptor number, with no effect on the affinity constant (47). The up-regulation of the T3 receptor in hypothyroidism also bears a temporal relationship to the expression of the protooncogenes c-erbA α and c-erbBβ in brain regions. For example, preliminary experiments indicate slight increases in the levels of c-erbAα1 and β1 transcripts, at least in cerebellum and brainstem (but not cerebral cortex), during hypothyroidism (Fig. 1) (62). On the other hand, c-erbBα2 mRNA is unchanged in all brain regions (Fig. 1) (62), indicating perhaps that the thyroid hormones may play a role in the regulation of splicing of specific primary transcripts, including those encoding the TR. In contrast, other workers have reported that levels of brain c-erbAα1, α2 (80) and Rev-ErbAα (55) mRNAs are unaffected by thyroid status, although their data may indicate an increase in the latter transcript during hypothyroidism (55). Interestingly, the intracellular thyroid hormone environment also appears to impinge upon the expres-
Adult-onset dysthyroid states and the brain

a) Morphology

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

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

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

b) General biochemistry

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

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

Studies of lysosomal enzymes indicate that thyroid hormone action is both parameter-selective and region-specific in the adult brain. Thus asial sulphatase A activity is reduced in cerebellum, and acid phosphatase activity is deficient in cerebellum and medulla of hypothyroid rats, whereas the activities of aryl sulphatase B and a range of glycosidases are normal (1). Furthermore, non-lysosomal alkaline phosphatase is deficient in sub-
cortex and midbrain (1), although other phosphohydrolases (Na,K- and Mg2+-ATPases) remain unaffected (unpublished observations). Despite the preferential localization of both aryl sulphatase A and acid phosphatase in neurons (109), general loss/compromise of this cell population is unlikely, since other lysosomal neuronal marker enzymes (β-D-glucosidase, N-acetyl-β-D-glucosaminidase and especially β-D-galactosidase) (109) are unchanged (1). The normality of N-acetyl-β-D-galactosaminidase activity (1) also argues against any gross effects on glia (110). Indeed, similar conclusions can be drawn from the normal expression of cell marker genes in the adult hypothyroid brain (50 to 60% reduction and restricted to only cerebellum (1)). Nevertheless, other workers have reported region-specific changes in myelin-associated marker enzymes; 2',3'cyclic nucleotide phosphodiesterase being reduced in whole forebrain and myelin-associated 5'-nucleotidase being increased in medulla (76), albeit proteolipid protein mRNA levels remain normal (54).

c) Metabolism

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

d) Neurotransmitter function

Chronic and acute hypo- and hyperthyroidism are associated with a spectrum of psychobehavioural disorders which are indicative of derangements in neurotransmitter metabolism/function. Indeed, it is generally accepted that hyperthyroidism is associated with an increase and hypothryoidism with a decrease in the catecholamine and indoleamine turnover rates in whole brain (reviewed in [92]), although contradictory evidence has been presented concerning the influence of thyroid status on regional levels of particular monoamines, as well as their synthetic rates (49, 92). For example, hypothyroidism has been reported to increase the rate of dopamine synthesis in midbrain, brainstem and cerebrum, with hyperthyroidism producing a similar effect in midbrain. The rate of noradrenaline synthesis has been found by some workers to be independent of thyroid status, at least in brainstem, midbrain, striatum and cerebrum, whereas others have reported changes in discrete regions of hypothalamus (49, 92). Chronic T3 treatment however, produces an increase in the bulk phase serotonin synthesis rate, independent of changes in brain tryptophan accumulation (3).

The influence of thyroid hormone on the metabolism of a variety of substrates in confluent neuronal and astrocytic cells in culture has been well documented. Thus, neuronal uptake of 2-deoxyglucose has been reported to be enhanced by T3 in a dose-dependent manner (Fig. 2) (43), whereas astrocytic uptake is independent of the thyroid hormones but regulated by insulin (unpublished data). Similarly, leucine uptake and incorporation into protein has been shown to be controlled by T3 in neurons (90), as is the uptake and incorporation of fucose into glycoprotein in astrocytes but not neurons (unpublished observations and [53]).

In general, supraphysiological amounts of T3 are required to elicit the enhancement of cellular metabolism. It may be noted that the cells used in these experiments were confluent and fully differentiated, analogous to, it is assumed, mature cells in vivo. If such observations can be extrapolated to the in vivo situation, it would appear that thyroid state influences substrate metabolism in a cell-specific manner, consequently bulk-phase metabolism may appear unaffected. The thyroid hormone dependence of membrane-related processes such as glucose and amino acid uptake may indicate a role for the membrane T3 binding sites in this process. The failure of T3, even at supraphysiological doses, to elicit such effects in synaptosomal particles prepared from adult euthyroid rats (90, 91) would tend to rule out this possibility, although adult-onset hypothyroidism appears to induce T3-responsiveness in vitro (91).
are influenced by T3 treatment (101).

With respect to the dopaminergic system, the levels of D2 receptors are decreased in striatum as a result of hypothyroidism (22), although they are apparently unaffected during hyperthyroidism (3).

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

Opiate receptor binding is also known to be dependent upon thyroid status. The number of naloxone binding sites is increased in mice made hyperthyroid by T4 treatment (33). Conversely, Lofentanil (a morphine agonist) binding sites are lacking on the synthetic enzyme, choline acetyltransferase, and cholinergic receptor systems. Compromise of the cholinergic system is feasible in view of the observation that the number of dendritic spines on the cerebral pyramidal neurons are reduced, since these cells are normally connected by afferent cholinergic fibres from the basal nuclear system.

PKA activity is elevated in all regions studied (Table 1).

Table 1. Specific activities of PKA and PKC in different brain regions from euthyroid (N) and hypothyroid (Tx) adult rats.

<table>
<thead>
<tr>
<th>Brain region</th>
<th>PKA</th>
<th>PKC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cerebral cortex</td>
<td>0.638 ± 0.016</td>
<td>0.360 ± 0.015*</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>0.552 ± 0.011</td>
<td>0.245 ± 0.013</td>
</tr>
<tr>
<td>Brain stem</td>
<td>0.492 ± 0.010</td>
<td>0.234 ± 0.006</td>
</tr>
</tbody>
</table>

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

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

e) Gene expression

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

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

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

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

f) Psychobehavioural function

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

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

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

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

Conclusion

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

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

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Thyroid hormone action in rat brain from fetal to adult life

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I. INTRODUCTION
A role for thyroid hormone [TH] in brain development is well established based on investigation of a range of dysthyroid animal models, in particular the rat. As in man, secretion of TH in the rat commences during fetal life, when the major phase of neuroblast proliferation is complete. However the rat brain is relatively immature at birth, corresponding to the second trimester human brain. Most past studies have concentrated on hypothyroidism induced during late fetal/early neonatal life [congenital hypothyroidism models], and this has been shown to adversely affect most stages of postnatal brain development, as evidenced by molecular biological, biochemical, histological and behavioural changes [reviewed in (1-6)]. Many of the effects are irreversible if TH replacement therapy is delayed beyond 20-25 days of postnatal life. Few studies have investigated TH action in the fetal brain, largely because of an assumption of placental impermeability to maternal thyroxine [T4] and 3,5,3'-triiodothyronine [T3]. Considerable recent evidence - again derived mainly from the rat - contradicts this, and indicates a role for maternal T4 in fetal neurogenesis. Similarly, an increasing number of reports, demonstrating largely reversible effects of thyroid status on adult brain, conflict with the view that once fully mature, the central nervous system [CNS] ceases to be TH-responsive. The supposition of a critical period of brain dependency on TH limited to the first two to three weeks of postnatal life is therefore highly questionable.

II. THYROID HORMONE HOMEOSTASIS
It is now generally accepted that both maternal T4 and T3 are transferred to the rat fetus (7-9), maternal T4 being quantitatively more important than T3 (7,10). Transfer of maternal T4 is most active in early pregnancy [detectable as early as 9/10 days gestation] so that, before the onset of fetal TH synthesis [17.5 days gestation], circulating TH is derived solely from the mother. Maternal TH transfer declines as the onset of fetal TH synthesis approaches (7,8); nonetheless maternal T4 still accounts for some 18% of the fetal extrathyroidal T4 pool near term (11). The postnatal animal is almost entirely dependent on its own thyroid activity, albeit a small proportion of circulating TH may be derived from the mother's milk during the suckling period (12).

Thyroid hormones are present within the fetal brain from at least 13 days gestation (9); they are detectable in other tissues but comparison of fetal to adult tissue ratios indicates that the brain is especially favoured (9,13). Various studies indicate that throughout development, most brain T3 is generated via local deiodination of T4 rather than from direct uptake of circulating T3 [reviewed in (12,14)]. Brain 5'-deiodinase [predominantly type II; 5'D-II] activity has been studied from 17 days gestation and exhibits a 6-fold increase by term (15,16). After birth, 5'D-II activity declines to low levels by 4 to 5 postnatal days, then increases again reaching maximal levels by day 12, these being slightly higher than those at term. Activity then gradually declines to low adult values (16,17). Since 5'D-II predominantly converts T4 to T3, it is not surprising that brain T3 levels also rise during early development (15,16). Indeed, T3 levels mature much earlier in brain when compared with many other tissues, in particular the blood (15,18).

The fetal brain also exhibits 5-deiodinase [5'D-III] activity at 3 to 6-fold higher levels than adult brain (19). As in the adult (20), early fetal brain reverse T3 concentrations are low relative to T4 and T3 (9). The activity of 5'D-III increases 1.5-fold between 14 and 19 days gestation (19), with a more pronounced increase during the first few days of postnatal life. Activity then declines to adult values by 20 postnatal days (17).

