EFFECTS OF HYPOTHYROIDISM ON CELL SIGNALLING IN THE DEVELOPING RAT BRAIN

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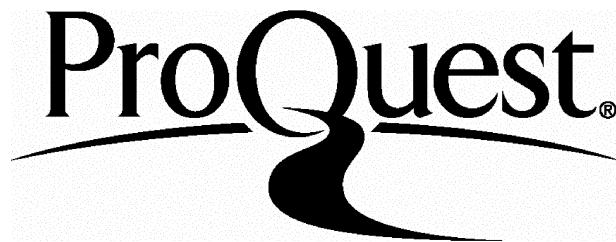
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ABSTRACT

Rat pups born of dams maintained on a propylthiouracil/low iodine diet regime were used to assess the effects of hypothyroidism on signalling components in the developing brain.

The success of the animal model of perinatal hypothyroidism was confirmed following measurements of free T_3 and T_4 plasma levels. Alterations in development and behaviour noted in hypothyroid pups were similar to those observed previously by other groups. Plasma metabolite concentrations indicated that the impairments in body and brain development noted in the hypothyroid state, were not the result of serious under-nutrition.

Crude and synaptosomal membranes were isolated from forebrain and hindbrain regions of euthyroid and hypothyroid animals at various ages. Changes in α-subunit abundance of G_q, G_o, G_i1 and G_i2 were measured via quantitative immunoblotting.

No differences in α-subunit levels of euthyroid and hypothyroid crude membranes were seen in either brain region. Compared to euthyroid animals, between 10 and 20 days postpartum a number of significant alterations were detected in synaptosomal membranes isolated from forebrains of hypothyroid pups; with the exception of $G_i2\alpha$, all of these changes were that of subunit up-regulation. By contrast, the hindbrain showed very few hypothyroid-induced changes; the only significant effects were seen in $G_i1\alpha$ at day 10 (total hindbrain) and day 25 (medulla oblongata). Hypothyroidism also altered the normal developmental profiles of all four α-subunits in both forebrain and hindbrain regions.

Synaptosomal membranes of 15 day old pups were also used to assess hypothyroid-induced effects on adenylyl cyclase activity. Changes in the pattern of

activation/inhibition of adenylyl cyclase by GTP were observed in hypothyroid forebrain and hindbrain membranes in the presence of forskolin. Furthermore, a significant decrease in specific cyclase activity was observed in hypothyroid forebrain membranes. By contrast, in the hindbrain, a significant increase in cyclase activity was evident.

Together, the changes observed may contribute to some of the neurological defects seen during perinatal hypothyroidism.

ACKNOWLEDGEMENTS

Scientists....peeping-toms at the keyhole of Mother Nature.

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ABBREVIATIONS

HR	Hormone-Receptor complex
CNS	Central nervous system
CTX	Cholera toxin
DAG	Diacylglycerol
DIT	Di-iodotyrosine
EGL	External granular layer
IP_3	Inositol 1,4,5-trisphosphate
MIT	Mono-iodotyrosine
PAGE	Polyacrylamide gel electrophoresis
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PKC	Protein kinase C
PLC	Phosphatidylinositol specific phospholipase C
PTU	6-n-propylthiouracil
PTX	Pertussis toxin
SDS	Sodium dodecyl sulphate
T_3	Triiodothyronine
T_4	Thyroxine

CHAPTER 1
INTRODUCTION

INTRODUCTION

1.1   THE BRAIN

The brain to all intents and purposes bears a striking resemblance to a cauliflower with a thick rope suspended beneath it. However this mass of specialised cells acts as a control centre for the rest of the body and has the daunting responsibility of collecting and processing information as well as co-ordinating and regulating the body's movements and activities. The brain governs our emotions; shapes our thoughts, hopes, dreams and gives rise to our imagination which makes us all so unique from one another. It determines the type of person we are.

The brain is complex in structure and organisation. It is divided into many regions, each possessing its own role in the overall function of the brain.

1.2   ANATOMY OF THE HUMAN BRAIN

It is convenient to divide the brain into three regions: forebrain, midbrain and hindbrain. The hindbrain emerges from the spinal cord and encompasses the medulla oblongata, cerebellum and pons. The midbrain denotes the top of the brain stem and is situated above the pons in the human. The forebrain is the most prominent part of the brain and can be subdivided into two gross areas; cerebrum (basal ganglia and cortex) and diencephalon (thalamus and hypothalamus). The brain stem, which lies above the spinal cord, acts as a bridge allowing messages to pass between the brain and the rest of the body. The anatomical and functional aspects of the brain may be found in the majority of anatomy and physiology text books available (e.g. Carpenter, 1990; Dowling, 1992a; Seeley et al., 1992). A general description of the various regions and their functions of the brain is outlined below.

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1.2.1   MEDULLA OBLONGATA AND PONS

The medulla and pons are the most primitive parts of the brain. The medulla, situated rostrally to the brain stem, contains numerous ascending and descending nerve tracts. Discrete nuclei (clusters of neurons) with specific functions including balance, control of head and facial features, and regulation of body functions (e.g. heart rate, respiration, gastrointestinal functions) are also housed in this region of the brain. The medulla is capable of exerting wide spread influences on virtually all parts of the brain by virtue of the vast axonal extensions of a separate group of neurons known as the *reticula formation*. The pons, resembling a thick bulge, is a portion of the brain stem just superior to the medulla oblongata. The pons as its name suggests (bridge), connects the medulla to the more developed areas of the brain. Discrete nuclei in the anterior portion of the pons relay information from the cerebrum to the cerebellum. Nuclei present in the posterior section are associated with cranial nerves. Like the medulla, the pons also contains ascending and descending nerve tracts. The pons is involved in control of cardiac functions and houses the sleep and respiratory centres.

1.2.2   CEREBELLUM

Attached to the medulla and pons is the cerebellum (little brain), a highly regulated structure whose main function is to co-ordinate and integrate motor activity. It receives input from the cerebral cortex concerning initiation of voluntary movement. The cerebellum integrates the information and relays it to the descending motor system along with sensory inputs to ensure smooth overall movement. Lesions in the cerebellum result in jerky, unco-ordinated movement which may be delayed or erratic.

1.2.3 MIDBRAIN

Situated just superior to the pons, the midbrain denotes the top of the brain stem and is a point of integration of various impulses. The roof of the midbrain consists of four mounds (*colliculi*) collectively termed *corpora quadrigemia*. There are two *superior* and two *inferior colliculi*. The latter are involved in hearing and are an integral part of the auditory pathways whereas the former are concerned with visual reflexes and receive input from the eyes, *inferior colliculi*, skin and cerebrum. The midbrain also plays a role in motor neuron stimulation responsible for eye and head turning.

1.2.4 HYPOTHALAMUS AND THALAMUS

Within the forebrain is "buried" the thalamus (from the Greek word for inside chamber) and the hypothalamus (under). The former acts as a relay station for the majority of information that passes to and from the brain. The hypothalamus contains several small nuclei and nerve tracts with functions that include regulation of olfactory reflexes, eating, drinking and sexual activity. A stalk protruding from the floor of the hypothalamus connects it to the pituitary gland. Consequently the hypothalamus is crucial in endocrine system regulation as it controls hormone secretion from the pituitary. Functions as diverse as metabolism, reproduction, autonomic (heart rate) muscle control and temperature regulation are all influenced by the hypothalamus. Moreover, pituitary hormones possess the ability to effect hormone release from endocrine glands elsewhere in the body (e.g. thyroid, adrenal), constituting yet another level of regulation by the hypothalamus. The thalamus, resembling something similar to a yo-yo, consists of numerous prominent nuclei which modify and relay sensory and motor activity information to the cerebral cortex. Functions of the thalamus involve general body movements and influence of mood.

Auditory, visual and sensory impulses congregate in this particular area of the brain.

1.2.5   BASAL GANGLIA

The basal ganglia are five prominent nuclei positioned around the thalamus concerned with movement (initiation and execution) and posture. They receive input from the cortex and emit signals to the thalamus. Lesions lead to movement dysfunction characterised by tremor, repetitive and slow movements. Parkinson's disease and Huntington's chorea are both results of impaired basal ganglia function.

1.2.6   CEREBRAL CORTEX

The outer layer of the cerebrum is called the cortex (shell). For humans and many other mammals, the cerebral cortex houses the majority of the higher nervous systems. Functions localised in this particular brain region include skilled movement, sensation, consciousness, memory and intelligence to name but a few. Cortical functions are arranged topographically on the cerebral cortex. The cortex itself invaginates many times to form grooves called *gyri*, resulting in a much greater surface area. In addition the cortex has evolved into two separate sections termed hemi-spheres which can be further subdivided into four lobes. Neurons in the cortex lie close to the surface. Specific neural functions are carried out by the lobes. The frontal lobes are involved primarily with movement and olfaction. The parietal lobes deal with somatic sensation, the occipital lobes with vision, and the temporal lobes with hearing and memory. The lobes receive information from the thalamus and initiate specific movements. Also situated within the cerebrum beneath the temporal lobes, are two hippocampi, concerned with long term memory. Removal leads to loss of function but surprisingly, this is not the case if only

Figure 1.1   LONGITUDINAL SECTION OF THE PRIMATE BRAIN
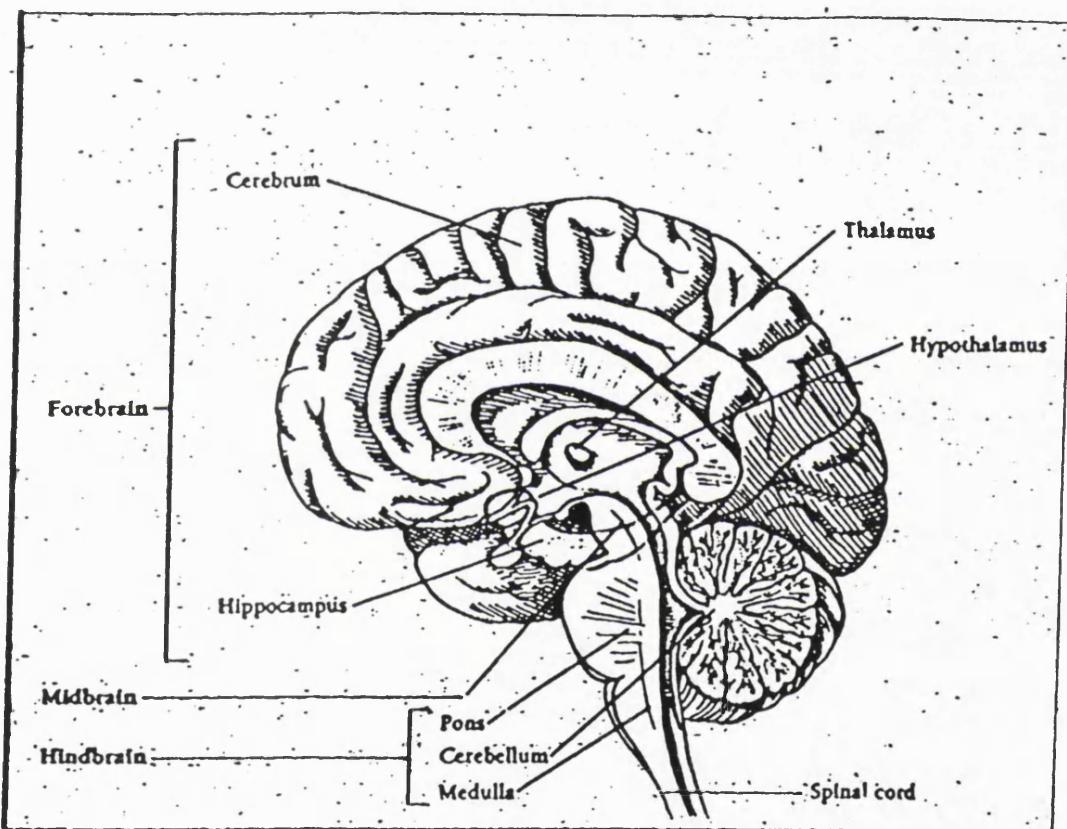
one is removed. Each cerebral hemisphere controls and receives inputs from the opposite side of the body. In the majority of people, the left side of the brain is dominant controlling speech and analytical skills; the right side deals with spatial and musical abilities.

Figure 1.1 depicts the locations of the major regions of the primate brain.

1.3   DEVELOPMENT OF THE HUMAN BRAIN

In the human, the first signs of the brain appear over the first three weeks of gestation. A single cell formed at conception gives rise to an embryo, and it is from a layer of tissues (neural plate) on the dorsal surface of the embryo that the central nervous system (CNS) develops. The lateral sides of the plate form neural crests which move forward and fuse together creating the neural tube. The expanded anterior portion of the tube goes on to form the brain. Constrictions in this region serve to separate the future fore-, mid- and hindbrain. The narrower posterior end of the neural tube gives rise to the future spinal cord. Alongside and separate from the neural tube lie crest cells from which the majority of the peripheral nervous system will develop.

Once the tube has formed, cell proliferation begins. What starts off as a single layer soon develops into a multi-layer of cells. Cell division takes place in specialised areas known as germinal zones located in the inner surface of the neural tube. Spongioblasts give rise to glia whereas neuroblasts are the precursors of neurons. As the cells migrate they form temporary contacts with one another but once at their final destination, differentiate into specific cells with precise properties and connections. Further development involves growth, axon and dendrite maturation and synaptogenesis. A small proportion of cells

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proliferate after migration, specifically those found in the basal ganglia and granule cell of the cerebellar cortex.

By five weeks gestation in the human, the major regions of the brain (forebrain, midbrain and hindbrain) are recognisable albeit in a primitive form. The forebrain divides into the telencephalon (cerebrum) and the diencephalon. The midbrain remains as a single structure whilst the hindbrain gives rise to the pons, cerebellum and medulla oblongata. More detailed descriptions of brain ontogeny can be found in Lund, 1978; Carpenter and Sutin, 1983; Naula and Fiertag, 1986; Dowling, 1992a.

1.4   <u>CELLS</u>

Interactions between nerve cells underlie much of the brains functions and accomplishments allowing the animal to learn, behave, remember and create. They communicate chemically by means of electrical signals relayed by neurons. There are two major types of cell in the brain: neurons and glia.

1.4.1   NEURONS

Neurons or nerve cells, are responsible for processing and receiving signals from sensory organs and other neurons, integrating that information and transmitting it. In addition to the same intracellular components of other cells, neurons possess unique features such as distinct cell shape and specialised structures known as synapses. There are about 10^{11} neurons in the human brain each composed of three parts:

(1) cell body - contains the nucleus, enzymes and components essential for the cell's survival;

(2) dendrites - tube-like extensions branching out from the cell body giving it a tree like

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appearance and are the sites where the neuron receives signals from other cells;

(3) axon - an extension of the cell body and the means by which signals travel to and from the cell body to other cells of the brain.

The axon may be branched and is frequently myelinated along its course increasing its efficiency as a conducting unit for electrical impulses/ signals (Palay and Chan-Palay, 1977). Neurons communicate with each other by conducting these electrical signals and are able to do so for long distances without loss of signal strength. Neurons can be excitatory, inhibitory and modulatory in effect, or motor, sensory and secretory in function. They can also be classified according to the number of processes they have e.g. unipolar (one axon), bi-polar (two axons), multi-polar (several axons). Neurons differ from other cells in the body in that once differentiated, possess a limited ability to regenerate following injury due to the high degree of differentiation.

As mentioned earlier, neurons can come in various forms and sizes. Each part of the brain is made up of certain types of neurons pertaining to that region's particular function. In other words, neurons in the cerebellum are different from those located in the cerebral cortex. Neurons can be divided into two broad classes: Golgi type I and Golgi type II (Dowling, 1992b).

Golgi type I neurons from one part of the brain can carry information to another part of the brain, spinal cord or an effector organ (e.g. muscle). They possess dendrites which receive information from other cells and effectors, and long axons which branch near the terminus forming synapses with other neurons or effectors. Golgi type I neurons possess nodes of Ranvier, points at which they are not myelinated along their axon, for rapid relay of information.

Golgi type II neurons have characteristically short or sometimes no axons. As a result,

these neurons are confined to one area of the brain and are involved in local interactions between nerve cells. They are often called association neurons and unlike Golgi type I cells, both axons and dendrites are capable of transmitting information. Golgi type II are believed to be an important part of subtle neuronal interaction.

Neurons within a particular brain region may be classified even further. The cerebellum is made up of five different neuronal cell types whereas in the cerebral cortex, two major types have been identified. Microscopic studies have revealed neurons and nerve terminals in areas of the brain such as cerebral cortex and cerebellum, are arranged in distinct layers. In the cerebral cortex, six layers can be distinguished labelled I-VI starting from the outermost layer. Pyramidal cell bodies predominate in layers II, III, V and VI whereas stellate cells though present in all layers predominate in layer IV. The relative abundance of the different neuronal cell types differ from region to region and reflect the various functions of that region in terms of input and output requirements.

The cerebellum in contrast, consists of three principal layers: molecular (outermost) layer; Purkinje cell (intermediate) layer and the granule (inner) cell layer. Purkinje cells of the cerebellum serve a similar role to that of pyramidal cells located in the cerebral cortex. The granule cell layer consists of densely packed small neurons whose axons penetrate the molecular layer and form contacts with Purkinje cell dendrites (parallel fibres). The Purkinje cell layer aside from Purkinje cells and parallel fibres, consists of basket cells and stellate cells. Stellate cells and basket cells are inhibitory interneurons. They along with Golgi cells, can be found in the granule layer and contain γ-amino butyric acid (GABA) or some other inhibitory neurotransmitter. Stellate and basket cells located in the molecular and Purkinje cell layers respectively, both receive inputs from the granule cell layer (via parallel fibres). Stellate cell axons form synapses with Purkinje cell dendrites

whereas the axons of basket cells synapse around the initial portion of Purkinje axons, thus enabling effective inhibition of the Purkinje cell layer if so required. Inputs to the cerebellar cortex are termed mossy and climbing fibres. The former terminate on granule cells whilst the latter terminate on groups of Purkinje cells whose activity they control (Strange, 1992).

1.4.2   GLIA

In 1846, Virchow came across a tissue that was not neuronal in nature. It possessed no synaptic connections and occupied all remaining space in the brain not already taken up by nerve cells. He named the tissue glia meaning glue, pertaining to its first assigned function, to hold the neuron network together. Though they do not appear to participate in the integration and processing of information, glia are ten times more abundant than neurons and can be divided into two categories: macroglia (astrocytes and oligodendrocytes) and microglia (similar to macrophages). Despite the extent of their numbers, their exact functions are uncertain but they appear to have several roles from protection of the brain against electrical/ionic environment changes, to the uptake and metabolism of released neurotransmitters or excess chemicals, e.g. K^+. In addition, they participate in CNS repair and provide structural and metabolic support for the vast array of neurons. During the early stages of the developing mammal, glia are required for guiding neuronal migration in certain regions of the brain (Hatten, 1990). Astrocytes are believed to possess connective and supportive functions as well as being involved in repair as they are seen to proliferate subsequent to trauma. They have also been assigned a putative role in transport within the blood brain barrier system and a firmer one in the regulation of pH and local ionic balance. Oligodendrocytes are responsible for the

production of myelin. The majority of cell axons are encased in myelin which acts as an insulator and also serves to increase the rate at which nerve impulses can travel along the cell with respect to an unmyelinated cell. Oligodendrocytes are distinguishable under electron microscopy from neurons and found to predominate in white matter, the colour characteristic to the presence of myelin. It can take several oligodendrocytes to encase an axon with myelin depending on the size of the axon. As mentioned earlier, the myelin sheath is not continuous. Unmyelinated points along the axon, known as nodes of Ranvier, allow signals to jump along the length of the axon resulting in rapid communication and relay of signals. Oligodendrocytes possess poor regenerative properties due in part to their slow mitotic rates. CNS diseases which target myelin result first in the capitulation of these vulnerable cells. The interaction between neurons and glia is an important one especially during the development, functioning and maintenance of the nervous system (Palayand Chan-Palay, 1977).

1.5   SYNAPSES

Brain function relies on the relay of information between cells. Axons and dendrites emerging from different or the same neurons communicate at specialised sites. Sherrington in 1897 first proposed a name for these contact points, from the Greek word for connection, synapses. A typical neuron can have anything from 1000 to 10,000 synapses and is capable of receiving information from 1000 other neurons. Briefly, the arrival of the nerve impulses at the synapse cause vesicles to fuse with the cell of the pre-synaptic membrane, releasing the contents into the intervening gap (synaptic cleft) that separates the axon from the neighbouring neuron (Heuser and Reese, 1977). Neurotransmitters regulate the opening of channels on the post synaptic membrane thereby

relaying the signal to the next neuron. There are many types of neurotransmitters: acetylcholine; catecholamines (adrenaline, dopamine) and amino acids (glutamate, GABA, 5-hydroxytryptamine). The function of neurotransmitters in the brain is an important one, controlling the numerous actions of the body from memory to movement. Neurotransmitters can also act like hormones; by initiating a chain of events via G-protein mediated signalling pathways, they are capable of regulating a vast array of metabolic and physiological processes in the body.

There are two different types of synapses: chemical and electrical (Dowling, 1992b). Chemical synapses are distinguishable by their pre-synaptic vesicles which cluster near sites of neurotransmitter release. Two classes of chemical synapses have been identified. Type I synapses are excitatory in nature and found mainly on dendrites. They possess spherical synaptic vesicles and widened synaptic clefts between the pre- and post- synaptic membranes. Type II chemical synapses are inhibitory and predominate in cell bodies. Like type I synapses, they are distinguishable by virtue of their flattened vesicles and narrow synaptic clefts. Type I vesicles contain excitatory neurotransmitters such glutamate and acetylcholine whereas type II contain inhibitory neurotransmitters which include GABA and glycine.

The most common synapses are those present between axon terminals and dendrites which are usually excitatory in nature. Inhibitory synapses occur between axon terminals and cell bodies.

Electrical synapses allow direct continuity between the interior of connecting cells thereby permitting ions and small molecules (<1.2 kDa) to pass from one cell to another. Electrical changes in one cell can be transmitted to the adjacent cell almost instantaneously with flow of molecules being bidirectional. In some cases (rectifying junctions) flow is

restricted to one direction.

1.6 THE THYROID

1.6.1 HISTORICAL ASPECT

The importance of thyroid hormones for normal growth, development and maintenance of the brain and body has been established for well over a century. It was Fagge in 1871 who first proposed the link between cretinism and thyroid dysfunction, whilst Coindet discovered the benefits of iodine administration to goitrous patients following the success of eating seaweed which is rich in iodine. The thyroid gland was confirmed to contain iodine in 1896 by Bauman, and in 1914 Kendall purified an iodine containing substance from extracts of thyroid tissue, later found to be thyroxine (T_4). Some forty years later in 1952, Pitt-Rivers isolated the more biologically active thyroid element, triiodothyronine (T_3). Numerous extensive studies over the years have revealed that a wide variety of biological and biochemical activities are dependent, either directly or indirectly, on thyroid hormone during all stages of life.

1.6.2 STRUCTURE OF THE THYROID

The thyroid gland, situated in the anterior neck and caudal to the larynx, consists of two pear shaped lobes. Thomas Wharton in 1656 first described and named the organ pertaining to its appearance (Greek for shield-like). In the new-born human, it weighs about 1.5 g, increasing with size and age to about 20 g in the adult. It is one of the most vascular organs gram for gram with a flow rate of 5 ml/g/min, and is served by four main arteries and a rich lymphatic supply. The role of the thyroid is to concentrate iodide from the blood stream and synthesize thyroid hormones. Consequently the thyroid is dependant

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upon a constant supply of dietary iodide and when this element is scarce, the gland enlarges in response to demands for the gland to trap more iodide. This visible swelling in the neck is called a goitre. The cells of the thyroid responsible for production and secretion of hormone are arranged into structures called follicles and known as follicular cells. A single layer of follicular cells surrounds a mass of colloidal material composed of hormone stores incorporated into a protein called thyroglobulin. Microvilli project from the apical end of each cell into the colloid. Depending on conditions such as iodine availability, physiology and pathological stimulations, follicle diameters vary from 100-1000 μm diameter, though on average they are 200-300 μm. The smallest follicles are the more active, with 20 to 50 follicles forming lobules encased in a fine mesh of connective tissue. The internal structures of follicles are typical of any cell involved in protein formation and secretion, i.e. mitochondria, rough E.R., Golgi Apparatus. In humans, follicular cells are able to trap iodine by the 12th week of gestation with hormone production occurring soon after (reviewed in McDougall, 1992).

1.7   THYROID HORMONES

Together, triiodothyronine (T_3) and thyroxine (T_4) constitute the thyroid hormones. The former is more important in terms of biological activity; the latter, less active, is present at higher concentrations and considered a precursor (or prohormone) of T_3. Both are iodothyronines, incorporating two iodinated phenyl rings joined together by an ether link, and differ in the number of iodine atoms as shown in Figure 1.2.

Figure 1.2   STRUCTURE OF THYROID HORMONES
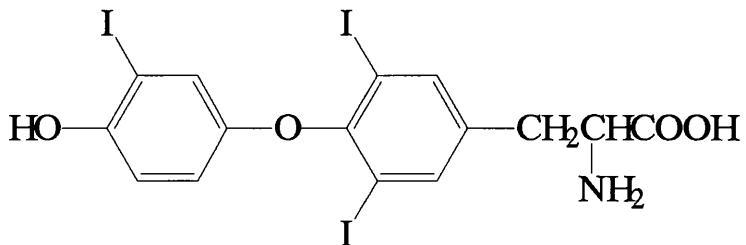
Figure 1.2a Triiodothyronine (T$_3$)
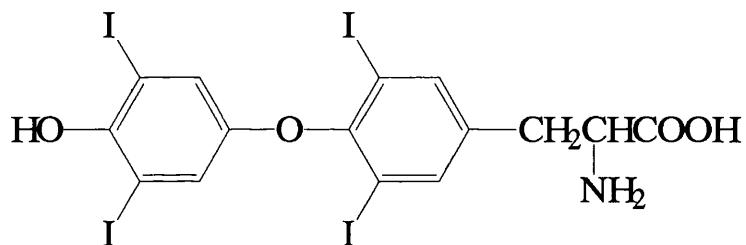
Figure 1.2b Thyroxine (T$_4$)

1.7.1   THYROID HORMONE SYNTHESIS

Iodide in the bloodstream is actively transported into the follicular cells against a chemical and electrical gradient. The trapped iodide is organified in the colloid which involves the iodide combining with tyrosine previously incorporated into thyroglobulin molecules. Thyroglobulin is the main constituent of the colloid and acts as a repository for thyroid hormones and their precursors. Prior to organification, the iodide is first oxidised by the action of thyroid peroxidase in the presence of hydrogen peroxide (H_2O_2) at the interface of the cell and colloid (Taurog, 1970). Of the 120 tyrosines per thyroglobulin, thirty are iodinated and six to eight available for thyroid hormone synthesis. Mono-iodotyrosine (MIT) and Di-iodotyrosine (DIT) are tyrosine molecules with one and two iodine atoms

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respectively. Two DIT molecules couple to form T_4 whereas one DIT together with a MIT produce T_3. The coupling step occurs via the same peroxidase involved in the organification procedure. At times of sufficient iodine in the diet there are 7-10 MIT, 5-10 DIT, 2 T_4 and 1 T_3 for every thyroglobulin molecule (Van Herle *et al.*, 1979). A more extensive account of hormone synthesis can be found in McDougall (1992).

1.7.2 SECRETION OF THYROID HORMONES

Before secretion of T_3 and T_4, thyroglobulin is first hydrolysed by lysosomes to release the active hormones along with MIT, DIT and amino acids. T_3 and T_4 enter the circulation; MIT and DIT are deiodinated. The iodide is reused with a small fraction lost from the thyroid (Dunn and Dunn, 1982a; Dunn and Dunn, 1982b).

1.7.3 THYROID HORMONES IN THE SERUM

T_3 and T_4 travel through the serum reversibly bound to carrier proteins. A minute proportion of hormone is free/unbound. Bound hormone is metabolically inert and acts like a hormone store as well as a buffer, providing a means for maintaining the amount of free hormone. In the serum, 3 out of 1000 T_3 and 3 out of 10,000 T_4 are free which means that 99.97% of T_4 and 99.7% of T_3 are bound. As a result of binding there is no loss of hormone via the urine except in cases of extreme proteinuria. Three types of carrier proteins carry thyroid hormone in the serum; thyroid binding globulin (TBG), thyroid binding prealbumin (TBPA) and albumin.

Although TBG is present at the lowest concentration of the three (1-2 mg/dl) it possesses the highest affinity for T_3 /T_4, carrying 70% of both hormones. A single chain glycoprotein produced by the liver, it contains one binding site per molecule.

TBPA is also synthesized in the liver and hastwo binding sies for T_4 molecules though only one site is ever occupied. TBPA carries 10-20% of T_4 and no or very little T_3. Albumin, with the weakest affinity for hormone, is present in the highest quantity transporting approximately 10% and 40% of T_4 and T_3 respectively.

Levels of these proteins are affected by a number of conditions (both psychological and pathological) and any deviation of affinity for the hormones results in change of total thyroid hormone. Free hormones enter the cell and dictate thyroid function. An increase in thyroid binding protein, for example, indicates a fall in T_4. This is recognised at the level of the pituitary and T_4 levels are adjusted accordingly (reviewed in Robbins and Bartalena, 1986; McDougall, 1992).

1.8   REGULATION OF THYROID FUNCTION

1.8.1   THYROID STIMULATING HORMONE (TSH)

Thyroid stimulating hormone (TSH) or thyrotrophin, is produced and secreted by specific cells (called thyrotrophes) in the anterior pituitary. TSH regulates the formation and release of T_3 and T_4.Levels of free T_3 and T_4 act as negative feedback controls for TSH secretion. Thyrotrophe nuclei possess T_3/T_4 receptors; the affinity for T_3 being 10-20 times greater than that for T_4. The cells are rich in 5'deiodinase II, responsible for the conversion of T_4 to T_3 (Larsen, 1982). Approximately 50% of the T_3 in the cell arises as a consequence of 5'deiodinase II action and it is therefore intra-thyrotrophe T_3 and not serum T_3 that acts as a regulator. High T_3 levels in the mouse lead to a decrease in the mRNA's of TSH subunits. Normally, receptor occupancy by T_3 is 50%, but when this figure reaches 90%, complete inhibition of mRNA is observed (Chin *et al.*, 1985). The net result of TSH action on the thyroid is to increase the synthesis of thyroid hormone

stores, with some of the newly synthesized hormone released within the hour (O'Riordan *et al.*, 1988).

Certain factors such as somatostatin and dopamine, inhibit TSH production and consequently its release. Oestrogen increases the levels of TSH by augmenting the regulatory action of TRH (thyrotrophin release hormone). The time immediately following birth sees a sudden rise in TSH level (which subsides after 24 to 48 hours) and precedes an increase in total and free thyroid hormone levels. The rise in thyroid hormone is believed to protect the newborn from the new, cold environment by stimulating an increase in hormones involved in heat production. TSH release is itself under control, by thyrotrophin release hormone (TRH) secretion from the hypothalamus.

1.8.2 THYROTROPHIN RELEASE HORMONE (TRH)

The existence of a hypothalamic factor governing anterior pituitary function was proposed over 40 years ago by Green and Harris (1947). Lesions in the hypothalamus led to organ failure whilst electrical stimulation of specific sites resulted in the release of hormone from the pituitary. In 1968, TRH was isolated, characterised, synthesized and shown to act both under *in vivo* and *in vitro* conditions (review in McDougall, 1992). As mentioned earlier, TRH controls the release of thyroid stimulating hormone from the pituitary.

Guillemin (1978) obtained 1mg pure TRH from the hypothalami of 5 million sheep. TRH is a tri-peptide (propylglutamyl-histidyl-prolinamine) and made in the hypothalamic cells. Other sites of synthesis include other areas of the brain, as well as the spinal cord and pancreas. After synthesis it is transported, via the blood stream, to the anterior pituitary where it interacts with high affinity specific receptors on the thyrotrophes invoking TSH

Figure 1.3

Simplified diagram of the control of thyroid function
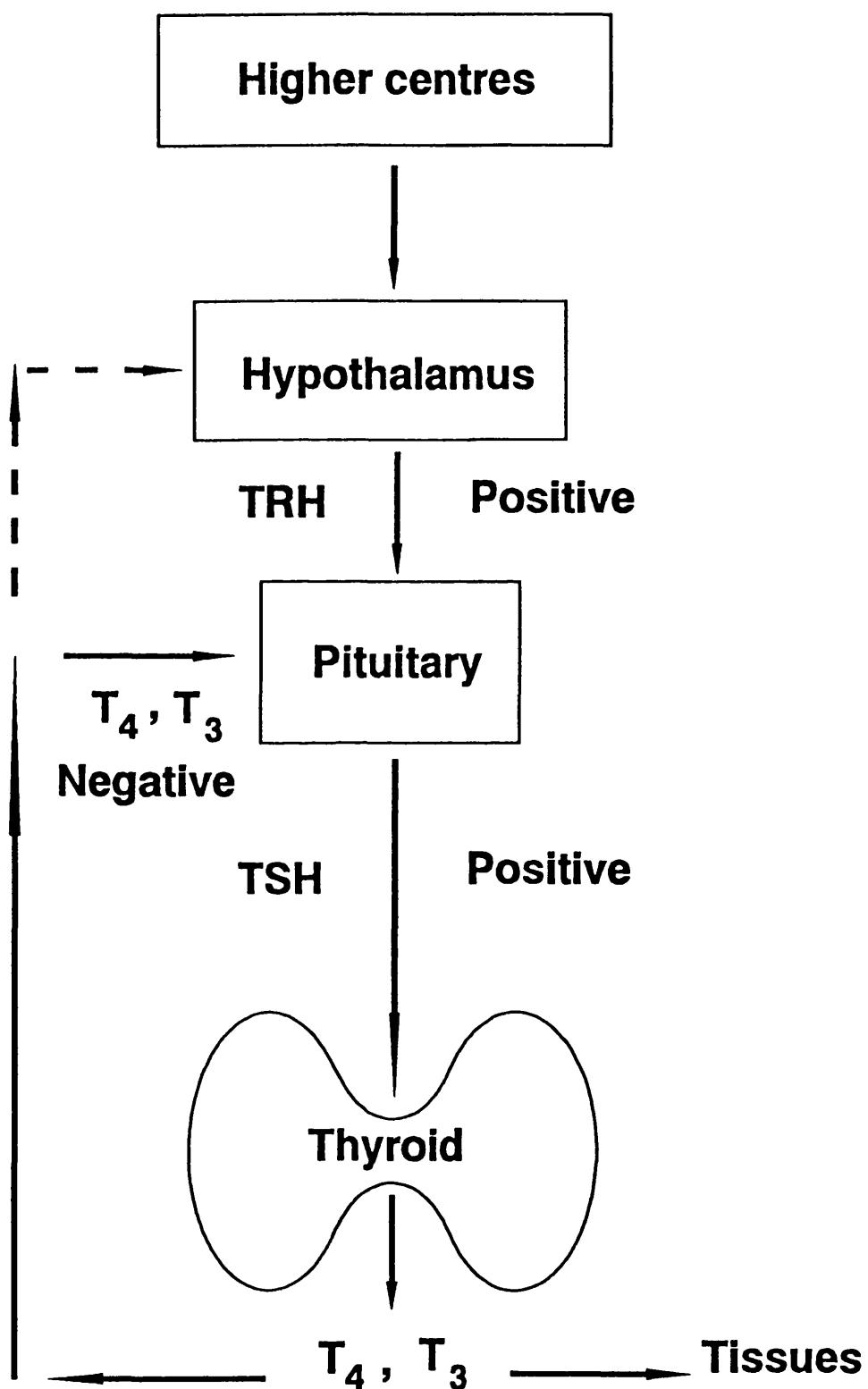
production. The mechanism proposed suggests the involvement of IP_3 signalling following receptor binding, resulting in a rise of intracellular Ca^{2+} which stimulates a cascade of events culminating in TSH-containing vesicles discharging their contents into the circulation (Kolesnick and Gershengorn, 1985). TRH has a very short half life of around 5 minutes and is excreted in the urine. TRH is present in other areas of the body and brain where it is thought to function as a neuromodulator.

In summary, the function of the thyroid gland is governed by a number of factors secreted by various organs. Thyroid hormone production is under the control of TSH secreted from the pituitary which in turn is regulated by TRH produced by the hypothalamus. The hypothalamus is under less defined control from higher centres in the brain. In addition, the thyroid gland itself is able to autoregulate hormone synthesis and release, though this is less important than TSH control (Figure 1.3).

1.9   ACTION OF THYROID HORMONES

Thyroid hormones are involved in a vast number of developmental and physiological events. For many years, the general consensus of opinion maintained that the mode of thyroid hormone action was confined to the nucleus only. This view has been revised following the discovery of thyroid hormone binding sites at locations external to the nucleus in a variety of cell types. As a result thyroid hormones have been assigned an extranuclear role which though minor in comparison, is of no less importance in the overall effect they exert in the body.

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1.9.1   NUCLEAR ACTION

The nuclear action of thyroid hormones is similar to that observed with steroid hormones. This is hardly surprising given the homology these two exhibit in their receptors. Thyroid hormone action is mediated via nuclear receptors which in turn regulate the transcription of specific mRNA in target cells. Virtually all of the physiological effects of thyroid hormones are believed to occur in this manner.

Briefly, after entering the cell, thyroid hormone binds to receptors present in the nucleus. Two genes encode thyroid receptor proteins; c-erb Aβ and c-erb Aα found on chromosome 3 and 17 respectively. They possess the same affinity for thyroid hormones. 50% of the receptor sites are occupied under normal conditions with 80% containing T_3. Binding initiates modulation of gene expression generating positive or negative effects on transcription. Consequently, it is usually several hours before a biological response is observed. The genes affected encode a range of biologically important components ranging from enzymes to hormones and receptors (Baxter and Eberhardt, 1979; Samuels et al., 1982). More extensive accounts detailing the nuclear action of thyroid hormones can be found in numerous reviews (Oppenheimer et al., 1987; Samuels et al., 1989; Brent et al., 1991; De Groot, 1991).