From as early as 17 days gestation [probably earlier (9)] the brain is able to respond to reduced circulating T4 levels to maintain tissue T3 concentrations (12). Putative homeostatic mechanisms include increased T4 uptake (12), down-regulation of 5'D-II activity (21,22) and a rapid increase in 5'D-II activity (15,16). Such observations support an important role for TH in brain throughout life.

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The primary mechanism of TH action is thought to be the modulation of target gene transcription mediated by specific nuclear T3 receptors (TRs) (23-25). The nuclear TRs belong to a superfamily of ligand-activated transcription factors which includes receptors for steroid hormones and retinoids (23-25). Multiple TR isoforms exist, encoded by the c-erbAα and c-erbAβ genes. Alternative splicing of the 3′-most exon of the c-erbAα gene, coupled with the usage of alternative splice acceptors, results in the production of mRNAs encoding three proteins: TRα1, TRα2, and TRα3. Only TRα1 binds TH and can be considered a true TR. The erbAα gene, encoding three proteins: TRα1, TRα2, and TRα3, both of which bind TH. Studies of mRNA, protein and nuclear T3 binding activity indicate that all TR isoforms are expressed in rat brain and are subject to developmental regulation.

### a. Nuclear T3 binding studies

Nuclear T3 binding has been studied in some detail by saturation techniques which, although limited by their ability to discriminate between various T3 binding receptor isoforms, have yielded important insights into TH action in the brain.

**Saturable T3 binding** has been detected in nuclear extracts of whole brain at 14 days gestation [earliest stage investigated] (26). Maximal binding capacity [MBC] increases three-fold by 16 days gestation, then remains stable throughout the remainder of fetal life. A further three-fold elevation occurs in the early postnatal period, maximal levels being attained by 5 - 6 postnatal days. This phase is followed by a steady [but slight] decline to the 20 - 30 postnatal day level. Similar changes in MBC in salt-extracted or intact nuclei from postnatal cerebral cortex have been reported (27-29), while in the cerebellum, receptor ontogeny appeared delayed, as expected, and receptor levels were lower than in cerebral cortex at all ages (27).

Several authors have reported developmental changes in the affinity constant [Kd] of T3 binding in cerebral cortex (28,29), but only when assayed in intact nuclei (30). Reconstitution experiments with salt-extracted nuclei have implicated chromatin-associated factors in this regulation (30), however their identity remains to be determined.

### b. Gene expression of T3 receptor isoforms

Strait et al. have determined molar concentrations of various TR isoform mRNAs by quantitative Northern hybridisation analysis (39). During brain development, both c-erbAα1 and -α2 were found to be coordinately expressed, with the latter species predominating (10-fold higher mRNA levels). Concentrations doubled between 19 days gestation and birth, with a further doubling by 4 postnatal days [maximum levels]. After day 10, a decline was observed, with adult values only slightly higher than those at 19 days gestation. More dramatic changes were observed for c-erbAβ3, mRNA levels were very low [<10% c-erbAα1] at 19 days gestation, but increased 40-fold to reach those of the adult by 10 postnatal days. This isoform exceeded c-erbAα1, from around 15 postnatal days onward, being 3 to 4 times higher at adulthood. Similar findings with respect to c-erbAα1, and -β3 mRNAs have been reported by other workers (40-42), although there is some disagreement regarding the ontogeny of c-erbAα2, with evidence both for (41) and against (40,42) co-ordinate expression with c-erbAα1, Rev-ErbAα mRNA is not detectable in brain until 15 postnatal days, with an increased level apparent at 60 days (42).

The ontogenic pattern of brain nuclear T3 binding capacity is not related to combined c-erbAα1 and -β3 mRNA molar levels [although some correlation with c-erbAα, mRNA alone is apparent] (39). These observations, coupled with evidence from other tissues, led to the suggestion that the various TR isoforms may be subject to differential translational/post-translational control (39). Indeed, this is supported by other evidence (section IIIc) however the existence of extranuclear TR-related proteins may also need to be taken into account.
The regional distribution of TR isom form mRNAs has also been examined in both neonatal and adult rat brain by quantitative Northern analysis (39,43). Levels of c-erbAa mRNAs vary ca. 3-fold between regions [highest in frontal cortex, lowest in diencephalon and brain stem] irrespective of age, with adult levels around half those in neonates. In whole brain, c-erbBaa mRNA concentrations were higher [5-fold] than those of c-erbBaa. More marked regional variation was noted for c-erbBbb, mRNA; the frontal cortex contained >50-fold higher levels than the cerebellum, irrespective of age. Adult c-erbBbb, mRNA levels were 4-fold higher than those of neonates in all brain regions, and exceeded those of c-erbBaa, in frontal cortex and cortex (43).

Others have also noted very low levels of c-erbBbb, mRNA in cerebellum relative to cerebrum (44). Although early studies indicated that c-erbBaa, mRNA was highly restricted to pituitary [detectable by Northern hybridisation] (45) and hypothalamus [detectable by RT-PCR] (46) in adult brain, more sensitive approaches [RT-PCR combined with Southern hybridisation] have demonstrated this species in cerebrum and possibly cerebellum (47,48).

Use of in situ hybridisation has allowed more detailed examination of the spatial expression of the c-erbB mRNAs (49,50). Both c-erbBaa, and -a, transcripts are detectable in the neural tube at 11.5 days gestation [earliest age studied], and all five major subdivisions of the brain [especially the telencephalon] at 12.5 days gestation (50). c-erbBbb, mRNA is also detectable at this latter age, but only within ventral areas of the hind brain and diencephalon (50). At subsequent developmental stages, including adulthood, both c-erbBaa, and -a, mRNAs are widely expressed [levels of the latter are generally highest], whereas c-erbBbb, mRNA is restricted to areas of c-erbBaa expression (49,50). The expression of c-erbBbb, is highly localised to pituitary [first detectable at 13.5 days gestation], with low levels also present in the late fetal striatum and hippocampus, and the postnatal neocortex, where it is limited to sub domains of c-erbBbb, expression (50).

During the remainder of fetal life, high levels of c-erbBaa, transcripts were seen in the olfactory bulb, piriform cortex, thalamus, caudate putamen, inferior colliculus, spinal cord, cerebellum, hippocampus and neocortex. Especially intense labelling of postmitotic neuronal cell layers in the latter three regions was noted. In general, the expression of c-erbBaa, [and -a] transcripts reaches maximal levels by the end of the first postnatal week, then declines by adulthood, with prominent labelling of the olfactory bulb, caudate putamen, hippocampus [dentate gyrus granule cells, CA1 and CA3 pyramidal cells], neocortex [main layers 2, 3, 5 and 6] and cerebellum [Purkinje cells, internal granular layer and deep cerebellar neurons].

Expression of c-erbBbb, transcripts is very limited during fetal life, moderate to high levels being seen only in caudate putamen [limited to rostral regions] and hippocampus [CA1 field]. However, levels increase markedly during the first postnatal week and peak one to two weeks later than c-erbBaa, (49,50). During this time labelling of the olfactory bulb, caudate putamen [rostral regions], neocortex [mid cortical layers] and hippocampus [pyramidal cell layer CA1] becomes particularly intense.

c) Expression of T3 receptor protein isoforms

The expression of TR proteins in brain has not been extensively documented. Falcone et al. have performed an immunohistochemical study using polyclonal antibodies specific for TRβ, and TRα (51). TRα, was inferred from differences in the labelling pattern between the latter antibody and a TRα common antisera. Several differences were apparent compared to studies of TR transcripts: TRα, appeared to be the predominant TRα isoform from 14 - 17 days gestation; TRβ, was detectable at low to moderate levels at 14 days gestation, up-regulated between 17 and 21 days gestation, but remained fairly stable through the first 3 weeks of postnatal life; the temporal pattern of TRα, expression was similar to that of TRβ, rather than TRα,.

TR isoform expression in adult cerebellum has also been investigated (43,44). Puymirat et al. reported that TRβ, was localised to Purkinje cells only, immunostaining being apparent in either the nucleus or the perinuclear region and cytoplasm (44). Strait et al. observed nuclear staining only with antisera specific for TRβ, and TRα, - the former isoform was localised to Purkinje cells [strongly stained] and granule cells [weakly stained], whereas the latter isoform was mainly localised to granule cells (43). Immunoprecipitation studies have demonstrated that TRβ, accounts for some 30% of nuclear T3 binding activity in this brain region (43), despite low mRNA levels - in adult brain, the TRβ, protein:mRNA molar ratio was calculated to be 7-fold higher in cerebellum than in cerebrum (43).

Immunocytochemical studies have demonstrated TRβ, expression in the adult pituitary, hypothalamus, piriform cortex, neocortex [especially layers 3, 5 and 6], amygdala, thalamus, hippocampus, parts of the septum and the cerebellum [especially Purkinje cells] (47). Although predominantly nuclear in localisation, cytoplasmic staining was also apparent in Purkinje cells and certain neocortical pyramidal neurons. Saturation techniques following immunoprecipitation indicated that TRβ, accounts for ca. 10% of nuclear T3 binding capacity in adult brain; the remainder being due to TRα, [61%] and TRβ, [29%] (48). In fetal brain however, only TRα, specific antisera was able to
An earlier study indicated that TRβ may contribute some decrease [by 90%] nuclear T3 binding activity (48); an earlier study indicated that TRβ, may contribute some 5% of nuclear T3 binding at 19 days gestation (52).

Anomalous findings with respect to glial cells, deriving from immunocytochemical studies, have been reported (53,54). Carlson et al., using TR isoform-specific antibodies, observed TRα, -α, -β, and -β immunoactivities in oligodendrocytes in adult cerebellum and cerebral cortex (53) and found that the latter showed no binding of all isoforms (54). Besnard et al. employed a monoclonal antibody immunoreactive for TRβ, -α, and -α isoforms, and observed labelling of both astrocytes [not noted above] and selective oligodendrocytic subtypes [medium oligodendrocytes] in corpus callosum of developing and adult rats (53). Oligodendroglial staining was not observed until 20 postnatal days, following which the proportion of labelled cells declined (53). TR expression in oligodendrocytes in culture is also related to morphological maturity - progenitor cells express only TRα isoforms, whereas mature oligodendrocytes express both TRα and TRβ (53,55).

Astrocytes in culture contain significant nuclear T3 binding capacity (36,57,58), as well as c-erbα, -α, and -β mRNAs (56). TRβ, protein has also been detected in astrocytes in culture [predominantly type 1 fibrous astrocytes] but immunoreactivity was localised to perinuclear and cytoplasmic regions (56). Leonard and coworkers have demonstrated the presence of c-erbβ, and -α [but not -β] mRNA in these cell types, as well as TRα, [but not TRβ,] immunoreactivity (57), whereas Carlson and colleagues could only detect TRβ, mRNA and protein in neuronal nuclear localisation (58). Type 2 astrocytes [found only in culture and not in vivo] were also examined and were shown to express mRNA and protein of all isoforms (58). The discrepancies with respect to astrocytes both in vitro and in vivo remain unresolved.

d) Possible roles for nuclear TR isoforms

The respective roles of the TRs and their non-T3 binding-related isoforms remain uncertain. With respect to TRs, the differential spatial and temporal expression in brain suggests quite distinct functions. From the foregoing it is clear that TRα predominates early in development, when cell proliferation is active, whereas TRβ, [and possibly TRβ,] is up-regulated concomitant with terminal differentiation. Similar relationships also hold in nervous tissue (59), oligodendroglial (55,60) and astrocytic (56) cultures. A role for TRα, in blast cell proliferation is consistent with studies in chick optic lobe which demonstrate that antisense oligonucleotides against c-erbβ block neuroblast proliferation whereas those against c-erbβ are ineffective (61). The TRα, protein may also be important in initial stages of cell differentiation since observations of developing neocortex, cerebellum and hippocampus demonstrate marked enhancement of c-erbβ, mRNA expression once cell migration is complete (50), notwithstanding the problems inherent in extrapolation from c-erbβ mRNA to protein.

Relevant to this suggestion are observations in rat PC12 pheochromocytoma cells and E18 immortalised brain neuroblasts stably expressing the chick c-erbα, gene, demonstrating modulation of neurotrophin-dependent neuronal differentiation (62). Indeed, mRNA levels of four TH-responsive genes [calbindin, inositol triphosphate receptor, PCP-2, myelin basic protein] are markedly up-regulated in the early postnatal cerebellum coincident with increases in TRα, and tissue T3 (64). In hypothaloid pups, these mRNA species exhibit slower developmental increases than in T3-treated animals. Transient cotransfection studies suggested T3-independent regulation of the PCP-2 gene promoter by TRβ, alone (64), however these events were later attributed to the parent vector (65). Although primary cell culture studies also support an important role for TRβ, in terminal differentiation of neuronal progenitors; as well as the usual difficulties in resolving this issue.

With respect to the non-T3 binding isoforms, it has been suggested - on the basis of transient transfection studies - that these may serve as dominant negative regulators of TRα, and TRβ, function, via competition at the level of the TH response element (66). In brain, TRα appears to be ubiquitously expressed at high levels throughout development, and is likewise found at high levels in TH-responsive cell cultures (59). It is uncertain, however, whether TRα, is present in the same cells as T3-binding isoforms in vivo. Recent evidence suggests that the phosphorylation state of TRs is important; phosphorylation attenuates the DNA binding activity of TRα, (67), but enhances T3-dependent transcriptional activation by TRα, and TRβ, (68). Although intriguing, the physiological relevance...
of TR phosphorylation remains to be demonstrated. The role of the Rev-ErbAα protein remains highly speculative. It has been suggested that Rev-ErbAα mRNA may regulate alternative splicing of c-erbAα gene transcripts (69), yet spatial expression of Rev-ErbAα mRNA in adult brain differs from that of c-erbAα transcripts (70).

IV EXTRANUCLEAR BINDING SITES

The brain, like many other TH-responsive tissues, possesses a range of extranuclear iodothyronine binding sites, but these have not been as intensively studied as the nuclear receptors. Consequently, their contribution to TH action remains poorly defined.

a) Synaptic membrane binding sites

Synaptosomes possess two classes of T3 binding sites, characterised by high affinity \([3 \times 10^{-9} \text{M}^{-1}]\) and low capacity, and low affinity \([4 \times 10^{-9} \text{M}^{-1}]\) and high capacity (71,72). Fractionation studies indicate a synaptic membrane localisation for the higher affinity site (72). Levels of both sites show regional variation and are developmentally regulated; cerebro-cortical levels increasing in parallel with synaptogenesis.

Radiotracer studies have demonstrated the preferential accumulation of T3 within synaptosomes as a consequence of direct uptake of extracellular T3 by dendrites and nerve terminals, and axonal transport of cell body T3 (reviewed in (73)). The T3 binding sites may serve as binding proteins to maintain a high synapticosomal:cortical T3 ratio (73) or as membrane transporters (74).

Membrane T3 binding sites in non-neural tissues have been implicated in extranuclear-mediated effects of TH on sugar and amino acid uptake; these effects are modulated via interaction with adrenergic agents (75).

The existence of similar mechanisms in brain has not been thoroughly investigated. Leucine uptake in synaptosomes from euthyroid adult rats is not subject to direct regulation by T3 (76), whereas uptake of o-aminoisobutyric acid and 2-deoxyglucose into nerve endings of hypothyroid (but not euthyroid) mouse cerebro-cortical slices is T3-regulated (77). Acute stimulation of 2-deoxyglucose uptake into adult rat synaptosomes exhibits similar thyroid status-dependency (78). T3 enhances the fast phase of depolarisation-induced Ca\(^{2+}\) uptake and Ca\(^{2+}\)-dependent GABA release in synaptosomes from euthyroid adult rats (79,80), synaptic T3 itself being subject to depolarisation-dependent release by a Ca\(^{2+}\)-dependent process (81). These observations, coupled with the existence of discrete synaptic T3 processing systems, are consistent with a direct role for T3 in neurotransmission (73,79-81). By analogy with the catecholamines, Dratman and colleagues further speculate that synaptic T3 may also act as a trophic factor, regulating late stages of neuronal differentiation and plasticity in adult brain (73). Further investigation of the roles of synaptic T3 and its receptors are therefore warranted.

b) Cytosolic binding sites

Early studies described the existence of a single class of low affinity cytosolic T3 (82,83) and T4 (84) binding sites in postnatal brain which were expressed in a region-specific manner and developmentally regulated (Table 1) (82-84). Discrepant findings with respect to the ontogeny of these sites may be related to the different methodologies employed for separation of bound and free hormone. Using a dextran-coated charcoal method, cytosolic T3 binding in developing brain was characterised by a fairly stable MBC but variable Ka - this declined during cerebellar development (82) but transiently peaked between 12 and 15 postnatal days in cerebrum (83). However, other studies of T4 binding demonstrated that dextran-coated charcoal removed a significant fraction of bound tracer (84). Using a Dowex resin separation procedure, an ontogenic decline in the MBC of T4 binding [undetectable in cerebellum by 30 postnatal days] was observed, whereas Ka remained unchanged (84).

Examination of iodothyronine binding in cytosol prepared from primary neural cell cultures has revealed the existence of two classes of binding site for both T4 and T3 (Table 1) (85,86). In the case of T3 binding, MBCs were highest in neuron-enriched cultures and declined concomitant with glial proliferation and differentiation (Table 1). Binding activity was very low in cytosol of glial cell subcultures (86). In neuron-enriched cultures, the two classes of binding site appear to bind both T4 and T3, with T3 being the preferred ligand (85).

In more recent years, a high affinity [ca. 10^{-9} \text{M}^{-1}], 58 kDa cytosolic T3 binding protein (CTBP) has been purified from adult rat cerebral cortex (87). This protein exhibits many similar properties to an extensively characterised 38 kDa kidney CTBP (88-90). Pretreatment of the brain CTBP with charcoal attenuates T3 binding, reactivation occurring upon the addition of NADP, NADPH plus dithiothreitol, or thioredoxin plus diithiothreitol (87). Maximal activation is observed with 25 nM NADPH or 10 - 50 nM NADP [higher concentrations of the latter are inhibitory] and is associated with changes in both MBC and Ka.

The ontogeny of such CTBPs has been examined in a variety of tissues (91). Saturation of T3 binding sites was conducted with cytosolic extracts which had been preincubated with charcoal, then maximally activated with NADPH. T3 binding activity was detectable during fetal life at the earliest age point studied [17 days gestation] in brain alone, though activity appeared in other tissues perinatally. The MBC peaked around the time of birth in cerebrum and cerebellum and declined
TABLE I. Cytosolic thyroid hormone binding sites in developing rat brain and primary cell cultures.