1.9.2   EXTRANUCLEAR ACTION

The majority of extranuclear binding studies have been focused on the plasma membranes of several tissues including human placenta (Alderson et al., 1985), rat synapses (Mashio et al., 1983), and rat and human erythrocytes (De Mendoza et al., 1977; Holm and Jacquemin, 1979). Two separate sites, differing in K_D , have been identified. Both play an important role in the determination of intracellular thyroid hormone concentration and

as a result, in the ability of the cell to respond to the hormone (Krenning and Doctor, 1986).

Thyroid hormones stimulate glucose transport in cells within minutes, a time course not consistent with a nuclear effect involving transcription. Instead plasma membrane sites are thought to be directly involved. Furthermore, interaction with the plasma membrane may be the only route possible for anuclear erythrocytes to respond to thyroid hormones. Sterling *et al.* (1977) demonstrated the presence of thyroid hormones in the inner mitochondrial membrane and linked them with immediate rises seen in O_2 consumption, again implicating a direct effect of thyroid hormones. Mowbray and co-workers have shown that the lowered ADP/O ratio observed in hypothyroid mitochondria could be completely restored within 15 minutes after administration of a near physiological dose of T_3 (Crespo-Armas and Mowbray, 1987). Subsequent studies by the same group suuggest that mitochondrial processes under rapid thryoid hormone control may be mediated by ADP-ribosylation (Thomas and Mowbray, 1987; Hardy and Mowbray, 1992). The cytosolic binding sites have been assigned putative roles of storage and supply to organelles such as the nucleus (Francon *et al.*, 1985). Thyroid hormones can also regulate enzyme activity in a manner which excludes nuclear receptor involvement. One example is the effect of thyroid hormones on deiodinase II action leading to the possibility of similar modes of action in other biological processes (Silva and Leonard, 1985).

During recent years the information on extranuclear roles of thyroid hormones have grown. Only a few have been mentioned here, a more detailed account can be found in Segal and Ingbar (1986).

1.10   RECEPTORS

Both nuclear and cytosolic binding sites for T_3 and T_4 have been identified in the brain and other tissues.

The cytosolic site is about 70 kDa with a greater affinity for T_4. Its suggested roles include that of transporter and subunit of the nuclear receptor involved with chromatin (Baxter and Eberhardt, 1979). The nuclear receptor is 50-70 kDa and is associated with DNA and chromatin (Oppenheimer *et al.*, 1974, Eberhardt *et al.*, 1978).

In the rat embryo, nuclear T_3 binding sites are present in the brain at 14 days gestation. The number increases 3-fold between day 14 and 15 gestation before remaining constant up until birth. After birth, T_3 sites rise again peaking after a week before falling to adult levels (Schwartz and Oppenheimer, 1978). The nuclear binding capacity in the neonate is double that of the adult. As with other situations, the number, distribution and density vary according to brain region (Coulombe *et al.*, 1981).

Bernal and Pekoven found high affinity T_3 nuclear receptor concentrations dropped after 10 weeks gestation but rose 10-fold by week 16 gestation in the human feotus (Bernal and Pekonen, 1984).

Current knowledge of thyroid hormone levels and receptors in both the developing and adult states is reviewed recently by Puymirat (1992).

1.11   CLINICAL ASPECTS OF THYROID HORMONES

As previously mentioned, thyroid hormones govern numerous biological and metabolic events in virtually all parts of the body throughout all stages of life. Consequently any imbalance will give rise to a variety of disorders linked to these events, the degree of damage varying according to the severity of imbalance and time of onset and treatment.

The two extremes of abnormal thyroid status are hypothyroidism and hyperthyroidism. The former arises as a result of deficient amounts of thyroid hormone or insufficient action of thyroid hormone to meet the body's requirements; the latter is caused by over activity of the thyroid.

1.11.1   HYPOTHYROIDISM

Thyroid hormone deficiency is usually caused by a disease of the thyroid (primary hypothyroidism). However, it can also arise from lack of stimulation at the level of the pituitary or hypothalamus (secondary and tertiary hypothyroidism respectively). Severe hypothyroidism involving skin disorder is known as myxoedema.

Autoimmune disorders, medication, radiotherapy and iodine deficiency are all causes of hypothyroidism. 1% of the adult population are afflicted with the disease with women being 5-10 times more susceptible (McDougall, 1992). Generally, the symptoms mirror that of reduced function. They include sluggish/tired behaviour, weight gain, cold intolerance, depression and reduced brain activity. The effects are easily reversed with hormone replacement. If left untreated, myxoedema coma can result culminating in death. Causes of hypothyroidism in children include dysgensis of the thyroid, iodine deficiency and inborn enzyme defects in, for example, organification, thyroid hormone action and iodine trapping (Hutchinson, 1980).

In neonates the most extreme case of hypothyroidism leads to cretinism and the effects are irreversible due to the vulnerable state of the developing brain at this time. Three different forms of cretinism have been identified on basis of their etiology.

Congenital hypothyroidism (also known as neonatal/sporadic cretinism) results when the feotus is unable to produce its own thyroid hormone due to genetic abnormalities,

abnormal thyroid development or exposure to goitrogens and /or antithyroid drugs (Malvaux, 1981; Foley, 1983). This form of cretinism may also result as a consequence of TSH or TRH shortage and inborn disorders. Severe impairment of neurological development, mental retardation, poor co-ordination and balance, abnormal motor movements, speech defects, tremor and spasticity are all indicative of the disease. It affects 1 out of every 4000 births with girls twice as likely to acquire it. If treated within 6 weeks, some recovery of intelligence quotient (I.Q.) occurs thus assuaging the effect on mental capabilities (MacFaul and Grant, 1977).

Endemic cretinism ensues from dietary deficiency of iodine inflicting profound mental deficiency along with irreversible abnormalities ranging from neuromuscular disorders to deaf mutism (McDougall, 1992). It is still prevalent in a number of countries like Brazil, China and Pakistan. There are two classes of the disease: hypothyroid/ myxoedematous cretinism and neurological cretinism. The former is caused by severe hypothyroidism during the very early stages of life (Konig, 1981). The clinical symptoms are similar to that of congenital hypothyroidism and can be avoided if mothers are given iodine replacement by the 20th week of gestation. The neurological form differs in terms of clinical manifestation in that it is due to iodine deficiency *in utero*. It is averted only if the mother is treated in the very early stages of pregnancy, ideally before conception as seen in the rat and sheep animal models (Hetzel and Hay, 1979). In addition to the symptoms seen in the other forms of neonatal cretinism, deaf mutism and spastic diplegia are also observed (Fierro-Benitez *et al.*, 1974; Querido *et al.*, 1978). As many as a billion people live in iodine deficient areas and nearly all suffer from some degree of goitre. The offspring of these women are the most at risk from endemic cretinism with 10% exhibiting the severest form of the disease. In contrast, hypothyroidism occurs in 1 out

of every 5000 births in the Western countries.

Disorders resulting from hypothyroidism (and hyperthyroidism) in the later periods of life are reversible with appropriate hormone treatment. This is not the case during the developmental period as previously mentioned. Although treatment can help alleviate some of the effects, they cannot fully correct the damage already done. In order for irreversible damage to be avoided, there is growing evidence that therapy should begin in the first trimester of pregnancy as the thyroid state of the mother is just as important as that of the growing feotus (Pharoah *et al.*, 1971; Porterfield and Hendrich, 1993).

1.11.2 HYPERTHYROIDISM

Hyperthyroidism is attributed to over activity of the thyroid gland. In cases where there are toxic levels of thyroid hormone, the condition is known as thyrotoxicosis. Treatment involves the administration of antithyroid drugs such as PTU or carbimozole, radio-iodine or removal of the gland (thyroidectomy). As for hypothyroidism, there are many causes and forms of the disease (McDougall, 1992). The most common is Graves' disease and is the consequence of an autoimmune disorder. Briefly, antibodies to the TSH receptor (TRAb) mimick and prolong the action of TSH resulting in over-production and over-secretion of thyroid hormones (Kriss *et al.*, 1964). Many of the symptoms are akin to that of elevated basal metabolic rate and hence generally the reverse of those observed in hypothyroidism. Common signs are nervousness, weight loss despite no change or increase in calorific intake, heat intolerance, fatigue and goitre. The majority of cases of hyperthyroidism in pregnancy and children are usally a result of Graves' disease. If treated at an early enough stage with antithyroid drugs, the effects are reversible in children. Treatment involves the administration of antithyroid drugs but there is danger

to the neonate in the form of malformation and spontaneous abortion by the mother. Furthermore, the possible transfer of the antibodies from the mother to the neonate via the placenta during pregnancy, increases the neonate's chances of developing Graves' disease (Momotani *et al.*, 1984).

1.12 THYROID HORMONES AND THE DEVELOPING BRAIN

The majority of data gathered on the role of thyroid hormones during brain development was obtained from studies carried out on neonatal rats though an appreciable amount of data have also come from sheep and mice.

The value of the data obtained from these studies however, is frequently debated in relation to its significance regarding human situations. Most of the information obtained from these experiments would be unavailable from humans, and the rat remains as a good model providing equivalent human and rodent developmental stages are compared.

1.12.1 NEUROLOGICAL DEVELOPMENT IN RATS AND HUMANS

Compared to the human, the rat brain is immature in terms of development at birth. At birth, the rat brain is at the same developmental stage as the human brain at 5 to 6 months gestation Eayrs (1968). At 10 days post partum (after birth), the rat brain is equivalent to the human brain at birth (Bass *et al.*, 1977). In other words, some developmental events that occur *in utero* (before birth) in the human, occur after birth in the rat. This facilitates manipulation of the environment around the developing rat brain during this important period in life. An important matter to consider is that though the same sequence of events takes place in every region, the time that this occurs differs from one area of the brain to the next. This is illustrated in the cerebrum and cerebellar regions.

The cerebellar cortex (hindbrain) develops later then the cerebrum (forebrain) with nearly 80% of the neurogenesis in the rat occurring in the former after birth (Balazs and Richter, 1973). In contrast, by birth rat cerebral neurogenesis is more or less over; the majority occurring between day 12 gestation and birth (Balazs, 1973; Berry, 1974; Stein *et al.*, 1989). In the human, cerebral neurogenesis is virtually complete after the second month of pregnancy (Zamenhof and Van Marthens, 1971).

In the rat gliogenesis begins at birth, continuing into adult life (Berry, 1974) with the same event occurring *in utero* in humans (Dobbing and Sands, 1973).

1.12.2   STAGES IN BRAIN DEVELOPMENT IN RELATION TO THYROID HORMONES

Hamburgh *et al.* (1971) and workers alikened thyroid hormone function to "time clocks" in the developing nervous system, governing the precise timing of events that occur. Brain development can be separated into distinct stages when viewed in relation to thyroid hormone influence.

Stage I embraces the first 10 to 12 weeks gestation in the human and the first 17 days gestation in rat. It is the period of development which occurs before feotal thyroid hormone synthesis and therefore the only source of thyroid hormone during this time is maternal in origin. Recent evidence indicates that thyroid hormones have an impact on early embryogenesis (see later). At this time cerebral neurogenesis takes place along with a degree of neuronal migration.

Stage II picks up from the end of stage I and terminates at birth in both rat and human. During this time, thyroid hormone is synthesized and released by the feotus with some contribution from the mother. T_3 and T_4 concentrations increase 10-fold at this time, the

Figure 1.4

Brain Neurological Development in Relation to Thyroid Hormones in Rat and Human

RAT

PHASE I
Maternal
TH
(0-17 dpc)

PHASE II
Maternal
+ Fetal TH
(17 dpc-birth)

PHASE III
Neonatal TH
(Birth - 20 days)

0   17 days   Birth   10 days   20 days

Cerebral neurogenesis and migration
(predominantly 10-18 dpc) (Phase I, II)

Neuronal differentiation, axonal outgrowth , dendritic ontogeny and synaptogenesis (primarily Phase III);
Cerebellar neurogenesis (primarily postnatal); Gliogenesis (predominantly 4-16 dpn)

Myelinogenesis (10-45 dpn) (Phase III)

HUMAN

PHASE I
(0-12 wks)

PHASE II
(12 wks-term)

PHASE III
(Birth - 1 year)

0   12 weeks   Birth   1 year

Cerebral neurogenesis and migration
(5-24 weeks) (Phase I, II)

Neuronal differentiation, axonal outgrowth , dendritic ontogeny and synaptogenesis (Phases II and III);
Cerebellar neurogenesis (predominantly prenatal); Gliogenesis (predominantly late fetal to 6 months

Myelinogenesis (2nd trimester - 2 years) (Phase II, III)

TH - thyroid hormones;   dpc - days post conception;   dpn - days postnatal

former accompanied by a parallel rise in 5'deiodinase II activity.

The time from birth to the first 30 days post partum in the rat, and to the second year in the human, is termed as stage III. From this moment on, the brain is solely dependent on thyroid hormone secreted by the neonatal thyroid. In the rat, stage III encompasses the majority of cerebellar neuronal proliferation, myelination and differentiation. In the forebrain although these events are essentially complete (having occurred in the latter period of stage I and all of stage II), neuronal maturation, outgrowth, synapse and myelin formation is observed. As mentioned earlier, the human brain is more developed at birth than the rat with the bulk of cerebellar events occurring in stage II and continuing into the earlier part of stage III. Myelination begins in stage II and continues throughout stage III.

Figure 1.4 summarises these events in the rat and human.

1.12.3   THYROID HORMONE EFFECTS ON BRAIN DEVELOPMENT

The literature covering thyroid hormone effects on brain development is extensive with many excellent reviews available (Morreale De Escobar *et al.*, 1983; Porterfield and Hendrich, 1993; Pasquini and Adamo, 1994).

The most important times during development transpire when the brain undergoes what is termed as an active growth spurt where there is quite literally, an explosion of cellular events occurring. As thyroid hormone requirements are critical at this time, inappropriate levels will, unsurprisingly, distort the final structure and organisation of the brain. The vulnerability of the brain at this time also means that any damage done at this point is irreversible. Even subsequent replacement therapy can never fully correct the damage regardless of speed of treatment. In humans, this period is considered to start at the end

of gestation and extending to the second year of age, whereas in the rat it encompasses day 18 gestation to day 21 post partum (Oklund and Timiras, 1977).

Axon and dendrite proliferation, synaptogenesis, gliogenesis and myelin production are but a few of the many incidents occurring in the cerebrum at this time. These same events occur at a later stage in the cerebellar region, along with the bulk of cell proliferation.

Growing evidence for placental thyroid hormone transfer and its importance in determining the extent of neurological damage, indicate that thyroid hormones are crucial throughout the majority of development from early embryogenesis to after birth (Porterfield, 1985; Morreale De Escobar *et al.*, 1988a; Morreale De Escobar *et al.*, 1990). Thyroid hormones affect all aspects of brain structure and function, and below some of the morphological, biochemical, electrophysiological and behavioral implications are discussed.

1.12.4   MORPHOLOGICAL EFFECTS

Work carried on a variety of animal models has shown that hypothyroidism causes a vast range of morphological changes in the developing brain. A weight decrease in both the cerebral and cerebellar regions is observed though for different reasons. The decrease in the cerebrum is due partly to a reduction in cell size with a concomitant increase in packing density and not due to a change in cell number though the final architecture of the cells are undoubtably affected. In contrast, the cerebellum exhibits a reduction in cell size and number coupled with a rise in packing density (Nicholson and Altman, 1972). The diminished number of cells is the result of impaired cell proliferation, leading to subsequent delay in the disappearance of the external granular layer (EGL), and an

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increase in cell death. The different reasons are most likely due to the different timings in development of the various regions as mentioned earlier. Just as different regions of the brain possess different chronologies of development, the same is true of the various cell types in terms of proliferation and differentiation. In the cerebellum, the maximum differentiation rate of basket and granule cells vary considerably; day 2-6 post natal and day 15-21 post partum respectively (Balazs and Richter, 1973). Precise thyroid hormone levels are required for the co-ordination of these events and a decrease or lack of thyroid hormones lead to supression of nerve cell maturation culminating in interactive failure between neurons. Abnormal thyroid hormone levels will therefore lead to specific damge in the brain depending on time and duration of insult. For example, a drop in thyroid hormone soon after birth for a few days, will affect Purkinje cell maturation to a greater extent than granule cell development in the cerebellum due to their different proliferation and differentiation times. Thyroid hormone effects on cell migration in the cerebellum (Lauder, 1979) are believed to be the result of modifications in parallel fibre growth rather than cell mobility (Lauder, 1977).

Purkinje cells show a decrease in size though not in number (Clos and Legrand, 1973). The glia/neuron ratio increases reflecting a change in the overall number of the different cell types.

Perhaps the most dramatic consequence of hypothyroidism is the hypoplastic neuropile, i.e. a reduction in brain electrical activity. This was first observed in the cerebral cortex by Eayrs in 1960 and in the cerebellum by Legrand in 1967. Pyramidal and Purkinje cell differentiation is disturbed with a drop in number, length and degree of branching observed (Nunez, 1984a; Nunez, 1984b) leading to consequential loss of neuronal contacts and electrical activity (Poddar and Sarkar, 1993). Furthermore, the consequences of a

hypoplastic neuropile may also have some bearing on synapse distribution and rate of formation in the aforementioned brain regions. Poddar and Sarkar (1993) working on rat cerebellum, suggest delayed tyrosination of α-tubulin in parallel fibre axons is also partly responsible for impaired synaptogenesis.

Abnormal glia are observed in the young thyroid hormone deficient rat in that they lack normal peripheral processes (Pesetsky, 1973). Myelin deposition is affected as well in the hypothyroid neonate. Again this is ascribed to the hypoplasia of the neuropile (Balazs *et al.*, 1969; Clos and Legrand, 1970) and is believed to be the result of impaired activity of myelin synthesizing enzymes (e.g. cerebrosides, sphingomyelin, sulfatides). Consequently there is a delay in myelination which normally occurs between day 14 and day 24 postpartum in the rat (Tsujimura *et al.*, 1971; Rosman and Malone, 1977). Clos and Legrand (1970) proposed that normal synthesis and deposition of myelin occurs only if neurite structure is itself normal.

The reduction in cell number seen during hypothyroidism at this time, is more likely the consequence of reduced synaptogenesis arising from impaired cell migration and differentiation, as it is a generally accepted view that a neuron that cannot make contact, dies (Nunez, 1984a). Abnormal synapse distribution may also account for the changes seen in the final location of receptors in the brain. Dupont *et al.* (1981) detected altered distribution of catecholamine receptors in certain areas of the brain.

In addition to their proposed role of regulators of growth factors (Walker *et al.*, 1979; Binoux *et al.*, 1985; Miller *et al.*, 1987), it has also been suggested that thyroid hormones act in conjunction with the aforementioned compounds (Timiras and Nzekwa, 1989). Growth factors (e.g. nerve, epidermal and insulin-like) perform important functions in neurological development and could disrupt brain development during hypothyroidism.

In other words the neurological damage seen in the hypothyroid state may be augmented by altered levels of these and possibly other crucial non-thyroid effectors.

The effects of hypothyroidism are mainly |discussed here. The effects of hyperthyroidism are essentially opposite to those of hypothyroidism, though the final consequences are ultimately similar, i.e. reduction in brain activity and size, and abnormal cellular organisation. A vast amount of work has been concentrated on neuronal development during times of thyroid hormone deficiency. Less attention has been paid to the thyroid hormone excessive state due partly to the difficulty in reproducing the conditions and to the less drastic clinical consequences as compared to hypothyroidism.

1.12.5 BIOCHEMICAL EFFECTS

The question of whether thyroid hormones have an effect on biochemical systems is answered by the multiple metabolic changes observed during an abnormal thyroid status in the brain.

During times of deficit thyroid hormone levels, DNA and RNA levels rise and fall respectively. The latter is due to an increase in turnover without a corresponding increase in synthesis (Balazs and Cocks, 1967; Geel and Timiras, 1967a) and as protein synthesis itself falls, the protein/DNA ratio diminishes also (Balazs et al., 1968). Effects on protein synthesis are mediated at transcription and translation levels due to the role of thyroid hormones in the regulation of protein synthesis (Munoz et al., 1991) (see also section on nuclear effects). Furthermore, thyroid hormone effects on amino acid metabolism and transport into the cell may also be contributory factors in the fall of protein levels observed during the hypothryoid state (Geel et al., 1967; Daniel et al., 1975; Ford and

Cramer, 1977). Maternal hypothyroidism results in abnormally low perinatal amino acid uptake, protein synthesis and brain DNA in the growing feotus (Hendrich *et al.*, 1982; Porterfield and Hendrich, 1982). In addition thyroid hormone influences ribosome synthesis and function, as well as mRNA stability though these changes may be secondary rather than primary thyroid hormone effects (Porterfield and Hendrich, 1993).

Maturation of the brain from a biochemical point of view is delayed. Cocks *et al.* (1970) observed a delayed ability of the growing hypothyroid rat to convert glucose into amino acids. A reduction in succinic and glutamate dehydrogenase activity and in the development of enzymes involved in oxidative processes have also been noted (Balazs and Richter, 1973; Sokoloff, 1977).

Thyroid hormone effects on amino acid metabolism may also have some bearing on the complement of neurotransmitters (Timiras, 1988). Tyrosine, for example, is utilized for catecholamine production, while glutamate, GABA and glycine have major roles in transmission. Furthermore, enzymes involved in transmitter turnover may mediate the reduction of neurotransmitter activity associated with hypothyroidism (Geel and Timiras, 1967b; Pesetsky and Burkart, 1977; Vaccari *et al.*, 1983). Lack of thyroid hormone also leads to a reduction in catecholamine, muscarinic, β-adrenergic, acetylcholine and GABA receptor numbers (Patel *et al.*, 1980; Smith *et al.*, 1980; Valcana and Timiras, 1981). Decreased activity of more general metabolic enzymes such as acetylcholinesterase, NADPH diaphorase, Na^+/K^+-ATPase and glucose-6-phosphodehydrogenase are but a few of the various biochemical parameters affected by low thyroid hormone levels in the developing brain. Not all enzymes are affected, e.g. lactate dehydrogenase and glutamate dehydrogenase, indicating a degree of selectivity of thyroid hormone effects (reviewed in Ford and Cramer, 1977).

Tubulin, neuro-filament proteins and microtubule associated proteins (MAPs) play an important role in neurite outgrowth and synaptogenesis. As thyroid hormones are required for the normal appearance and development of these proteins, it comes as no surprise that altered thyroid state results in abnormal assembly, stabilization and composition (Nunez, 1984a; Stein et al., 1991). Hypothyroidism in neonates hinders maturation of the unique brain proteins D1 and D2 which have been implicated in early stages of synaptogenesis (Patel et al., 1985). Balazs et al. (1969) observed a drop in the levels of myelin constituents such as the cerebrosides and phospholipids, in conjunction with their associated enzymes. Altered myelin composition and deposition during hypothyroidism further contribute to the final abnormal structure of cells and their functions (Rossman and Malone, 1977).

Other hormones, e.g. growth factors, act in conjunction with thyroid hormones and therefore the mature brain is the result of a summation of these factors (Binoux et al., 1985). Rapid changes occur at this time with the developing brain changing day to day anatomically and biochemically.

1.12.6   ELECTROPHYSIOLOGICAL EFFECTS

Hypothyroidism leads to a number of changes in electrophysiological activities of the brain during the early stages of life (review in Ford and Cramer, 1977). Decreases in alpha rhythm are observed in hypothyroid children (Anderson, 1966). Hearing loss, common in human endemic cretinism and also in rats rendered hypothyroid at birth, has been attributed to poor middle ear development as seen by impaired ossification and irregular formation and function (Glorio and LeVecchio, 1983; Herbert et al., 1985). Speech control is affected contributing to the deaf-mutism also characteristic of various

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forms of hypothyroidism (Querido *et al.*, 1978; Frost, 1986). Other electrical evoked control systems, e.g. neuromuscular co-ordination and motor ability are also affected (Timiras, 1988).

1.12.7   BEHAVIOURAL EFFECTS

Eayrs observed that hypothyroid rats exhibited more frequent errors in straight maze tests, delayed acquisition of the air-righting response and diminished exploratory behaviour (Eayrs and Taylor, 1951; Essman *et al.*, 1968).

Hypothyroid children exhibit lower than average (I.Q.), poor memory retention, concentration and learning capabilities. Appropriate therapy however, can help alleviate some of these effects if introduced at an early enough stage. When treatment was initiated in subjects at or younger than 3 months, a higher degree of recovery compared to subjects treated at 7 months was observed (Dussault and Ruel, 1987).

1.13   <u>MATERNAL HYPOTHYROIDISM AND EFFECTS</u>

The thyroid state and hormonal contribution of the mother were originally thought to be irrelevant to events in the developing feotus. This is because the critical periods of brain development were assumed to occur following the onset of feotal thyroid function, and that all events antecedent to this were independent of thyroid hormone control. Pharoah *et al.* (1971) deduced that iodine deficiency during the first trimester of pregnancy was critical to development, as iodised oil administered prior to conception prevented neurological damage. Ferreiro *et al.* (1987) proposed that the consequences of maternal iodine deficiency were more severe than those of congenital hypothyroidism. He reasoned that as the developing embryo is thyroid hormone deficient until onset of foetal thyroid

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function at the expense of thyroid iodothyronine, the foetus would be unable to synthesize and store adequate amounts of thyroid hormone for use soon after birth. As a result, the decrease of maternal thyroid hormones during times of maternal iodine deficiency abolishes the protective effects otherwise gained during congenital hypothyroidism, leading to greater injury in the growing foetus.

In order for maternal thyroid hormones to have an influence they must traverse the placenta, and this issue of placental transport has been debated for many years (review in Porterfield and Hendrich, 1993). Radioimmunoassay studies (RIA) indicate the presence of thyroid hormones in brain embryonic tissue by the second week of gestation, well before initiation of feotal thyroid function (Obregon et al., 1984; Porterfield and Hendrich, 1992). Ekins et al. (1985) on administering $[^{125}I]T_4$ to pregnant rats, detected a large percentage of radioactivity in the feotus. Deiodination forms one basis of argument against thyroid hormone transfer during pregnancy, the proposal being that the majority of thyroid hormone is deiodinated en route due to placental inner ring monodeiodinase activity (Roti et al., 1982).

It must be noted that evidence against transfer arose at a time predating modern RIA and immunoradiometric assay (IRMA) techniques, and when levels were detected in the brain, they were usually considered too low to be of physiological significance.

The little data available from human feotuses show the presence of T_3 and occupied receptors before the onset of feotal thyroid function (Bernal et al., 1985; Ferreiro et al., 1988). Timing of these events correspond with those observed in the developing rat and therefore thyroid hormones detected in the early human feotus could be of maternal origin (Myant, 1958).

Since the advent of modern detection methods, there is an accumulation of evidence in

favour of placental thyroid hormone transfer (for reviews see Morreale de Escobar *et al.*, 1993; Porterfield and Hendrich, 1993). During congenital hypothyroidism, the mother exhibits normal levels of T_4 and the growing foetus develops normally. The foetal brain is therefore "protected" until birth but from that point onwards, neonatal thyroid hormone levels are insufficient to compensate for foetal thyroid failure. This is the reason why the symptoms of congenital hypothyroidism increase dramatically after birth. The damage is avoidable only if the condition is identified in time, emphasizing the urgency of early detection and treatment. In iodine deficient situations, pregnant women have low iodine and T_4, and normal T_3 levels. If T_3 is low, the chances of still birth and/or infant birth increase as the foetus, bereft of maternal T_4 protection, is hormone deficient throughout gestation. Vulsma's group claim that placental transport occurs in sufficient amounts to decrease the symptoms of congenital hypothyroidism in the foetus (Vulsma *et al.*, 1989). Maternal contribution of thyroid hormones continues even after foetal thyroid function has begun, with a significant proportion of transfer occurring in late gestation and just before birth (Geloso, 1967). As much as 17.5% of foetal thyroid hormones may come from the mother and if the antithyroid drug methimazole is administered, both maternal and foetal T_3/T_4 are suppressed (Morreale de Escobar *et al.*, 1988b; Morreale de Escobar *et al.*, 1990). Placental deiodinase-III activity takes over from deiodinase-II at day 15 gestation in the rat, just prior to foetal function. The enzyme is responsible for generating metabolically inactive rT_3 (3,3',5'-triiodothyronine) and iodine from T_4 (Woods *et al.*, 1984; Emerson 1989). This led to the suggestion that the iodine produced is taken up by the foetus for its own use in thyroid hormone production. Plenty of evidence supports this idea, e.g. McDonald *et al.* (1988) detected ^{125}I in the foetus soon after the administration of [125I]T_4 into pregnant goats.

The exact cause of foetal impairment resulting from maternal hypothyroidism is as yet unknown. However, evidence strongly indicates the importance of thyroid hormones in brain development during the early stages of gestation.

1.14   TRANSMEMBRANE SIGNALLING

Our ability to think, taste, see, move and basically exist depends on the ability of cells to communicate with each other and respond accordingly. They do so via chemical signals which act as extracellular messengers, most of which are impermeable to the barrier of the cell's plasma membrane. Instead, the signals elicit an intracellular response by a process known as transmembrane signalling whereby intermediaries in the cell bring about the required effect. The signals rely on a group of guanine nucleotide binding regulatory proteins (G-proteins) to convey the message inside the cell. G-Proteins are a part of a larger family of GTPases which include factors involved in protein synthesis, for example elongation factor Tu (EF-Tu), and the ever expanding group of "small" (20-35kDa) GTPases such as the ras proteins (Bourne *et al.*, 1990; Bourne *et al.*, 1991).

The function of G-proteins are described in more detail later but briefly, upon signal detection by receptors which span the plasma membrane, the information is relayed to a series of second messenger effector systems that ultimately bring about the response in the cell. This relay of signal is brought about via G-protein action and they can therefore be likened to telephones, allowing the communication between signals outside the cell and effector systems.

From the plethora of research carried out over the years, it has become increasingly apparent that aberrations in transmembrane signalling can contribute to diseases ranging from cholera to Alzheimers disease; and that one day, treatment of such diseases may be

focused at the level of specific G-proteins.

1.15   G-PROTEINS

It was 1971 when the first report documenting the requirement of GTP for hormone linked

signal transduction appeared (Rodbell *et al.*, 1971). Since then the knowledge gleaned

from numerous studies has established that the basis of this GTP prerequisite reflects a

stage in the signal transduction process involving G-proteins (for review see Gilman,

1987; Linder and Gilman, 1992). The properties and roles of G-proteins are discussed

below and those of the other components associated with them, later.

1.15.1   STRUCTURE AND PROPERTIES

G-Proteins are heterotrimeric structures, composed of three subunits (α, β and γ) which

are separable by SDS-PAGE. To date, 21 different α-subunits (39-52 kDa on SDS-

PAGE) have been identified; the products of 17 genes. In addition, 5 types of β-subunits

and at least 6 forms of γ, migrating at 35-36 kDa and 8-11 kDa on SDS-PAGE

respectively, have been described. These numbers are expected to rise as it is believed

that there are many more of each subunit yet to be discovered.

The unique α-subunit of each G-protein is currently used to distinguish between the

heterotrimers though functional differences in some β- and γ-subunits have been proposed

(Cerione *et al.*, 1987).

Although they possess no obvious region for lipid bilayer attachment, G-proteins are

normally associated with the cytoplasmic surface of the plasma membrane with the

exception of transducin which is found on the intracellular retinal disc membrane. The

hydrophobic β- and γ-subunits usually exist as a tightly associated complex requiring

detergents for solubilization whereas the more hydrophilic α-subunits do not (Neer *et al.*, 1984; Sternweis, 1986). Sternweis, after observing that interactions of $G_s\alpha$ and $G_o\alpha$ with $G\beta\gamma$ (β and γ dimer) incorporated into phospholipid vesicles was abolished in the absence of the dimers, proposed that α-subunit attachment to the pasma membrane was mediated by $G\beta\gamma$ anchoring (Sternweis, 1986). Fatty acid modifications are present on a number of G-protein α- and γ-subunits. For certain G-proteins, myristoylation of the α-subunit, along with $G\beta\gamma$ association, is required for attachment to the plasma membrane (Mumby *et al.*, 1990; Linder *et al.*, 1991). In the case of $G_s\alpha$ which lacks lipid modification, the subunit may be attached directly to its effector, adenylyl cyclase (Arad *et al.*, 1984; Levitzki, 1987). The γ-subunit modification is one of prenylation and, combined with their hydrophobic nature serves to augment their role of anchor to the plasma membrane (Clapham and Neer, 1993).

The precise site of α and $\beta\gamma$-subunit interaction is as yet unidentified, but the N-terminal region of the α-subunit is believed to be involved as cleavage prevents its association to $G\beta\gamma$s in the plasma membrane (Eide *et al.*, 1987; Neer and Clapham, 1988). In addition to containing the binding sites for bacterial toxin substrates and guanine nucleotides, the α-subunit also functions as an enzyme due to its intrinsic GTPase activity. This ability to hydrolyse the terminal phosphate group of bound GTP to yield bound GDP and free inorganic phosphate (P_i), is an integral part of their role in mediating and controlling signal transduction.

Cholera toxin catalyses the ADP-ribosylation of a specific Arg residue in certain α-subunits (G_s, G_{olf}, G_t) while pertussis toxin ADP-ribosylates (G_i, G_t, and G_o) on a Cys residue (Gilman, 1984). The action of cholera toxin leads to constitutive activation of the α-subunits by reducing/inhibiting their intrinsic GTPase activity (Cassel and Selinger,

Table 1.1 SUMMARY OF G-PROTEIN PROPERTIES

G-Protein	α-subunit (kDa)[a]	Tissue Expression	Substrate for	Receptors	Function [b]
$G_{s(s)}$ * $G_{s(l)}$ *	45 - 52	Ubiquitous	CTX	β-AR, Glucagon, ADO, others	Adenylyl cyclase (+) Ca^{2+} channels (+) Na^+ channels (-)
G_i1 G_i2 G_i3	40 - 41	Brain, all tissues	PTX	α_2-AR, ADO, M_2-Cho, others	Adenylyl cyclase (-) Ca^{2+} channels (-) PLC (+)
G_oA G_oB	39 - 40	Brain, others	PTX	D_2-Dop, α_2-AR, others	K^+ channels (+)
G_q	42	Nearly ubiquitous	-	M_1-Cho, α_2-AR, others	PLC-β (+)
G_t1 G_t2	40	Retinal rods Retinal cones	CTX, PTX	Rhodopsin Cone opsin	cGMP PDE (+)
G_{olf}	45	Olfactory cells	CTX	Odorant	Adenylyl cyclase (+)

a: migration position of α-subunits on SDS-PAGE

b: (+) stimulatory ; (-) inhibitory

*: denotes two splice variants

CTX: Cholera toxin ; PTX: Pertussis toxin ; PLC: Phospholipase C ; PDE: Phosphodiesterase.

AR: adrenergic receptor ; ADO: adenosine receptor ; M-Cho: muscarinic cholinergic receptor ; Dop: dopaminergic receptor

1977; Abood *et al.*, 1982) and ultimately prolonging the activation of the effector system. Modification by pertussis toxin also leads to a reduction in GTPase activity, but in this case by promoting uncoupling of the G-protein α-subunit from receptor (Hsia *et al.*, 1984; Ui, 1984; Van dop *et al.*, 1984).

A general overview of G-protein subunit structure and function can be found in Neer (1995). A summary of the properties of some G-proteins is shown in Table 1.1.

1.15.2   MECHANISM OF G-PROTEIN ACTION

G-Proteins couple receptors to effector systems. Their functions are determined by the cyclic pattern of guanine nucleotide association and hydrolysis. GTP binding leads to the "switching on " of the G-proteins followed by consequent activation of the effector. Hydrolysis of GTP to GDP results in deactivation or "switching off ". The precise mechanism of G-protein mediated receptor-effector signalling is documented in a number of excellent reviews (Gilman, 1987; Milligan, 1988; Taylor, 1990a; Kaziro *et al.*,1991) and a brief summary of events is outlined here.

In the basal/resting state, the G-protein exists as a heterotrimer with GDP occupying the guanine nucleotide binding site of the α-subunit. G-Protein and receptor (R) interaction is catalysed upon binding of the receptor with agonist/hormone (H). G-Protein association with the activated HR complex promotes exchange of GDP for GTP on the α-subunit resulting in its subsequent activation. The GTP bound α-subunit is able to dissociate from the βγ complex to go on and activate the effector which is responsible for the generation of the second messenger inside the cell. Hydrolysis of GTP to GDP via the GTPase activity of the α-subunit, deactivates the subunit allowing its reassociation with the βγ dimer and restoring the G-protein to a state ready for another cycle of events.

Figure 1.5

G-Protein Mediated Transmembrane Signalling
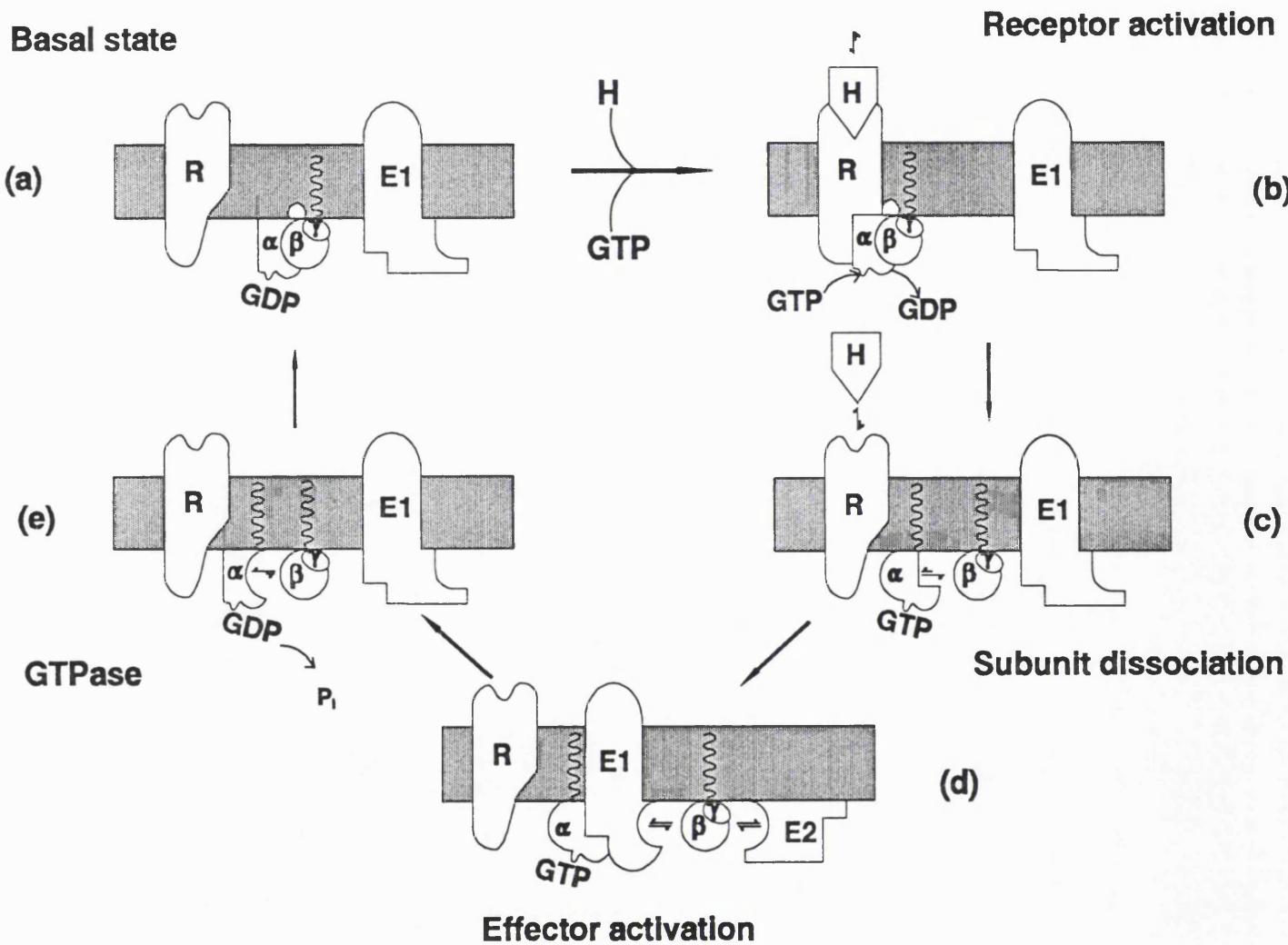
Adapted from Tang and Gilman (1992).