<table>
<thead>
<tr>
<th>Age</th>
<th>$K_a$ ($M^{-1}$)</th>
<th>MBC (pmol/mg prot)</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Brain region</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cerebellum</td>
<td>10 pnd - adult</td>
<td>0.07 - 0.64 x 10^4</td>
<td>0.79 - 0.87</td>
</tr>
<tr>
<td>Cerebrum</td>
<td>3 pnd - adult</td>
<td>0.58 - 2.94 x 10^4</td>
<td>1.97 - 2.74</td>
</tr>
<tr>
<td>B. Mixed cell culture</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neuron-enriched</td>
<td>7 div</td>
<td>i. 1.2 - 3.7 x 10^6</td>
<td>i. 1.8 - 3.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ii. 0.8 - 1.4 x 10^6</td>
<td>ii. 10 - 15</td>
</tr>
<tr>
<td>Glial-enriched</td>
<td>14 div</td>
<td>i. 1.7 x 10^6</td>
<td>i. 0.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ii. 0.7 x 10^6</td>
<td>ii. 3.6</td>
</tr>
</tbody>
</table>

Affinity constant ($K_a$) and maximal binding capacity (MBC) were determined by Scatchard analysis. Ages are postnatal days (pnd) for brain regions or days in vitro (div) for mixed cell cultures. The latter were derived from fetal cerebral hemispheres at 16 - 17 days gestation.

concomitant with the main phase of glial proliferation. A further increase was apparent in the late postnatal period [4 to 6 weeks] in cerebrum alone. Cerebellar extracts exhibited lower MBC than cerebral cytosol at all stages, whereas $K_a$ was similar [ca. 1.1 x 10^4 M^-1] throughout development.

The role of the 58 kDa brain CTBP remains to be explored in detail. In vitro studies of the kidney protein indicate that although the NADP- and NADPH-activated forms are readily interconvertible, they may possess distinct functions (90,92). The NADP-activated form may enhance T3 delivery to the nucleus, whereas the NADPH-activated form appears to inhibit this process, via retention of T3 within the cytosol (92), and may even facilitate T3 delivery to the mitochondrion (90). The physiological relevance of this mechanism in regard to the brain has, however, been questioned (87).

Although not studied in brain, two other cytosolic 58 kDa proteins - pyruvate kinase subunits types M1 and M2 - exhibit T3 binding with similar affinities [ca. 10^4 M^-1] to the low affinity class of brain cytosolic T3 binding sites (93,94). Monomers, and not tetramers, possess T3 binding activity. Transient transfection studies indicate that such proteins may serve to integrate cellular metabolism with nuclear activity: promotion of tetramer to monomer conversion of endogenous subtype M2 by glucose starvation inhibits T3-dependent transcriptional activity of transfected human TRβ, (95). Additional CTBPs, related to pyruvate kinase subtype R, have been reported in human erythrocytes (96). Tetrameric forms bind T3, with high affinity [10^8 M^-1], but binding is cold-sensitive. These observations are of interest since brain cytosol is usually prepared at 4 °C, and T3 binding assays are routinely conducted at 0 °C.

c). Mitochondrial binding sites

The presence of a high affinity [$K_a$: 2 x 10^6 M^-1], 28 kDa, T3 binding protein has been demonstrated in the inner mitochondrial membrane in various rat tissues (97-99). Although not extensively characterised, similar sites were found in rat brain, but only up to 12 days of postnatal life (97,98). A role for the protein in mediating direct TH action on mitochondria has been postulated (97,98), as evidenced by rapid, protein synthetic-independent effects of T3 on oxidative phosphorylation in dispersed liver cells and mitochondrial vesicles from hypothyroid rats (97,100). Nevertheless the existence of mitochondrial T3 binding sites, their identity, and the direct effects of T3 on mitochondria, remain highly controversial [reviewed in (75,101,102)], and their physiological relevance in brain, in particular, remains unexplored.

Using immunochemical approaches, two groups have recently demonstrated the presence of nuclear TR-
related proteins in rat liver mitochondria (103,104). Somewhat discrepant results were obtained, however, which may in part be related to the different antibodies employed. Ardail et al. reported the presence of TRβ and TRβ-related proteins [48 and 55 kDa, respectively] (103), whereas Wurtaniak et al. detected a TRβ-related protein [43 kDa species] but no TRβ-related species (104). The TRβ-related protein could not be detected in mitochondria from adult brain, but no data were presented with respect to developing brain (104). Further characterisation of the protein revealed that it bound to natural and synthetic TH response elements, and TRβ-related proteins [48 and 55 kDa, respectively] as well as a sequence within the mitochondrial promoter, indicating a potential role in the regulation of expression of the mitochondrial genome. It is of interest, therefore, that the expression of mitochondrial-encoded genes in brain has been shown to be influenced by thyroid status (105,106), as discussed in detail below [see sections VII and VIII].

V. MATERNAL TH AND BRAIN DEVELOPMENT

Significant transfer of maternal T4 to the fetus well before the onset of TH synthesis, its accumulation and metabolism within the fetal brain, coupled with the expression of boma-fide TRs [mainly TRβ], suggest that early neurogenesis is regulated by maternal thyroid status. In order to examine the role of maternal TH in brain development, various thyroidectomised rat-dam models have been developed [see (107-110)]. These differ with respect to the method of thyroidectomy [surgical, radiiodine ablation, or a combination of these] and the degree of thyroid hypofunction. In our model, dams are partially thyroidectomised (Tx) by surgical means [parathyroid-spared] and time-mated with normal males when circulating total T4 levels are ≤15 nM. At 15 days gestation, maternal T4 levels are typically reduced to around 30% of the euthyroid control dam values, with T3 levels reduced by some 50% [e.g. see (109)]. Similar reductions in maternal serum TH levels, as a consequence of radiothyroidectomy, depress fetal T4 and T3 levels by only 50% (9). This is in contrast to congenital and adult-onset hypothyroidism models which are generally characterised by severe brain hypothyroidism. We have avoided studying severely hypothyroid rat dams, for several reasons: they exhibit decreased fertility, often appear unwell near term, and may experience difficulty in giving birth. The latter two effects would render studies of late fetal and postnatal progeny largely meaningless.

a) Adult progeny

Our early work concentrated on 7-month-old adult progeny of Tx dams [i.e. end point of development] and demonstrated brain regional compromise of neural cell-specific and general metabolic parameters [Table 2]. Affected lysosomal enzyme activities included several with preferential neuronal localisation, especially β-galactosidase and aroy sulphatase (114). Discordant changes were noted in more selective neuronal markers, suggesting that maternal TH influences the development of specific neuronal populations. For example, the activity of the cholinergic neuronal marker, choline acetyltransferase, was affected in the subcortex alone (Table 2), whereas GABAergic and glutamatergic neuronal markers were unaffected (115, unpublished observations). Gial markers such as N-acetylgalactosaminidase [general marker (116)] and glutamine synthetase [astrocytic marker] are unchanged (111,115). However enzymes with exclusive [CNPass] or preferential [oleate esterase] oligodendroglial localisation are affected [Table 2]. Such effects are consistent with the identification of TRs in neurons and oligodendroglial progenitor cells [see section III].

β-Galactosidase and aroy sulphatase, as well as other affected enzymes [Table 2], participate in myelin turnover. Deficits in galactolipids further indicate compromised myelination in adult-Tx dam progeny. Whether maternal TH regulates oligodendrocytic progenitor cells, or the effects occur secondary to neuronal changes, is unknown. The fact that most affected parameters are normal in the cerebellum [Table 2] is consistent with the existence of a TH-requiring window early in uterine life.

Behavioural studies indicate impairment of brain function in adult experimental progeny at 2 - 3 months. Such animals take longer to emerge from a box placed in an open field and exhibit reduced activity within the open field (117). Both males and females rear less frequently than controls, while female progeny alone demonstrate decreased baseline locomotor activity. When a novel object is introduced into the open field, however, only male Tx dam progeny exhibit deficient locomotor behaviour.

In contrast, Hendrich et al. have reported that male and female adult progeny of radiothyroidectomised dams exhibit increased spontaneous activity, as measured by placing cages on stabilimeters (118). These workers also observed that adult progeny displayed deficient learning behaviour in a Lashley alley maze (118). Indeed, similar conclusions can be drawn from our animal model; progeny require three to four times the number of trials compared with control progeny to reach 50% criteria when tested in a food reward paradigm [Skinner box] (113).

b) Developing progeny

In order to further examine the existence of a TH-requiring window in early development, our recent work has focussed on fetuses [both before and after the onset of TH synthesis] and early postnatal rats. Aspects of this work are discussed below, together with the findings of other groups, where pertinent. Finally possible limitations of the animal models are addressed.

We have observed significant depression [ca. 20%] in
TABLE 2. Biochemical changes in brain regions of adult (7-month-old) progeny of hypothyroxinemic rat dams.

<table>
<thead>
<tr>
<th>Brain region</th>
<th>CB</th>
<th>MD</th>
<th>MB</th>
<th>CC</th>
<th>SC</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Macromolecules</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein concentration</td>
<td>±</td>
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<td>Lactate dehydrogenase</td>
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Only enzyme activities which exhibit statistically significant (P < 0.05; Student's t-test) changes in one or more brain regions (cerebellum: CB; pons-medulla: MD; midbrain: MB; cerebral cortex: CC; subcortex: SC) are shown:

- ± no change
- 1 11 - 30% decrease, 11 31 - 50% decrease, 111 >50% decrease
- T T 11 - 30% increase, T T 31 - 50% increase, TTT >50% increase

Adapted from (111-113).

Body and brain weights of fetuses from Tx dams, but only at the earliest age point studied (15 days gestation) (109). Throughout the remainder of fetal life (19 and 21 days gestation), and through the early postnatal period to adulthood these values are normal (109,111,112). In general, weights of gross anatomical regions are also normal in postnatal and adult animals. Other groups, using different thyroidectomised rat dam models, have reported body and brain weight reductions during late fetal life (107,118-121) which may persist well into the postnatal period (118-121).