The entire process is highly regulated and controlled intrinsically. The liberation of GDP from the G-protein is the rate limiting step in the whole procedure (reviewed by Gilman, 1987). G-Protein interaction with HR promotes GDP release and, due to its high physiological concentration in the cell, GTP readily fills the empty site. Following activation by GTP binding, the G-protein dissociates from the HR complex which results in a reduction of agonist affinity for receptor. The HR complex disassociates and the receptor and agonist are free for further rounds of activation (Figure 1.5).

The relatively slow rate of GTP hydrolysis allows activation of effector to occur before hydrolysis ensues and hence determines the life time of the active α-subunit and the effector system.

At first, G-protein affinity for guanine nucleotides appears excessive given the high levels of GTP present in the cell, but is explained as it results in a relatively slow exchange rate of GTP for bound GDP thereby ensuring that spontaneous activation of G-proteins does not occur.

Mg^{2+} is a critical requirement for virtually all aspects of G-protein action and is extensively reviewed by Gilman (1987).

Activated receptors have high affinity for heterotrimeric G-proteins with the guanine nucleotide binding site empty, (Wessling-Resnick et al., 1987) and therefore not only promote GDP release but hold open the guanine nucleotide binding site for GTP (May and Ross, 1988).

Traditionally, the α-subunit has been regarded as the active end of the G-protein; while the $\beta\gamma$ dimer functions as the inhibitor or anchor, maintaining the G-protein in its inactive resting conformation (Gilman 1987). As mentioned earlier, GDP bound α-subunits alone are incapable of interacting with receptor and therefore need to reassociate with G$\beta\gamma$

before participating in another cycle of events. Furthermore, β is essential for presenting the α-subunit to the receptor prior to activation (Fung *et al.*, 1983; Weiss *et al.*, 1988; Florio and Sternweiss, 1989). Recently it has become increasingly apparent that the $\beta\gamma$ dimers have an even greater role in G-protein function, acting as regulators themselves and this will be discussed later on.

Finally, the whole process of heterotrimeric G-protein signal transduction is one of amplification such that a single agonist is capable of activating the catalytic actions of several effector molecules in the cell (Taylor, 1990a).

1.16 α-SUBUNITS

As mentioned earlier, the various α-subunits though responsible for regulating diverse signalling pathways, share common features in terms of amino acid sequences and overall signalling mechanism. Several highly conserved regions in the primary amino acid sequences have been observed between the α-subunits. They encode domains which are common to all G-protein functions. The site of receptor interaction has been assigned to the C-terminal of the α-subunit following evidence from mutational, peptide competition and pertussis toxin studies which prevented association between receptor and G-protein (Kaziro *et al.*, 1991). Recently, Conklin *et al.* (1993) found that the substition of just 3 amino acids in the C-terminal region of $G_q\alpha$, switched its receptor specificity to that of $G_i\alpha$. The C-terminal however, is not the sole determinant in receptor specificity as exemplified by the two splice variants of $G_o\alpha$ which couple to different receptors despite their identical 8 C-terminal amino acids (Kleuss *et al.*, 1991). The N-terminal region besides being the site of myristoylation, is also the site of $\beta\gamma$ binding as seen from proteolytic cleavage experiments. Finally, α-subunits posses sites for guanine nucleotide

and Mg^{2+} binding, ADP-ribosylation by bacterial toxin, and effector interaction (Kaziro *et al.*, 1991).

A more detailed account of the individual α-subunits follows.

1.16.1 $G_s\alpha$

Four distinct forms of $G_s\alpha$ have been identified, two short and two long. Cloning of the complete $G_s\alpha$ gene from the human genomic library established that all forms arise from the alternative splicing of a single gene transcript (Bray *et al.*, 1986; Kozasa *et al.*, 1988). The gene consists of 13 exons all of which are incorporated into the long forms, with exon 3 excluded from the short forms. In addition, the presence or absence of a serine at the start of exon 4 distinguish between the two short forms and between the two long forms of the α-subunit. The splice variants are most likely responsible for the diverse migration rates of $G_s\alpha$ observed on SDS-PAGE (45-52 kDa). $G_s\alpha$ participates in the hormonal stimulation of adenylyl cyclase which is responsible for generating the second messenger cAMP in a wide range of tissues. The subunit is a target for ADP-ribosylation by cholera toxin (CTX) which leads to its permanent activation. This trait, along with reconstitution studies involving $G_s\alpha$ and cyc⁻ S49 cell membranes (lacking in $G_s\alpha$), have led to the elucidation of the role of $G_s\alpha$ in signal transduction (Bourne *et al.*, 1975; Gill and Meren, 1978). The presence of non-hydrolysable GTP analogues also results in the persistent activation of adenylyl cyclase by the α-subunit (Ross *et al.*, 1978). Activation of $G_s\alpha$ is a slow, Mg^{2+}-dependant process, such that increases in concentration of the divalent ion are accompanied by a rise in rate and extent of adenylyl cyclase activation (Iyengar, 1981). It was first purified from rabbit liver by its ability to reconstitute cyc⁻ membrane adenylyl cyclase activity (Northup *et al.*, 1980) and has since been isolated

from other sources (Hanski *et al.*, 1981; Codina, 1984).

$G_s\alpha$ also activates voltage gated Ca^{2+} channels in cardiac and skeletal muscle (Imoto *et al.*, 1988; Yatani *et al.*, 1988a; Mattera *et al.*, 1989) and has been implicated in the inhibition of cardiac Na^+ channels in neonatal rat ventricles (Ono *et al.*, 1989).

The G-protein G_{olf}, involved in odorant signal transduction and found solely in olfactory cilia (Jones *et al.*, 1989), also activates adenylyl cyclase and shows 88% homology in amino acid sequence to $G_s\alpha$. Forskolin stimulation of adenylyl cyclase is potentiated by $G_s\alpha$, and vice versa (Darfler *et al.*, 1982) and will be discussed later.

1.16.2 $G_i\alpha$

The G-protein G_i was first identified by its role of inhibitor in the dual regulation of adenylyl cyclase. Three distinct $G_i\alpha$ cDNAs have been identified ($G_i1\alpha$, $G_i2\alpha$ and $G_i3\alpha$), their amino acid sequences showing more than 88% homology. All three forms are encoded by separate genes and are not the result of alternative splicing. They are of similar size with $G_i1\alpha$ and $G_i3\alpha$ migrating at 41 kDa, and $G_i2\alpha$ at 40 kDa on SDS-PAGE. G_i is as ubiquitously distributed as G_s though in greater abundance (Gilman, 1987), and their $G\beta\gamma s$ are structurally identical. Unlike $G_s\alpha$, the $G_i\alpha$ members are sensitive to modification by pertussis toxin (PTX) and though their precise roles are as yet undetermined, they all possess the ability to regulate potassium channels of cardiac myocytes (Yatani *et al.*, 1988b).

$G_i1\alpha$ is highly expressed in brain and neuronal cell lines. It has been implicated in the activation of Phospholipase C (PLC) and potassium channels, and in the inhibition of calcium channels (Kaziro *et al.*, 1991).

Distribution of the other two forms of G_i is also broad, with a degree of tissue specificity

as determined by Northern blot analysis (Kaziro *et al.*, 1991). $G_i2\alpha$ has been associated with the inhibition of adenylyl cyclase in some cell types following studies involving antisense oligonucleotides and specific antibodies. Moxham *et al.* (1993) observed a reduction in neonatal growth of transgenic mice following the introduction of $G_i2\alpha$ specific antisense RNA. Suppresion of the α-subunit was accompanied by a rise in cAMP concentration and loss of receptor mediated inhibition of adenylyl cyclase. A similar study focusing upon the role of $G_i2\alpha$ in PLC regulation, revealed an increase in basal and hormone mediated PLC activity (Watkins *et al.*, 1994). This implied that the G-protein regulated two distinct pathways, adding further support to the ever growing opinion of cross talk among G-proteins.

Light *et al.* (1989) found evidence for $G_i3\alpha$ mediated regulation of the renal epithelial cell line (A6) amiloride sensitive sodium channel via an indirect mechanism. $G_i3\alpha$ has also been implicated in events within the cell. Stow *et al.*, (1991) detected localisation of this α-subunit in the Golgi Apparatus and that over expression led to a decrease in the rate of vesicle transport and packaged protein secretion. In addition, this effect was abolished in the presence of PTX.

The $G_i\alpha$s are substrates for protein kinase A (PKA) and protein kinase C (PKC) (Watanabe *et al.*, 1988; Pyne *et al.*, 1989; Houslay *et al.*, 1991). Yatomi *et al.* (1992), working on platelets, proposed phosphorylation of $G_i\alpha$ by PKC as a potential inhibitory mechanism of receptor mediated intracellular Ca^{2+} mobilization systems. This feed back regulation of platelet activation would be essential to ensure minimal haemorrhaging at vascular injury sites which would otherwise lead to thrombosis or atherosclerosis. Work by Houslay and workers have implicated G_i phosphorylation as a key feature in Insulin resistant states (see section 1.20).

1.16.3 $G_o\alpha$

G_o was so named to distinguish it from G_s and G_i (O for other). It was first identified in bovine brain and is a member of the PTX sensitive G-proteins (Neer *et al.*, 1984; Sternweis and Robishaw, 1984). Two variant forms of the α-subunit have been identified termed $G_oA\alpha$ and $G_oB\alpha$. They result from the alternative splicing of a single gene and differ in the C-terminus. They migrate at 39-40 kDa on SDS-PAGE, and though not as widely distributed as other G-proteins, predominate in the CNS and the heart (Kaziro *et al.*, 1991; Watson and Arkinstall, 1994). G_o constitutes up to 2% of the total membrane protein in the brain and is responsible for the majority of guanine nucleotide binding seen in this organ (Sternweiss and Robishaw, 1984). There may be more forms of G_o however, as Katada's group have purified four G_o α-subunits from rat brain via anion exchange chromatography (Inanobe *et al.*, 1990).

Like other G-proteins, the α-subunits show selective interaction with $\beta\gamma$ dimers. They inhibit Ca^{2+} channel activity and, following PTX and reconstitution studies, appear to be implicted in PLC regulation and K^+ channel activation (Brabet *et al.*, 1990). The two $G_o\alpha$ splice variants are regulated during neuronal development with a transient increase in $G_oA\alpha$ observed in differentiating neurons (Asano *et al.*, 1992).

A $G_o\alpha$-like protein distinct from the major species and named $G_o*\alpha$, has also been identified in bovine brain, heart muscle and the adrenal medulla (Goldsmith *et al.*, 1988).

1.16.4 $G_q\alpha$

$G_q\alpha$ is a member of the Gq class of G-proteins which also include G_{11}, G_{14}, G_{15} and G_{16}. They are all insensitive to pertussis and cholera toxin, and appear as 42-43 kDa bands on SDS-PAGE. G_q is widely expressed in tissues and cell lines though absent in T-cell lines

(Simon *et al.*, 1991; Watson and Arkinstall, 1994).

$G_q\alpha$ and $G_{11}\alpha$ are very similar, exhibiting more than 88% homology in their amino acid sequences, and have been purified together from bovine brain and liver (Pang and Sternweis, 1990; Taylor *et al.*, 1990b). $G_q\alpha$ was first linked to the PLC signalling pathway after a novel 42 kDa, PTX non-sensitive, partially purified protein which activated PLC was found to have an identical sequence to $G_q\alpha$. Furthermore $G_q\alpha$, as well as $G_{11}\alpha$, was specific for the β iso-forms of PLC and not the γ or δ forms (Smrcka *et al.*, 1991; Taylor *et al.*, 1991). More evidence for G_q regulation of PLC came about following studies involving the expression of a constitutively active $G_q\alpha$ mutant which resulted in continuous phosphatidylinositol hydrolysis by PLC (Wu *et al.*, 1992a).

The other members of the G_q family also show particular distribution and activitation properties. For example, G_{11} and G_{14} are widely distributed with the former being particularly high in brain. In contrast, G_{15} and G_{16} are found only in the haematopoietic cells. Both $G_{11}\alpha$ and $G_{16}\alpha$ activate PLC isoforms $\beta 1$, $\beta 2$ and $\beta 3$, whereas G_{14} and G_{15} only activate PLC-$\beta 1$ (Watson and Arkinstall, 1994).

1.16.5   OTHER Gα SUBUNITS

G_t or $G_{transducin}$ exists as two forms, $G_t 1\alpha$ and $G_t 2\alpha$. They are highly expressed in retinal rod and cone cells respectively, where they play an important role in visual signal transduction. Both activate rod /cone specific cGMP phosphodiesterases following photoactivation (Stryer 1986; Stryer, 1991).

Gusducin (G_{gus}) is a relatively new G-protein expressed only in taste buds where it is probably involved in the perception of taste. It shows 80% homology with G_t specially

in the areas of receptor and effector interaction (McLaughlin *et al.*, 1992).

$G_z\alpha$ /$G_x\alpha$ is a 41 kDa protein found mainly in brain and neuronal cells exhibiting unique biochemical properties from the other G-protein α-subunits. $G_z\alpha$ exhibits much slower rates of guanine nucleotide exchange and its GTPase activity is 200-fold slower (Kcat = 0.05 min^{-1}) than other α-subunits (Watson and Arkinstall, 1994). $G_z\alpha$ is a target for phosphorylation by PKC and mediates the inhibition of adenylyl cyclase in a PTX/CTX insensitive manner (Wong *et al*, 1992). The α-subunit has also been implicated in PLC and K$^+$ channel regulation (Kaziro *et al.*, 1991).

G_{12} and G_{13} are other members of the PTX resistant class of G-proteins and although the mRNAs of their α-subunits are ubiquitously expressed, little is known of their function (Strathman and Simon, 1991; Watson and Arkinstall, 1994).

1.17 G$\beta\gamma$ SUBUNITS

At present, four forms of β and six of γ have been identified. The β-subunits run at 35 kDa (β1 and β4) and 36 kDa (β2 and β3) on SDS-PAGE; the γ-subunits between 8-11 kDa. They are widely distributed though there is some tissue specificity, e.g. β1 is found primarily in retina and rod cells where it associates with the α-subunit of transducin, β4 is abundant in the brain and the lungs (Simon *et al.*, 1991). Since the discovery of the various forms, it has become increasingly apparent that although a number of β and γ combinations are possible, there is selective coupling between the two subunits with each possessing a particular role(s) (Pronin and Gautam, 1992; Clapham and Neer, 1993; Iñiguez-Lluhi *et al.*, 1993). For example,β2 does not associate with γ1 and β2γ5 is capable of activating PLC-β2.

The γ-subunits are sites of lipid attachment and carboxymethylation and appear to be more

vital than β in determining the precise role of the dimer (Clapham and Neer, 1993).

Initial studies on G-protein mediated cell signalling centred around the α-subunits, but there is evidence that G$\beta\gamma$ plays a part too. Their first proposed role was the indirect inhibition of adenylyl cyclase by interacting and deactivating $G_s\alpha$ and later, studies on receptor and G-protein interaction showed that the $\beta\gamma$ complex was essential for guanine nucleotide exchange. The first sign that G$\beta\gamma$ could regulate effectors came from Logothetis' group. They found that the dimer was capable of activating heart myocardial K^+ channels (Logothetis et al., 1987) and since then, it has been accepted that G$\beta\gamma$ have a role of their own in regulating a wide range of receptor activated signalling pathways (Birnbaumer, 1992; Clapham and Neer, 1993; Iñiguez-Lluhi et al., 1993). Pitcher (1992) demonstrated that G$\beta\gamma$ could directly interact with the C-terminal region of β-adrenergic receptor kinase (βARK) thereby promoting localisation of the kinase to the membrane bound receptors. Phosphorylation of receptors by βARK lead to their desensitization, an important event in controlling the system's response to agonists. The same mode of desensitization is seen in other receptor types and appears to be another aspect of G$\beta\gamma$ involvement in cell signalling (reviewed in Haga et al., 1994).

G$\beta\gamma$s regulate the various isoforms of adenylyl cyclase in a direct manner with varying effects (review in Tang and Gilman, 1992). G$\beta\gamma$ also activates Phospholipase A2 (Jelsema and Axelrod, 1987), Phosphoinositide 3 kinase (Thomason et al., 1994) and is postulated to be the basis of the PTX sensitive activation of PLC observed in some cell systems (Katz et al., 1992).

The fact that both the $\beta\gamma$ complex and α-subunit serve as determinants for signal transduction, implies great diversity and cross talking in the regulation of hormone mediated signalling pathways.

1.18   EFFECTOR SYSTEMS

Cells, protected from their external environment by virtue of a plasma membrane, are capable of preventing hormonal and neurotransmitter signals from entering. Instead these signals are translated into second messengers which are generated inside the cell by a group of effector systems. Effector systems include adenylyl cyclase, PLC and ion channels. The second messengers produced elicit the appropriate response within the cell, as determined by the external messenger.

1.18.1   ADENYLYL CYCLASE

Adenylyl cyclase catalyses the formation of the second messenger cAMP from ATP. In the mammalian system cAMP is of major importance, regulating a wide range of events which include gene transcription, mitogenesis and metabolism. The second messenger is a short lived entity. It activates a cAMP dependent kinase (protein kinase A) in an allosteric manner which then goes on to phosphorylate a variety of specific enzymes in the cell thus yielding the appropriate agonist response. Six distinct mammalian forms of the cyclase have been identified and cloned (Watson and Arkinstall, 1994). All exhibit 35-67% homology and share common topology. There do however differ in terms of regulation (see later).

Though all of the forms are activated by $G_s\alpha$, the precise mechanism is unclear. Levitzki and colleagues maintain that G_s, or at least the α-subunit, remains associated with the enzyme throughout the cycle of signalling events. This stems from biochemical and kinetic studies which establish that G_s and adenylyl cyclase association is not rate limiting and that adenylyl cyclase activation is a first order reaction. This would not be the case if the two entities were separate (Tolkovsky *et al.*, 1982; Arad *et al.*, 1984; Levitzki,

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Figure 1.6

Regulation of Mammalian Adenylyl Cyclases
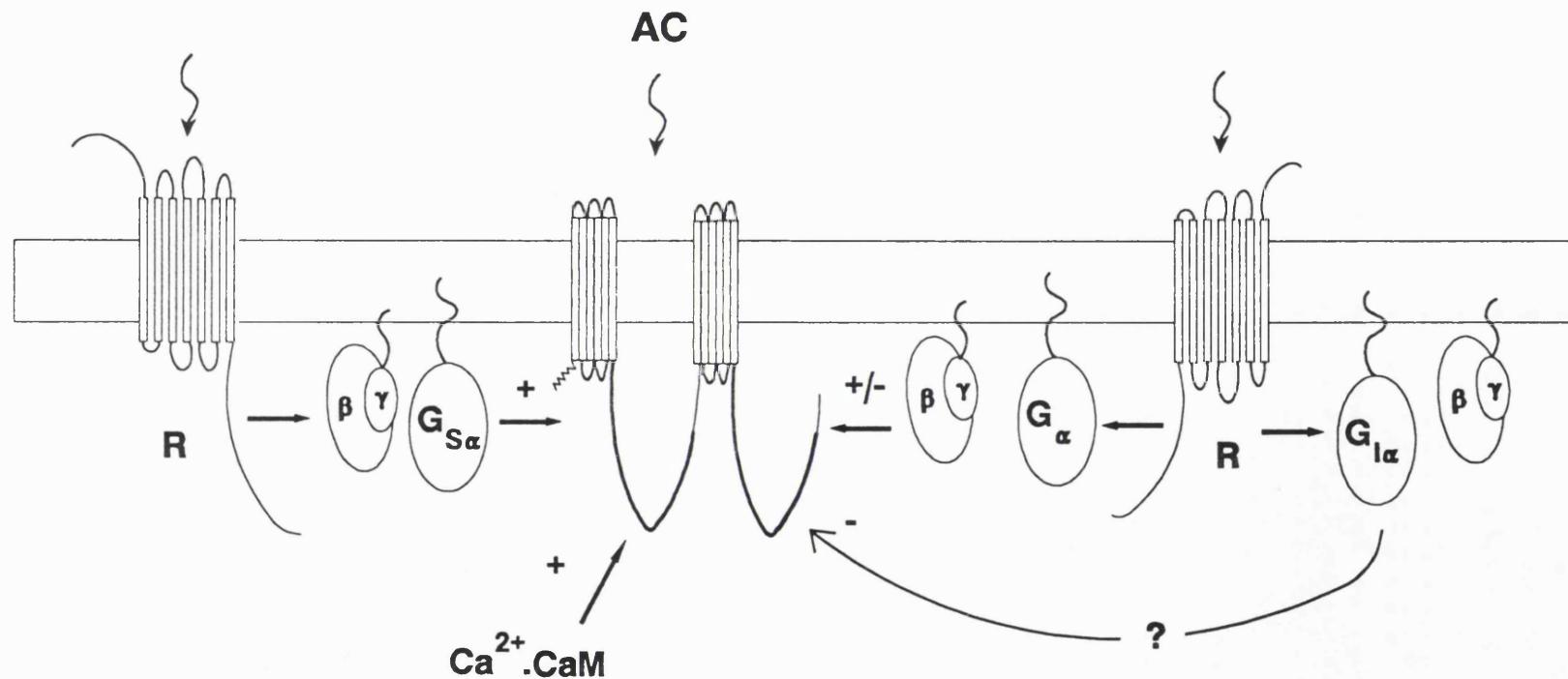
R, G protein-coupled receptors; G_s and G_i, G proteins that stimulate and inhibit adenylyl cyclase, respectively; $G\alpha$, any G protein α subunit.

Adapted from Tang and Gilman (1992).

1988). Their model of adenylyl cyclase activation incorporates inhibition of the system directly by G_i via its α-subunit, and indirectly by the release of their $G\beta\gamma$s which "mop" up $G_s\alpha$, deactivating it. The $G\beta\gamma$s of both G_s and G_i are found to be functionally interchangeable (Manning and Gilman, 1983).

Other groups, mainly Gilman's, disagree on the basis of G_s behaviour in detergent. They claim that the components in the system are all separate at the start of the cycle and that it is only after activation of G_s by GTP binding, that association of $G_s\alpha$ with adenylyl cyclase occurs. Once hydrolysis transpires, the two dissociate and the α-subunit reassociates with $G\beta\gamma$ (Gilman, 1987). This model also incorporates the same type of G_i mediated inhibition of adenylyl cyclase as proposed in Levitski's scheme. Direct inhibition by $G_i\alpha$ is thought to be minor due to failure to show a G_i-adenylyl cyclase interaction in reconstituted systems (Smigel, 1986) and the fact that $G\beta\gamma$ is more effective at inhibiting adenylyl cyclase in the presence of $G_s\alpha$ (Katada *et al.*, 1984).

To further add to the confusion, Levitski's group have found that purified Gpp[NH]p-activated adenylyl cyclase retains the β-subunit (Marbach *et al.*, 1990) supporting the hypothesis that dissociation is not part of adenylyl cyclase activation.

A diagrammatical representation of mammalian adenylyl cyclase regulation is shown in Figure 1.6.

The various types of mammalian adenylyl cyclase identified to date, all possess unique characteristics (reviewed by Tang and Gilman, 1992). Tang and Gilman (1991) verified that $G\beta\gamma$ stimulation of adenylyl cyclase types II and IV was direct. By using α-subunits of other G-proteins they found they could prevent activation and proposed that other G-proteins can mediate cAMP levels in the cell, providing $G_s\alpha$ was present. Federman *et*

Table 1.1 <u>PROPERTIES OF MAMMALIAN ADENYLYL CYCLASE</u>

Isoform	Expression	Effect of $G_s\alpha$	Effect of $\beta\gamma$	Effect of Ca^{2+}-calmodulin	Effect of Forskolin
I	Brain	+	-	+	+
II	Brain, Lung	+	+	o	+
III	Olfactory	+	o	-	+
IV	Brain, Others	+	+	o	+
V	Brain, Heart, Others	+	o	o	+
VI	Brain, Heart, Others	+	o	o	+

+ (stimulatory) ; - (inhibitory) ; o (no effect) ;

Adapted from Tang and Gilman (1992).

al. (1992) confirmed this idea from studies involving PTX. The toxin partially blocked $\beta\gamma$ stimulation of adenylyl cyclase implicating a PTX sensitive G-protein, i.e. G_i or G_o. These findings suggest that G_i could stimulate adenylyl cyclase by virtue of its $G\beta\gamma$ in the presence of activated $G_s\alpha$. Recently Taussig *et al.* (1994) detected type-specific regulation of adenylyl cyclase isoforms by G_i and G_o α-subunits. Compared to G_i, G_o was found to possess a lower affinity for the effector but as mentioned earlier, this G-protein is more abundant in the brain. Adenylyl cyclase types V, VI and the brain specific, Ca^{2+}-calmodulin sensitive type I isoform were found to be the most susceptible to this kind of inhibition. This has led to the idea that certain adenylyl cyclases, particularly types I and II, in addition to possessing binding sites for the α-subunits of specific G-proteins also have sites for $\beta\gamma$. In summary, adenylyl cyclase regulation appears to be more complicated than first imagined due mainly to the features of the different isoforms and to the added demonstration of $G\beta\gamma$ involvement. Agonists binding to receptors can therefore stimulate one effector pathway through α-subunits and a different pathway through $G\beta\gamma$ generating complex pathways in their intracellular response. Table 1.2 summarises the properties of the different adenylyl cyclase types.

1.18.1a   Forskolin and Na^+ effects

Adenylyl cyclase is under the dual regulation of two types of G-proteins (Rodbell, 1980). When the enzyme is assayed with respect to increasing concentrations of GTP, the result is a stimulatory phase followed by an inhibitory one. This bimodal pattern reflect the different GTP requirements of $G_s\alpha$ and $G_i\alpha$ for activation. In the fat cell system, the maximum stimulatory response occurs at approximately $0.05\mu M$ GTP and the inhibitory response at 0.1-$1\mu M$ GTP (Limbird, 1981; Cooper, 1982; Jakobs *et al.*, 1984a).

All isoforms of adenylyl cyclase are activated by forskolin, a diterpene from the root

Coleus forskohlii (Daly, 1984). The precise mode of activation is unclear. Stimulation

in the absence of G_s indicates a direct effect though for maximal activation the G-protein

is required (Seamon and Daly, 1981; Barovsky and Brooker, 1985). The diterpene can

act synergistically with agonists of the adenylyl cyclase system (Seamon and Daly, 1986)

with approximately 100 mM forskolin required for synergistic adenylyl cyclase activation,

a considerably lower concentration (10-100 fold) than that required for direct activation

of enzyme in the absence of G_s (Nelson and Seamon, 1988). In brain membranes,

forskolin has two types of affinity for adenylyl cyclase; high affinity interaction requires

G_s whilst the low affinity does not (Nelson and Seamon, 1986). The GTP non-

hydrolysable analogue Gpp[NH]p and other G_s activating agents (e.g. NaF, Mg^{2+}) increase

the maximum binding capacity (B_{max}) for forskolin at the high affinity site without

affecting K_D (Seamon and Daly, 1985). Nelson and Seamon (1986) proposed that this site

associates with activated G_s-adenylyl cyclase complex.

Under conditions of no agonist, monovalent cations ($Na^+ > Li^+ > K^+$) abolish the inhibitory

effect of GTP on adenylyl cyclase and the response to the guanine nucleotide resembles

that of a stimulatory system (Londos et al., 1981). If agonist is present, the salt has no

effect and the biphasic response of the system to GTP remains unaltered (Jakobs et al.,

1984b).

1.18.2   PHOSPHOLIPASE C

Phosphoinositide specific phospholipase C (PLC) is also linked to heterotrimeric G-

proteins. The second messengers generated play an important role in mediating the

cellular response to hormones, growth factors and neurotransmitters.

Agonist activated PLC catalyses the hydrolysis of membrane phosphotidylinositol 4,5

bisphosphate (PIP_2) to yield two messengers, inositol 1,4,5 trisphosphate (IP_3) and

diacylglycerol (DAG). IP_3 binds to specific receptors triggering release of Ca^{2+} from

intracellular stores. DAG activates protein kinase C (PKC) isoforms in conjunction with

membrane phospholipids and Ca^{2+} (for reviews see Berridge, 1987; Cockcroft and

Thomas, 1992; Nishizuka, 1992). Together, the two messengers initiate a chain of events

that result in the culmination of the cellular response.

PLC has been identified in both the cytosolic and membrane compartments of the cell

(Baldassare et al., 1989; Meldrum et al., 1989). Several isoforms of PLC have been

identified and are currently classified according to sequence homology though they can

also be distinguished by their enzyme purification and biochemical properties (Cockcroft

and Thomas, 1992). Although low overall sequence homology is observed among the

different isoforms (20-55%), two domains of higher homology have been identified (Rhee

et al., 1991). Proteolytic and mutation studies suggest these domains to be critical for

catalytic activity. Park et al. (1993a) found removal of the C-terminal region of PLC-β1

which is predominant in brain, led to loss of activation by $G_q\alpha$.

PLC-γ activation involves tyrosine kinase whilst that of PLC-δ is unknown. Only the β-

forms of PLC are under regulation by G-proteins. Evidence of G-protein involvement in

PLC function came from studies on membranes and permeabilized cells which

demonstrated a requirement for guanine nucleotides (Litosch, 1990). The α-subunits of

the G_q family activate PLC in a PTX insensitive manner, in particular PLC-β1 and PLC-

β3 (Taylor et al., 1990c; Smrcka et al., 1991; Wu et al., 1992b; Park et al., 1993b). This

however did not explain the PTX sensitive PLC regulation detected in some cells. The

discovery that G$\beta\gamma$s from PTX sensitive G-proteins could activate PLC-β2 (Katz et al.,

1992), led to the suggestion that this may be the basis of this aspect of PLC activation (Camps et al., 1992; Iñiguez-Lluhi et al., 1993). Current evidence suggests that $G\beta\gamma$ activates with the following efficacy, PLC-$\beta3$ > PLC-$\beta2$ > PLC-$\beta1$. In contrast, $G_q\alpha$ activates in the order PLC-$\beta1$ > PLC-$\beta2$ > PLC-$\beta3$, with PLC-$\beta4$ completely unaffected by both (Clapham and Neer, 1993). The most likely source of these $G\beta\gamma$s are G_o and G_i, though direct evidence is lacking. Other possible candidates for PTX sensitive PLC regulation are the α-subunits of G_o and G_i themselves (Brandt et al., 1985; Ohta et al., 1985; Moriaty et al., 1990; Blitzer et al., 1993). Furthermore, purified G_o and G_i have been reported to reactivate PLC activity after inactivation of endogenous G-proteins by PTX (Kikuchi et al., 1986; Banno et al., 1987) and Padrell et al. (1991) detected PLC activation by G_o in frog oocytes.

Like adenylyl cyclase, PLC regulation is far from simple. In a single cell, different agonists can stimulate PLC via PTX sensitive and insensitive pathways. In addition, different members of the G_q family selectively regulating the β-isoforms provide specific yet subtle differences in activation characteristics and which can be tailored to meet the requirements of a particular cell. The question of dual control has already begun to be answered from studies showing $G\beta\gamma$ and G-protein mediated inhibition of PLC (Moriaty et al., 1988; Litosch, 1989; Bizzarri et al., 1990; Van Geet et al., 1990).

1.18.3   ION CHANNELS

A large number of G-protein α- and $\beta\gamma$-subunits have been linked to ion channel regulation (Birnbaumer et al., 1990). Ion channels are present in all cell plasma membranes, particularly in excitable tissues, mediating many cellular events from synaptic transmission to secretion. The majority of studies involve patch clamping techniques and

whole cell recordings.

The first evidence that G-proteins could directly activate ion channels came from studies on Ca^{2+} and K^+ channels in myocytes, consequently promoting the idea of ion channels as effector systems. Ion channel regulation by agonist activated G-proteins occurs in a variety of tissues and is excellently reviewed by Hille (1992) and Clapham (1994). Breitweiser and Szabo (1985) activated muscarinic gated heart K^+ channels using non-hydrolysable GTP analogues and found the system to be PTX sensitive. Furthermore, activation of these channels in neurons resulted in the inhibition of post synaptic potentials (North, 1989). Schubert *et al.* (1989) demonstrated a decrease in Na^+ currents mediated by G_s in neonate rat ventricles. Ono *et al.* (1989) however, attributed this phenomenon to an indirect effect via cAMP rather than a direct one by the G-protein. In neuronal and pituitary cells, PTX sensitive G-proteins mediate the inhibitory response of voltage-gated Ca^{2+} channels to agonists and neurotransmitters (Linder *et al.*, 1990; Schmidt *et al.*, 1991). Van Dongen (1988) partially characterised four types of K^+ channels and demonstrated their activation by $G_o\alpha$. Kleuss *et al.* (1991), using anti sense oligonucleotides against G_o1 and G_o2, were able to inhibit Ca^{2+} channels in rat pituitary GH3 cells. Work focusing on $G\beta\gamma$s have revealed their involvement in cardiac muscarinic gated K^+ channels (Codina *et al.*, 1987; Logothetis *et al.*, 1987). Codina *et al.* (1987) further demonstrated activation by α-subunits occurred at a lower concentration than that needed for $G\beta\gamma$ mediated activiation, and that the two effects were not additive. Kim *et al.* (1989) suggest that $G\beta\gamma$ activation is not direct, but rather the result of Phospholipase A_2 action. Finally, as mentioned earlier, all three $G_i\alpha$s are able to activate K^+ channels (Yatani *et al.*, 1988b).

In neuronal and pituitary cells, agonists and transmitters are responsible for voltage

dependent Ca^{2+} channel inhibition which in turn is crucial for neurotransmitter release. This is believed to occur, depending on the type of receptor, via PTX sensitive and insensitive pathways (Heschler *et al.*, 1987; McFadzean *et al.*, 1989). In patch reconstitution studies, G_s was able to restore activity to run down L-type Ca^{2+} channels whilst G_o and G_i inhibited dihydropyridine sensitive Ca^{2+} channels (Yatani *et al.*, 1987; Schmidt *et al.*, 1991).

The central nervous system relies on transmitter action and alterations at that level would affect the brain in terms of cell signalling via membrane de- and re-polarization, and synaptic transmission. Finally, although Dolphin's group and others have provided sound evidence for G-protein coupling between neurotransmitter receptors and gated Ca^{2+} channels (Scott *et al.*, 1992) no firm proof of direct G-protein coupling to ion channels exists and until pure channel and G-protein preparations can be reconstituted in lipid bilayer systems, is unlikely to be forthcoming.

1.19 RECEPTORS

Receptors are widely distributed and respond to a diverse range of agents from amino acids and lipid analogues to specialised stimuli such as light and odour. Agonists elicit intracellular changes by first binding to appropriate cell membrane receptors. More than 100 G-protein coupled receptor subtypes have been cloned and sequenced enabling extensive molecular and biochemical studies to be performed. The discovery of conserved amino acid domains within the primary sequences suggests common ancestry and more than likely pertain to their shared functions and/or structures (reviewed by Strosberg, 1991). Each receptor family consists of several members which can themselves be further sub-divided. At least 5 types of muscarinic and dopamine receptors have been identified

along with several adrenergic, purinergic and adenosine receptors. All members of this superfamily contain 7 hydrophobic regions, each 20-25 amino acids in length. These regions span the membrane in the form of α-helices and are believed to be involved in agonist binding (Lefkowitz and Caron, 1988). The N-terminal and C-terminal regions house sites for N-linked glycosylation and palmitoylation respectively (Ovchinnikov et al., 1988; O'Dowd et al., 1989; Rands et al., 1990). Attempts to identify the region involved in G-protein coupling have been unsuccessful. This may reflect the specificity of receptors and hence there is no conserved region for this feature. Furthermore, it is possible that the overall 3-dimensional structure is necessary and cannot be deduced from primary sequence alone. A review of general receptor function and structure can be found in Savarese and Fraser (1992).

All G-protein-coupled receptors exhibit desensitization upon continuous exposure to agonist. This ability to adapt is mediated at the level of receptor and has been extensively characterised for the β_2-adrenergic receptor (β_2-AR) and the visual pigment receptor, rhodopsin (Dohlman et al., 1991). Long-term desensitization involves receptor internalisation and /or internalisation. In contrast, short-term desensitization is mediated by phosphorylation of specific serine and threonine residues. For β_2-AR, phosphorylation is effected via the actions of β-ARK and/or PKA (Benovic et al., 1986; Sibley et al., 1987). Similar modes of desensitization are seen in other receptor types (reviewed by Haga et al., 1994). Furthermore, Milligan and colleagues have noted that cellular levels of $G_s\alpha$, $G_o\alpha$ and $G_i\alpha$ are regulated in certain cell systems following agonist activation of their associated receptors (Mullaney et al., 1993). This feature may be a part of the down regulation phenomenon that occurs in cells following continuous activation by agonists. The recent realisation that these receptors are transcriptionally regulated by the second

messengers that they generate, also indicates another aspect of their control (Collins *et al.*, 1992).

There are increasing suggestions that a single receptor type can activate G-proteins of more than one family. Parker *et al.* (1991) detected coupling between M_2 muscarinic receptors and G_o, G_i and G_z while Bernstein *et al.* (1992) observed activation of M_1 type receptors elevated IP_3 and cAMP production, implying coupling to G_q and to G_s. Receptors are precise in their actions and though several G-proteins may converge onto a single effector, the extent of their modulation by individual receptors is highly specific. A detailed account of all the various receptors and their agonists is reviewed by Watson and Arkinstall (1994).

1.20   G-PROTEINS AND PATHOLOGICAL STATES

Given the vast array of cellular events mediated by G-proteins, it would be logical to assume that certain pathological states may in part be the result of abberations in G-protein function and/or expression. Several functional and structural assays have been developed to look for such changes. Functional studies include detection methods for GTPase activities, binding and reconstitution studies and experiments using non-hydrolysable guanine nucleotide analogues. Structural assays employed toxins (CTX and PTX) to radio-label the α-subunits as a means of quantitation. This unreliable procedure has been superseded by the advent of immunological probes; specific antibodies raised against defined areas in each G-protein subunit. The probes allow quantitation via immunoblotting and ELISA techniques. Oligonucleotide probes employed in Northern blot assays permit identification and quantitation of G-protein mRNAs in a similar manner.

Hormones are capable of regulating both G-protein levels and activities. Dysfunctions in signal transduction have been implicated in a number of disease and pathological states which include diabetes, thyroid disorders and alcoholism. Work carried out on induced disease states have provided insight into their causes and paved the way forward for possible methods of treatment.

In the diabetic state, loss of G_i mediated inhibition of adenylyl cyclase has been observed in liver and adipose tissues. In hepatocytes, this is attributed to a decrease in abundance of $G_i2\alpha$ and $G_i3\alpha$, and to the elevated levels of $G_i2\alpha$ phosphorylation by PKC (Katada *et al.*, 1985; Bushfield *et al.*, 1990). Tissue-specific changes in the mRNAs of $G_i\alpha$-subunits have also been detected in the diabetic rat (Griffiths *et al.*, 1990). Houslay (1989) has speculated that phosphorylation may be characteristic in many insulin resistant states and it remains to be seen whether it is a consequence or a contributing factor of the disease itself.

Abnormal G-protein levels have been detected in the brain tissues of Alzheimer patients (Cowburn *et al.*, 1992; Warpman *et al.*, 1993) and may underlie some of the effects of ethanol in the brain and other organs (Gordon *et al.*, 1992).

Extensive studies have been performed on abnormal thyroid states in a variety of tissues. In adipocytes, an impairment in lipolysis occurs. Saggerson (1986) showed that during hypothyroidism, adipocytes exhibit an increased response to anti-lipolytic agents (adenosine, prostaglandin E_1 and nicotinic acid). This has been ascribed to increased levels of the inhibitory α-subunits ($G_i\alpha$) of adenylyl cyclase (Ros *et al.*, 1988; Milligan and Saggerson, 1990). In ventricular membranes, Levine *et al.* (1990) observed increased levels of $G_i2\alpha$ and $G_i3\alpha$ in the hypothyroid state with no discernible affect on adenylyl cyclase activity. Adrenergic responsiveness is also altered in a range of tissues during

abnormal thyroid states and is reviewed by Bilezikien and Loeb (1983).

Indirect evidence for altered G-protein levels in the CNS during thyroid deficient times came from studies by Mazurkiewcz and Saggerson (1989). Working on synaptosomal membranes from rat forebrain, they demonstrated an increased response by adenylyl cyclase to the inhibitory agonist N^6-L-phenylisopropyladenosine (PIA) with no change in receptor number or basal and forskolin stimulated activity. This suggested lesions at the G-protein level and was later confirmed by Orford *et al.* (1991) who detected increases in synaptosomal membrane $G_i1\alpha$, $G_i2\alpha$ and $G_o\alpha$ levels in six anatomical brain regions of the adult hypothyroid rat. All three G-protein α-subunits were significantly increased in membranes from the cerebral cortex and the striatum whereas in the medulla oblongata and hippocampus only the abundances of $G_i2\alpha$ and $G_o\alpha$ were significantly elevated. By contrast, changes in $G_o\alpha$ and $G_i2\alpha$ were noted in the cerebellum and hypothalamus respectively. Subsequent measurements by the same group showed decreased levels of $G_i1\alpha$ and $G_i2\alpha$ in synaptosomal membranes isolated from cerebral cortex of adults rats following short-term administration of T_3 (Orford *et al.*, 1992). In the heart Michel-Rehter *et al.* (1993) observed a significant increases in $G_s\alpha$ during times of excess thyroid hormone.

Wong *et al.* (1994) performed similar G-protein studies on the developing rat cortex. A decrease in $G_s\alpha$ was observed with no discernible changes in $G_i\alpha$, $G_o\alpha$, $G_q\alpha$ or $G\beta$.

1.21   AIMS OF PROJECT

As G-proteins play such a central role in many signal transduction mechanisms, it is reasonable to assume that normal expression and subsequently function/activity of these signalling components play an important part in the successful programming of brain

development. In light of the observations of perturbed G-protein expression in various regions of the adult hypothyroid and hyperthyroid rat (Orford *et al.*, 1991: Orford *et al.*, 1992), it was decided to further the study of the altered thyroid hormone state on G-protein mediated signal transduction cell signalling processes at an earlier stage in life. The overall aim of the project was to investigate the effects of hypothyroidism on cell signalling components in the developing brain. This involved the development of an animal model of perinatal hypothyroidism. Success of the animal model was assessed via regular monitoring of animals and measurements of plasma thyroid hormone and metabolite levels. The use of specific G-protein α-subunit antisera enabled insight into the ontogenic profile of G-protein α-subunits in synaptosomal membranes of forebrain and hindbrain regions as well as providing immunological evidence of hypothyroid-induced effects on their expressions at various times of postnatal development. Altered G-protein abundance/function during the hypothyroid state were investigated further by assessing GTP effects on adenylyl cyclase activities of synaptosomal membranes from euthyroid and hypothyroid animals at day 15 postpartum to ascertain any perturbations in the activation/inhibition profile of the enzyme during the hypothyroid state.

CHAPTER 2
MATERIALS AND METHODS

2.1   MATERIALS

All reagents used were of the highest grade and, unless stated below, were purchased

from BDH Ltd., Poole, Dorset, U.K. and Sigma Chemical Company, Poole, Dorset, U.K.

In particular:

Adenosine-5-triphosphate (ATP), ammonium persulphate, acetylcholine iodide, fatty acid

poor bovine serum albumin (BSA), 4-chloro-1 naphthol, chloramine T, 5,5' dithiobis- (2-

nitrobenzoic acid) (DTNB), 2', 3'-cyclic NADP, Dowex 1 x 8-200 chloride, dimethyl

sulphoxide (DMSO), chromatographic alumina neutral type WN3, forskolin, gelatin, 3-

isobutyl-1 methyl xanthine (IBMX), 2-(4-iodo phenyl)-3-(4-nitrophenyl)-5-phenyl

tetrazolium chloride (INT), (2-N-morpholino) ethene sulphonic acid (MES), papavarine,

6-n-propylthiouracil (PTU), sodium dodecyl sulphate (SDS), SDS-PAGE prestained

markers, Tween 20, triethanolamine, tyrosine, Triton-X-100 were purchased from Sigma

Chemical Company, Poole, Dorset, U.K.

Acrylamide, N, N ' - methylene bisacrylamide, EDTA (disodium), glycine, bromophenol

blue, β- mercaptoethanol, N, N, N', N', - tetramethylethylenediamine (TEMED) were

obtained from BDH Ltd., Poole, Dorset, U.K.

Creatine kinase (from rabbit muscle), creatine phosphate (disodium), glucose-6-phosphate,

glucose-6-phosphate dehydrogenase (from yeast), guanosine-5'-triphosphate were

purchased from Boehringer Mannheim, Lewes, East Sussex, U.K.

Anti - rabbit IgG and horse-radish peroxidase-conjugated anti - rabbit IgG were purchased

from ICN Biomedicals, High Wycombe, Bucks., U.K.

Sodium [^{125}I]-iodide and cAMP enzyme immunoassay kit systems were bought from Amersham International PLC, Little Chalfont, Bucks., U.K.

Ficoll was obtained from Pharmacia LKB, Uppsala, Sweden.

Nitrocellulose sheets were purchased from Schleicher and Schuell (Dassel).

Hydrogen peroxide was bought from Fisons Scientific Apparatus, Loughborough, Leics., U.K.

Amerlex-M Free T_3 RIA Kit were purchased from Kodak Clinical Diagnostics Ltd., Amersham, Bucks, U.K.

VIDAS FT_4 assays were obtained from bioMérieux, Lyons, France.

WAKO Kits were supplied by Alpha Laboratories, Eastleigh, Hants, U.K.

ELISA 96 well microtitre plates and lids were bought from Flow Laboratories, Inc. McLean, Virginia, U.S.A.

Polyclonal G-protein α - subunit antibodies were a generous gift from Dr. G. Milligan, Department of Biochemistry, University of Glasgow, Scotland.

2.2   ANIMALS

Male and female rats of the Sprague Dawley strain were used. All animals had constant access to food and water *ad libitum* and were maintained at 21°C on a light/dark cycle of 13hr/11hr with light from 06:00 to 19:00hr. Animals were fed on Rat and Mouse No. 3 Breeding Diet (Special Diet Services, Witham, Essex, U.K.) which contained 21% digestible crude protein, 4% digestible crude oil and 29% starches and sugars (w/w). The weights of the mothers and pups were monitored during and after the pregnancy period.

2.2.1   INDUCTION OF HYPOTHYROIDISM

Animals rendered hypothyroid were fed on an iodine deficient version of the Rat and Mouse No. 3 Breeding Diet and drank water containing 0.01% (w/v) 6-n-propylthiouracil (PTU). Ethanol was used to dissolve the PTU in tap water and was present at 0.25% (w/v) final concentration (Chohan *et al.*, 1984). The PTU solution was replaced every other day with a freshly made batch.

2.2.2   MOTHERS

Female adult rats which had already experienced at least one pregnancy and weighing a minimum of 280 g, were used for breeding purposes. Early studies had shown that animals of this nature were better able to cope with the combined rigours of the hypothyroid regime and pregnancy. Approximately one day after conception (as judged by vaginal plugs), dams to be made hypothyroid were put on the low iodine diet (see section 2.2.1); euthyroid dams were maintained on normal diet and drinking water. These feeding regimens were continued throughout pregnancy and the suckling period.

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2.2.3　　PUPS

Rat pups used in the developmental studies were bred at the Institute of Neurology. Hypothyroid pups were born of adult female rats maintained on the low iodine/PTU regime described in section 2.2.2. Control pups were born of similarly weight matched mothers on normal diet and drinking water.

All litter sizes were standardised to eight pups per litter soon after birth and allowed to nurse off the mothers until reaching the appropriate age for study. Litter standardisation was necessary to ensure that all animals had the potential to develop at the same rate. It has been noted that during the time of brain development, even small variations in litter size are enough to produce wide differences in the developmental age of animals despite being the same chronological age (Dobbing, 1964; Irvine and Timiras, 1966).

2.3　　<u>BRAIN DISSECTION PROCEDURE</u>

After the animals were killed, intact brains were rapidly and carefully removed, and placed on a sheet of filter paper wetted with ice cold isolation media in a petri dish. Excess blood was removed by blotting and various brain regions were dissected as required. Forebrain and hindbrain regions were obtained from animals of 1, 10 and 15 days of age. For animals of 20 and 25 days of age, forebrain, cerebellum and medulla oblongata regions were dissected out.

The dissection procedure was based on the method detailed by Glowinski and Iverson (1966). The forebrain and hindbrain (cerebellum and medulla oblongata) were separated with a transverse section as shown in Figure 2.1a. When the cerebellum and medulla oblongata were required the hindbrain was further subdivided as shown in Figure 2.1b. The cerebellum was distinguishable from the medulla oblongata by its darker colour. It

should be noted that the forebrain separated out here is not homogeneous in nature and consists of many sub-regions (Figure 2.1b).

All brain regions were weighed and used to prepare membrane fractions as detailed in section 2.4.

2.4 PREPARATION OF SYNAPTOSOMAL, MITOCHONDRIAL AND MYELIN FRACTIONS FROM RAT BRAIN.

The earliest methods for the isolation of synaptosomes from brain employed sucrose gradients and required long high speed centrifugations. Due to the high concentrations of sucrose however, the final state of the synaptosomes obtained was less than satisfactory from a metabolic view point. The introduction of Ficoll as a gradient forming medium offered two advantages over previous methods in that: (1) it was possible to retain iso-osmolality during the gradient step; (2) the time of centrifugation was considerably reduced. The separation procedure employed in the present study for isolating synaptosomes (and subsequently synaptosomal membranes), relies on a flotation technique in a discontinuous Ficoll/sucrose gradient based on the method of Booth and Clark (1978) with a few modifications. All procedures were carried out on ice or at 4°C.

2.4.1 ADULTS

The following protocol was used to prepare fractions from three pooled adult forebrains. Following death of the animals by cervical dislocation and decapitation, peripheral plasma samples were collected (see section 2.6) and brains rapidly removed. Forebrains were dissected out and placed in ice-cold isolation medium (10 mM Tris-HCl buffer [pH 7.4] containing 0.32 M sucrose and 1 mM EDTA). The weighed tissue was finely chopped with scissors, and blood and other debris removed by carefully decanting off the excess

isolation medium and adding fresh isolation medium. The washing procedure was repeated until the excess isolation medium was fairly clean.

The chopped tissue was homogenised by hand in a glass-glass Dounce type homogeniser (8-12 up and down strokes, total clearance 0.1 mm) and diluted with isolation medium to give a final homogenate of 10-15% (w/v). The homogenate was centrifuged for 3 minutes at 1500 g_{av} in a Beckman J2-21/E high speed centrifuge fitted with a JA20 rotor. The supernatant was carefully decanted into fresh tubes, avoiding contamination by the nuclear pellet and centrifuged in the same rotor at 18,000 g_{av} for 10 minutes, producing the crude mitochondrial/synaptosomal pellet (P2).

The pellet was resuspended in isolation medium to give a final volume of 6ml and added to 31 ml 12% Ficoll/sucrose medium (10 mM Tris-HCl buffer [pH 7.4] containing 12% [w/v] Ficoll, 0.32 M sucrose and 50 mM EDTA) to give a 10% Ficoll suspension. 18 ml aliquots of this suspension were dispensed into two 37 ml polycarbonate centrifuge tubes. This was overlayed with 9 ml of 7% Ficoll/sucrose medium (10 mM Tris-HCl buffer [pH 7.4] containing 7% [w/v] Ficoll, 0.32 M sucrose and 50 mM EDTA) followed by 9 ml isolation medium forming a discontinuous Ficoll/sucrose gradient. The gradients were centrifuged in a SW28 swing out rotor at 110,000 g_{av} for 45 minutes in a Beckman L8 high speed ultra centrifuge. This resulted in the myelin and synaptosomal fractions separating out and banding at the first and second interfaces respectively, with the mitochondria forming a pellet at the bottom of the tube.

The myelin fraction was aspirated using a glass pipette, resuspended in 50 mM Tris-HCl (pH 7.4) and centrifuged at 105,000 g_{av} for 60 minutes in a Beckman L8 high speed ultra centrifuge fitted with a 70.1 Ti rotor. The resulting pellet was resuspended using a small loose fitting Potter-type homogeniser in 50 mM Tris-HCl (pH 7.4) and stored at -70°C.

The synaptosomal band was also removed and resuspended in 5 mM Tris-HCl (pH 8.0). The suspension was subjected to sonication for 30 seconds and then left on ice for 30-45 minutes. The combination of sonication and osmotic shock ensured lysis of the synaptosomes resulting in synaptosomal membranes. The membranes were then centrifuged at 105,000 g_{av} in the same rotor as for the myelin fraction and the pellet obtained resuspended in 50 mM Tris-HCl (pH 7.4) and stored at -70°C in 0.2 ml aliquots. The mitochondrial pellets were resuspended in isolation medium and stored at -70°C.

2.4.2   PUPS

Synaptosomal membrane preparations used in developmental studies were obtained from the brain regions of animals at 10, 15, 20 and 25 days of age and essentially followed the same procedure used for adult brains as detailed in section 2.4.1. In all preparations, tissue samples from one litter (eight pups of either sex) were pooled. At days 10 and 15 post partum brains were dissected into forebrain and hindbrain regions; at days 20 and 25 postpartum the hindbrain region was further dissected to yield cerebellum and medulla oblongata (section 2.3). Consequently, as the overall procedure involved smaller quantities of material, the volumes used were adjusted accordingly. In addition, the use of 13 ml polycarbonate tubes and buckets were employed in the Ficoll/sucrose stage.

A diagrammatical representation of the protocol is shown in Figure 2.2.

Figure 2.2

Synaptosomal Membrane Preparation

Brains mince

↓

Homogenise in
isolation media

1,500 g x 3 mins

S1 ———— P1

18,000 g x 10 mins

S2 (discard) ———— P2
(crude synaptosomal/
mitochondrial pellet)

Dilution with 12% Ficoll ⟶

2.5 ml isolation medium

2.5 ml 7.5% Ficoll

5 ml crude synaptosomes in 10% Ficoll

110,000 g

60 min

myelin band

synaptosomal band

mit. pellet

Synaptosomes resuspended in 5 mM Tris/HCL (pH 8.0)

↓ 30 sec sonication
30 min incubation on ice

Synaptosomal membranes

↓ 110,000 g x 60 mins

Synaptosomal membrane pellet
resuspended in 50 mM Tris/HCL (pH 7.4)

2.5 PREPARATION OF CRUDE MEMBRANES

Crude membranes were prepared from animals at 1 day of age. The pups used for this stage of study were those culled for standardisation purposes when litters were reduced to eight pups per litter.

Pups were rapidly decapitated and blood plasma obtained as detailed in section 2.6. The brains were carefully removed and dissected into forebrain and hindbrain regions (see section 2.3). The weighed tissue was finely chopped with scissors. Blood and other debris were removed by carefully decanting off excess isolation medium and adding fresh isolation medium. The washing procedure was repeated until the excess isolation medium was fairly clean. The chopped tissue was homogenised by hand in a glass-glass Dounce-type homogeniser and diluted to give a final homogenate of 10-15% (w/v) with isolation medium.

The homogenate was centrifuged for 3 minutes at 1500 g_{av} in a Beckman J2-21/E high speed centrifuge fitted with a JA20 rotor. The supernatant was carefully decanted into fresh tubes to prevent contamination by the nuclear pellet. The supernatant was centrifuged in the same rotor at 18,000 g_{av} for 10 minutes, thereby producing the crude mitochondrial/synaptosomal pellet (P2). The pellet was resuspended in 5 mM Tris-HCL (pH 8.0), sonicated for 30 seconds and left on ice for 30-45 minutes. The membranes were then centrifuged at 105,000 g_{av} for 60 minutes in a Beckman L8 high speed ultra centrifuge fitted with a 70.1 Ti rotor. The resulting pellets were resuspended in 50 mM Tris-HCl (pH 7.4) and stored at -70°C.

2.6 COLLECTION OF PLASMA

Plasma samples were obtained from all animals used in the study. Animals were killed via cervical dislocation, decapitated and peripheral blood collected in 10 ml siliconised glass beakers containing 50 μl heparin to prevent coagulation. The blood was transferred into 1.5 ml plastic Eppendorf tubes and spun on a bench top centrifuge for 2 minutes at 7000 g_{av}.

The resulting supernatant (plasma) was carefully aspirated using a disposable plastic syringe and needle, transferred into fresh Eppendorf tubes and stored at -70°C.

2.7 HORMONE AND METABOLITE MEASUREMENTS OF PLASMA

All plasma hormone and metabolite measurements and procedures were performed via specific programmed protocols set up on a COBAS FARA automated analyzer. This facility was generously provided by Dr J.M. Land (Department of Clinical Biochemistry, Institute of Neurology, London, U.K.).

2.7.1 FREE TRIIODOTHYRONINE (T_3)

Free T_3 levels were assayed using the Amerlex-M Free T_3 RIA kit from Kodak Clinical Diagnostics Ltd. The assay relies on the competition between a $[^{125}I]$-T_3 derivative and T_3 for a limited number of binding sites on an antibody. The amount of T_3 derivative bound is therefore related to the concentration of free T_3 in the plasma sample.

Reagents and samples were prepared according to manufacturer's recommendations. 500μl of $[^{125}I]$-T_3 derivative solution was dispensed into tubes containing 100 μl aliquots of standard and samples. To this was added 500 μl of Amerlex-M T_3 antibody suspension and the tubes mixed thoroughly. After incubation for 2 hours at 37°C the bound antibody

fraction was isolated via magnetic separation, and counted in a gamma counter. The free T_3 concentration was obtained from the standard curve generated during the course of the assay.

2.7.2 FREE THYROXINE (T_4)

Free T_4 levels were measured using the VIDAS FT_4 assay manufactured by bioMérieux. The method combines an enzyme immunoassay procedure with an enzyme linked fluorescent assay (ELFA) detection technique.

The kit included ready to use FT_4 strips which contained all the reagents necessary for the assay. 100 μl of sample, calibration or control were pipetted into the first sample well of the strip and the assay carried out on COBAS FARA.

2.7.3. 3-HYDROXYBUTYRATE

3-Hydroxybutyrate was assayed using a modified method by Harrison *et al.* (1988).

3-Hydroxybutyrate reacts with NAD^+ in the presence of 3-hydroxybutyrate dehydrogenase (HBD) forming acetoacetate and NADH.

The reagents used consist of a working enzyme buffer (2.5 mg NAD^+ and 80 μl HBD in 10 ml buffer; 0.1 M Tris [pH 8.5], 5% [v/v] hydrazine hydrate and 5 mM EDTA) and a 1 mM 3-hydroxybutyrate working standard. The assay was carried out via a programmed protocol on COBAS FARA.

2.7.4 LACTATE

Lactate measurements were obtained by following the increase of NADH formed by lactate dehydrogenase (LDH) action on lactate using a modified method of Wahlefeld

(1983). The reagents employed in the assay consisted of working buffer (20 mg NAD^+ in 10 ml buffer; 0.5 M glycine [pH 9.0], 5 mM EDTA, 2% (v/v) hydrazine hydrate) and a 2 mM lactate working standard solution. The working standard and samples were diluted 1:1 with 0.8 M and 0.4 M percholic acid respectively before testing on COBAS FARA.

2.7.5   NON ESTERIFIED FATTY ACIDS (NEFA)

NEFA levels were assessed using the WAKO kit supplied by Alpha Laboratories. The assay procedure, based on the method of Schimizu *et al.* (1980) was carried out on COBAS FARA. Briefly, NEFA in the presence of ATP and Mg^{2+} is acted upon by acylCoA synthetase to form acylCoA, AMP and pyrophosphate. AcylCoA reacts with acyl CoA oxidase to form hydrogen peroxide, which goes on to react with antipyrene in the presence of peroxide. The resultant purple colour can be measured at 550nm.

2.7.6   GLUCOSE

Glucose was measured by following the rate of its oxidation by glucose oxidase (Ziegenhorn *et al.*, 1977). Hydrogen peroxide formed as a result, reacts with 4-aminophanazone to produce a quinoneimine dye measurable at 520nm .

2.8   ESTIMATION OF PROTEIN

Protein concentrations were measured by the method of Lowry *et al.* (1951).

Tissue samples were made up to 0.2 ml with 0.1 M sodium hydroxide. Bovine serum albumin was used as a protein standard. Concentrations of 0, 20, 40, 60, 80 and 100 μg were used to construct a standard curve in a final volume of 0.2 ml. To each sample was

added 2 ml of solution A which consisted of 2% (w/v) sodium carbonate in 0.1 M sodium hydroxide, 5% (w/v) sodium potassium tartrate and 1% (w/v) copper sulphate [98:1:1(v/v)]. Tubes were mixed thoroughly and 50 μl undiluted Folin-Ciolcalteu reagent was added. After mixing, the tubes were left to stand for 30-60 minutes at room temperature allowing colour development to occur.

Absorbance measurements were carried out against a reaction blank containing water in place of tissue and albumin, on a Kontron Uvicon 940 spectrophotometer at 660nm. Protein concentrations of the samples were determined from the standard curve obtained.

2.9   QUANTITATIVE   IMMUNO-DETECTION   OF   G-PROTEIN α-SUBUNITS

The detection and quantitation of the G-Protein α-subunits involved a combination of various techniques incorporating SDS-Polyacrylamide gel electrophoresis (SDS-PAGE), Western blotting and immunostaining.

2.9.1   POLYACRYLAMIDE GEL ELECTROPHORESIS

Polyacrylamide gel electrophoresis was carried out in the presence of the dissociating agent sodium dodecyl sulphate (SDS), employing the method of Laemmli (1970) with minor modifications.

Gel slabs (14 cm x 13 cm) were formed in clean glass gel cassettes assembled from two glass plates separated by 1.5 mm thick spacers and held together by metal spring clips. The gel cassette assembly was clamped in an upright position and the bottom sealed with a quick setting gel before casting of the main resolving gel occurred. The resolving gel solution (10% [w/v] acrylamide, 0.27% [w/v] N, N'-bis-methylene acrylamide, 0.375 M

Tris-HCl [pH 8.8], 0.1% [w/v] SDS) was polymerised by the addition of N, N, N', N' - tetramethlethylenediamine (TEMED) and ammonium persulphate, both at final concentrations of 0.1% (v/v). The solution was gently mixed and poured into the gel cassette leaving sufficient space at the top for the stacking gel. A flat meniscus was obtained by carefully overlaying the top of the resolving gel with water-saturated-butanol and the gel left to polymerise for 45 to 60 minutes. Prior to casting the stacking gel, the overlay solution was completely washed off with distilled water to remove all traces of butanol. Polymerisation of the stacking gel (4.5% [w/v] acrylamide, 0.12% [w/v] N, N'-bis-methylene acrylamide, 0.125 M Tris-HCl [pH 6.8], 0.1% [w/v] SDS) was initiated as for the resolving gel. Sample wells were formed in the stacking gel during polymerisation by inserting a sample comb capable of forming ten wells at the top of the gel cassette. Care was taken to avoid the presence of any air bubbles and the gel left for a minimum of 45 minutes. After the gel had set, the comb was carefully removed thereby forming the sample wells.

During the simultaneous detection of $G_i1\alpha$ and $G_i2\alpha$ with the antibody SG2, a different resolving gel was employed. This resolving gel (12.5% [w/v] acrylamide, 0.063% [w/v] N, N'-bis-methylene acrylamide, 0.375 M Tris-HCl [pH 8.8], 0.1% [w/v] SDS) allowed the separation of the two different α-subunits during electrophoresis (Mitchell *et al.*, 1989). The 12.5% resolving gel was polymerised, overlayed and stacking gel applied as described above for the 10% resolving gel.

Prior to loading and running the gel, the gel cassette was attached to a conventional vertical electrophoresis gel apparatus and both electrode chambers filled with electrode buffer (0.025 M Tris [pH 8.3], 0.192 M glycine, 0.1% [w/v] SDS). Samples and prestained molecular weight markers prepared as described in section 2.9.3, were loaded

in the wells and the apparatus connected to a power supply. Electrophoresis was carried out under conditions of maximum voltage (450 V) and constant current of 40 mA per gel. Once the samples had entered the resolving gel the current was increased to 50 mA per gel.

2.9.2   MINI GELS

When appropriate, mini gels (5cm x 6 cm) were cast and run using the Mini-PROTEAN II® dual slab cell system from Biorad. Gels were cast in the same manner as for the larger sized gels i.e. resolving and stacking gel, with ten sample wells. Electrophoresis was carried out at 200 volts constant voltage for 45 to 60 minutes as recommended by the manufacturers.

2.9.3   PREPARATION OF SAMPLES FOR GEL ELECTROPHORESIS

Membrane samples were added to sample buffer (0.125 M Tris-HCl [pH 6.8], 2% [w/v] SDS, 5% [w/v] β-mercaptoethanol, 10% [w/v] glycerol, 0.001% [w/v] bromophenol blue, final concentration) and heated in a water bath at 100°C for 30 minutes to ensure complete denaturing of proteins. Samples were allowed to cool to room temperature before being loaded into the sample wells of the gel.

Prestained molecular weight markers were subjected to the same treatment and run on each gel. The molecular weight markers consisted of : α_2 - macroglobulin (180 kDa), β - galactosidase (116 kDa), fructose-6-phosphate kinase (84 kDa), pyruvate kinase (58 kDa), fumarase (48.5 kDa), lactate dehydrogenase (36.5 kDa) and triosephosphate isomerase (26.6 kDa).

2.9.4   WESTERN BLOTTING

The process whereby proteins, separated on a gel, are electrophoretically transferred and immobilised onto a support matrix (such as nitrocellulose) is known as Western blotting. Once transferred, the proteins are more readily accessible for analysis by a variety of techniques. The method employed here is that described by Towbin *et al.* (1979).

Sheets of Whatman 3MM filter paper and nitrocellulose membrane (0.45 μm pore size) were pre-soaked in Transfer Buffer (192 mM glycine, 25 mM Tris [pH 8.3]). A soaked Scotch-Brite pad was placed upon a rigid plastic grid followed by two sheets of filter paper and finally by the nitrocellulose sheet. The gel was recovered from the glass cassette assembly and placed on top of the nitrocellulose ensuring that all air bubbles were excluded. After trimming the nitrocellulose to the size of the gel, a further two pieces of filter paper were overlayed followed by a second Scotch-Brite pad. Finally, the top plastic grid was placed in position and the entire ensemble secured by elastic bands forming the transfer sandwich. The tight and even pressure between gel and nitrocellulose resulting from such an assembly ensured that efficient transfer of proteins occurred. The transfer sandwich was placed in a conventional electrophoresis tank filled with transfer buffer with the gel facing the anode. A power supply was connected and electrophoresis carried out for 1 hour at 50 volts.

2.9.5   IMMUNOLOGICAL DETECTION OF WESTERN BLOTS

The immunological detection of particular proteins which have been transferred onto a support matrix is known as immunoblotting. The technique relies upon the precise binding of antibody to certain proteins or parts of proteins. Nitrocellulose sheets that had undergone Western blotting as described in section 2.9.4 were subjected to

immunoblotting.

In order to minimize any non-specific binding of antiserum to the matrix, the nitrocellulose sheet was first blocked with a non-specific protein. This was done by incubating the sheet in 3% gelatin (w/v) in Tris-buffered saline (TBS; 20 mM Tris-HCl [pH 7.4], 500 mM NaCl) at 30°C for 90 minutes. The gelatin buffer was poured off and replaced with primary antiserum in 1% (w/v) gelatin-TBS at a dilution of 1:100 for $G_o\alpha$, $G_i1\alpha$ and $G_i2\alpha$, and at 1:500 for $G_q\alpha$. This was left overnight in a shaking water bath at 25°C. The following day the antiserum was removed and the nitrocellulose sheet washed extensively with distilled water followed by two 15 minute washes with TBS containing 0.05% (v/v) Tween-20 (TTBS) and then two further 15 minute washes with TBS. Secondary antiserum was added and left for 2 - 3 hours at 25°C. The secondary antiserum used consisted of a mixture of [^{125}I]-iodine labelled antibody (section 2.11) and horse-radish peroxidase conjugated antibody at final dilutions of 1:50 and 1:100 respectively in 1% (w/v) gelatin-TBS. Removal of the secondary antiserum was followed by the same sequence of washes of the nitrocellulose sheet as previously described. The blot was developed with 2.8 mM 4-chloro-1-naphthol, 0.5% (v/v) hydrogen peroxide in TBS as substrate for the peroxidase, thus enabling visualisation of cross reacting bands.

2.9.6   QUANTITATION OF G-PROTEIN α-SUBUNITS

The radioactive [^{125}I] - iodine label of the secondary antiserum enabled quantitation of the bands obtained. Following development, the nitrocellulose sheets were washed with water and TBS and left to air dry. Cross reacting bands were excised and radioactivity determined in a Nuclear Enterprise NE 1600 gamma counter. Equivalent sized unstained regions were also excised and counted to determine background which was subtracted

Figure 2.3 QUANTITATION OF G-PROTEIN α-SUBUNITS WITH RESPECT TO AMOUNT OF PROTEIN RESOLVED.

(a) $G_q\alpha$;   (b) $G_o\alpha$;   (c) $G_i\alpha$.

Synaptosomal membranes from forebrains of adult euthyroid rats were resolved by SDS-PAGE. G-protein α-subunits were quantitated as described in section 2.9 to demonstrate the linearity of the system.

$G_q\alpha$, $G_o\alpha$ and total $G_i\alpha$ were detected with antisera CQ1, OC1 and SG2 respectively. Quantitative experiments were performed using protein levels falling within the region of these curves. The values represent the means of two separate experiments.

Figure 2.3a $G_q\alpha$

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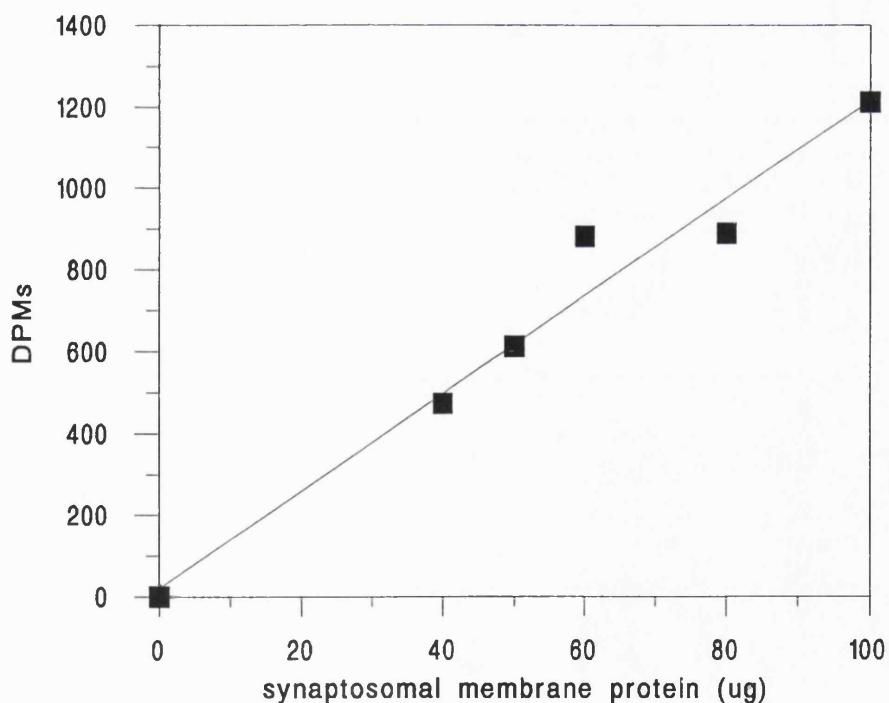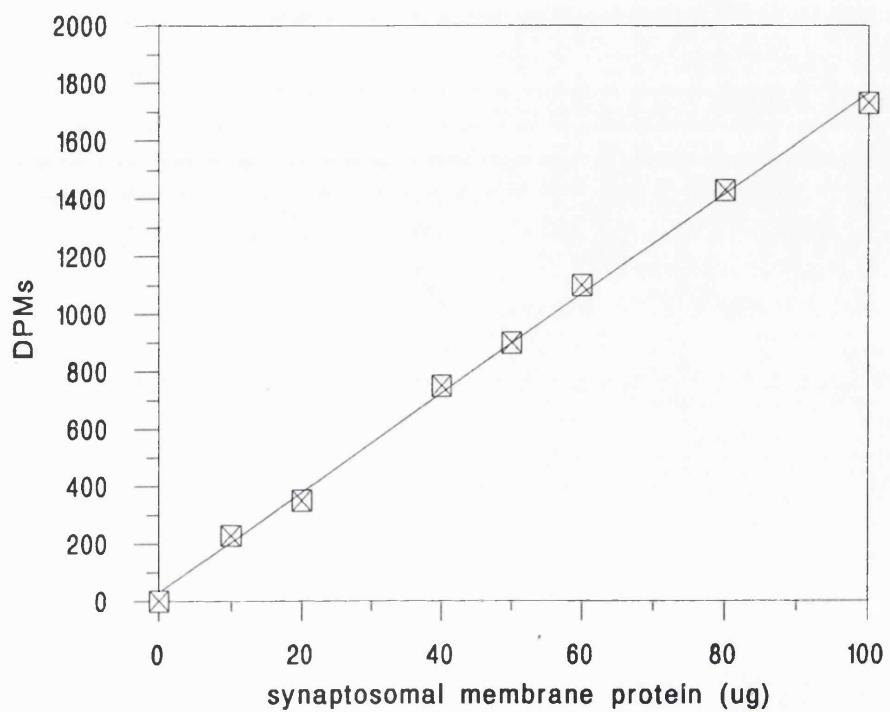
Figure 2.3b $G_o\alpha$

Figure 2.3c   Total $G_i\alpha$
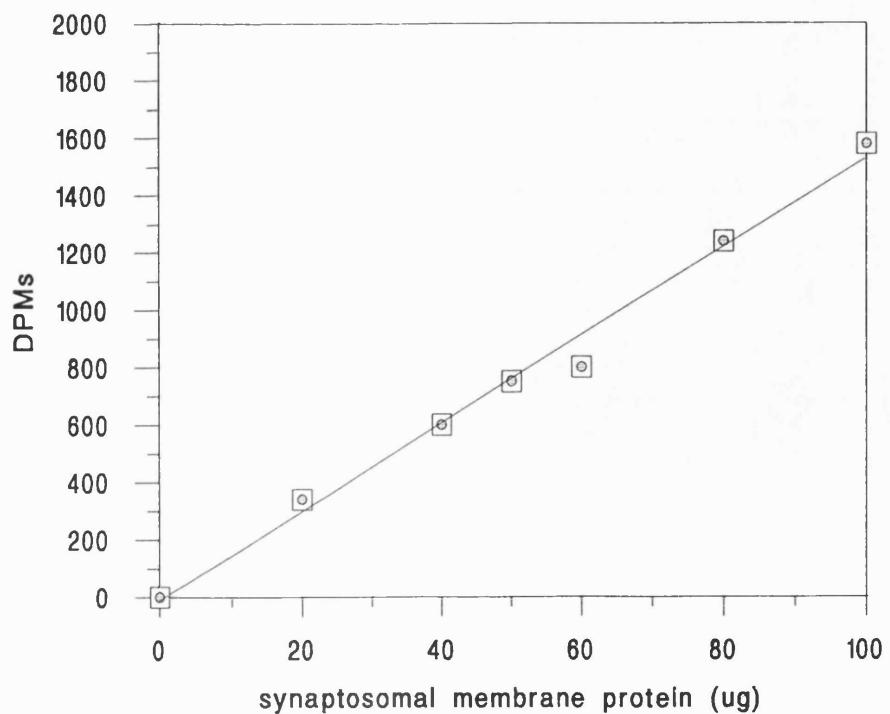
from all values. With the exception of data from day 1 membranes, membrane levels of the various α-subunits were expressed on a relative basis. In order to compensate for possible variations in the efficiency of electroelution onto nitrocellulose and to take into account of day-to-day variations in the specific radioactivity of ^{125}I-labelled secondary antibody, an aliquot of one particular synaptosomal membrane preparation isolated from the forebrains of 15 day old euthyroid pups was run as a reference standard on every gel. For each G-protein α-subunit band, (except those of crude membranes) this reference value was taken arbitrarily as 1.0 per unit quantity of membrane protein and other measurements from the same gel expressed relative to this. Crude membranes prepared from fore- and hindbrain regions of animals age 1 day postpartum were expressed as DPMs. For each antiserum, preliminary experiments established that quantification was linear with respect to amount of electrophoresed protein over the range tested (Figure 2.3).