In our own model, the reduction in brain weight at 15 days gestation in Tx dam progeny is associated with decreased DNA content, indicative of a deficit in cell number (109). 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. These parameters were normal in...
postnatal brain regions, at least from 5 to 14 days. Total protein concentrations were also normal in both fetal brain and postnatal brain regions in experimental progeny (109). In other studies, encompassing 16 days gestation through to 2 postnatal months, brain total protein concentrations were unaffected in Tx dam progeny, although isolated differences of generally low statistical significance were observed [e.g. see (122, 123)]. These findings contrast with data in 7-month-old progeny (Table 2).

Work with other Tx dam models (107,120,124) has demonstrated similar changes in fetal brain DNA content, DNA concentration and protein:DNA ratio during early development, albeit the effects tend to be more prolonged, analogous to the findings with respect to brain and body weights. Additionally, reductions in whole brain ganglioside concentrations at term and 5 postnatal days in the radiothyroidectomised rat dam model, are consistent with delayed neuronal maturation (124).

With regard to brain protein concentrations, Morreale de Escobar et al. observed these to be normal at 20 days gestation but increased at 21 days gestation in experimental progeny (107). In contrast, Porterfield and Hendrich observed deficits at term and at 1, 5 and 60 postnatal days (6,108,118). Parallel changes occur in whole brain protein synthetic activity, as studied in a cell-free system, with deficient brain uptake of amino acids also evident up to 5 postnatal days (108).

The polyamines are important regulators of cellular growth and differentiation. Extensive study has shown that ornithine decarboxylase [ODC] activity is a highly sensitive marker for the detection of abnormalities in perinatal brain maturation arising from a wide range of insults (125), including neonatal thyroid dysfunction (126-128). We have reported a complex pattern of change in this enzyme as a consequence of maternal thyroid status. In prenatal brain, activity was reduced at 15 days gestation, normal at 19 days gestation [after the onset of fetal TH synthesis], but elevated near term. Further deficits were apparent in the early postnatal period in the brain stem, subcortex and cerebral cortex [but not cerebellum], with activity becoming normal by 14 postnatal days.

The glucose transporter isoform GLUT1 is also expressed at high levels in rapidly developing tissues and up-regulated upon trophic stimulation (129). Like ODC activity, its expression is deficient in brain of early Tx dam fetuses. Western blotting of fetal brain microsome preparations with a GLUT1-specific antibody has shown a large deficit [ca. 40%] in a subtype of this protein, but only at 16 days gestation (130). Although GLUT1 is predominantly localised to the blood-brain barrier (BBB) at this stage of development (131-133), this reduction is unlikely to reflect a generalised compromise in BBB function, since another marker, alkaline phosphatase activity, appears normal (134).

The polyamines regulate the development of catecholaminergic nerve pathways and the acquisition of sensorimotor function (125). This, coupled with the well characterised effects of postnatal thyroid status on the ontogeny of catecholaminergic and cholinergic metabolic enzymes (1,2,4,5), and the proposed neurotrophic activities of these neurotransmitters (2,135, 136), prompted us to examine several of these parameters in the Tx dam model (137). In experimental progeny, the early ontogeny of DOPA decarboxylase was aberrant in fetal whole brain, exhibiting a similar pattern of change to that of ODC. In addition, activity was elevated in postnatal cerebral cortex and, in particular, brain stem. Monoamine oxidase activity was also deficient in fetal brain but elevated in postnatal brain stem. Of the cholinergic metabolic enzymes, the prenatal ontogeny of choline acetyltransferase was affected, whereas acetylcholine esterase appeared normal. This latter observation is somewhat surprising in view of the widespread deficiencies we have observed in this enzyme activity in adult progeny (Table 2). Several other enzyme activities [including β-galactosidase, aryl sulphatase and acid phosphatase] which show widespread changes in 7-month-old brain, appear normal at earlier developmental stages (110).

Studies of the calmodulin-dependent phosphatase, calcineurin, indicate that the development of the cerebellum may also be dependent on maternal thyroid status (122,123). Whole brain neutral and alkaline components of this phosphatase activity have been shown to be markedly reduced in neonatal Tx dam progeny (125). However, at later stages of development [2 months], only the cerebellum is affected, the activities of neutral and acidic components being elevated (122). Thus although postnatal cell acquisition (109) and the development of specific nerve pathways (137) appear normal in cerebellum, more subtle effects on neurite outgrowth cannot be discounted (138).

Together, these studies provide compelling evidence that maternal TH is necessary for brain development. In thyroidectomised dam models, brain development is compromised not only before the onset of fetal TH synthesis but also in the late fetal/postnatal period, culminating in irreversible brain dysfunction.

During early fetal life, brain and body weights are reduced, and neuroblast acquisition appears deficient, consistent with the proposed role of T3, in cell proliferation. The expression of several proteins [ODC, GLUT1] which play essential roles in proliferative growth is also compromised at this stage. These early changes may reflect a general delay in brain development. However, in our model, body and brain
weights, cell number and GLUT1 protein expression become normal soon after the onset of fetal TH synthesis and remain so near term. Selective compromise of other parameters is apparent, however, pointing to asynchronous [rather than delayed] development of the late fetal/postnatal brain.

The damage appears to be localised, at least at the gross anatomical level, and is confined to phylogenetically older regions of the brain, in which major phases of neuroblast division precede fetal TH synthesis and are thus dependent on the maternal T4 supply. Although several fetal abnormalities [DNA concentration, protein:DNA ratio] are corrected in the early postnatal period, others [ODC, calcineurin and catecholamine metabolic enzymes] persist beyond the active phase of synaptogenesis and myelogenesis. The perturbation of polyamine and catecholamine metabolic enzymes suggests an impact on multiple neurotrophic systems which may underlie the complex pattern of changes seen in the adult brain.

In regard to the adult brain, several widespread abnormalities [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. Such effects may indicate degenerative changes in the brain, although longitudinal studies are required to clarify these observations.

Other groups have reported similar changes in early brain development as a consequence of maternal thyroidectomy, albeit the effects are more prolonged than those seen in our model. The reasons for these discrepancies are as yet unresolved. Porterfield & Hendrich have reported numerous metabolic changes in both mothers and fetuses, and that peri- and postnatal thyroid function is disturbed in progeny (108). The observation that postnatal cerebellar growth and biochemical functions [in particular ODC] are largely unaffected in our model would argue against a role for these and other mechanisms, such as nutritional disadvantage during suckling, in our model (109).

Placental weight and gross indices of placental cellular development, including ODC activity, also appear normal in Tx dams (109). Changes in the glycosylation of placental GLUT1 protein have been observed, but only near term (130). Together, these findings are consistent with low levels of expression of TR mRNAs and nuclear T3-binding activity in rat placenta [manuscript in preparation]. Furthermore, although impaired during early fetal life, body and brain weights become normal by 19 days gestation and remain so at term. These observations indicate that placental dysfunction is unlikely to contribute significantly to the fetal brain damage.

VI. CONGENITAL HYPOTHYROIDISM

Although there is a vast literature (1-6) detailing the deleterious effects of neonatal hypothyroidism on rat brain development, the precise mechanisms underlying the damage are unknown. Given the complexity of the brain, and the interdependence of the various events that constitute development, it is difficult to distinguish between direct and indirect effects of TH on this tissue. The recent application of molecular biological techniques to this problem [e.g. see (139)] has allowed identification of several candidate genes which may be primary targets for transcriptional regulation by T3. Several of these genes are discussed in further detail below.

a) RC3/Neurogranin

The RC3 gene product [neurogranin] is a protein kinase C substrate and is localised in dendritic spines and the cell bodies of postnatal forebrain neurons [e.g. see (140)]. It has been implicated in postsynaptic calcium second messenger function, in particular long-term potentiation. Combined maternal/fetal/neonatal hypothyroidism results in reduced expression of RC3 mRNA and protein from 5 postnatal days onwards in several brain regions, the striatum being most severely affected (141). This effect was reversible by TH replacement therapy, but if left untreated, adult control levels were never reached. In situ hybridisation studies have confirmed these findings and further indicate that thyroid status influences late phases of RC3 maturation in a complex manner (140). Changes in the RC3 gene and flanking sequences, however, have so far failed to demonstrate a TH response element, although retinoic acid and steroid hormone response elements were identified (142).

b) Immediate early genes

Several studies have implicated thyroid status in the regulation of immediate early gene expression in developing brain. The basal expression of c-jun mRNA is increased in postnatal hypothyroidism but whether this is a primary or secondary effect is unclear (139). NGFI-A has been more extensively studied. Expression of NGFI-A mRNA and protein has been shown to be T3-responsive in developing rat brain [but not adult brain], in an age- and region-dependent manner (143,144). These latter effects do not appear to be related to differential T3 receptor isoform expression (144). A putative TH response element has been identified upstream of the promoter region in the mouse gene, suggesting that T3 may directly regulate NGFI-A expression at the transcriptional level (145). Indeed a rapid increase in NGFI-A mRNA levels is observed when T3 is administered to hypothyroid rats (143). Furthermore, a recent study of neuro-2a cells over-expressing human TR8, indicates direct transcriptional regulation of the NGFI-A gene [but not c-jun] by T3 (146). However, indirect mechanisms cannot be ruled out, since NGFI-A is induced by a variety of growth factors (147,148), which may themselves be under TH control. Nevertheless, the postulated roles of NGFI-A in the regulation of cell proliferation, differentiation
and synaptic plasticity (149-152) are consistent with the effects of congenital hypothyroidism on the brain.

c) Myelin genes
Neonatal hypothyroidism results in severe delays in myelination, and various myelin proteins are suspected of being under TH control (3-6). Indeed, an early differential screening study identified a number of oligodendroglial genes whose expression was markedly reduced in postnatal hypothyroid animals (139). Of these genes, myelin-associated glycoprotein [MAG] and, in developing brain, myelin basic protein [MBP] have been studied in some detail at the mRNA level. A TH response element has been identified in the promoter region of the MBP gene (153-155). This, coupled with the demonstration of TR isoforms in oligodendrocytes, at least during active myelogenesis [see section IIc] - suggests that MBP is a direct target for transcriptional regulation by T3. Other studies however point to effects of TH on MBP mRNA stability (156,157), suggesting that TH may regulate myelin gene expression at multiple levels. With respect to MAG, neonatal hypothyroidism delays mRNA and protein expression by several days, whereas hyperthyroidism accelerates ontogeny (158). Regional differences were apparent, cerebral cortex and hippocampus being more severely affected than striatum and mesencephalon; however by adulthood all regions were normal. Nuclear run-on assays failed to show a T3-induced increase in MAG gene transcription, indicating TH acts at a post-transcriptional level in this instance - possibly by increasing mRNA stability (158).

d) Neurotrophins and their receptors
In a recent comprehensive study, Alvarez-Dolado and coworkers observed alterations in gene expression of a number of neurotrophins and neurotrophin receptors as a consequence of neonatal hypothyroidism (159). Thus nerve growth factor [NGF] mRNA was decreased in most brain regions [except striatum]; its high affinity receptor [trkA] was reduced in striatum; and its low affinity receptor [p75NGFR] was increased in cerebellum (159). Acute treatment of hypothyroid pups with T3 rapidly normalised hippocampal NGF mRNA levels, whereas cortical levels remained depressed. Neurotrophin-3 [NT-3] mRNA was elevated in most regions except cerebellum in neonatal hypothyroidism, whereas gene expression of brain-derived neurotrophic factor, trkB and trkC receptors appeared normal (159).

In normal brain, both NT-3 and p75NGFR mRNAs are down-regulated during the postnatal period; therefore the effects of neonatal hypothyroidism are indicative of a developmental delay (159). This study is in agreement with the elevated mRNA and protein levels of p75NGFR previously noted in cerebellum in hypothyroid neonatal rats (160,161), although the latter authors were unable to detect any effect of hypothyroidism on NGF protein (160). On the other hand, an early study of the cerebellum found neonatal hypothyroidism to result in decreased NT-3 mRNA levels; T3 administration resulting in increased NT-3 gene expression in vivo and in primary cultures of cerebellar granule cells (162). The reasons for these discrepancies are as yet unclear, as is the mechanism of TH-mediated regulation of neurotrophin/neurotrophin receptor gene expression. Further clarification of this latter point is required, given the fundamental role that neurotrophins play in brain development, modulating the proliferation, differentiation and survival of specific neuronal populations.

e) PCP-2 gene expression
PCP-2 is a Purkinje cell-specific gene of unknown function. It accumulates in the cerebellum of normal rats during the postnatal period reaching maximum levels around day 15, consistent with the period of Purkinje cell differentiation. In hypothyroid animals, the rise in PCP-2 mRNA is delayed, normal adult levels being attained between 15 and 45 postnatal days (64). T3 replacement normalises this process. The presence of T3 response elements in the promoter region of the PCP-2 gene suggests the possibility of direct transcriptional regulation (163).

f) Mitochondrial genes
The expression of several mitochondrial genes [cytochrome c oxidase subunit III [COX-III], 12S and 16S rRNAs] in brain has been shown to be regulated by T3 (105). Combined maternal/fetal/neonatal hypothyroidism was associated with a significant decrease in the steady state levels of 16S rRNA even in the fetal brain (19 days gestation), with the fundamental role that PCP-2 gene expression suggests the possibility of direct transcriptional regulation (163).

TH action in rat brain

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TH action in rat brain
changes in gene expression may simply reflect, for example, cell loss or abnormal cellular differentiation. In other cases, post-transcriptional mechanisms may be important, affecting mRNA stability. How TH may regulate mRNA turnover is as yet unknown, but a recent study of neuro-2a cells over expressing TRβ, has implicated a cytosolic serine/threonine protein kinase pathway in the T3-dependent stabilisation of acetylcholine esterase transcripts (146). Whether similar mechanisms are operative in vivo remains to be demonstrated.

VII ADULT-ONSET HYPOTHYROIDISM

The adult brain is capable of TH uptake and metabolism, and expresses high levels of nuclear TRs [preferentially distributed within neuronal cells]. An increasing number of biochemical studies support a role for TH action in this tissue [reviewed in (113)]. However, many of the genes which appear TH-responsive [either directly or indirectly] in the neonatal brain do not appear so in adulthood. For example, of the mitochondrial-encoded genes, only 12S rRNA changes as a consequence of adult-onset hypothyroidism (105,106). Similarly Alvarez-Dolado et al. (159) showed that, of the neurotrophin/neurotrophin receptors genes, only NGF and trkA mRNAs were reduced by this treatment. This contrasts with the findings of Giordano et al. (164) who found that peripherally administered TH increased the expression of NGF and NT-3 mRNAs in the hippocampus (164). RC3 mRNA expression in adult brain is decreased upon hypothyroidism and this can be reversed by T3 replacement therapy (140). Since this gene possesses a retinoic acid response element, the observations that hypothyroidism differentially modulates RXRβ and γ isoforms in adult brain (165,166), may be pertinent. These latter effects were rapidly reversible by T3 administration, with T3 exerting direct transcriptional regulation on RXRβ [but not RXRγ] expression (166).

The reasons for the restricted responsiveness of adult brain gene expression to thyroid status are unknown. Observations on the PCP-2 gene indicate the possible presence of silencing sequences which may attenuate the effects of T3 at maturity (163). A further possible factor may be the changes in chromatin structure that accompany brain development (167,168). These may conceivably serve to regulate the access of TRs to their response elements. Interestingly, chromatin rearrangement per se may be subject to regulation by thyroid status (169,170).

VIII REFERENCES


TH action in rat brain

(abstract).


Maternal hypothyroxinemia disrupts neurotransmitter metabolic enzymes in developing brain

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Abstract
Maternal hypothyroxinemia disrupts neurotransmitter metabolic enzymes in developing brain

Introduction
Maternal hypothyroidism is associated with reduced monoamine oxidase (MAO) activity in fetal whole brain at 16 and 19 days gestation (dg). A similar trend was observed for choline acetyltransferase (ChAT) activity. In contrast, DOPA decarboxylase (DDC) activity was markedly elevated at 21 dg. Further study of these enzymes at 14 dg showed no differences between normal and experimental progeny – suggesting they become TH sensitive after this age. Tyrosine hydroxylase (TyrH) and acetylcholinesterase (AChE) activities were unaffected prenatally. During postnatal development, the activities of TyrH, MAO, DDC and, to a lesser extent, AChE were increased in a brain region- and age-specific manner in experimental progeny.

The prenatal disturbances noted in this study may have wide-ranging consequences since they occur when neurotransmitters have putative neurotropic roles in brain development. Furthermore, the chronic disturbances in enzyme activity observed during postnatal life may affect neurotransmission, thereby contributing to the behavioural dysfunction seen in adult progeny of hypothyroxinemic dams.

Introduction
Maternal thyroid hormone (TH), notably thyroxine (T4), is transferred to the fetus prior to the onset of fetal thyroid function in both humans and rats. The transferred TH accumulates within the fetal brain – coincident with the expression of TH metabolic enzyme activities and 3,5,3'–triiodothyronine (T3) nuclear receptors (Porterfield & Hendrich 1993, Pickard et al. 1997). It is possible therefore, that the critical period of TH-dependency of brain development begins prior to fetal thyroid function, when an adequate maternal TH contribution is crucial. Indeed, field studies in iodine-deficient endemias have shown that maternal serum T4 levels in hypothyroxinemic pregnancies correlate with subsequent motor and cognitive function in the children (Pharoah & Connolly 1989). Furthermore, impaired intellectual and motor function are also apparent in children born to hypothyroxinemic women in iodine-sufficient environments (Man et al. 1991).

Previous studies utilizing thyroidectomized (Tx) rat dam models have shown that adult progeny exhibit impaired motor performance, cognition and learning ability (Hendrich et al. 1984, Attree et al. 1992, Sinha et al. 1994), suggesting underlying neurotransmitter dysfunction. Furthermore, brain region-specific alterations in acetylcholinesterase (AChE), choline acetyl transferase (ChAT) and several myelin metabolic enzyme activities have been described in adult experimental progeny (Pickard et al. 1997). It is thought that the compromise in adult brain function stems from the insult incurred during fetal life. Indeed, the brains of fetal and postnatal Tx dam progeny exhibit a range of biochemical abnormalities, including changes in cellular protein and DNA concentrations, and ornithine decarboxylase activity (Morreale de Escobar et al. 1985, Pickard et al. 1993, Porterfield & Hendrich 1993).