2.10 ANTISERA

The primary antisera used in the immunodetection of Western blots were a generous gift from Dr. G. Milligan (Department of Biochemistry, University of Glasgow). The polyclonal antisera were raised in New Zealand white rabbits against synthetic peptides representing internal sequences of the various pertussis toxin sensitive G-Protein α-subunits.

Antiserum OC1 was raised against the decapeptide of amino acids 345-354 (ANNLRGCGLY) of $G_o\alpha$ and recognised both splice variants, $G_o1\alpha$ and $G_o2\alpha$.

The antiserum CQ1 used to detect $G_q\alpha$ was raised against the decapeptide QLNLKEYNLV and represents an internal sequence of both $G_q\alpha$ and $G_{11}\alpha$.

Figure 2.4   IMMUNOBLOTS OF SYNAPTOSOMAL MEMBRANE G-PROTEIN α-SUBUNITS VISUALISED USING SPECIFIC POLYCLONAL ANTISERA.

Crude and synaptosomal membranes were prepared from fore- and hindbrain regions of animals of various ages. Membrane G-Protein α-subunits were separated via SDS-PAGE and transferred onto nitrocellulose sheets. $G_q\alpha$, $G_o\alpha$ and $G_i\alpha$ were detected with antisera CQ1, OC1 and SG2 respectively and cross-reacting bands were visualised with a mixture of [^{125}I]-iodine labelled antibody and horseradish-peroxidase conjugated antibody.

Figure 2.4a   Immunoblot depicting visualisation of $G_q\alpha$ in crude and synaptosomal membrane fractions from 1 and 20 day-old euthyroid rat pup brain regions following detection with CQ1 antiserum.

C indicates Crude Membranes; F indicates Forebrain; MO indicates Medulla Oblongata.
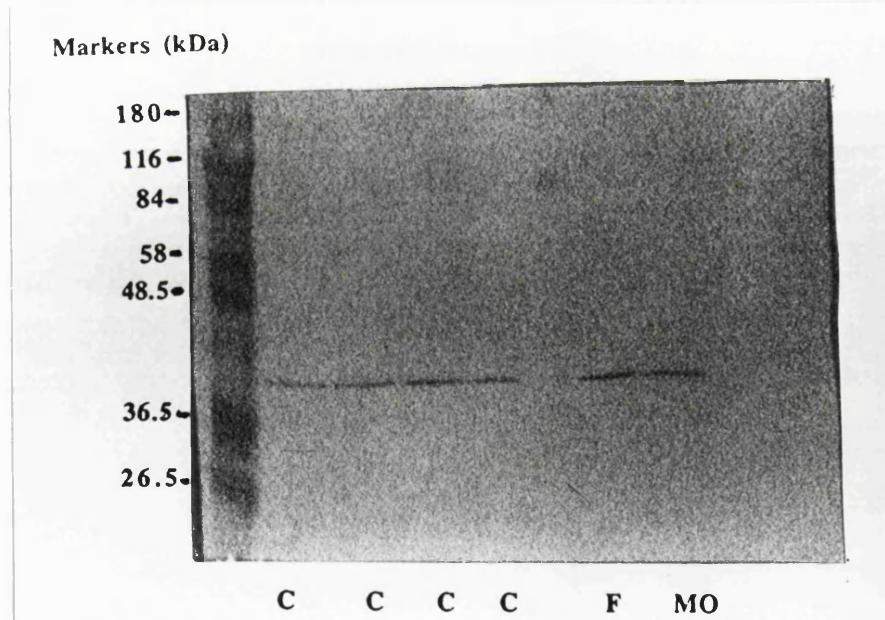
Figure 2.4b   Immunoblot depicting visualisation of $G_0\alpha$ in synaptosomal membranes of 10 day-old euthyroid and hypothyroid rat pups following detection with OC1 antiserum.

E indicates Euthyroid ; H indicates Hypothyroid.
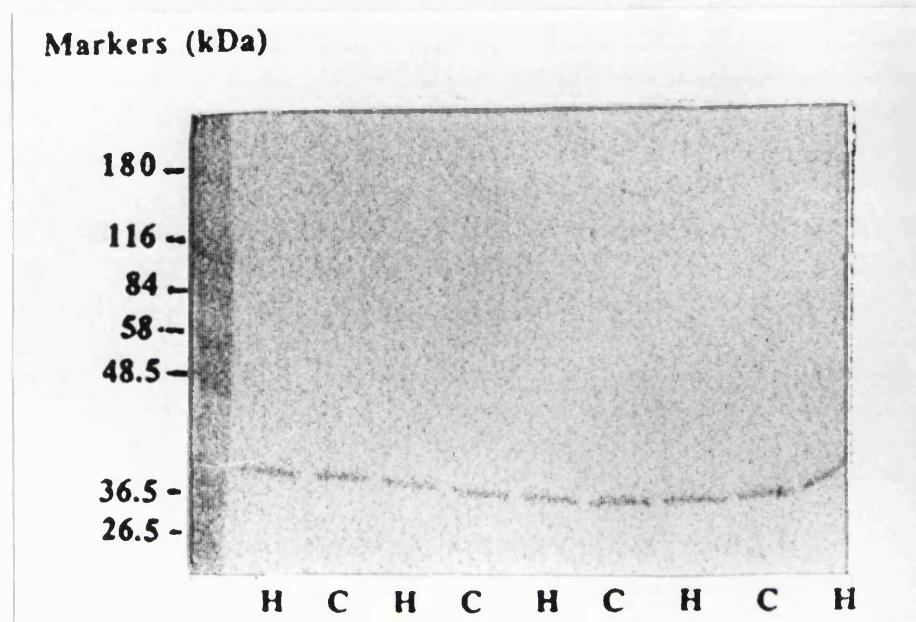
Figure 2.4c   Immunoblot depicting visualisation of $G_i1\alpha$ and $G_i2\alpha$ in crude and synaptosomal membranes of 15 day-old euthyroid and hypothyroid rat pups following detection with SG2 antiserum.

CF indicates Control Forebrain Reference; E indicates Euthyroid; H indicates Hypothyroid.

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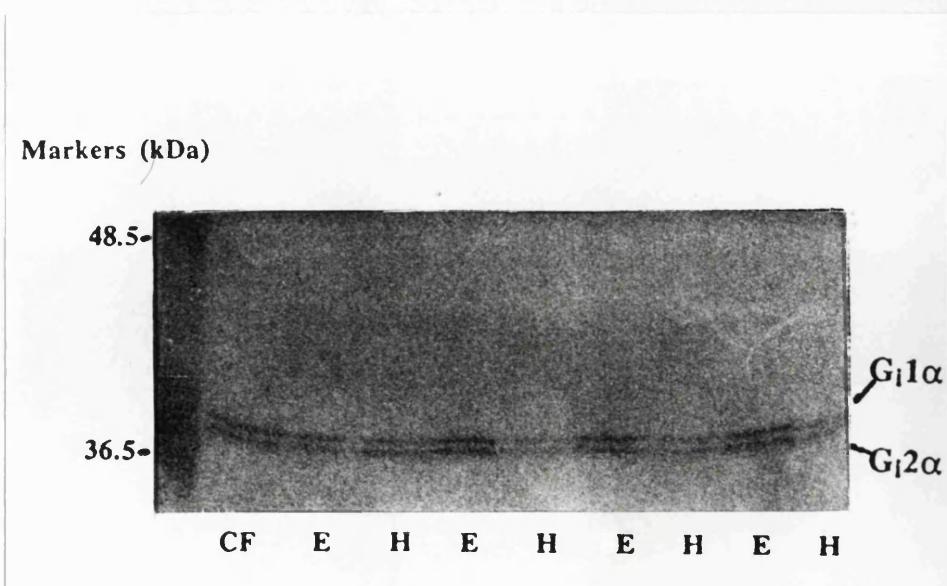
Antiserum SG2 was raised against a decapeptide sequence (KENLKDCGLF) of the transducin α-subunit and recognises both $G_i1\alpha$ and $G_i2\alpha$.

The α-subunits of the various G-Proteins were resolved on 10% acrylamide gels and detected with the appropriate antiserum with the exception of SG2. For adequate resolution and simultaneous detection of $G_i1\alpha$ and $G_i2\alpha$, a 12.5% acrylamide/0.063% bisacrylamide was employed as detailed in section 2.9.1.

An example of the immunoblotting results obtained with each of the G-protein α-subunit polyclonal antisera can be seen in Figure 2.4.

2.11   **IODINATION OF IMMUNOGLOBULINS**

Radioactive iodine labelled IgG was prepared via a modified method by Greenwood *et al.* (1963).

2.11.1   **IODINATION PROCEDURE**

An anion exchange medium, consisting of a slurry of Dowex 1 x 8-200 (chloride form) mixed with distilled water, was poured into disposable columns prepared from 3 ml plastic Pasteur pipettes. The Pasteurs were trimmed of their tops and plugged with glass wool. A sufficient amount of exchange media was poured into the Pasteur pipettes to give a final bed volume of 1ml (9-10 cm in height).

The columns were equilibrated with a minimum of 8 column volumes of 0.1 M sodium phosphate buffer (pH 7.5) containing 0.2% (w/v) BSA. The stock solution of sodium $[^{125}I]$- iodine was diluted down to 1 mCi/ml with distilled water.

Into small plastic disposable vials was added 100 μl diluted sodium $[^{125}I]$- iodine followed by 20 μl anti-rabbit IgG antibody and 10 μl 0.1 M sodium phosphate buffer (pH 7.5).

Addition of 10 μl of 18 mM chloramine T (dissolved in phosphate buffer) initiated the reaction, which was allowed to proceed for 2 minutes before being terminated with 50 μl 2.2 mM tyrosine (dissolved in phosphate buffer). The contents of the vial were then applied to a column along with 2 ml sodium phosphate buffer (pH 7.5), thereby allowing the elution of the iodinated protein and retention of the iodinated tyrosine complex.

2.11.2   VALIDATION OF IODINATION PROCEDURE

The iodination success rate was checked by measuring the ratio of bound iodine to free iodine.

10 μl of the eluate was diluted to 1 ml with phosphate buffer of which 10 μl was added to 200 μl BSA solution (100 mg/ml BSA in phosphate buffer) in Eppendorf tubes and mixed.

To this was added 1 ml of ice-cold 10% (w/v) trichloroacetic acid to precipitate protein. The tubes were spun in an Eppendorf bench top centrifuge at 7,000 g_{av} for 5 minutes. The supernatant was carefully removed without disturbing the pellet, and the radioactivity of both the supernatant and the pellet determined on a Nuclear Enterprise NE 1600 gamma-counter.

The percentage of conjugated iodine was determined as follows:

$$\frac{\text{counts of the pellet}}{(\text{counts of the pellet} + \text{counts of the supernatant})} \times 100$$

A figure in the 95-99% range indicated a successful iodination process. In general, specific activity of the iodinated protein fell in the 30-40 million cpm/ml range for

immunoglobulins.

2.12 SPECTROPHOTOMETRIC ENZYME ASSAYS

2.12.1 ACETYLCHOLINESTERASE (EC 3.1.1.7)

The synaptic membrane marker, acetylcholinesterase, was assayed spectrophotometrically via the method of Ellman *et al.* (1961).

The reaction follows the rate at which the yellow anion of 5-thio-2-nitro benzoic acid is formed when the 5: 5-dithio bis-2-nitro benzoate ion reacts with thiocholine. The latter is formed as a result of the action of acetylcholinesterase on acetylthiocholine.

The assay mixture, consisted of 0.2 M potassium phosphate buffer (pH 8.0), 10 mM dithio bisnitro benzoic acid (DTNB), 10% Triton -X -100 (w/v), 10 - 50 μl tissue extract, in a final volume of 1 ml. Addition of 10 μl 75 mM acetylcholine iodide initiated the reaction which was followed at 25 °C on a Kontron Uvikon 940 spectrophotometer in 1 ml plastic cuvettes. Absorbance changes were measured against a reagent blank containing all assay constituents with the exception of acetylcholine iodide at 412nm.

Acetylcholinesterase activity was calculated from the molar extinction coefficient for 5-thio-2-nitro benzoic acid of 13.6μmol^{-1}ml at 412nm.

2.12.2 2', 3' - CYCLIC NUCLEOTIDE 3' - PHOSPHOHYDROLASE (CNP) (EC 3.1.4.37)

The myelin marker 2', 3' - cyclic nucleotide 3' - phosphohydrolase (CNP) was assayed spectrophotometrically via the coupled assay described by Sogin (1976).

CNP acts upon 2', 3'-cyclic NADP, forming the 2' phospho compound which is subsequently utilized in the dehydrogenation of glucose-6-phosphate. The NADPH

formed as a result is then measured at 340nm.

The assay mixture, consisted of 0.2 M MES buffer (pH 6.0), 1 mM 2 ', 3'-cyclic NADP, 5 mM glucose-6-phosphate, 0.6 units/ml glucose-6-phosphate dehydrogenase, 30 mM $MgCl_2$, in a final volume of 1 ml. Addition of 20-50 μl tissue extract which had been diluted 30 fold with 1% Triton- X -100 (w/v) 0.2% BSA (w/v), initiated the reaction which was followed on a Kontron Uvikon 940 spectrophotometer at 25°C in 1 ml plastic cuvettes. Absorbance changes were measured against a reaction blank which contained all assay constituents with the exception of tissue extract at 340nm.

CNP activity was calculated from the molar extinction coefficient for NADPH of $6.22\mu mol^{-1}ml$ at 340nm.

2.12.3   SUCCINATE DEHYDROGENASE (EC 1.3.99.1)

The mitochondrial inner membrane marker enzyme succinate dehydrogenase (SDH) was assayed spectrophotometrically via the method of Jenkins and Peters (1978).

The assay uses 2(p-iodophenyl) - 3 - (p-nitrophenyl- 5- phenyl tetrazolium chloride) (INT) as a synthetic electron acceptor.

The reaction was carried out in glass test tubes containing 0.1 ml 0.3 M sodium succinate (pH 7.4) and 0.1 ml 0.3 M sodium phosphate (pH 7.4) containing 4 mg/ml INT. The tubes were preincubated at 37 °C for 5-10 minutes before the addition of 100 μg of tissue in a volume of 0.2 ml. The tubes were then incubated for a further 15 minutes at 37 °C after which time the reaction was terminated by addition of 2.4 ml solvent mixture consisting of ethanol (v/v) : ethylacetate: 10% TCA (w/v) (130:200:20).

The tubes were mixed, centrifuged for 10 minutes at 1,500 rpm on a benchtop centrifuge to remove denatured protein and read in quartz cuvettes at 25 °C on a Kontron Uvicam

940 spectrophotometer. Absorbance changes were measured against a reagent blank containing all assay constituents with water instead of tissue at 420nm.

Succinate dehydrogenase activity was calculated using the molar extinction coefficient for INT of 20.1μmol^{-1}ml at 420nm.

2.12.4   CALCULATION OF ENZYME ACTIVITIES

Enzyme activities obtained from the spectrophotometric assays (section 2.12) were calculated using the Beer Lambert Law below and expressed as nmoles/mg protein/min.

$$A = \epsilon \,.\, c \,.\, l$$

where   A = change in absorbance

ϵ = molar extinction coefficient

l = optical path length (1 cm)

c = concentration.

2.13   ASSAY OF ADENYLYL CYCLASE (EC 4.6.1.1)

The adenylyl cyclase assay employed was a minor modification of the combined methods of Cooper and Londos (1979) and Sharma *et al.* (1982).

Adenylyl cyclase activity was assessed by measuring the amount of cAMP released via the cAMP assay detailed in section 2.14.2.

2.13.1   ADENYLYL CYCLASE ASSAY PROCEDURE

Adenylyl cyclase was assayed in a medium containing 25 mM triethanolamine (pH 7.4), 1mM EDTA, 5 mM MgSO$_4$, 10 μM papavarine, 1 mM ATP, 5 mM creatine phosphate, 26 units/ml creatine phosphokinase in a final volume of 0.2 ml. The reaction was initiated

Figure 2.5 <u>TIME COURSE OF BASAL AND FORSKOLIN-STIMULATED ADENYLYL CYCLASE ACTIVITIES IN BRAIN SYNAPTOSOMAL MEMBRANES OF 15-DAY OLD PUPS.</u>

(a) Forebrain ;   (b) Hindbrain.

Synaptosomal membranes were prepared from brain regions of 15 day old euthyroid and hypothyroid pups (section 2.4).   Adenylyl cyclase was assayed as described in section 2.14 in the presence and absence of 10 μM Forskolin at the times indicated.

FIGURE 2.5a   FOREBRAIN

◇ Euth basal   ♦ Hypo basal
□ Euth forskolin   ■ Hypo forskolin

FIGURE 2.5b   HINDBRAIN

◇ Euth basal   ♦ Hypo basal
□ Euth forskolin   ■ Hypo forskolin

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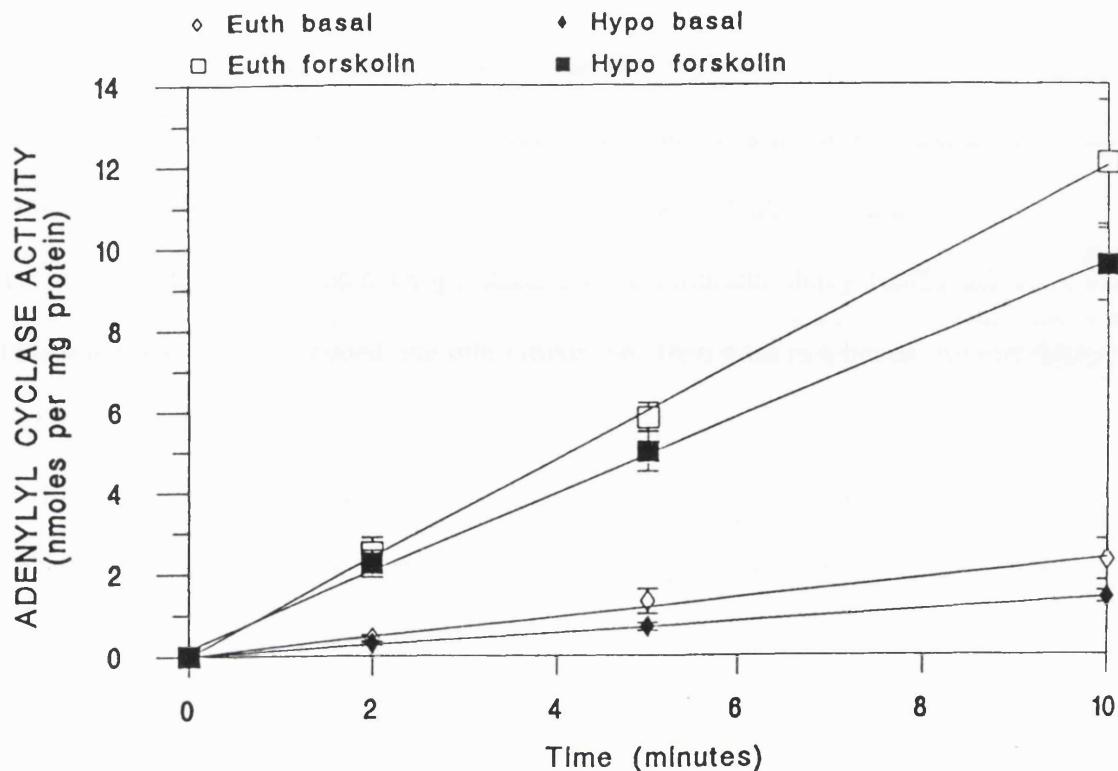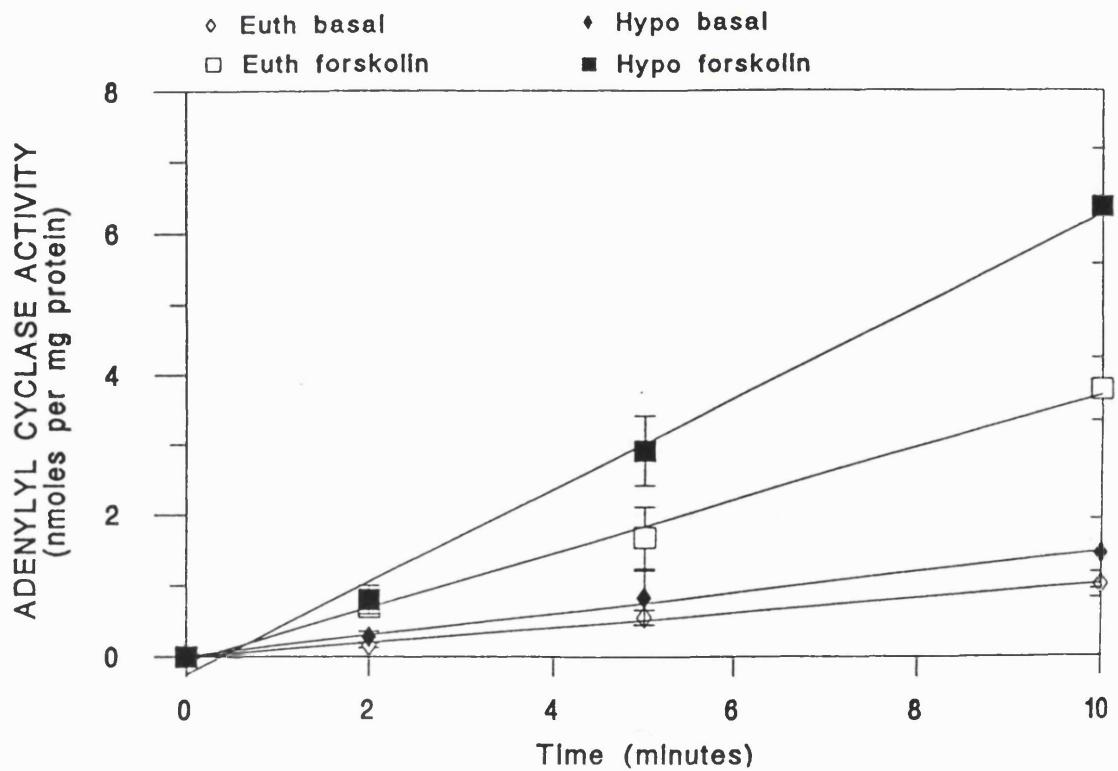
by the addition of either 2.5 μg protein (basal activity) or 1.0 μg protein (forskolin stimulated activity) and allowed to proceed for 5 minutes at 30°C. Reaction were terminated by immersing the reaction tubes in a 90°C water bath for 3 minutes. The tubes were allowed to cool to room temperature, 200 μl Alumina slurry (0.45g/ml 50 mM triethanolamine [pH 7.6]) added, the tubes mixed and then spun in a bench top centrifuge for 2 minutes at 7000 g_{av}.

50 μl aliquots of the supernatant were assayed for cAMP as described in 2.14.2.

The assay was used to look at the effects of GTP upon forskolin stimulated adenylyl cyclase activity in the presence and absence of Na^+.

Enzyme activity was established to be linear with respect to time under basal and forskolin stimulated conditions in both forebrain and hindbrain regions (Figures 2.5a and 2.5b).

2.14   cAMP ASSAY

cAMP was assayed using an enzymeimmunoassay (EIA) purchased from Amersham International PLC.

2.14.1   PRINCIPLE OF ASSAY

The assay relies upon the competition between unlabelled and labelled cAMP, for a limited number of binding sites on a cAMP specific antibody. As the amounts of antibody and peroxidase-labelled cAMP are constant, the amount of the peroxidase-labelled ligand bound to the antibody is related to the amount of added unlabelled ligand. The peroxidase ligand / antibody complex is immobilized onto microtitre wells which have been pre-coated with secondary antibody. Unbound ligand is removed by a series of washes. The addition of a tetramethylbenzine (TMB)/hydrogen peroxide substrate

ascertains the amount of peroxide labelled cAMP bound via a microtitre plate reader. A summary of the enzyme immunoassay procedure is shown in Figure 2.6.

2.14.2   cAMP ASSAY PROCEDURE

cAMP was assayed on 8 x 12 well microtitre plates coated with donkey anti-rabbit IgG antibody using the non-acetylation procedure (range 12.5 - 3200 fmol/well). Reagents were prepared according to manufacturer's recommendations and allowed to equilibrate to room temperature prior to use.

Working standards (12.5 - 3200 fmol) were set up via a series of successive doubling dilutions. 100 μl of each standard was pipetted into each well setting up the standard curve. Non - specific binding (NSB) and zero standard (Bo) values were determined by pipetting 200 μl and 100 μl assay buffer (0.05 M sodium acetate buffer [pH 5.8] containing 0.02% BSA) respectively into separate wells. In addition, two wells were left empty at the start of the plate and used to blank the machine at time of reading (blanks). 100 μl aliquots of unknown sample from the adenylyl cyclase assay (section 2.13.1) were pipetted into the rest of the wells.   All samples (standards and unknowns), were assayed in duplicate. 100 μl rabbit anti-cAMP was added into all wells except the blank and NSB wells. The plate was covered, gently shaken and incubated at 3 - 5°C for exactly 2 hours. Care was taken to ensure that the assay temperature did not exceed 5°C during the course of the assay by leaving the plate on ice throughout the incubation time. After such time, 50 μl cAMP-peroxidase conjugate was carefully pipetted into all wells except the blank wells. The plate was gently shaken and incubated for exactly 1 hour at 3 - 5°C. The wells were aspirated, washed four times with wash buffer (0.01 M phosphate buffer [pH 7.5] containing 0.05 % Tween 20) and blotted dry on tissue paper. Any residual liquid

Figure 2.6 — Summary of cAMP Ensyme immunoassay
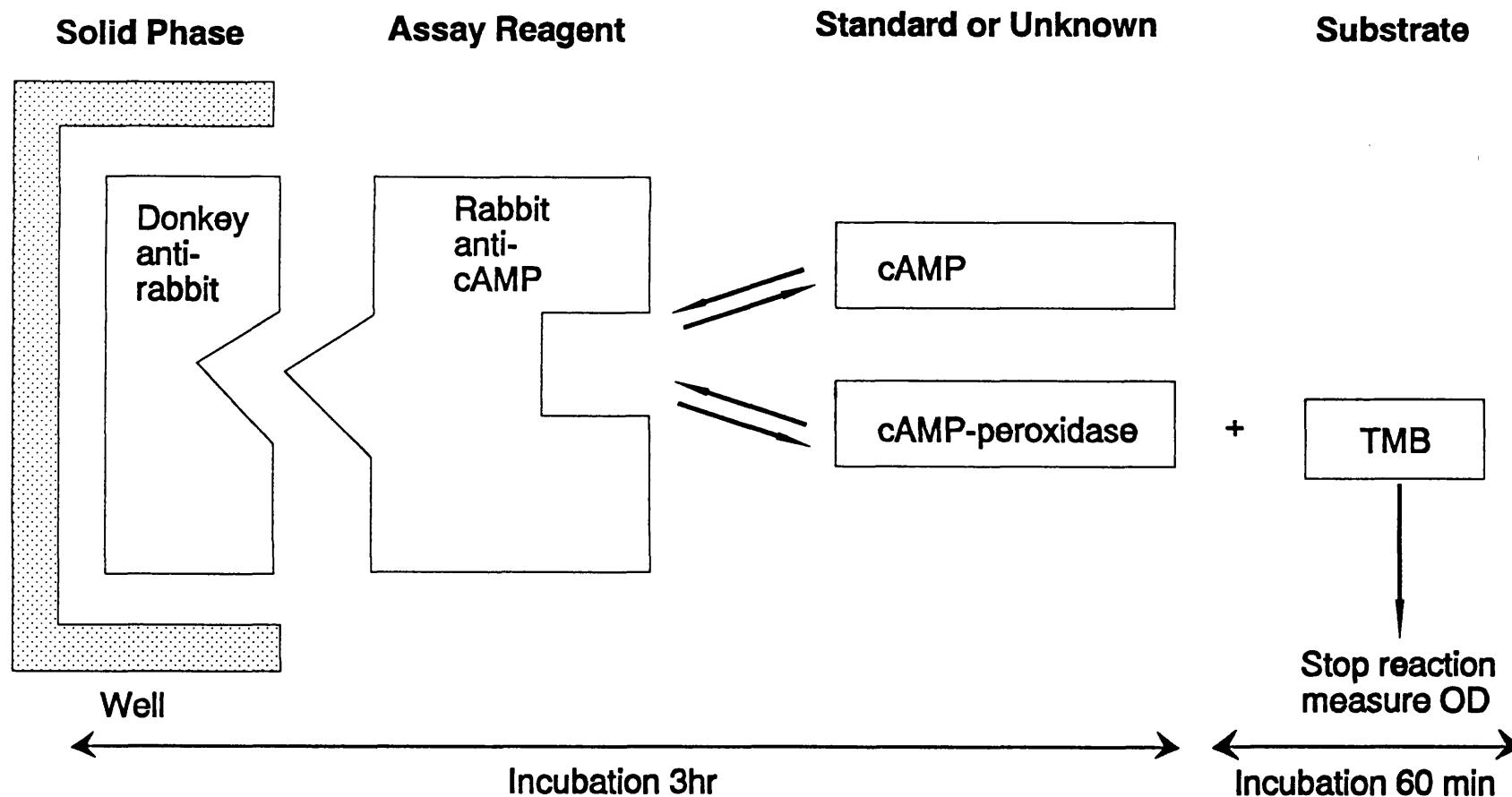
was removed by knocking the plate sharply onto tissues. 150 μl TMB/hydrogen peroxide substrate was immediately added to all wells, the plate covered and shaken gently on a plate shaker for 1 hour at room temperature allowing blue colour development. The reaction was stopped by the addition of 100 μl 1 M sulphuric acid into each well, the plate shaken and read at 450nm against the blank wells in a Titerek Multiscan MCC/340 microtitre plate reader.

Calculation of the results obtained were determined as follows:

1. Average optical density (OD) for each set of duplicate readings were calculated.

2. % Bound was calculated using the following equation :

$$\% \; (B \; / \; Bo) \; = \; \frac{\text{standard or sample OD - NSB OD}}{\text{Bo OD - NSB OD}} \; \times \; 100$$

A standard curve was generated using the appropriate readings from the plate and cAMP concentration of unknown samples (fmol/well) read directly from the graph..

2.15   TRIAL ELISA PROCEDURE

An attempt was made to duplicate the cAMP enzyme immunoassay kit used for the determination of cAMP (section 2.14.2) with a laboratory-made version in an effort to reduce the cost the assay.

Blank standard 96 well microtitre plates from Flow Laboratories were used and coated with antiserum in the following manner.

Goat anti - rabbit IgG was added to 0.05 M carbonate- bicarbonate buffer (pH 9.6) at the

required dilutions, and 200 μl pipetted into each well. The plate was left overnight at 4°C. The following day, unbound antibody was washed off with 4 washes of 0.15 M phosphate buffered saline [pH 7.2] (PBS). 250 μl 1% (w/v) blocking agent in PBS was added to each well and left for 1 hour. The plate was rinsed with two washes of PBS and the plate blotted dry by upturning and knocking it sharply onto tissue paper. A range of antibody dilutions (1:50, 1:100, 1;500, 1:1000 and 1:2000) were tested along with a variety of blocking agents (caesin, gelatin BSA and milk powder).

2.16   STATISTICAL ANALYSIS

Statistical evaluation of data was determined using mean and standard error mean (S.E.M.) values and calculated using Students t-test for unpaired samples.

All data shown are represented as mean ± S.E.M. Numbers of experiments (n) are from separate experiments with measurements carried out in duplicate unless stated otherwise. If no error bars are visible in a figure, they lie within the symbol.

CHAPTER 3
RESULTS AND DISCUSSION:
HYPOTHYROIDISM AND THE
DEVELOPING RAT

3.1   HYPOTHYROIDISM AND DEVELOPMENT

Numerous studies have focused upon the consequences of hypo- and hyper-thyroidism on the central nervous system (CNS) in many animal models. Investigations centred on adult animals enabled insight into the influence of thyroid hormones on neuronal function, whereas those concerned with pre- and post-natal animals have yielded knowledge of the role of thyroid hormones in the development and differentiation of the nervous system. Both deficient and excessive levels of thyroid hormones lead to disturbances in brain development with severe repercussions as the animal progresses into adulthood, assuming that the animal survives. The amount of data gleaned from these investigations is vast (reviewed in Ford and Cramer, 1977; Porterfield and Hendrich, 1993), with the majority of work carried out under hypothyroid conditions.

Several types of hypothyroidism have been identified, distinguished on the basis of their etiology (see section 1.8.1). Depending on onset of deficiency and magnitude of insult, the consequences vary in terms of severity and clinical manifestations (Porterfield and Hendrich, 1993). Hypothyroidism may result from any of several factors, e.g. iodine deficiency, exposure to goitrogens and /or anti-thyroid drugs, impaired function or development of the thyroid. Many different methods have been employed in the inducement of the hypothyroid state for both adult and developmental studies. Methimazole, propylthiouracil (PTU), low iodine feed, $[^{131}I]$-iodine treatment and thyroidectomy have all been used by various groups. In developmental studies, the situation is further complicated by onset (e.g. start or end of gestation, postnatal) and duration of insult. Care should therefore be taken when comparing data between different groups. In this study of development, hypothyroidism was induced in female adult rats immediately following conception via a protocol combining iodine-deficient feed and

PTU-supplemented drinking water, successfully employed for earlier adult studies (Chohan *et al.*, 1984). Pups born of these dams were therefore subjected to hypothyroid conditions throughout pre- and post-natal life, and hence are models of neurological cretinism resulting from maternal hypothyroidism. Euthyroid control animals were maintained on standard diet and drinking water.

3.2 EFFECTS OF HYPOTHYROIDISM ON THE DEVELOPING RAT.

Throughout the developmental period studied (1 to 25 days postpartum), animals were weighed and monitored on a regular basis. Litters were standardised at birth to 8 pups per litter in order to minimise deviations in the developmental age of animals at each age group. Variations in litter number affect the rate of development amongst individual litters, leading to confusion over the data obtained (Dobbing, 1964; Irvine and Timiras, 1966). On a few occasions it was necessary to foster pups from one litter to another to make up numbers of pups in each litter. Only animals of the same thyroid state were used, i.e. hypothyroid pups from one litter were introduced into a hypothyroid litter born of another dam. Fostering was only carried out soon after birth and when necessary, due to small litter size or death of newly born animals. Both control and hypothyroid pups were of similar body weights from birth through to 6 days postpartum. Thereafter, growth of the euthyroid pups accelerated whereas a retardation in growth was evident from the second postnatal week in hypothyroid pups, becoming more marked during the third week of life. By day 25 postpartum, control animal were approximately 3-fold heavier than their hypothyroid counterparts (Figure 3.1). Complete thyroidectomy results in reduction of pituitary acidophil cell numbers which are responsible for growth hormone secretion. Low doses of T_4, which restore body weight in young thyroidectomized animals, also
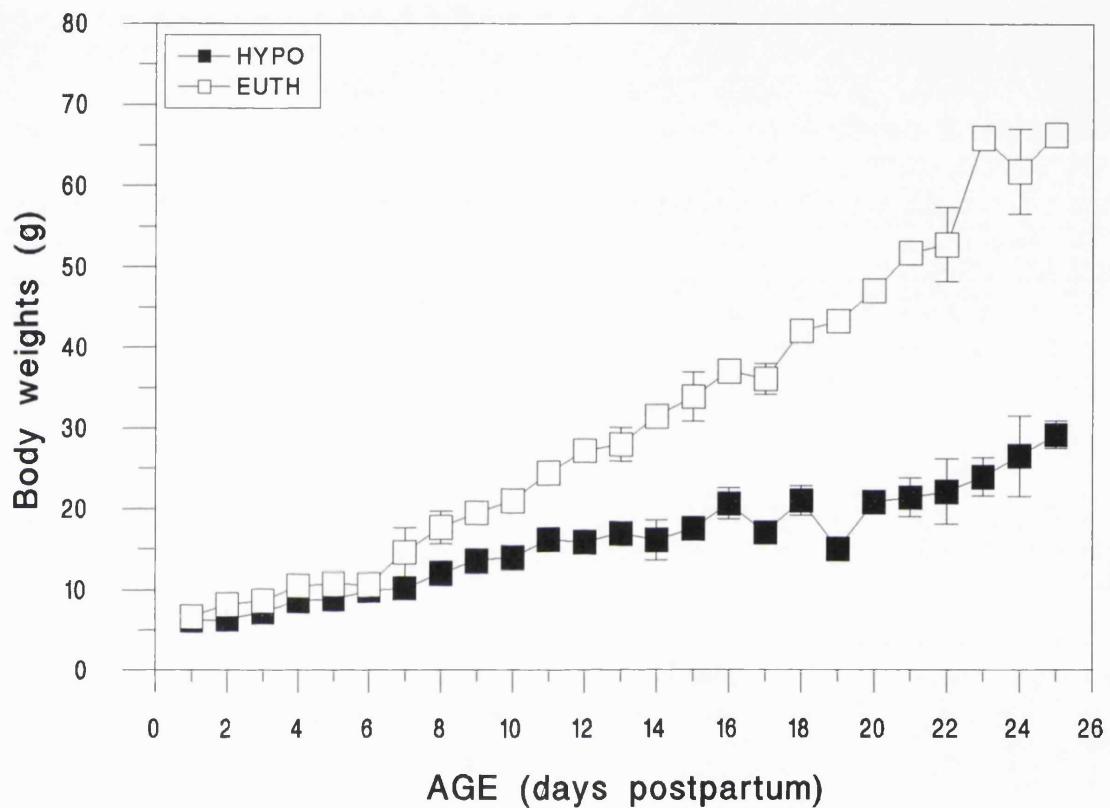
Figure 3.1 <u>**BODY WEIGHTS OF EUTHYROID AND HYPOTHYROID RAT PUPS DURING POSTNATAL DEVELOPMENT.**</u>

Animals were weighed regularly throughout the developmental period studied. The values were obtained by determining the mean weight of pups in each litter and then calculating the mean ± S.E.M. of those values (n=4-12 litters).