During postnatal development in the rat, neurotransmitter systems and synaptogenesis constitute major targets for TH action (Porterfield & Hendrich 1993). Cholinergic and aminergic neurotransmitter systems in rat brain are particularly susceptible to alterations in postnatal thyroid status, disruption occurring in neurotransmitter levels (Rastogi & Singhal 1976), metabolic enzyme activities
Singhal 1974, Gripois & Fernandez 1977a,b, Kalaria & Lenoir 1980, Safaei & Timiras 1985, Garza et al. 1988. Several of the affected neurotransmitter systems have been reported in rat neural cell culture models (Honegger & Lenoir 1980, Safaei & Timiras 1985, Garza et al. 1988). Whether such TH-responsive neurotransmitter systems are also targets for maternal TH action during early fetal brain development has not been investigated.

In this study we have therefore employed a partially Tx rat dam model to investigate the effects of maternal hypothyroxinemia on the pre- and postnatal ontogeny of several cholinergic and monoaminergic metabolic enzymes in brain. A preliminary report of these data has appeared elsewhere (Evans et al. 1995).

Materials and Methods

Materials

General reagents were obtained from Sigma Chemical Co. (Poole, Dorset, UK) and BDH-Merck Ltd (Lutterworth, Herts, UK). R-(−)-depenyl hydrochloride was obtained from ICN Biomedicals Ltd (Thame, Oxon, UK), and acetylcoenzyme A and 6,7-dimethyl-5,6,7,8-tetrahydropterine hydrochloride from Calbiochem—Novabiochem Ltd (Beeston, Notts, UK). [3H]Acetylcoenzyme A was purchased from Amersham International (Amersham, Bucks, UK), D,L-3,4-[alanine-1-''^C] dihydroyxphenylalanine and L-[1-''^C]tyrosine from NEN Dupont Ltd (Hounsow, Herts, UK) and Picofluor scintillant from Canberra-Packard Ltd (Pangbourne, Berks, UK). Total T4 and T3 radioimmunoassay kits were obtained from North East Thames Regional Immunnoassay Service (London, UK) and rat thyroid stimulating hormone (TSH) radioimmunoassay kit from Biocode Biotechnology (Liege, Belgium).

Animals

Sprague-Dawley rat dams, partially Tx by surgical removal of the left lobe and isthmus (parathyroid-spared), were mated with normal males when circulating T4 levels were <20 nM. The control group constituted euthyroid (N) dams mated with normal males. All animals were maintained at 22 °C on a cycle of 14 h light: 10 h darkness, with free access to an iodine-replete diet. The drinking water of the Tx dams was supplemented with calcium lactate (0.1% w/v). When pregnancy was allowed to continue to term, litters were standardized to seven pups on the day of birth.

Sample preparation

Pregnant dams and postnatal progeny were stunned and killed by cervical dislocation. A cardiac blood sample was taken from the dams immediately after killing for serum TH determination. Brains were dissected from fetal and postnatal progeny, placed on ice and cleaned of meninges and blood vessels. Postnatal brains were dissected into four gross anatomic regions — cerebellum, brain stem (comprising pons, medulla and midbrain), subcortex and cerebral cortex — whereas prenatal brain was used whole. Tissue was homogenized in 9 vol ice-cold 0.32 M sucrose and aliquots stored at −20 °C.

Enzyme assays

Monoamine oxidase (MAO) activity was assayed by the fluorometric method of Krajl (1965), using kynuramine as substrate. At several age points, 1 μM clorgyhne or 1 μM deprenyl hydrochloride was included to assess the activity of the MAO-A and -B isoforms respectively (Squires 1972). These concentrations of inhibitor were determined at 19 days gestation (dg) in preliminary experiments (data not shown). DOPA decarboxylase (DDC), tyrosine hydroxylase (TyrH) and ChAT activities were assayed radiometrically according to the methods of Okuno & Fujisawa (1982), Waymire et al. (1971) and Fonnum (1969, 1975) respectively. AChE activity was measured using the colorimetric procedure of Ellman et al. (1961).

Protein determination

Protein was assayed in tissue homogenates using Folin–Ciocalteau reagent (Lowry et al. 1951) with bovine serum albumin as standard.

Thyroid function

Total T3, total T4 and TSH were determined in maternal serum by radioimmunoassay using commercial kits.

Statistical analysis

All values are expressed as mean ± s.e.m. Statistical significance was determined either by two-way analysis of variance (ANOVA) with post-hoc analysis by Fisher’s PLSD test, or Student’s t-test, as indicated. In all cases, values of P<0.05 were taken to be significant. Where necessary, data was logaritmithically transformed prior to analysis to satisfy the criteria for ANOVA (Snedecor & Cochran 1980).

Results

Initially, the study was restricted to samples from 16 dg to 30 postnatal days (pnd) and these constitute the main
Table 1 Maternal serum TH levels, litter number and fetal body weight in normal (N) and partially Tx rat dam pregnancies. Values are means ± S.E.M., n ≥ 5. Treatment effects (two-way ANOVA) were observed for T4 (P<0.0005) T3 (P<0.001), fetal body weight (P<0.001), and litter number (P<0.005).

<table>
<thead>
<tr>
<th>Days Gestation</th>
<th>Dam Status</th>
<th>T4 (nM)</th>
<th>T3 (nM)</th>
<th>Litter number</th>
<th>Body weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>N</td>
<td>40.86 ± 2.21</td>
<td>1.19 ± 0.12</td>
<td>14.73 ± 0.94</td>
<td>0.500 ± 0.011</td>
</tr>
<tr>
<td></td>
<td>Tx</td>
<td>14.46 ± 1.22***</td>
<td>0.76 ± 0.14*</td>
<td>13.00 ± 1.11</td>
<td>0.424 ± 0.022**</td>
</tr>
<tr>
<td>19</td>
<td>N</td>
<td>29.50 ± 2.55</td>
<td>1.88 ± 0.34</td>
<td>15.12 ± 1.32</td>
<td>2.440 ± 0.080</td>
</tr>
<tr>
<td></td>
<td>Tx</td>
<td>16.93 ± 2.35***</td>
<td>1.03 ± 0.08*</td>
<td>12.14 ± 0.67</td>
<td>2.180 ± 0.075*</td>
</tr>
<tr>
<td>21</td>
<td>N</td>
<td>25.53 ± 2.64</td>
<td>2.12 ± 0.38</td>
<td>14.00 ± 1.28</td>
<td>5.008 ± 0.248</td>
</tr>
<tr>
<td></td>
<td>Tx</td>
<td>9.87 ± 1.29***</td>
<td>1.43 ± 0.31*</td>
<td>12.89 ± 1.02</td>
<td>4.613 ± 0.177</td>
</tr>
</tbody>
</table>

*P<0.05, **P<0.01 and ***P<0.005 compared with N dam progeny (Fisher’s PLSD).

section of the results below. Additional samples were later collected at 14 dg to assay those enzyme activities showing disturbed prenatal ontogeny in Tx dam progeny and these are presented separately.

Animal model

The experimental dams in this study were moderately hypothyroid (Table 1); total T4 levels were reduced to 40–60% of controls, while total T3 was less severely deficient. A further series of dams (n ≥ 4) with comparable total T4 levels (Tx vs N dam; 11.68 ± 2.50 vs 5.14 ± 6.14 nM at 16 dg; 15.74 ± 2.61 vs 5.90 ± 2.61 nM at 19 dg; and 11.51 ± 2.28 vs 2.86 ± 4.68 nM at 21 dg) were also evaluated for maternal serum TSH levels. These were found to be markedly elevated at all stages of pregnancy studied (P<0.0005; Fisher’s PLSD test). Values (Tx vs N dam) were: 18.94 ± 2.84 vs 2.82 ± 0.21 ng/ml at 16 dg; 22.99 ± 5.34 vs 5.95 ± 1.18 ng/ml at 19 dg; and 27.10 ± 3.89 vs 2.09 ± 0.33 ng/ml at 21 dg. Litter sizes were marginally lower throughout gestation in Tx dams relative to controls (Table 1). Fetal body weight was reduced in Tx dam progeny at 16 and 19 dg but normal near term (Table 1). Brain weight and protein concentration (in terms of whole brain and by region) were normal at all stages of development (data not shown).

Monoaminergic metabolic enzyme activities

The ontogenic profiles of TyrH specific activity in fetal brain from N and Tx dam progeny were very similar, both displaying a 1-4-fold increase between 16 and 21 dg (data not shown). During postnatal development however, TyrH specific activity was 32% higher in the cerebral cortex of Tx dam progeny at 10 pnd and although the overall treatment effect was significant, the difference had disappeared by 20 pnd (Fig. 1). A similar increase (by 28%) was observed in the subcortex at 20 pnd, although no overall treatment effect was found for this region (Fig. 1).

In normal fetal brain, the specific activity of DDC declined linearly by 58% between 16 and 21 dg, whereas in experimental progeny this decline was absent (Fig. 2). Consequently, although the specific activity of DDC in Tx dam progeny at 16 dg was slightly lower than in controls, by 21 dg it was 58% higher (Fig. 2). Enhanced levels of DDC specific activity persisted in a region-specific manner during postnatal development in Tx dam progeny, with significant treatment effects apparent in cerebral cortex and brain stem (Fig. 3). Post-hoc analysis confirmed the statistical significance of the higher activities
in experimental progeny at 20 pnd in the cerebral cortex and at 20 and 30 pnd in the brain stem (Fig. 3).

In normal fetal brain, total MAO specific activity increased 1.5-fold between 16 and 19 dg, then remained stable (Fig. 4). The initial phase of induction was blunted in Tx dam progeny, with total MAO activity being reduced by ca. 25% at 16 and 19 dg. Using isozyme-specific inhibitors no treatment-related effect on MAO-A or -B specific activity (nmol product/h/mg protein) was apparent at 16 dg (data not shown), whereas MAO-A specific activity was significantly reduced (P<0.05 by Fisher's PLSD) at 19 dg (13.34 ± 0.64 vs 15.80 ± 0.65; n=5). Despite the apparent normalization of total MAO activity by 21 dg (Fig. 5), differences in activity were seen in brain stem (P<0.05 treatment effect for two-way ANOVA) in postnatal Tx dam progeny. Specific activity was ca. 20% higher in the experimental progeny at 10 (76.83 ± 8.10 vs 64.77 ± 3.37; n ≥ 6) and 20 pnd (78.23 ± 5.11 vs 65.11 ± 4.40; n ≥ 7), but only 7% higher at 30 pnd (84.52 ± 5.77 vs 78.69 ± 6.37; n ≥ 8).

Cholinergic metabolic enzyme activities

Following a very similar pattern to MAO, ChAT specific activity in normal fetal brain increased 1.5-fold between
Maternal T4 and neurotransmitter metabolic enzymes

Table 2 Whole brain DDC, MAO and ChAT specific activities in 14 dg normal (N) and partially Tx rat dam progeny. Values are means ± S.E.M., n ≥ 5. No significant differences were observed as determined by Student's t-test.

<table>
<thead>
<tr>
<th>Dam status</th>
<th>DDC (nmol DOPA/h/mg protein)</th>
<th>MAO (nmol product/h/mg protein)</th>
<th>ChAT (nmol acetylCoA/h/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>0.958 ± 0.223</td>
<td>9.963 ± 0.701</td>
<td>0.702 ± 0.053</td>
</tr>
<tr>
<td>Tx</td>
<td>0.694 ± 0.112</td>
<td>9.068 ± 0.354</td>
<td>0.635 ± 0.054</td>
</tr>
</tbody>
</table>

16 and 19 dg and then remained constant through to 21 dg (Fig. 5). In Tx dam progeny the profile was different in that the initial rise in activity continued from 19 to 21 dg (P<0.05, Fisher’s PLSD), although there was no overall treatment effect. After birth, ChAT activity was normal in all brain regions between 10 and 30 pnd (data not shown).

AChE specific activity increased 4-fold from 16 to 21 dg in normal fetal brain and this profile was unaffected by maternal thyroidectomy (data not shown). Postnatal development was also largely unaffected in Tx dam progeny, the only exception occurring in cerebral cortex at 10 pnd where the specific activity was significantly (P<0.05 by Fisher’s PLSD) raised (56.0 ± 9.9 vs 40.7 ± 2.4 nmol thiocholine/min/mg protein; n=5); this normalized by 20 pnd however and was not associated with any overall treatment effect.

Defining the onset of TH sensitivity

Having demonstrated that the prenatal ontogeny of certain enzymes, namely DDC, MAO and to a lesser extent ChAT, were disrupted as a consequence of maternal hypothyroxinemia, it was decided to investigate whether their ontogeny was disturbed even earlier in development. Subsequent experiments were therefore performed at 14 dg. As with the previous results (Table 1), serum total T4 and T3 levels were reduced (P<0.05 by Student’s t-test) in the Tx dams relative to controls (13.50 ± 1.86 vs 39.69 ± 1.70 nM and 0.71 ± 0.16 vs 0.89 ± 0.06 nM for T4 and T3 respectively; n ≥ 5). Litter sizes were not significantly reduced (13.50 ± 1.34 vs 16.00 ± 1.10; n ≥ 5), but followed the same trend as previously (Table 1). Similarly, brain weight and protein concentration did not differ from control values (data not shown).

In contrast to the 16 dg data, body weight was not affected (0.164 ± 0.003 vs 0.157 ± 0.005 g; n ≥ 5 for N and Tx dam progeny respectively). Furthermore, no significant differences were apparent in any of the enzyme activities measured at this age (Table 2).

Discussion

In this Tx rat dam model, fetal TH deprivation will be greatest prior to – but mitigated by – the onset of fetal TH secretion at 17.5 dg, since intrathecal TH is predominantly maternally derived before this time. At 16 dg, maternal serum total T4 in Tx dams was reduced by 65% relative to controls. Total T3 was less severely affected, therefore these dams were considered ‘hypothyroxinemic’. Serum TSH levels were significantly elevated in Tx dams, confirming thyroid dysfunction and the expected pituitary feedback response.

Maternal hypothyroxinemia disrupted the ontogeny of several neurotransmitter metabolic enzymes in fetal brain. Total MAO activity was reduced at 16 and 19 dg, but normal at 21 dg; ChAT activity exhibited a similar trend. These changes are consistent with the effects of TH insufficiency at later stages of brain development, as studied in in vivo and cell culture models. For example, total MAO activity is reduced by combined maternal-fetal hypothyroidism (Gripois & Fernandez 1977) or neonatal hypothyroidism (Gripois & Fernandez 1977). Furthermore, MAO-A activity is TH sensitive in neuroblastoma cell lines (Safaei & Timiras 1985) and was reduced by maternal hypothyroxinemia in this study. ChAT activity is also regulated by postnatal thyroid status (Ladinsky et al. 1980, Garza et al. 1988).

These results suggest that TH may regulate MAO and ChAT activities through common mechanisms both before and after the onset of fetal TH synthesis. In postnatal brain, TH is considered to act via nuclear TH receptors (TR). TRs are present at detectable levels in rat fetal brain from 14 dg (Perez-Castillo et al. 1985, Falcone et al. 1994), rising 3-fold by 16 dg (Perez-Castillo et al. 1985). Thus, MAO activity is disrupted after 14 dg, coincident with the rise in TR number. Further work is required therefore to examine TH-mediated transcriptional regulation of neurotransmitter metabolic enzymes.

TyrH and AChE are also regulated by postnatal thyroid status (Geel & Timiras 1967, Rastogi & Singh 1974) but were unaffected prenatally by maternal hypothyroxinemia. TyrH activity appears less TH-sensitive however than ChAT in an in vivo model (Kalaria & Prince 1985) or MAO-A in neuroblastoma cells (Safaei & Timiras 1985), while AChE is less responsive than ChAT to T3 in neuronal cultures (Honegger & Lenoir 1980, Garza et al. 1988). Prenatal changes in brain AChE and TyrH...
activities may have occurred if more severe maternal hypothyroxinemia had been induced. The interpretation of findings from overtly hypothyroid rat dam models is however confounded by factors such as severe maternal metabolic compromise and placental maldevelopment (Bonard & Herrera 1991). Thus severe maternal hypothyroidism produces permanent deficits in body and brain weight, and brain protein concentration (Henrich et al. 1997). The influence of such confounding factors in the present model appears to be minimal, since in Tx dam pregnancies: fetal body weight normalized near term; fetal brain weight and protein concentration were unaffected; effects on neurotransmitter metabolic enzyme activities were specific rather than general; and both MAO and ChAT normalized by 21 dg (i.e. appear to be corrected by fetal TH synthesis).

Maternal hypothyroxinemia also disrupted the prenatal ontogeny of DDC, but only near term when the intrauterine TH environment is determined largely by the fetus. This effect may be a progressive consequence of the earlier TH deficit. Indeed, postnatal dysthyroidism has little effect on DDC activity (Virgili et al. 1991). DDC activity remained elevated postnatally in brain stem and cerebral cortex, indicative of long term or, in the case of brain stem, permanent compromise to monoaminergic neurons. Chronic changes were also apparent for other enzyme activities, in particular TyrH in cerebral cortex and MAO in brainstem. Interestingly, all the enzymes affected postnatally showed increased activity, and disturbances were confined to brain regions in which neurogenesis occurs during early gestation. No changes were seen in cerebellum, possibly because this region develops largely after the onset of fetal TH synthesis. These results confirm and extend previous observations in postnatal Tx dam progeny (Pickard et al. 1993), and strongly support a prenatal origin for the postnatal disturbances. Neurotransmitters have putative neurotropic roles in early gestation (Lauder 1993, Leslie 1993) and it is therefore possible that neuronal differentiation is disturbed by maternal hypothyroxinemia thus leading to chronic brain maldevelopment and perhaps the observed postnatal effects. Alternatively, the early TH deficit may impact on other signals which regulate the development of monoaminergic neural pathways, such as polyamines (Slotkin & Bartolomé 1986). Indeed, ornithine decarboxylase, which regulates polyamine biosynthesis, is sensitive to maternal hypothyroxinemia in fetal and postnatal rat brain (Pickard et al. 1993).

Earlier work in adult progeny of Tx dams showed disturbances in brain ChAT and AChE activities (Sinha et al. 1994). These results seem to be at variance with the present study, however they may reflect covert disturbances which become apparent over time. A similar effect was noted regarding TyrH activity in this study. Alternatively, the more severe maternal hypothyroxinemia induced in the study of adult progeny (T4 levels were depressed to only 10% of controls (Sinha et al. 1994)), may be responsible.

In summary, this study demonstrates that maternal thyroid status regulates the ontogeny of monoaminergic and, to a lesser extent, cholinergic neurotransmitter metabolic enzyme activities in rat brain. These changes are evident during fetal life (from 16 dg) when the neurotransmitters concerned have putative neurotropic roles, and may therefore have long term repercussions for brain development. Compromise to monoaminergic metabolic enzymes during postnatal life, may impinge upon neurotransmission and contribute to the behavioural dysfunction seen in young adult progeny of Tx dams (Attrée et al. 1992, Sinha et al. 1994). These findings may be pertinent to humans, since maldevelopment of cholinergic and monoaminergic nerve pathways during the first half of gestation, when the intrauterine TH environment is determined by the mother, may contribute to the impaired cognitive and motor development reported in offspring of hypothyroxinemic women (Man et al. 1991).

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