Figure 3.2   <u>EUTHYROID   AND   HYPOTHYROID   PUPS   AT   1   DAY POSTPARTUM.</u>

Figure 3.2a   Euthyroid

Figure 3.2b   Hypothyroid
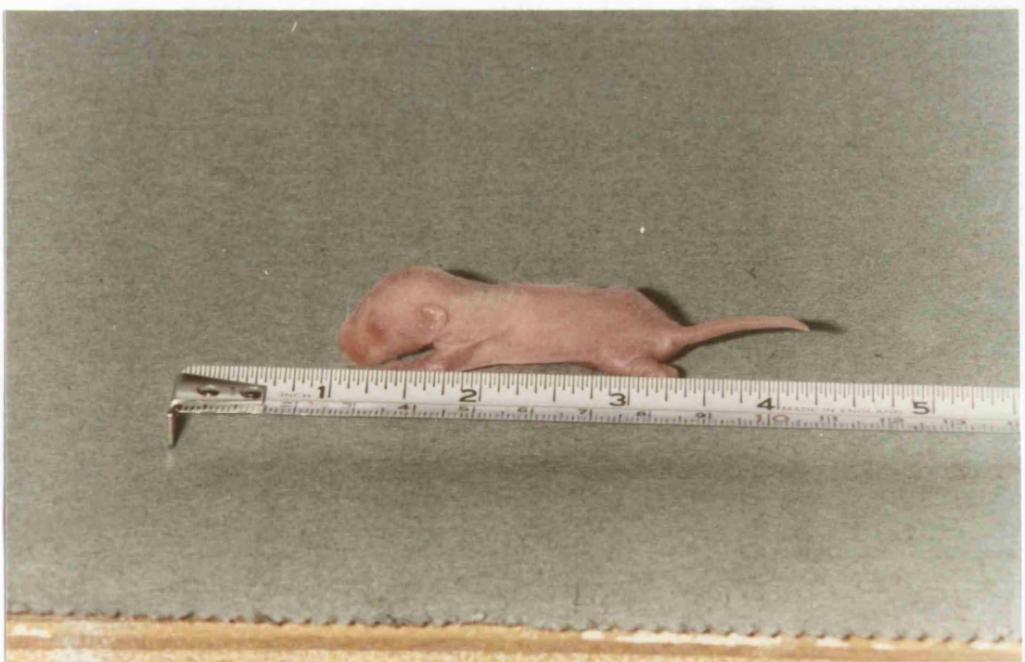
restore pituitary histology and levels of circulating growth hormones (Hervas *et al.*, 1975). It is therefore feasible that thyroid hormones influence somatic growth by stimulating production and release of pituitary growth hormone.

Animals which were hypothyroid during the early stages of life showed a reduction in body weight and morphology as well as delayed signs of physical development e.g. eye opening, pelage (coat) appearance. Control and hypothyroid pups at day 1 postpartum were indistinguishable in their visual appearance and behaviour (Figures 3.2a and 3.2b) though hypothyroid pups were slightly smaller in appearance. By day 10 postpartum, fur had begun to appear on control animals. This event was impaired in hypothyroid pups and though fur growth did occur, the overall appearance of the pelt was markedly different ("dull" and matted) from that of control animals which was thicker and healthier. Eye opening, another indication of development, was also delayed 2-3 days during hypothyroidism in growing pups. At day 15 control pups showed signs of high activity. Aware of their surroundings, the pups were alert and their movements co-ordinated (Figure 3.3a). By contrast, hypothyroid pups appeared listless and lethargic in manner at day 15. From Figure 3.3b, it was evident that hypothyroid pups possessed poor co-ordination, posture and stance reflecting abnormalities in muscle development and strength. Moreover, very little or no exploratory behaviour was observed; indeed, these pups displayed minimal activity and awareness of their surroundings. At day 20, hypothyroid animals were considerably smaller than their control counterparts. Levels of activity were more evident though still substantially less than those of control animals, and eye opening was obvious. By day 25, control animals resembled miniature versions of adults in terms of overall body shape. Figures 3.4a and 3.4b contrast the differences in appearance of both sets of animals at this age.

Figure 3.3   EUTHYROID   AND   HYPOTHYROID   PUPS   AT   15   DAYS POSTPARTUM.

Figure 3.3a   Euthyroid

Figure 3.3b   Hypothyroid

Figure 3.4 **EUTHYROID AND HYPOTHYROID PUPS AT 25 DAYS POSTPARTUM.**

Figure 3.4a **Euthyroid**

Figure 3.4b **Hypothyroid**

Hypothyroidism and its effects on development and behaviour have been extensively studied and the repercussions of the hypothyroid regime used in this study are similar to those documented previously (Hamburgh *et al.*, 1964; Eayrs, 1971; Legrand, 1984).

Oklund and Timiras (1977) found body growth was greatly impaired when PTU was administered in early gestation indicating a role of thyroid hormones in growth regulation during both pre- and post-natal periods of life. In contrast, no signs of neonatal growth retardation was observed when PTU treatment was initiated in late pregnancy (Hamburgh *et al.*, 1964; Legrand, 1969). The same groups found hypothyroidism induced during early fetal development leads to evident effects on body and brain growth by the last week of gestation. Foetuses of dams with low circulating T_3 and T_4 concentrations resulting from thyroidectomy, possess undetectable levels of both iodothyronines prior to onset of fetal thyroid function (Geloso and Bernard, 1967). Foetuses from such mothers suffer from numerous metabolic imbalances, with resulting progeny showing permanent alterations of brain protein and enzyme content, and behavioural defects (Hendrich *et al.*, 1984).

Disturbances in size and morphology of the developing hypothyroid rat are the consequences of disrupted skeletal and muscle growth. Lack of thyroid hormone in young rats lead to retardation in bone elongation and development of ossification centres (Becks *et al.*, 1950; Geloso *et al.*, 1968; Hamburgh, 1968). In human congenital and juvenile hypothyroidism, abnormalities in membranous and cartilaginous parts of the skeleton have been observed (Anderson, 1960; Wilkins, 1965). Foetal body length is usually unaffected with abnormalities becoming progressively more evident following birth; growth retardation of the limbs and head contribute to the morphological characteristics of hypothyroid dwarfism. In rats thyroidectomized at birth, disturbances in skeletal muscle

growth and size are seen (Scow, 1953) following disturbance in cellular composition and protein synthesis (Bacou *et al.*, 1980; Brown *et al.*, 1981). Delayed and abnormal appearance of the pelage (coat) in hypothyroid animals are due to disruptions in hair shaft and follicle formation, and thyroid hormone effects on skin development (Scow, 1951; Scow, 1959). The majority of defects seen in the developing rat during hypothyroidism are seen in the congenital and neonatal hypothyroid human (Thilly *et al.*, 1978; Legrand, 1986; Delong, 1989).

3.3 EFFECTS OF HYPOTHYROIDISM ON THE DEVELOPING BRAIN.

One of the first objectives was to decide on the extent to which the brain could be dissected to give sufficient amounts of material from small numbers of young rats. In the case of animals age 1, 10 and 15 days postpartum, the brain was separated into forebrain and hindbrain regions. At 20 and 25 days postpartum, the hindbrain was further subdivided into cerebellum and medulla oblongata regions.

Following death and decapitation of animals, brains were removed, dissected and weighed as detailed in section 2.3. Figure 3.5 shows the wet weight measurements of forebrain regions from euthyroid and hypothyroid pups at the developmental ages studied. At day 1 postpartum there was no discernible difference between the two sets of animals. By day 10, the difference in growth rates was more pronounced such that forebrain regions of hypothyroid animals showed a significant 20% reduction in wet weight from this time on. A similar trend occurred in the developing hindbrain though in this case, a significant reduction (25%) was observed from day 15 postpartum (Figure 3.6). These results confirmed the observations made by other groups working on hypothyroidism initiated during gestation (Nicholson and Altman, 1972; Balazs, 1973; Nunez, 1984a). As
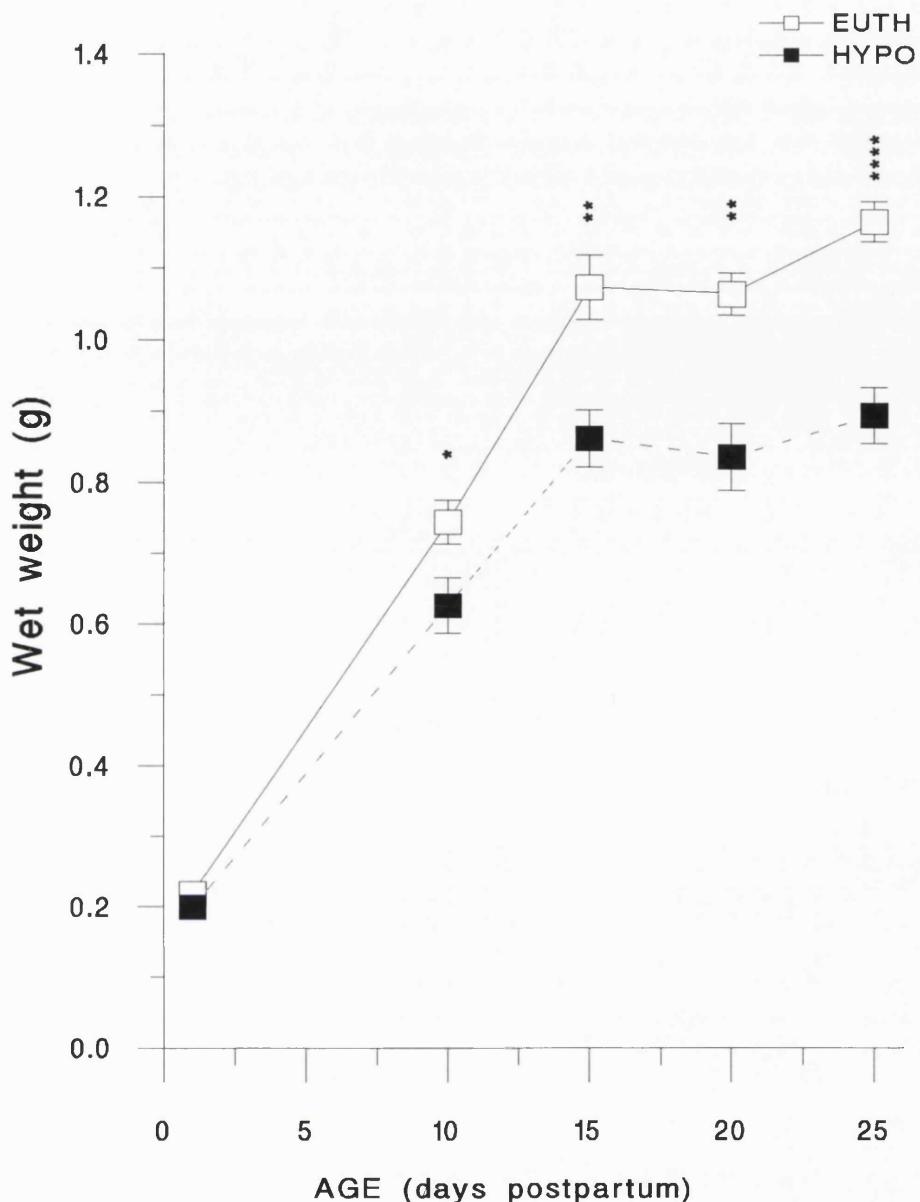
Figure 3.5 <u>**FOREBRAIN WEIGHTS OF EUTHYROID AND HYPOTHYROID PUPS AT VARIOUS STAGES OF DEVELOPMENT.**</u>

Forebrain regions from animals at 1, 10, 15, 20 and 25 days potpartum were dissected and weighed as described in section 2.3. Values are means ± S.E.M. of 6-8 separate determinations.

*, ** and **** indicate P<0.05, <0.02 and <0.001 respectively for comparison of hypothyroid (HYPO) and euthyroid (EUTH) states.
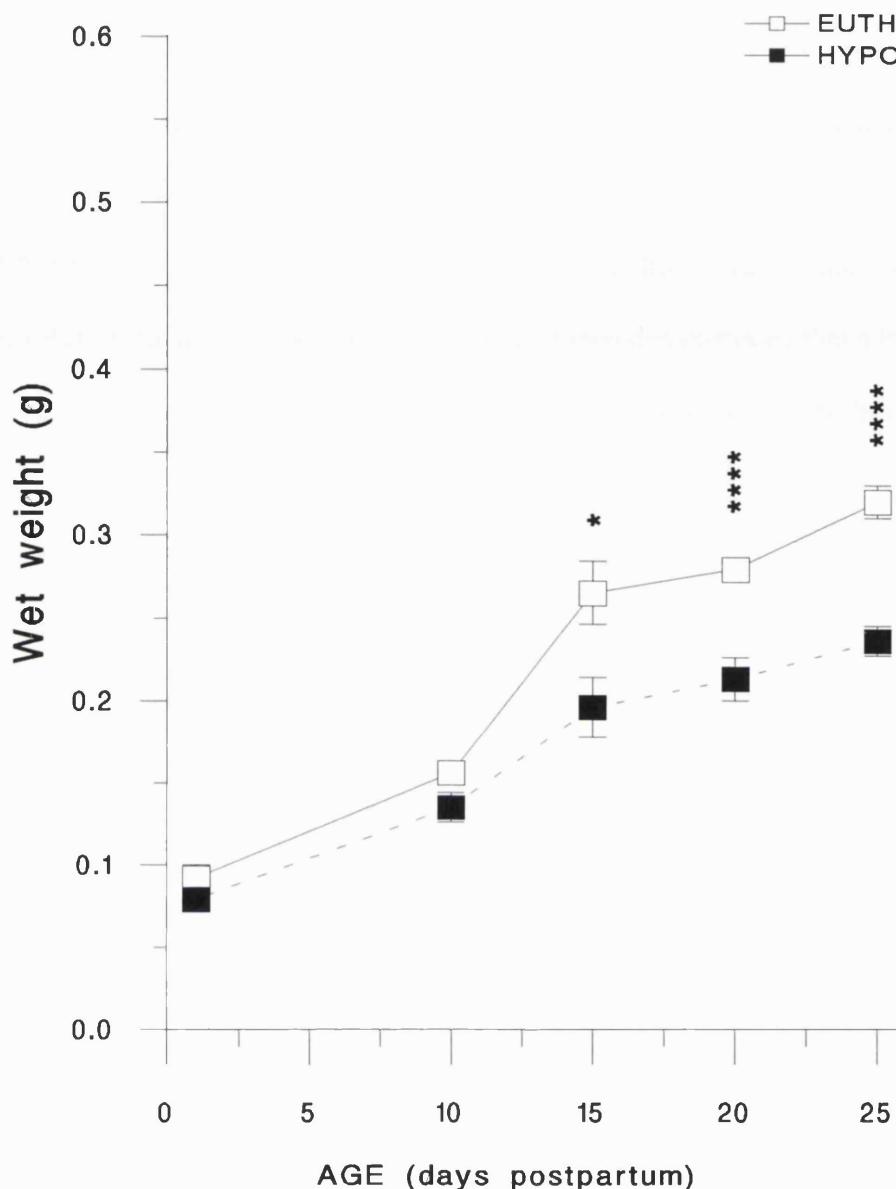
Figure 3.6 HINDBRAIN WEIGHTS OF EUTHYROID AND HYPOTHYROID PUPS AT VARIOUS STAGES OF DEVELOPMENT.

Hindbrain regions from animals at 1, 10, 15, 20 and 25 days postpartum were dissected and weighed as described in section 2.3. Day 20 and 25 values are the summations of cerebellum and medulla oblongata weights. Data are means ± S.E.M of 6-8 separate determinations.

* and **** indicate $P<0.05$ and <0.001 respectively for comparison of hypothyroid (HYPO) and euthyroid (EUTH) states.

mentioned in section 1.12.4, the weight decreases observed were the summation of many factors: cell size, packing density, cell number, migration and proliferation to name but a few (Balazs, 1973; Clos and Legrand, 1973; Himwich, 1973; Lauder, 1979). Narayanan and Narayanan (1985) observed a delay in cell acquisition in rat foetuses born of mothers given PTU from day 7 gestation. Potter *et al.* (1986) demonstrated that a lack of maternal thyroid hormones in early gestation led to a reduction in cerebrum and brain stem growth in the developing sheep foetus. An 8-fold drop in expression of the NGFI-A gene product is observed in hypothyroid rats (Pipaon *et al.*, 1992). This early response gene, induced by signals which initiate growth and differentiation, has been assigned a putative role in brain development. The fact that treatment with T_3 reversed the effect within one hour suggests the effect of thyroid hormone is a direct one. In summary, the brain is critically dependant on thyroid hormones for normal CNS development in all aspects (i.e. morphological, biochemical and functional). The hypothyroid brain is immature at stages when proper connectivity should already be established. Thyroid hormones appear to act as "time switches", synchronizing the sequence of developmental events in the brain; as a result a vast body of work has focused upon the various facets of neuronal development influenced by thyroid hormones (reviewed by Timiras, 1988).

3.4 PLASMA THYROID HORMONE AND METABOLITE MEASUREMENTS IN DEVELOPING EUTHYROID AND HYPOTHYROID RATS.

As mentioned above, animals born of hypothyroid dams showed a reduction in body weight. Maternal hypothyroidism may affect foetal development indirectly by altering maternal metabolism such that fetal nutrition is compromised (Porterfield *et al.*, 1975; Bonet and Herrera, 1988; Bonet and Herrera, 1991). Moreover, there is evidence of

perturbed milk secretion by lactating dams and suckling behaviour of neonates (Narayanan *et al.*, 1982; Tamasy *et al.*, 1984; Miyake *et al.*, 1989). Very little data is available on the influence of hypothyroidism on milk composition of nursing dams. Though no changes were detected in the mammary glands of lactating PTU-treated dams (Oklund and Timiras, 1977) it is surprising that the sole nutritional source of developing animals in the early stages of life has not been studied given the fact that lactation is very much under the influence of hormones. To ensure that the change in body weight was not the result of malnutrition in hypothyroid developing animals, plasma samples collected at time of death (section 2.3) were tested for various metabolites as detailed in Materials and Methods. In addition, levels of the thyroid hormones T_3 and T_4 were also determined to ensure the protocol employed for inducing hypothyroidism in developing pups was successful (sections 2.7.1 and 2.7.2).

Plasma levels of free T_3 and T_4 from euthyroid and hypothyroid pups at various ages are shown in Figures 3.7 and 3.8 respectively. In euthyroid pups free T_4 peaked between 10 and 20 days post partum with a slightly delayed peak in free T_3 observed. At all ages tested, free T_3 levels were 2-5 fold lower (P<0.05-0.001) in hypothyroid pups compared to values of age matched euthyroid pups (Figure 3.7). Moreover in the hypothyroid state, the postnatal rise in free T_3 was both delayed and lowered such that the peak level at 20 days post partum was only 22% of that seen in euthyroid pups. In hypothyroid pups free T_4 was essentially undetectable throughout the developmental period, falling below the sensitivity limits (<0.02 pM) of the assay employed to measure the hormone. 5'-deiodination of thyroid hormones is catalysed by type I and type II deiodinases. The former is present in the thyroid gland where it is highly active in the conversion of T_4 to T_3. Moreover, type I is also sensitive to inhibition by PTU and may account for the low
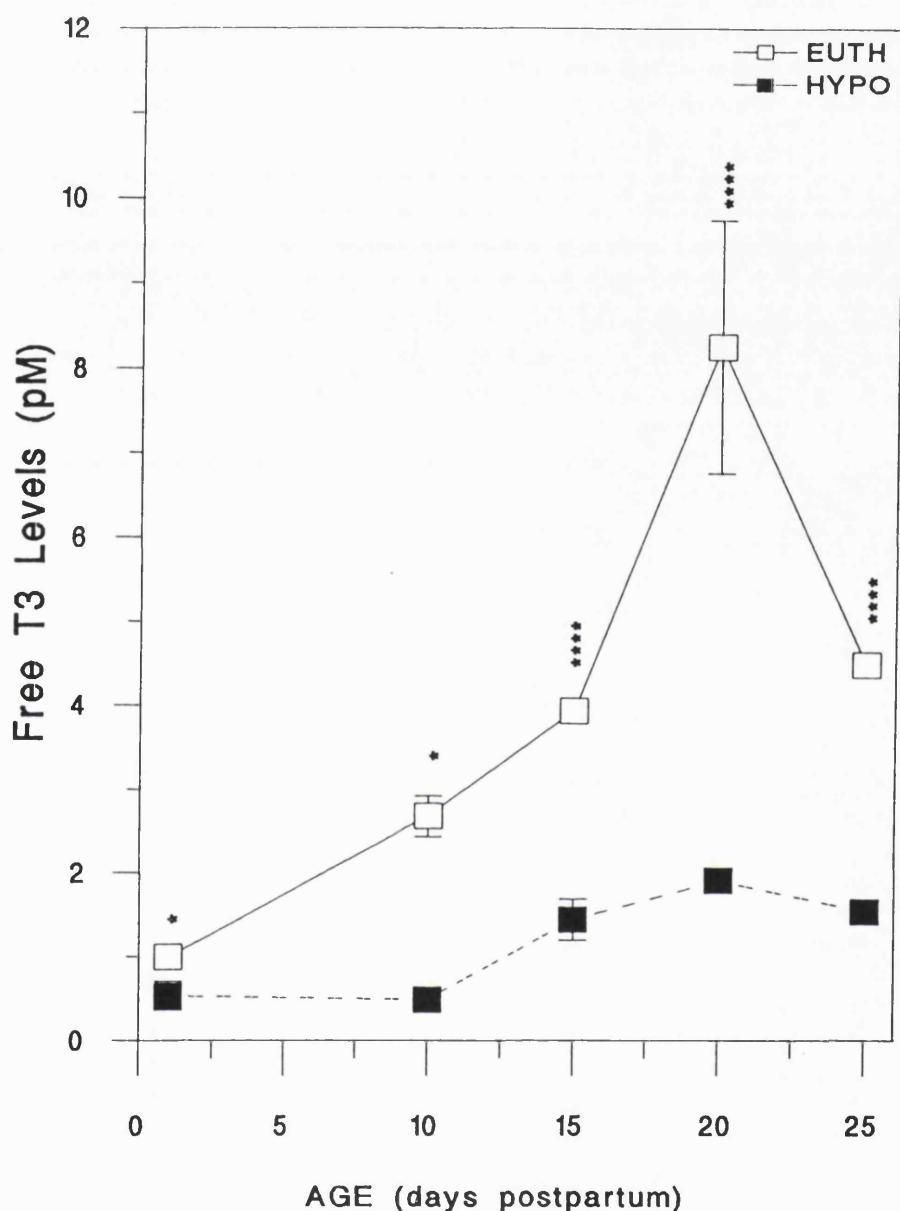
Figure 3.7 <u>PLASMA FREE T_3 LEVELS OF EUTHYROID AND HYPOTHYROID PUPS AT 1, 10, 15, 20 AND 25 DAYS POSTPARTUM.</u>

Plasma samples collected from animals at various ages (section 2.6) were assessed for T_3 as detailed in section 2.7.1. Values are expressed as means ± S.E.M. (generally n>4). * and **** indicate P<0.05 and <0.001 respectively for comparison of euthyroid (EUTH) and hypothyroid (HYPO) states.
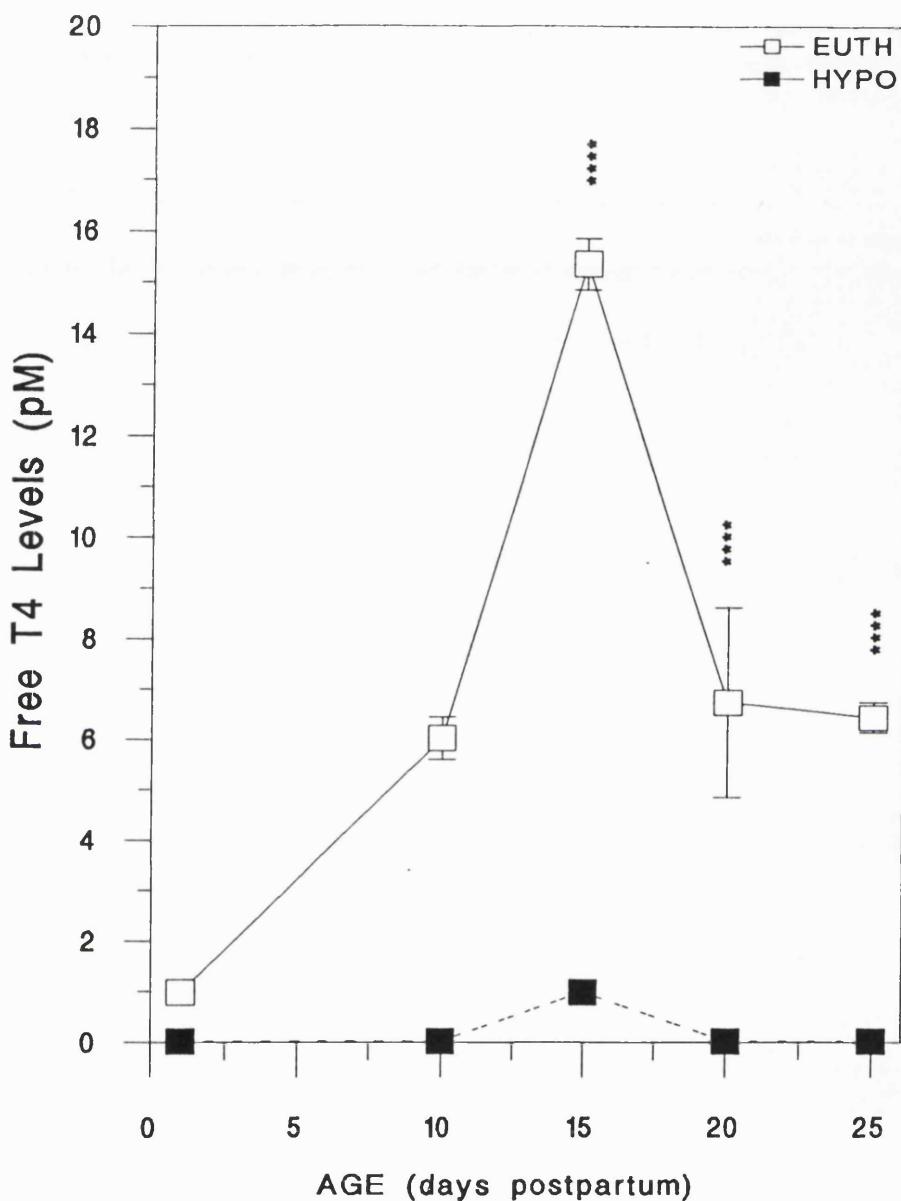
Figure 3.8 <u>PLASMA FREE T_4 LEVELS OF EUTHYROID AND HYPOTHYROID PUPS AT 1, 10, 15, 20 AND 25 DAYS POSTPARTUM.</u>

Plasma samples from animals at various ages (section 2.6) were assessed for T_4 as detatiled in section 2.7.1. Values are expresses as means $\pm$ S.E.M. (generally n>4).

**** indicates P<0.001 for comparison of euthyroid (EUTH) and hypothyroid (HYPO) states.

level of thyroid hormones observed (Leonard and Visser, 1986).

From the data obtained on the thyroid hormone state of both sets of animals (euthyroid and hypothyroid), it was clear that the regime used to induce hypothyroid pups for study of hypothyroidism during development was a successful one.

In humans, though gross maturation of the brain occurs *in utero,* several important events (glial proliferation, myelination) take place after birth. Brain growth is a non-linear process, occurring in "spurts" (Dobbing and Sands, 1979). During such times, the brain is particularly vulnerable. The rat brain at birth is neurologically immature. Postnatally, the rat brain becomes progressively active metabolically due to the wide range of cellular events that occur (see section 1.12.5). As a result the brain's energy requirements at this time are high. During suckling, the neonate experiences a high fat/low carbohydrate diet which changes during the weaning period (approximately 23 days) to a high carbohydrate/low fat diet. The brain depends mainly on a mixture of ketone bodies and glucose as a source of energy during the suckling period, maintained by the high fat milk diet supplied by the mother. Approximately 3 weeks after birth, the brain undergoes a transition from the immature neonatal brain state to the maturing adult brain. At this time, the brain has acquired the full complement of active enzymes needed for the full oxidation of glucose for its main energy requirements and it dependancy on ketone bodies as an additional energy source decreases (Girad *et al.*, 1992). Studies carried out on different brain regions support the hypothesis that there is a correlation between the maturation of enzymes involved in aerobic glycolysis and neurological competence and that this occurs at different times depending on brain region (reviewed in Clark *et al.*, 1993).

In addition to affecting the maturation of glycolytic enzymes, hypothyroidism delays the decline in ketone body metabolising enzymes characteristic of the weaning period (Patel,
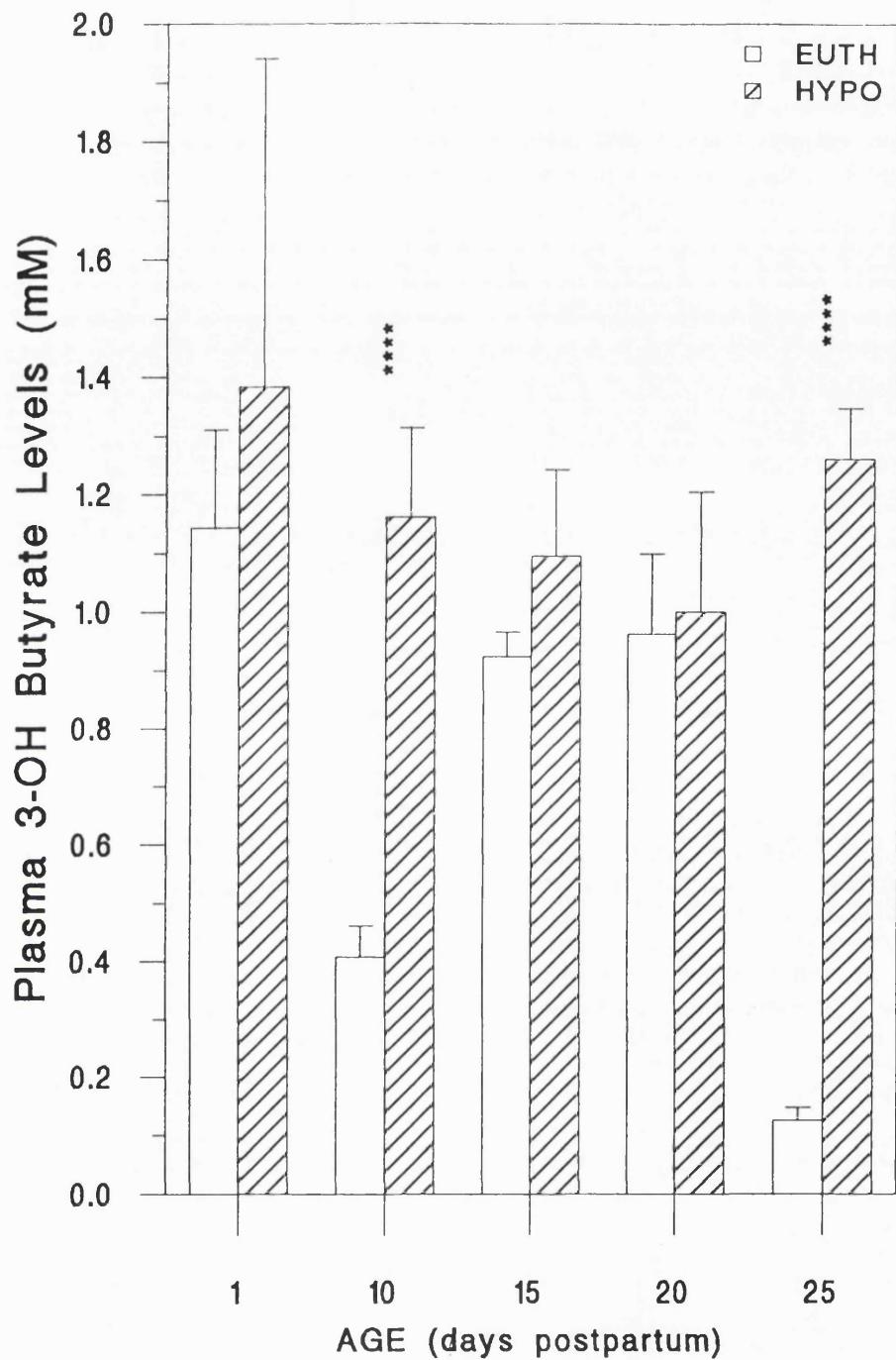
Figure 3.9 <u>PLASMA 3-HYDROXYBUTYRATE LEVELS OF EUTHYROID AND HYPOTHYROID RATS AT 1, 10, 15, 20 AND 25 DAYS POSTPARTUM.</u>

Plasma samples were collected as described in section 2.6 and 3-hydroxybutyrate levels determined as detiled in section 2.7.3.

Values are expressed as means ± S.E.M. In all cases n>4.

**** indicates P<0.001 for comparison of the euthyroid (EUTH) and hypothyroid (HYPO) states.

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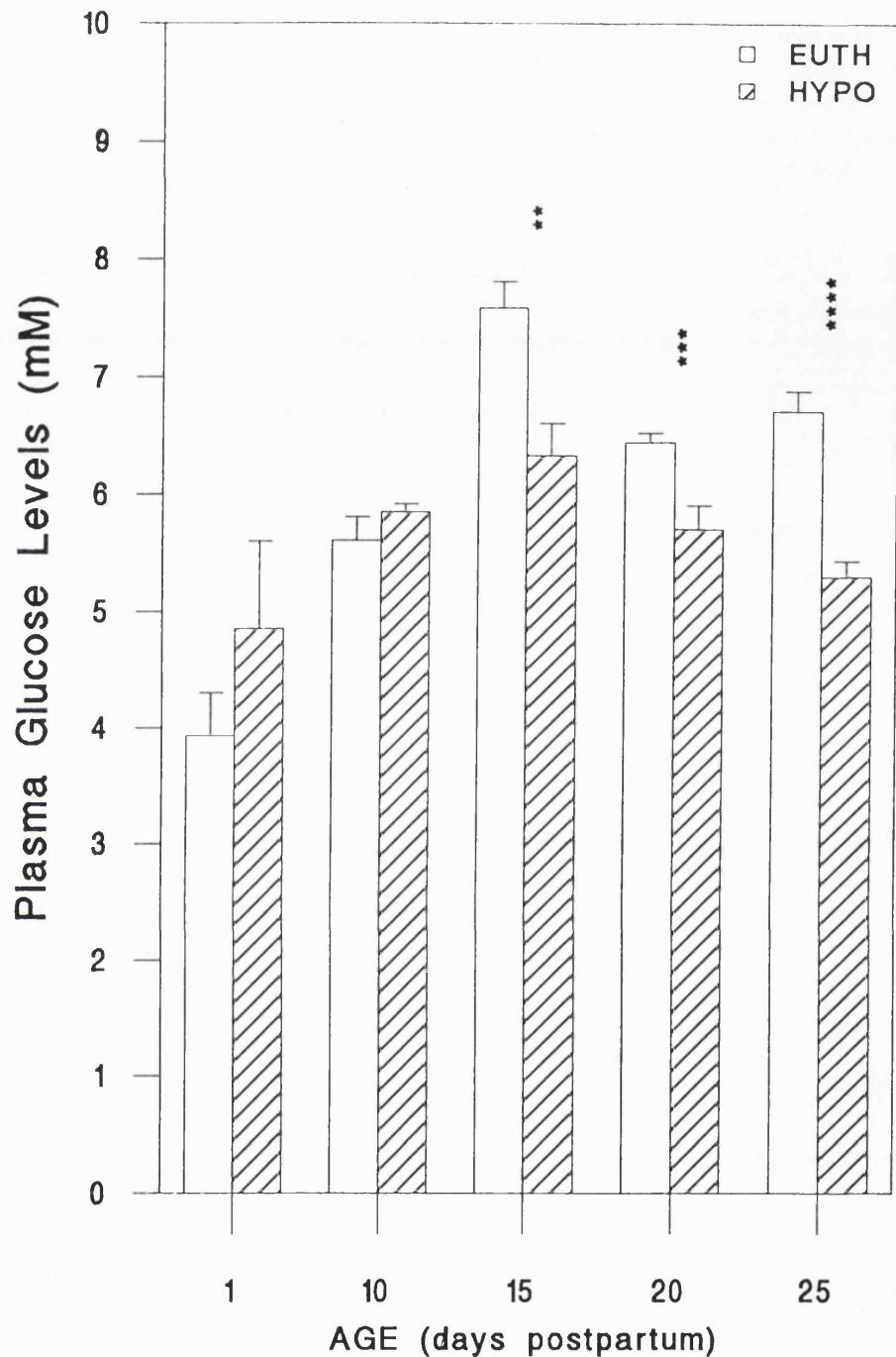
Figure 3.10 <u>PLASMA GLUCOSE LEVELS OF EUTHYROID AND HYPOTHYROID RATS AT 1, 10, 15, 20 AND 25 DAYS POSTPARTUM.</u>

Plasma samples were collected as described in section 2.6 and glucose levels determined as detailed in section 2.7.4.

Values are expressed as means ± S.E.M. In all cases n>4.

, *, **** indicate P<0.02, <0.01 and <0.001 respectively for comparison of the euthyroid (EUTH) and hypothyroid (HYPO) states.

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1980). Throughout the nursing period, hypothyroid animals as mentioned earlier were highly lethargic in nature. Unlike control pups which were eager to nurse off dams continuously, hypothyroid pups exhibited a decreased inclination to suckle. Plasma samples taken from hypothyroid and control animals at all ages studied were tested for 3-hydroxybutyrate (section 2.7.3) and glucose (section 2.7.6). At day 10 there was a significant elevation in plasma 3-hydroxybutyrate in the hypothyroid case (Figure 3.9). At days 15 and 20 the hypothyroid pups had slightly but significantly lower plasma glucose concentrations which were still in the "normal" range of glycaemia (Figure 3.10). Measurements of plasma lactate and non-esterified fatty acids (Figures 3.11 and 3.12) revealed that there were no significant differences between euthyroid and hypothyroid pups over the first 20 days post partum. Overall there were no changes in the plasma metabolite profiles over days 1 to 20 which were consistent enough or large enough to suggest that the impaired brain and body development of the hypothyroid pups could be due to serious undernutrition. As indicated in section 2.2.3, all pups remained with the dams until used for experiments. It was generally observed that euthyroid pups were eating the solid (low fat/high carbohydrate) diet between 20 and 25 days post partum; this was not seen in the less active hypothyroid pups which were still suckling. It was noted that in this day 20 to day 25 period, hypothyroid pups did not exhibit the significant increase in plasma lactate (Figure 3.11) or the considerable decrease in plasma 3-hydroxybutyrate (Figure 3.9) that were observed (presumably as a consequence of weaning) in euthyroid pups.
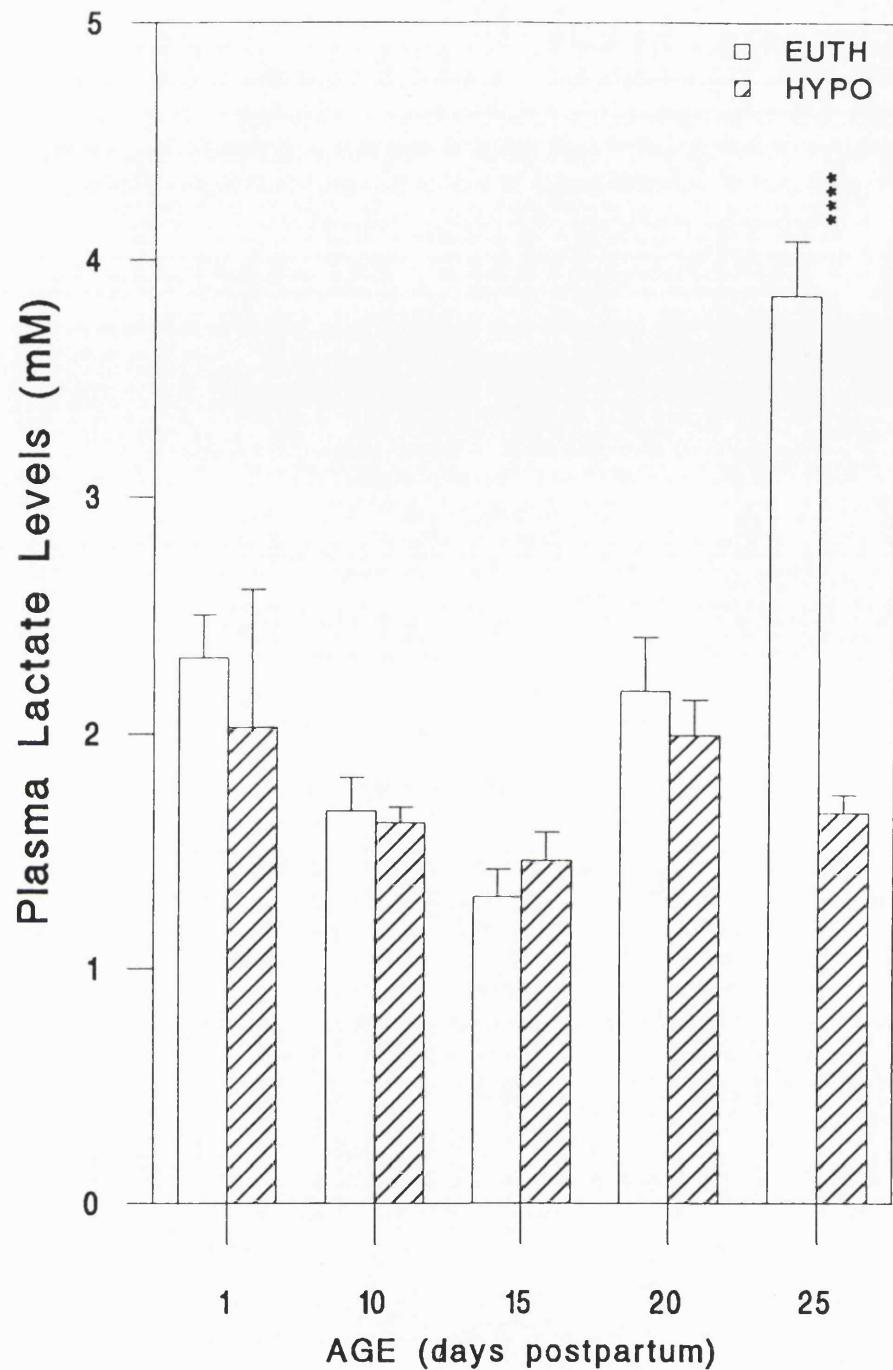
Figure 3.11 <u>PLASMA LACTATE LEVELS OF EUTHYROID AND HYPOTHYROID RATS AT 1, 10, 15, 20 AND 25 DAYS POSTPARTUM.</u>

Plasma samples were collected as described in section 2.6 and lactate levels determined as detailed in section 2.7.5.

Values are expressed as means ± S.E.M. In all cases n>4.

**** indicates P<0.001 for comparison of the euthyroid (EUTH) and hypothyroid (HYPO) states.

140
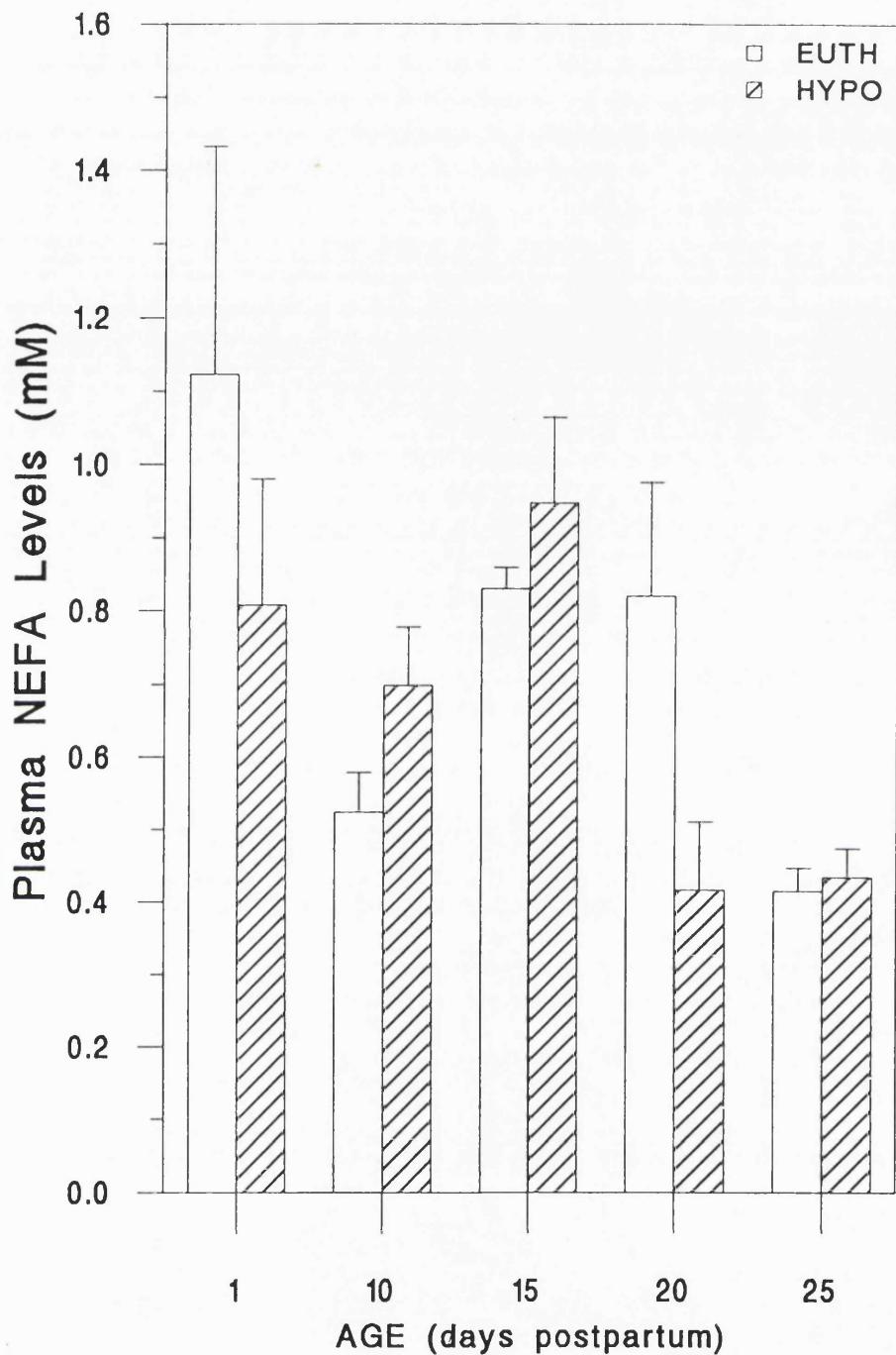
**Figure 3.12** <u>PLASMA NON-ESTERIFIED FATTY ACID LEVELS OF EUTHYROID AND HYPOTHYROID RATS AT 1, 10, 15, 20 AND 25 DAYS POSTPARTUM.</u>

Plasma samples were collected as described in section 2.6 and non-esterified fatty acid levels (NEFA) determined as detailed in section 2.7.6.

Values are expressed as means ± S.E.M. In all cases n>4.

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3.5 PLASMA THYROID HORMONE AND METABOLITE MEASUREMENTS OF EUTHYROID AND HYPOTHYROID DAMS.

Plasma metabolite measurements were also carried out on the female adult rats used for breeding purposes. Thyroid hormone measurements confirmed that dams maintained on the low iodine diet were hypothyroid, exhibiting significantly reduced free T_3 and free T_4 levels compared to control/euthyroid dams ($P<0.001$). With the exception of non-esterified fatty acids which were 50% lower in hypothyroid dams ($P<0.001$), glucose, lactate and 3-hydroxybutyrate levels were comparable between the two sets of mothers (Table 3.1). The drop in plasma non-esterified fatty acids may be readily explained by the firmly established effect of hypothyroidism on lipolysis. Hypothyroidism has been seen to impair mobilisation of fatty acid stores in adipose partly by inducing up-regulation of the inhibitory G-proteins of adenylyl cyclase, G_i. Increases in α-subunit abundance levels of all three G_i proteins have been observed in adult hypothyroid rats (Malbon et al., 1985; Milligan et al., 1987a; Ros et al., 1988; Milligan and Saggerson, 1990). Up-regulation of G_i coupled input to adipocytes leads to increased inhibition of cAMP production by adenylyl cyclase. cAMP contributes substantially to the regulation of lipolysis; a fall in levels of the cyclic nucleotide resulting in decreased fatty acid production via the inactivation of hormone-sensitive lipase (HSL). A rise in the levels of G-proteins responsible for adenylyl cyclase inhibition therefore, affects the breakdown of fat stores in the body which would in turn would have bearing on non-esterified fatty acid levels in the blood. The effects of hypothyroidism in adipose tissue is reviewed by Saggerson (1992).

**TABLE 3.1   PLASMA THYROID HORMONE AND METABOLITE LEVELS
OF EUTHYROID AND HYPOTHYROID DAMS.**

Plasma samples were collected from euthyroid and hypothyroid dams shortly after sacrifice of litters (section 2.3) and hormone and metabolite measurements carried out as detailed in section 2.7. Values are means ± S.E.M. with numbers of measurements shown in parentheses. * indicates $P<0.001$ for comparison of euthyroid and hypothyroid states.

Hormone/Metabolite	State	
	Euthyroid	Hypothyroid
FT3 (pM)	4.85 ± 0.28 (6)	0.77 ± 0.18 (7) *
FT4 (pM)	11.25 ± 0.55 (2)	<1.00 (7) *
NEFA (mM)	0.82 ± 0.05 (6)	0.47 ± 0.04 (7) *
GLUCOSE (mM)	7.63 ± 0.23 (4)	7.23 ± 0.27 (6)
LACTATE (mM)	1.12 ± 0.07 (4)	1.08 ± 0.29 (5)
3-OH BUT (mM)	0.08 ± 0.02 (4)	0.05 ± 0.01 (5)

FT3 : free T3

FT4 : free T4

NEFA : non-esterified fatty acids

3-OH BUT: 3-hydroxybutyrate

3.6   EFFECTS OF HYPOTHYROIDISM ON PREGNANCY.

The consequences of maternal thyroid dysfunction on progeny reflect the type of hypothyroidism experienced, i.e. congenital, iodine deficient (Ramsden 1977; Legrand, 1986; Porterfield and Hendrich, 1993). Bakke *et al.*, (1976) demonstrated that parental hypothyroidism in either the male or female parent could induce significant effects in their offspring. Progeny of thyroidectomized female rats mated with normal males showed persistent endocrine and metabolic abnormalities similar to those experienced by offspring resulting from matings between hypothyroid males and normal females.

Pregnancy is a period of regulated changes and events. Hormonal and metabolic demands of impending childbirth result in thyroid gland changes and consequently gestation may be affected by alterations in thyroid function (Burrow, 1972). Thyroidectomized dams experience small litters and high infant and maternal mortality rates; this is seen in both rats and humans (Greenman *et al.*, 1962; Pharoah, *et al.*, 1976).

In this study, following mating between normal male and female rats, the latter were maintained on an iodine deficient diet and PTU-supplemented drinking water as detailed in section 2.2.2. PTU in addition to possessing an inhibitory effect on thyroid hormone synthesis also blocks production of the more metabolically active thyroid hormone, T_3 from T_4 (Schwatz *et al.*, 1971; Oppenheimer *et al.*, 1972). Resulting progeny were employed in the investigation of hypothyroid effects on cell signalling during development.

Initial trials on the success of the aforementioned mating protocol were unsuccessful due to increased tendencies of dams to abort/reabsorb their litters during gestation, and higher maternal and neonatal mortality rates as experienced by other groups (Porterfield and Hendrich, 1975; Hendrich *et al.*, 1984). As mentioned previously, outcome of pregnancy

in untreated hypothyroid women is poor. An increase in thyroid function has been linked

with normal pregnancy (Dowling *et al.*, 1960). During this time, basal metabolic rate may

rise by as much as 20% and is associated with increased respiration of the fetal-placental

unit, and maternal cardiac output (Burwell, 1954; Chopra *et al.*, 1975). As thyroid

hormones are known to affect basal metabolic rates this may partly explain the low

success rate of hypothyroid pregnancies. Chances of abortion and still birth and perinatal

death of progeny are greatly increased, with the most severe cases occurring during

marked iodine deficiency (Pharoah *et al.*, 1976). This inevitably hindered the overall

study as without material from developing rats, investigations could not commence. The

problem was eventually overcome following the realisation that the few successful

hypothyroid pregnancies observed were experienced by dams of a greater body weight and

which had experienced at least one normal pregnancy. These animals appeared better

suited to cope with the combined rigours of hypothyroidism and pregnancy.

Consequently, females that were a minimum of 280 g in body weight, and had delivered

at least one litter were used for breeding purposes.

Throughout the pregnancy and suckling periods, body weights of hypothyroid and

euthyroid/control dams were monitored on a regular basis. Figure 3.13 shows the data

accumulated from these observations. Both sets of dams show weight gain throughout

pregnancy. Body weight increases dramatically towards time of parturition (birth) in

euthyroid controls whilst hypothyroid dams show a lesser degree of weight gain. Indeed,

a drop in body weight is observed a few days prior to parturition, possibly indicative of

fetal reabsorption or death. Following birth, the subsequent weight loss experienced by

dams was greater in hypothyroid animals than in controls. Body weights of thyroid

hormone-deficient and euthyroid dams fell below those at start of conception to 81% and

Figure 3.13 <u>BODY WEIGHTS OF EUTHYROID AND HYPOTHYROID DAMS</u>

<u>DURING AND AFTER PREGNANCY.</u>

Adult female rats used for breeding purposes were maintained either on a normal diet and drinking water (Euthyroid) or on an iodine deficient feed and drinking water containing 0.01% PTU (Hypothyroid) as described in section 2.2.2. Animals were weighed on a regular basis before and after birth of litters. Day 0 gestation denotes the day of mating of female rats; Day 0 postpartum denotes the day of parturition.

Values are means ± S.E.M. (n=6-10).

FIGURE 3.13   <u>BODY WEIGHTS OF EUTHYROID AND HYPOTHYROID DAMS DURING AND AFTER PREGNANCY.</u>
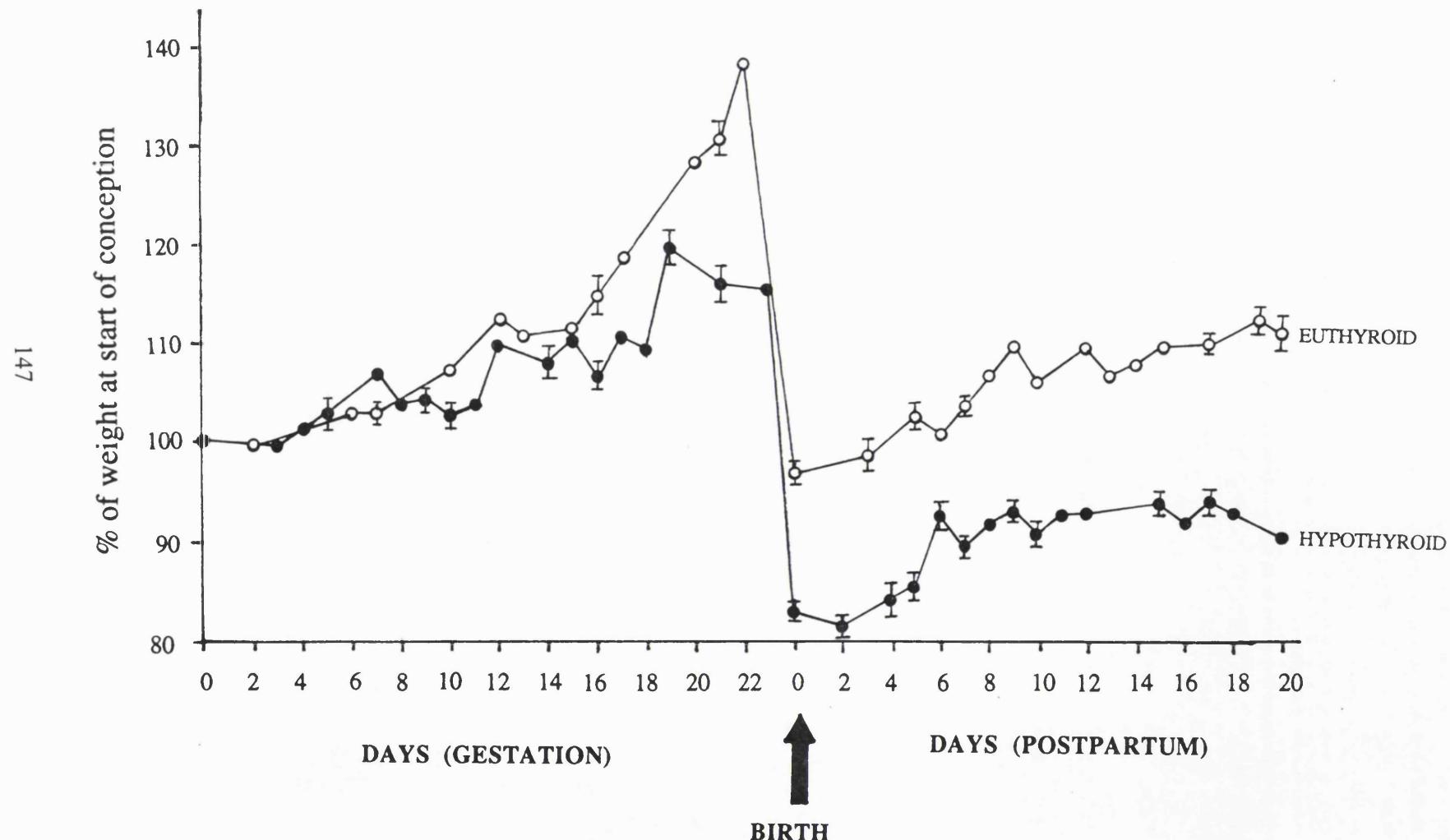
97% respectively. Over the suckling period, though increases in mother body weights are observed, in the case of hypothyroid animals these figures never exceed those at time of mating.

CHAPTER 4
RESULTS AND DISCUSSION:
<u>THE EFFECTS OF HYPOTHYROIDISM ON G-PROTEINS AND ADENYLYL CYCLASE IN THE DEVELOPING BRAIN</u>

4.1 EFFECTS OF HYPOTHYROIDISM ON G-PROTEINS AND ADENYLYL CYCLASE IN THE DEVELOPING BRAIN.

Much attention has been focused on the effects of various pathological states upon G-protein mediated cell signalling events. Changes at all levels of transmembrane signalling have been investigated, i.e. receptor, G-protein and secondary effector. Due to the advent of molecular biological techniques and immunological probes, many sensitive functional and structural assays have been developed to look for any changes in cellular function during abnormal states. The importance of thyroid hormones for normal development is well documented (see Introduction), and deviations from physiological levels lead to defective neurite growth, differentiation and synaptic connectivity in cerebral and cerebellar brain regions (Porterfield and Hendrich, 1993). The demonstration of enhanced inhibition of rat brain adenylyl cyclase by GTP, and the nucleotide's amplified ability to alter adenosine receptor to the low affinity state during times of adult thyroid hormone deficiency implied alterations at post receptor loci (Mazurkiewicz and Saggerson, 1989). This was confirmed by Orford et $al.$ (1991) who demonstrated up-regulation of $G_i 1\alpha$, $G_i 2\alpha$ and $G_o\alpha$ in synaptosomal membranes from various regions of the brain in a model of adult hypothyroidism via quantitative immunoblotting. Subsequent measurements by the same group showed decreased levels of $G_i 1\alpha$ and $G_i 2\alpha$ in synaptosomal membranes isolated from cerebral cortex of adult rats following short-term administration of T_3 (Orford et $al.$, 1992).

It would not be unreasonable to assume that normal expression of G-proteins is vital for the successful programming of brain development and that inappropriate levels at crucial times would lead to perturbed development and consequently, functioning of the brain. Furthermore, derailment of the normal pattern of developmental events is known to occur in pre- and postnatal hypothyroidism. In order to test the hypothesis that hypothyroidism-

induced effects on the brain is associated with altered signal transduction, changes in G-protein α-subunit levels and GTP effects upon adenylyl cyclase activity in synaptosomal membranes were investigated.

4.2 PREPARATION OF SUBCELLULAR FRACTIONS FROM DEVELOPING ANIMALS.

The protocol used to isolate synaptosomal membranes for study of the developing brain was based on a method originally designed for the preparation of viable synaptosomes from adult rat brains (Booth and Clark, 1978). Mazurkiewicz and Saggerson (1989) employed a slightly modified version of the same synaptosomal protocol for their work on synaptosomal membranes from adult rat brain regions. Preliminary experiments on marker enzymes showed that this modified method yielded synaptosomal membrane fractions that were relatively free from myelin and mitochondria. In the same study, Mazurkiewicz and Saggerson (1989) further established that hypothyroidism did not alter percentage distribution of the marker enzymes.

To ascertain whether the method was equally applicable for isolating fractions from younger developing animals, the purity of subcellular fractions obtained was assessed via enzyme marker assays. To recap, the method employed a Ficoll/sucrose gradient for the separation of the various subcellular fractions (myelin, synaptosomes and mitochondria) from a crude synaptosomal/mitochondrial pellet. Fractions were prepared from forebrain regions of euthyroid animals at the ages intended for later studies (10, 15, 20 and 25 days postpartum). In addition, subcellular fractions were prepared from adult rat forebrain (8 weeks) for comparison. Marker enzyme activities of all three fractions were measured to determine the extent of their distribution and consequently, the effectiveness of the

Table 4.1 DISTRIBUTION OF MARKER ENZYMES IN SUBCELLULAR FRACTIONS FROM RAT FOREBRAIN AT VARIOUS STAGES OF DEVELOPMENT.

Subcellular fractions were isolated as described in section 2.3 from rat forebrain regions at stages throughout development and assayed for marker enzymes (section 2.12). Values are means ± S.E.M. for 3-5 separate preparations, each repeated in triplicate.

Table 4.1a Acetylcholinesterase (synaptosomal membrane marker enzyme) activity (nmoles/min per mg of protein).

	Membrane Fraction		
Age (days)	Synaptosomal	Myelin	Mitochondria
10	106.51 ± 3.15	64.57 ± 0.41	30.32 ± 3.24
15	105.44 ± 9.14	68.14 ± 1.77	34.76 ± 9.59
20	117.31 ± 6.79	45.53 ± 2.61	28.81 ± 1.60
25	121.58 ± 6.91	58.16 ± 2.23	45.25 ± 2.19
8 weeks	193.00 ± 15.71	69.96 ± 1.77	51.19 ± 1.46

Table 4.1b 2', 3'- Cyclic nucleotide phosphohydrolase (myelin marker enzyme) activity (nmoles/min per mg of protein).

	Membrane Fraction		
Age (days)	Synaptosomal	Myelin	Mitochondria
10	242.62 ± 20.07	3051.53 ± 162.32	239.09± 52.62
15	119.53 ± 15.13	3434.95 ± 300.00	242.58 ± 4.15
20	2118.48 ± 52.76	8408.00 ± 231.5	452.42 ± 4.84
25	1591.70 ± 262.08	7592.64 ± 84.31	438.66 ± 0.67
8 weeks	1979.88 ± 309.48	8412.69 ± 46.71	319.58 ± 4.13

TABLE 4.1   **DISTRIBUTION OF MARKER ENZYMES IN SUBCELLULAR**
FRACTIONS FROM RAT FOREBRAIN CONTINUED.

Table 4.1c   **Succinate dehydrogenase (mitochondrial marker enzyme) activity**
(nmoles/min per mg of protein).

	Membrane Fraction		
Age (days)	Synaptosomal	Myelin	Mitochondria
10	19.88 ± 1.78	7.18 ± 0.18	33.95 ± 2.39
15	17.33 ± 0.76	10.03 ± 3.31	29.30 ± 0.04
20	14.33 ± 0.60	6.42 ± 0.08	53.21 ± 1.21
25	9.69 ± 0.60	9.47 ± 0.08	62.84 ± 2.12
8 weeks	13.36 ± 0.82	6.46 ± 0.27	61.21 ± 1.51

protocol on neonatal rat brains. Acetylcholinesterase has been shown to be a useful synaptosomal marker (Cotman and Matthews, 1971; Booth and Clark, 1978) and was used to give an indication of synaptosomal membrane distribution in each of the three fractions obtained (section 2.12). Measurement of the marker enzymes, 2', 3'-cyclic nucleotide phosphodiesterase (CNP) and succinate dehydrogenase (SDH) activities gave an indication of myelin and mitochondrial contamination respectively (sections 2.12.1 and 2.12.3). Table 4.1 shows the results obtained from marker enzyme measurements carried out on the different subcellular fractions. At all ages, 55-60% of total acetylcholinesterase activity (sum of activity in all three fractions at each age) was associated with the synaptosomal membrane fraction (Table 4.2a), and 75-90% of CNP activity with the myelin fraction (Table 4.2b). 55-75% of SDH activity was present in the mitochondrial fraction (Table 4.2c). The results demonstrated that a good degree of separation occurred with relatively high purity and little contamination by other subcellular fractions at all ages. Percentage distributions of the markers in each fraction from 8 week old animals (Table 4.2) were comparable to those obtained by Mazurkiewicz and Saggerson (1989) for control adult rats. In Table 4.1b, the dramatic rise of CNP activity in the myelin fraction between day 15 and day 20 corresponds to the onset of myelination that occurs during this time of development.

Table 4.2 % DISTRIBUTION OF MARKER ENZYMES IN SUBCELLULAR FRACTIONS FROM RAT FOREBRAIN AT VARIOUS STAGES OF DEVELOPMENT.

Values are expressed as percentage of total marker enzyme activity (sum of activity in all three fractions at each age).

Table 4.2a   Acetylcholinesterase (synaptosomal membrane marker enzyme).

	Membrane Fraction		
Age (days)	Synaptosomal	Myelin	Mitochondria
10	53%	32%	15%
15	51%	32%	17%
20	61%	24%	15%
25	54%	26%	20%
8 weeks	61%	22%	16%

Table 4.2b   2', 3'- Cyclic nucleotide phosphohydrolase (myelin marker enzyme).

	Membrane Fraction		
Age (days)	Synaptosomal	Myelin	Mitochondria
10	7%	86%	7%
15	24%	71%	5%
20	20%	76%	4%
25	17%	79%	4%
8 weeks	18%	79%	3%

Table 4.2c   Succinate dehydrogenase (mitochondrial marker enzyme).

	Membrane Fraction		
Age (days)	Synaptosomal	Myelin	Mitochondria
10	32%	12%	56%
15	31%	18%	52%
20	19%	9%	72%
25	12%	12%	76%
8 weeks	17%	8%	75

4.3 EFFECTS OF HYPOTHYROIDISM ON THE DEVELOPMENTAL PROFILE OF BRAIN SYNAPTOSOMAL MEMBRANE G-PROTEIN α-SUBUNITS.

Crude and synaptosomal membranes isolated from brain regions of rats at various stages of development were used to study the effect of hypothyroidism on G-protein α-subunit abundance levels. Membrane proteins were resolved in either 10% or 12.5% SDS polyacrylamide gels (section 2.9) before being transferred via Western blotting onto nitrocellulose sheets and probed with specific antisera for individual G-protein α-subunits (sections 2.9.4 and 2.9.5). Using a mixture of iodinated and horse-radish peroxidase conjugated secondary antiserum, cross-reacting bands corresponding to G-protein α-subunits were detected and quantified as described in section 2.9.6. With the exception of data from day 1 membranes, the synaptosomal membrane levels of the various α-subunits were expressed on a relative basis. In order to compensate for possible variations in the efficiency of electroelution onto nitrocellulose and to take into account of day-to-day variations in the specific radioactivity of ^{125}I-labelled secondary antibody, an aliquot of one synaptosomal membrane preparation isolated from the forebrains of 15 day old euthyroid pups was run as a reference standard on every gel. For each G-protein α-subunit band, (except those from crude membranes) this reference value was taken arbitrarily as 1.0 per unit quantity of membrane protein and other measurements from the same gel expressed relative to this. G-protein α-subunit levels in crude membranes prepared from forebrain and hindbrain regions of animals age 1 day postpartum culled as a result of litter standardisation procedures (section 2.4), were expressed as DPMs.

Table 4.3   **EFFECT OF HYPOTHYROIDISM ON G-PROTEIN α-SUBUNIT ABUNDANCE LEVELS IN CRUDE MEMBRANES FROM 1 DAY-OLD RAT PUP FOREBRAIN AND HINDBRAIN REGIONS.**

(a) $G_q\alpha$;   **(b)** $G_o\alpha$;   **(c)** $G_i1\alpha$;   **(d)** $G_i2\alpha$.

Crude membranes of forebrain and hindbrain regions of 1 day old pups were resolved in either 10% or 12.5% gels, transferred onto nitrocellulose sheets and probed with antisera as described in Materials and Methods. $G_q\alpha$, $G_o\alpha$ and total $G_i\alpha$ were detected with antisera CQ1, OC1 and SG2 respectively. Cross reacting bands were quantitated with ^{125}I-labelled secondary antibody and excised for gamma counting. Values are expressed as DPMs and represent means ± S.E.M. (n=7-8).

Table 4.3a $\underline{G_q\alpha \text{ (DPM/30 } \mu g \text{ of membrane protein)}}$

Brain Region	EUTHYROID	HYPOTHYROID
Forebrain	7852 ± 783	7462 ± 525
Hindbrain	8726 ± 489	10691 ± 1102

Table 4.3b $\underline{G_o\alpha \text{ (DPM/30 } \mu g \text{ of membrane protein)}}$

Brain Region	EUTHYROID	HYPOTHYROID
Forebrain	12649 ± 899	13136 ± 792
Hindbrain	10756 ± 644	11587 ± 1224

Table 4.3   EFFECT OF HYPOTHYROIDISM ON G-PROTEIN α-SUBUNIT ABUNDANCE LEVELS IN CRUDE MEMBRANES FROM 1 DAY-OLD RAT PUP FOREBRAIN AND HINDBRAIN REGIONS.

Table 4.3c $\underline{G_i1\alpha\ (DPM/30\ \mu g\ of\ membrane\ protein)}$

Brain Region	EUTHYROID	HYPOTHYROID
Forebrain	4222 ± 342	4212 ± 244
Hindbrain	4120 ± 468	4285 ± 659

Table 4.2d $\underline{G_i2\alpha\ (DPM/30\ \mu g\ of\ membrane\ protein)}$

Brain Region	EUTHYROID	HYPOTHYROID
Forebrain	4947 ± 217	4596 ± 181
Hindbrain	5436 ± 755	5984 ± 623

4.3.1 $\underline{G_{q/11}\alpha}$

Antiserum CQ1 was used to detect changes in $G_{q/11}\alpha$ abundance levels in synaptosomal membranes isolated from various brain regions throughout development. CQ1, raised against the decapeptide sequence QLNLKEYNLV, detected both $G_q\alpha$ and $G_{11}\alpha$. $G_q\alpha$ predominates over $G_{11}\alpha$ by 2-4 fold in crude membranes from various regions of adult rat brain (Milligan, 1993). Previously these two α-subunits have been resolved using gels containing 13% (w/v) acrylamide (Blank *et al.*, 1991; Milligan, 1993). Attempts to repeat this met with little success and throughout this study, under the conditions employed (10% SDS-PAGE; section 2.9), $G_q\alpha$ and $G_{11}\alpha$ co-migrated and were detected as one band following immunoblotting. Compared to euthyroid membranes, no change in the level of this particular G-protein α-subunit was detected in crude membrane fractions from forebrains of hypothyroid 1 day old animals. This was also the case for crude membranes isolated from hindbrain regions as shown in Table 4.3a (page 157).

Figure 4.1 shows the relative abundance levels of $G_{q/11}\alpha$ in euthyroid and hypothyroid synaptosomal membranes prepared from forebrains of rat pups at various stages of neonatal life. From a developmental point of view, $G_{q/11}\alpha$ immunoreactivity in forebrain synaptosomal membranes increased nearly 2-fold (P<0.05) over the 10 to 20 day period in euthyroid pups. Compared to euthyroid animals, a precocious significant doubling of $G_{q/11}\alpha$ was observed in synaptosomal membranes from forebrains of day 10 hypothyroid animals (P<0.01). Between days 15 and 25, levels of $G_{q/11}\alpha$ of hypothyroid animals were comparable with those of euthyroid animals with a slight increase in levels detected in the hypothyroid state.

Figure 4.2a shows the relative abundance levels of $G_{q/11}\alpha$ in synaptosomal membranes isolated from the euthyroid hindbrain. The ontogenic profile of this particular α-subunit
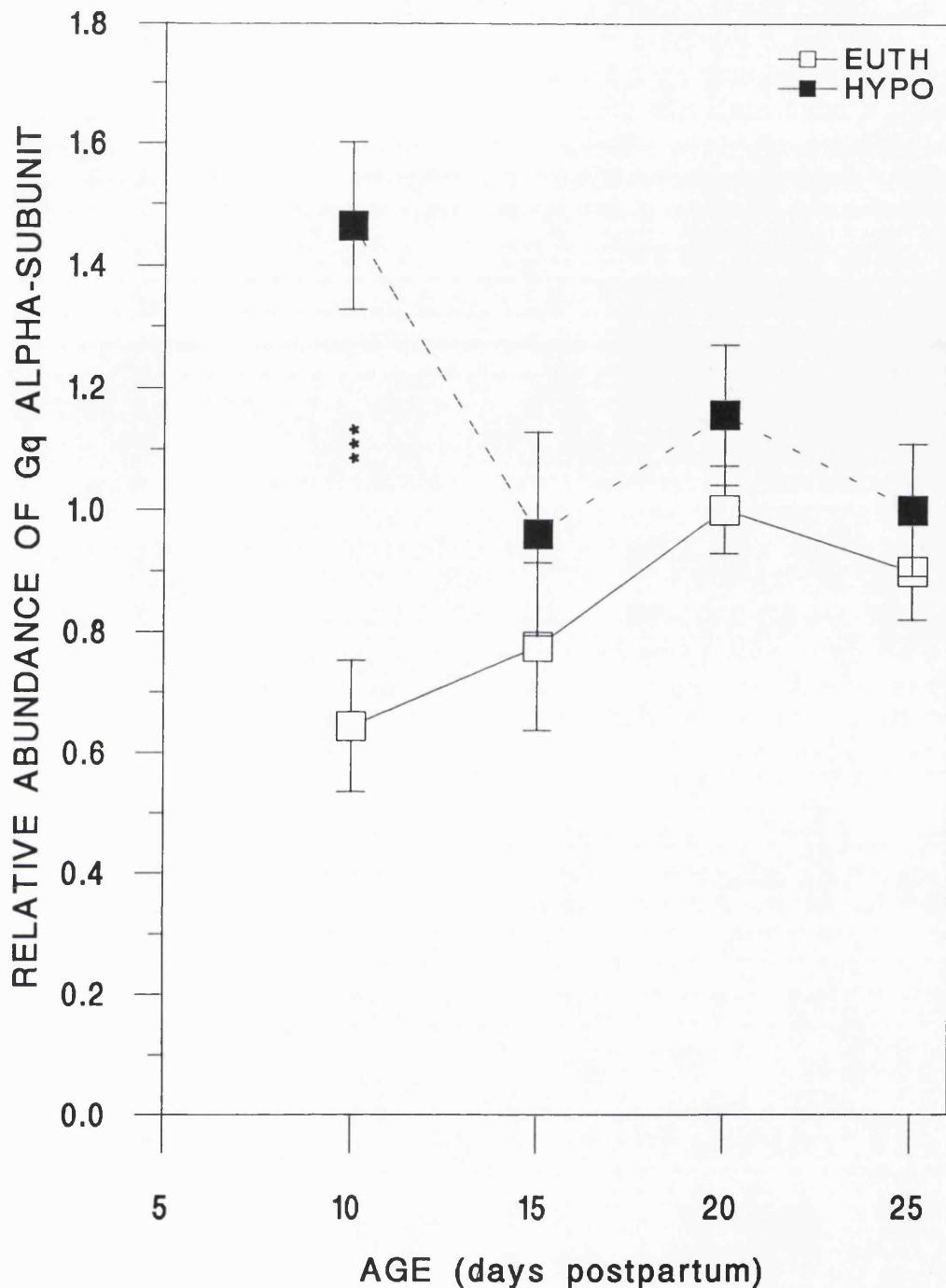
Figure 4.1 <u>EFFECT OF HYPOTHYROIDISM ON THE RELATIVE ABUNDANCE OF $G_{q/11}\alpha$ IN SYNAPTOSOMAL MEMBRANES FROM RAT PUP FOREBRAIN.</u>

Synaptosomal membranes (60 μg) were resolved in 10% SDS gels. Following transfer to nitrocellulose sheets, $G_{q/11}\alpha$ was probed with antiserum CQ1 and detected with ^{125}I-labelled secondary antibody as described in section 2.9.

Values are means ± S.E.M. (n=5-6) and expressed on a relative basis which is explained under the Materials and Methods section.

*** indicates P<0.01 for comparison of the hypothyroid (HYPO) and euthyroid (EUTH) states.

Figure 4.2 <u>**RELATIVE ABUNDANCE OF** $G_{q/11}\alpha$ **IN SYNAPTOSOMAL MEMBRANES FROM RAT PUP WHOLE HINDBRAIN AND HINDBRAIN REGIONS.**</u>

(a) Euthyroid ;   (b) Hypothyroid.

Synaptosomal membranes (60 μg) were resolved on 10% SDS-gels, electroeluted onto nitrocellulose sheets, and subjected to immunoblotting using CQ1 antiserum. Bands were excised and counted for radioactivity (section 2.9).

Values are means ± S.E.M. (n=4-6) and expressed on a relative basis which is explained under the Materials and Methods section.

HIND, CE and MO indicate hindbrain, cerebellum and medulla oblongata respectively. *** indicates P<0.01 for comparison of the hypothyroid (HYPO) and euthyroid (EUTH) states.

Figure 4.2a **EUTHYROID**
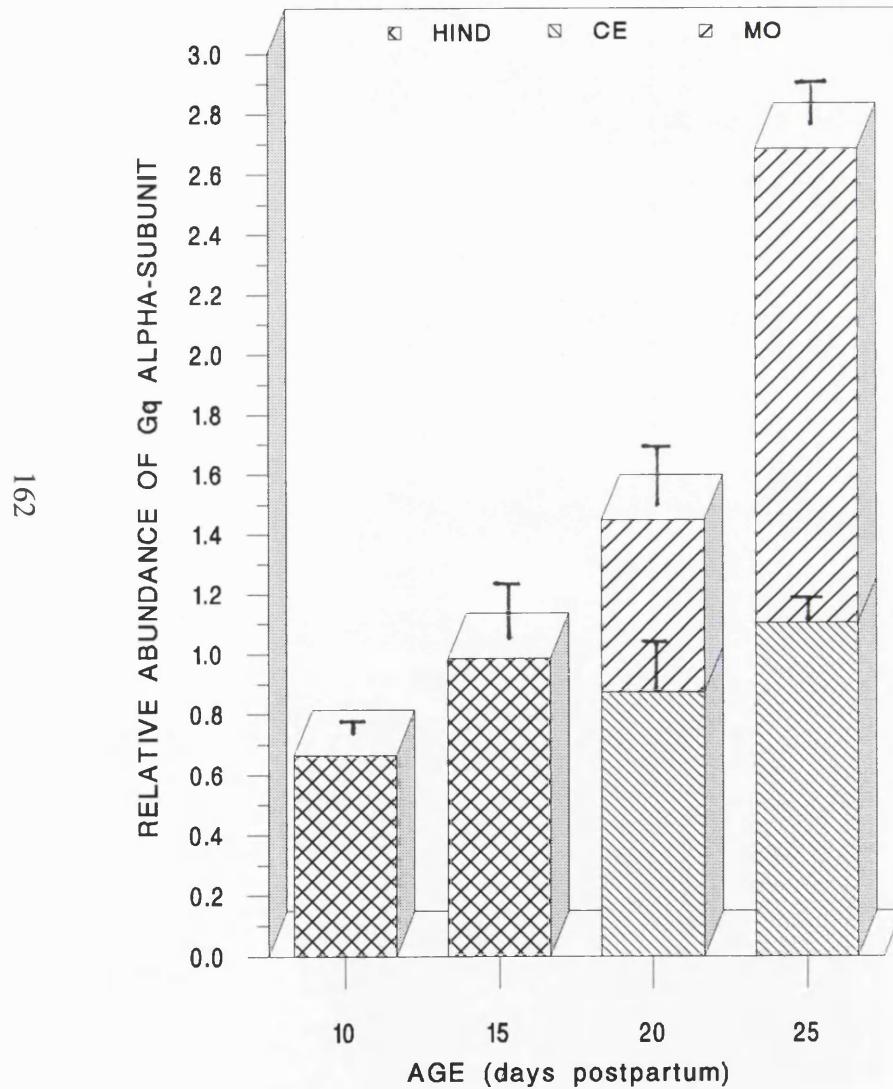
Figure 4.2b **HYPOTHYROID**

162
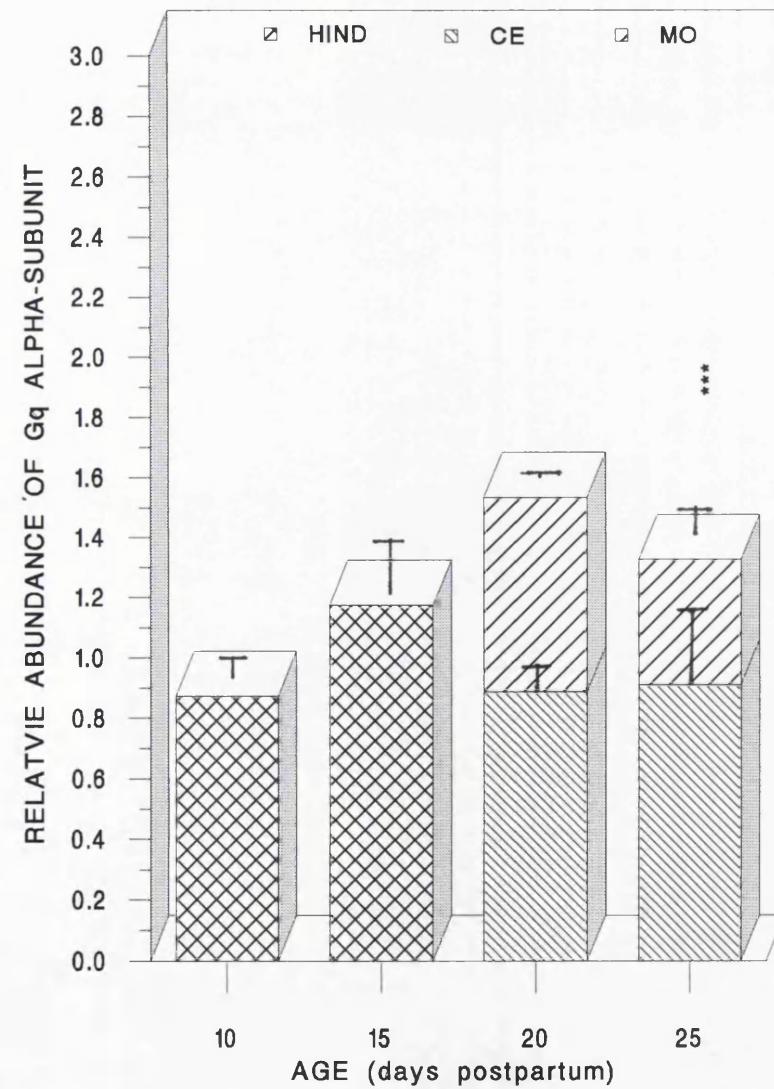
in the euthyroid hindbrain revealed a 48% increase over the 10 to 15 day period. At days 20 and 25 when the hindbrain was studied in more anatomical detail (cerebellum and medulla oblongata) the progressive rise in $G_{q/11}\alpha$ levels was still evident in the cerebellum and in the medulla oblongata.

Hypothyroidism altered the developmental profile of this α-subunit in the hindbrain region (Figure 4.2b). Increases of 31% and 19% (compared to euthyroid values) were seen in 10 and 15 day-old hypothyroid animals respectively. In general an upward trend through days 10 to 15 and days 20 to 25 was observed in the hypothyroid state. Only in the medulla oblongata was the upward trend reversed, leading to a 54% deficit ($P<0.01$) in $G_{q/11}\alpha$ relative to the euthyroid state at day 25. No changes as a result of hypothyroidism were evident in membranes prepared from the cerebellum at days 20 and 25.

4.3.2 $\underline{G_0\alpha}$

G_0 is highly abundant in the brain, constituting up to 2% of total membrane protein (Sternweiss and Robishaw, 1984). The antiserum OC1 used to detect changes in $G_0\alpha$ recognises the two splice variant forms (G_0A and G_0B) of the G-protein α-subunit but as in the case of $G_{q/11}\alpha$, under the conditions employed in this study the two forms migrate together and are detected as a single band on SDS-PAGE. As a result of its predominance in the brain, considerably lower amounts of synaptosomal membranes were used to look for changes in $G_0\alpha$ (10 μg compared to 100 μg protein).

At day 1, hypothyroidism caused no abundance change in this α-subunit compared to euthyroid values in both forebrain and hindbrain regions (Table 4.3b; page 157).

The developmental profile of forebrain synaptosomal membrane $G_0\alpha$ is shown in Figure 4.3. At day 15, levels of $G_0\alpha$ were dramatically below those seen in the other times
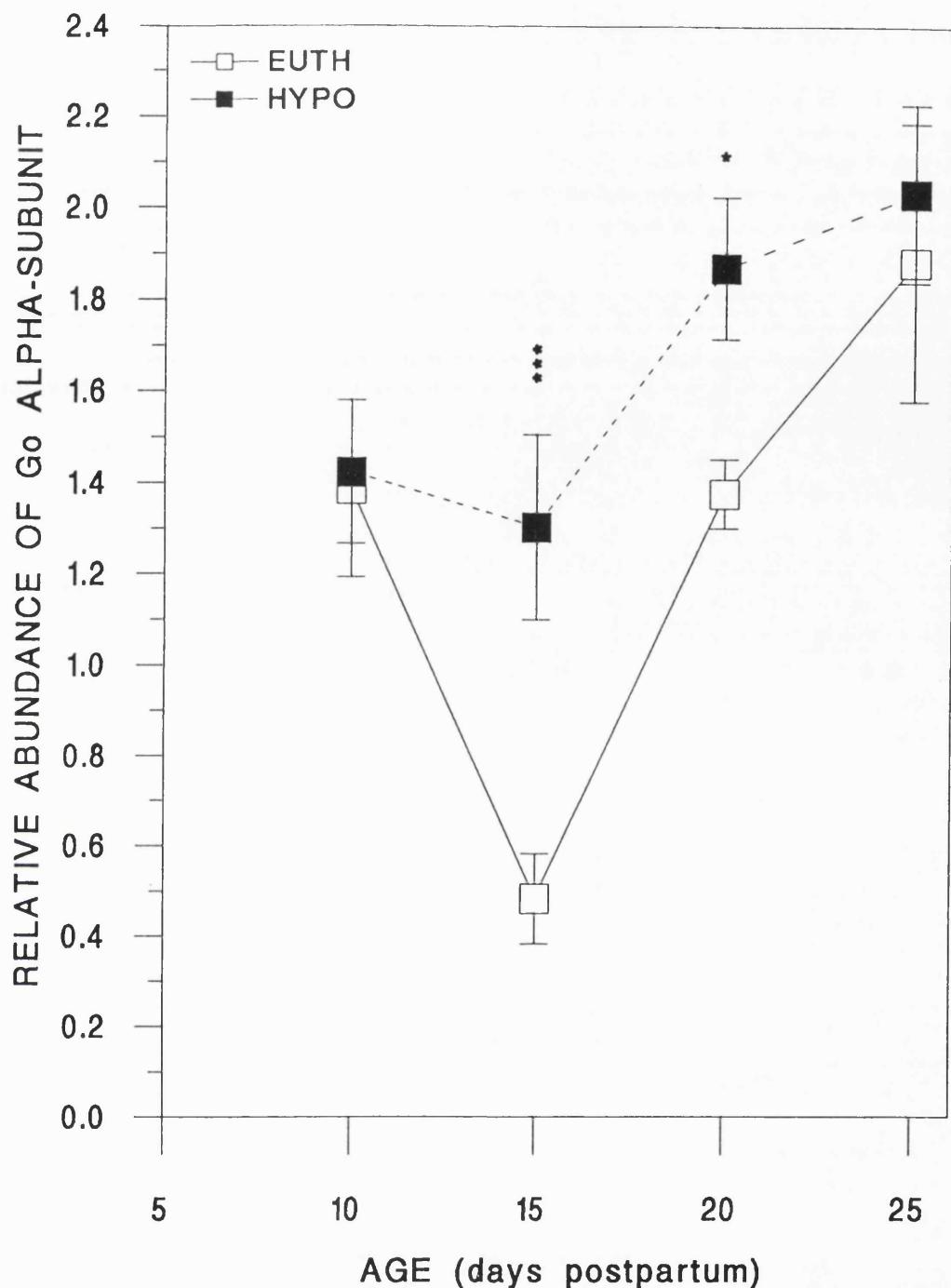
**Figure 4.3 EFFECT OF HYPOTHYROIDISM ON THE RELATIVE ABUNDANCE OF $G_o\alpha$ IN SYNAPTOSOMAL MEMBRANES FROM RAT PUP FOREBRAIN.**

Synaptosomal membranes (10 μg) were resolved in 10% SDS gels. Following transfer to nitrocellulose sheets, $G_o\alpha$ was probed with antiserum OC1 and detected with ^{125}I-labelled secondary antibody as described in section 2.9.

Values are means ± S.E.M. (n=5-6) and expressed on a relative basis which is explained under the Materials and Methods section.

* and *** indicate P<0.05 and <0.01 respectively for comparison of the hypothyroid (HYPO) and euthyroid (EUTH) states.

studied (P<0.05 compared with euthyroid day 10 and P<0.001 compared with euthyroid days 20 and 25). During hypothyroidism, a change in the ontogenic appearance of $G_o\alpha$ was observed (Figure 4.3). Relative abundance levels of this α-subunit were elevated in membranes isolated from hypothyroid animals. At days 15 and 20, hypothyroid membranes were significantly higher compared to control membranes (2.7-fold, P<0.01 and 1.5-fold, P<0.05 respectively). Furthermore, the drop in $G_o\alpha$ observed at day 15 in the euthyroid state was less pronounced during hypothyroidism.

In both the euthyroid and hypothyroid hindbrain, synaptosomal membrane $G_o\alpha$ abundance levels increased between the first 10 and 15 days of life though this was more pronounced in the former (76% increase, P<0.02) as seen in Figure 4.4. Between days 20 and 25, the upward trend was still evident in the cerebellum (in both states) and in the medulla oblongata in the euthyroid state. In contrast, abundance of $G_o\alpha$ in membranes from medulla oblongata regions of hypothyroid pups decreased by 46% (P<0.02) during this time period. Finally, as with $G_{q/11}\alpha$, hypothyroidism had no noteworthy effects upon $G_o\alpha$ levels in the hindbrain at all ages studied.

4.3.3 $G_i1\alpha$

The α-subunit of the G-protein G_i1 was detected simultaneously with that of G_i2 using the antiserum SG2. Raised against a decapeptide sequence common to both α-subunits (KENLKDCGLF), the two proteins were separable by SDS-PAGE on 12.5% gels as described in section 2.9.1 (Mitchell *et al.*, 1989).

Table 4.3c (page 158) shows the levels of $G_i1\alpha$ in crude membrane homogenates isolated from forebrain and hindbrain regions. As with the previous G-protein α-subunits, hypothyroidism had no discernible effect in either brain region with similar levels of the

Figure 4.4 <u>RELATIVE ABUNDANCE OF $G_0\alpha$ IN SYNAPTOSOMAL MEMBRANES FROM RAT PUP WHOLE HINDBRAIN AND HINDBRAIN REGIONS.</u>

(a) Euthyroid ;   (b) Hypothyroid.

Synaptosomal membranes (10 μg) were resolved on 10% SDS-gels, electroeluted onto nitrocellulose sheets, and subjected to immunoblotting using OC1 antiserum. Bands were excised and counted for radioactivity (section 2.9).

Values are means ± S.E.M. (n=4-6) and expressed on a relative basis which is explained under the Materials and Methods section.

HIND, CE and MO indicate hindbrain, cerebellum and medulla oblongata respectively.

Figure 4.4a **EUTHYROID**
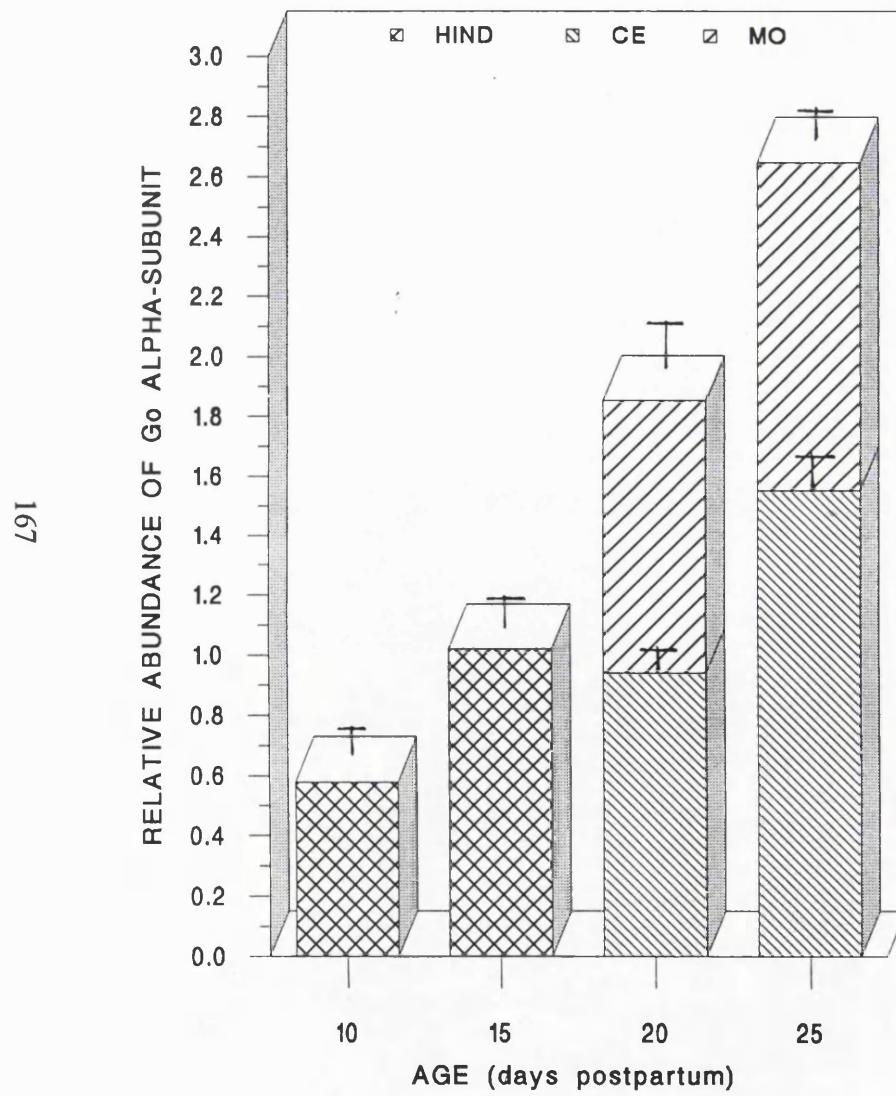
Figure 4.4b **HYPOTHYROID**
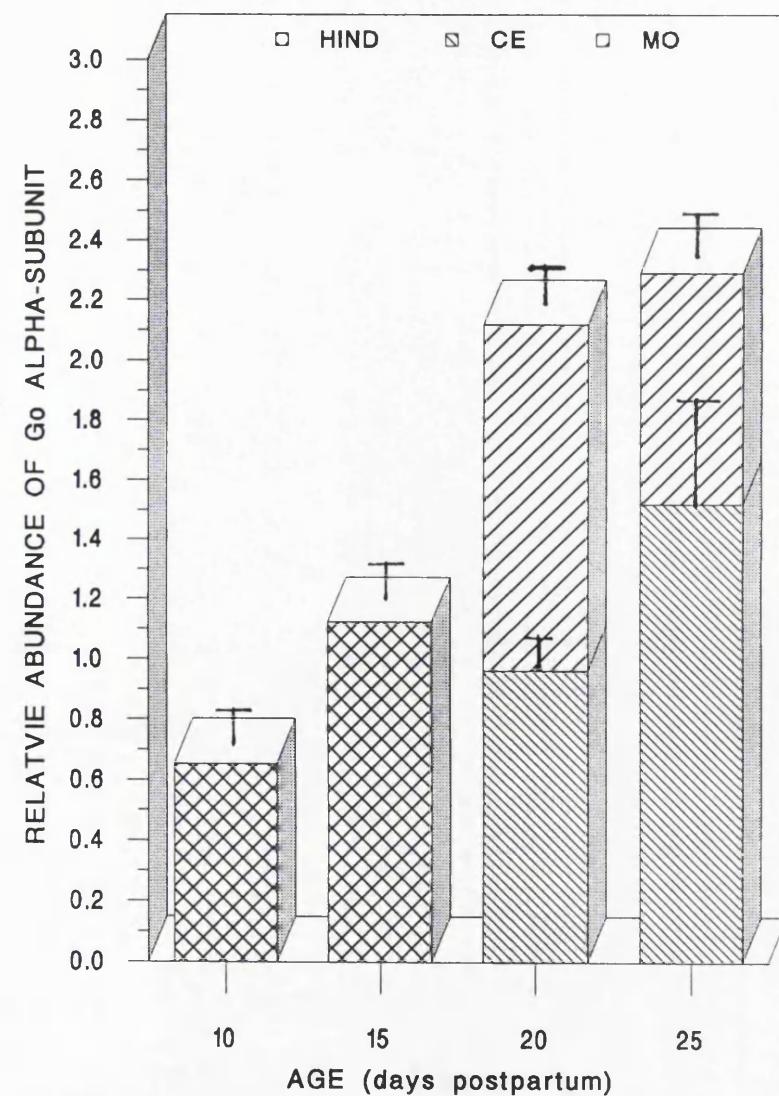
α-subunit detected between the two states at this stage of development.

When synaptosomal membranes were tested, hypothyroidism was seen to have perceivable effects on the levels of this α-subunit (Figure 4.5). Focusing first on the ontogenic profile, the level of $G_i1\alpha$ in both euthyroid and hypothyroid states "troughed" at day 15. Compared with day 15, values for this G-protein α-subunit at all other times were significantly different (euthyroid: day 10, $P<0.05$; day 20, $P<0.02$; day 25, $P<0.05$. hypothyroid: day 10 $P<0.02$; day 20, $P<0.001$; day 25, $P<0.02$). Hypothyroidism did not perturb the developmental profile of $G_i1\alpha$ with the exception of a significant 1.2-fold increase ($P<0.05$) compared to control at day 20.

In the hindbrain a different picture was seen. Figure 4.6 shows that between days 10 and 15, increases of 2.2-fold ($P<0.01$) and 1.4-fold ($P<0.05$) in euthyroid and hypothyroid membranes respectively were observed with levels of the α-subunit slightly elevated in the hypothyroid state. At day 10 the expression of $G_i1\alpha$ was significantly advanced in the hypothyroid state; compared to the euthyroid state, a 95% rise in the hindbrain level of $G_i1\alpha$ ($P<0.02$) was detected in hypothyroid pups at this time. Between days 20 and 25, decreases in the abundance of $G_i1\alpha$ were noted in the cerebellum and medulla oblongata regions in both states. With the exception of membranes isolated from the medulla oblongata at day 25 when a 20% drop ($P<0.05$) in $G_i1\alpha$ was noted, no discernible perturbations by hypothyroidism were evident.
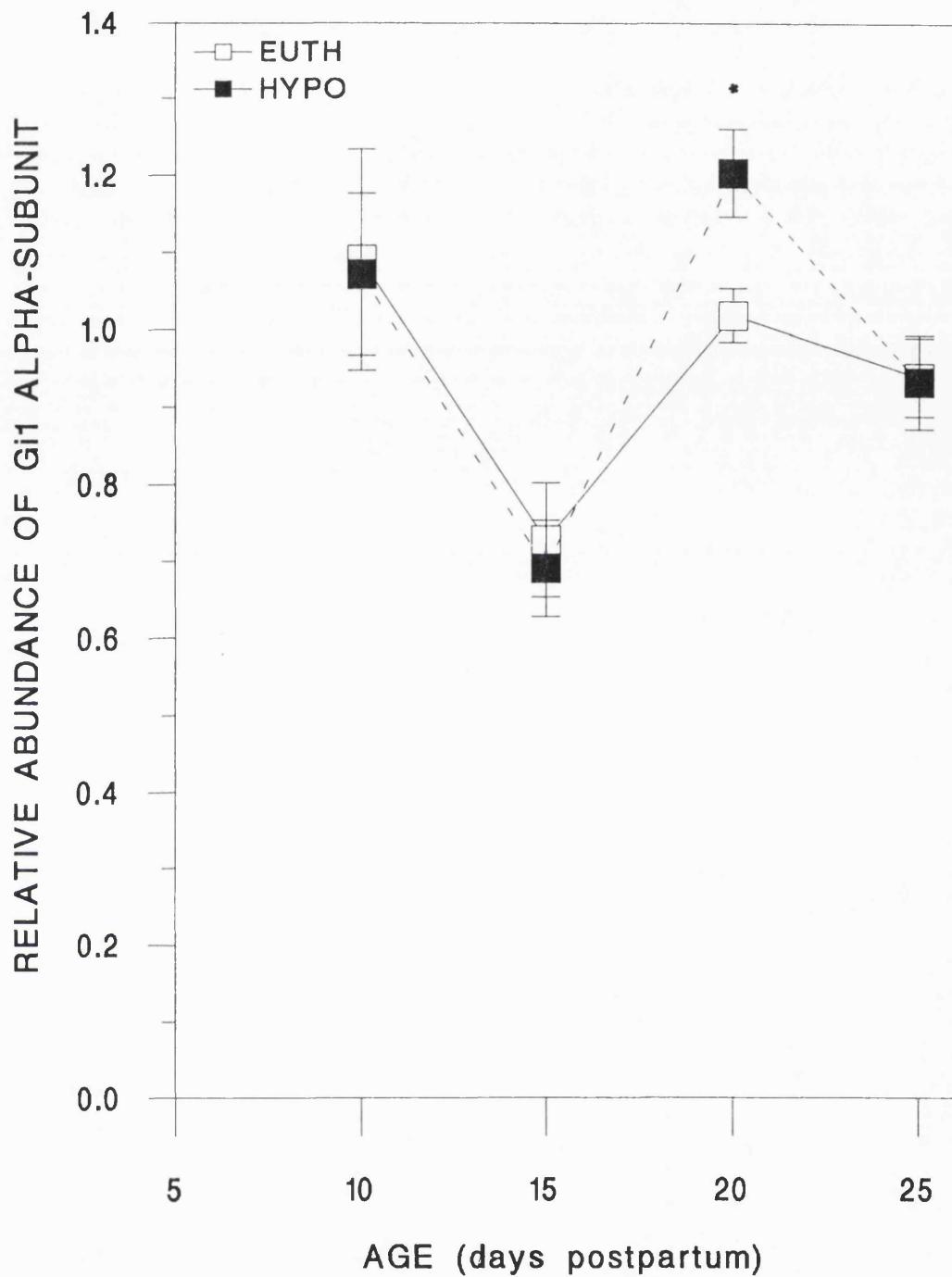
Figure 4.5 <u>**EFFECT OF HYPOTHYROIDISM ON THE RELATIVE ABUNDANCE OF $G_i1\alpha$ IN SYNAPTOSOMAL MEMBRANES FROM RAT PUP FOREBRAIN.**</u>

Synaptosomal membranes (100 μg) were resolved in 12.5% SDS gels. Following transfer to nitrocellulose sheets, $G_i1\alpha$ was probed with antiserum SG2 and detected with ^{125}I-labelled secondary antibody as described in section 2.9. The upper band of the doublet pertaining to $G_i1\alpha$ was consequently excised and quantitated via gamma counting.

Values are means ± S.E.M. (n=4-6) and expressed on a relative basis which is explained under the Materials and Methods section.

* indicates P<0.05 for comparison of the hypothyroid (HYPO) and euthyroid (EUTH) states.

Figure 4.6 <u>RELATIVE ABUNDANCE OF $G_i1\alpha$ IN SYNAPTOSOMAL MEMBRANES FROM RAT PUP WHOLE HINDBRAIN AND HINDBRAIN REGIONS.</u>

(a) Euthyroid ;   (b) Hypothyroid.

Synaptosomal membranes (100 μg) were resolved on 12.5% SDS-gels, electroeluted onto nitrocellulose sheets, and subjected to immunoblotting using SG2 antiserum. Bands were excised and counted for radioactivity (section 2.9). $G_i1\alpha$ was detected simultaneously with $G_i2\alpha$ as a doublet; the upper band pertained to $G_i1\alpha$.

Values are means $\pm$ S.E.M. (n=4-6) and expressed on a relative basis which is explained under the Materials and Methods section.

HIND, CE and MO indicate hindbrain, cerebellum and medulla oblongata respectively. ** indicates P<0.02 for comparison of the hypothyroid (HYPO) and euthyroid (EUTH) states.

Figure 4.6a

EUTHYROID

RELATIVE ABUNDANCE OF Gi1 ALPHA-SUBUNIT

AGE (days postpartum)

HIND   CE   MO

Figure 4.6b

HYPOTHYROID

RELATIVE ABUNDANCE OF Gi1 ALPHA-SUBUNIT

AGE (days postpartum)

HIND   CE   MO
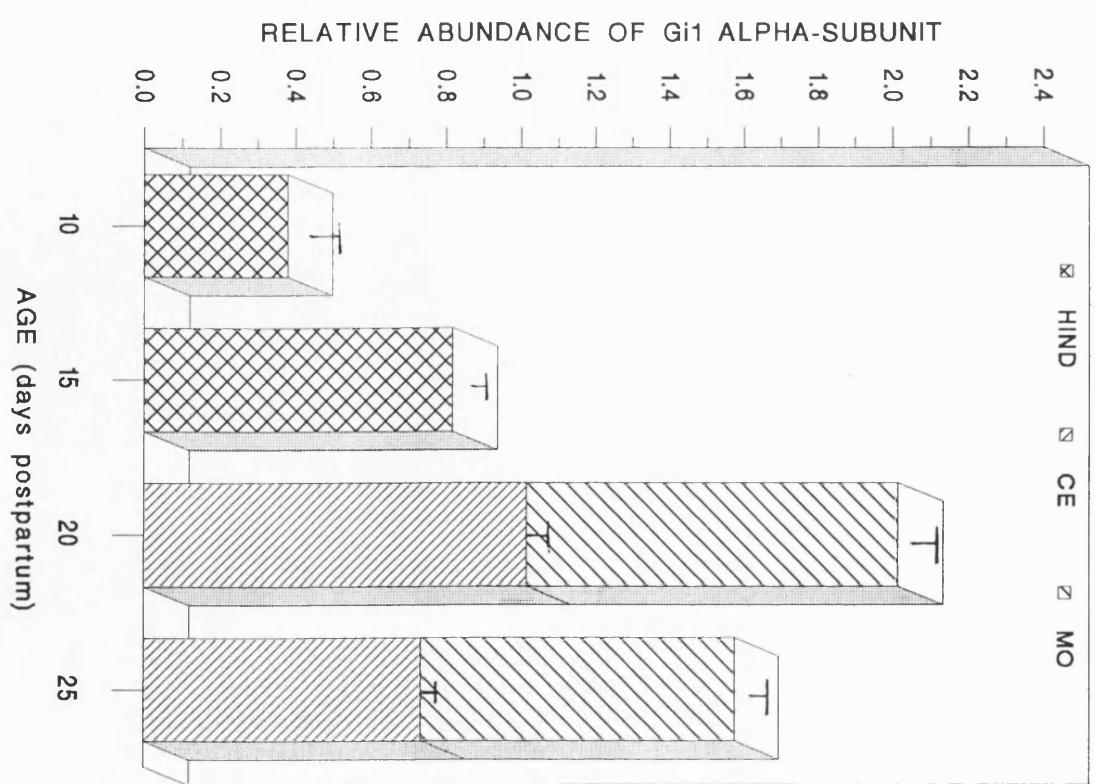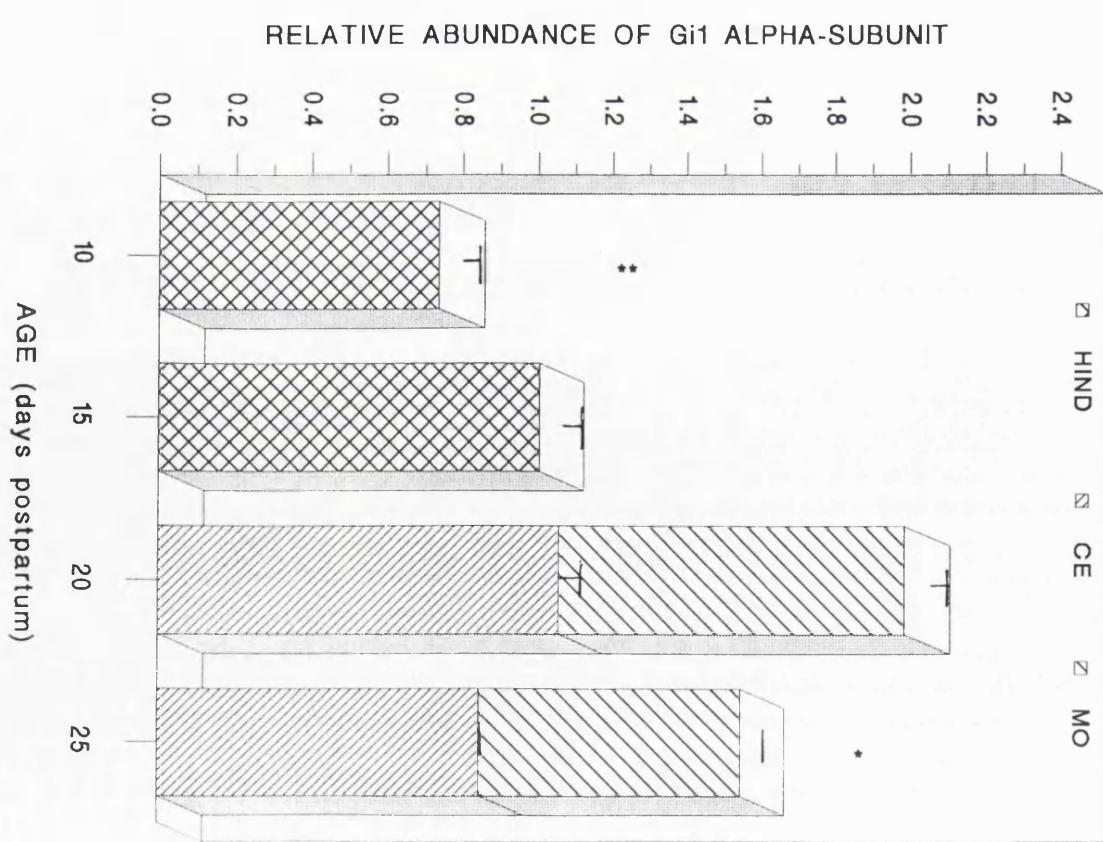
4.3.4 <u>$G_i2\alpha$</u>

As mentioned previously, G_i1 and G_i2 were detected simultaneously using the antiserum SG2 which recognised the decapeptide sequence KENLKDCGLF common to both proteins. The lower of the two bands which corresponded to $G_i2\alpha$ was excised and quantitated via gamma counting as described in section 2.9.

Hypothyroidism had the least effect on this particular α-subunit in all brain regions studied. At day 1 as for other G-protein α-subunits studied, no changes were observed between crude membrane fractions isolated from hypothyroid and euthyroid pups in both brain regions as seen in Table 4.3d (page 158).

Figure 4.7 shows the relative abundance levels of $G_i2\alpha$ in forebrains of euthyroid and hypothyroid animals at various stages of neonatal life. In contrast to the other α-subunits investigated, $G_i2\alpha$ abundance levels remained essentially constant throughout the developmental period studied (day 10 to day 25 postpartum).

The only change observed in the forebrain during hypothyroidism was a 36% decrease (P<0.01) at day 15 resulting in a dip in the developmental profile of $G_i2\alpha$ (Figure 4.7). This value was also significantly lower compared to other times in hypothyroid forebrain samples (P<0.02 versus days 10 and 20, P<0.01 versus day 25).

The relative abundance levels of $G_i2\alpha$ in hindbrain regions of euthyroid and hypothyroid pups are shown in Figure 4.8. A gradual rise in the α-subunit was detected in the euthyroid hindbrain between the 10 and 20 day period (taking cerebellum and medulla oblongata values together at day 20). This was also observed in membranes isolated from hypothyroid animals. When hindbrain regions at days 20 and 25 were treated as discrete regions, it was seen that with the exception of the cerebellum during the euthyroid state, levels of the α-subunit fell over the 20 to 25 day period. In the medulla oblongata this
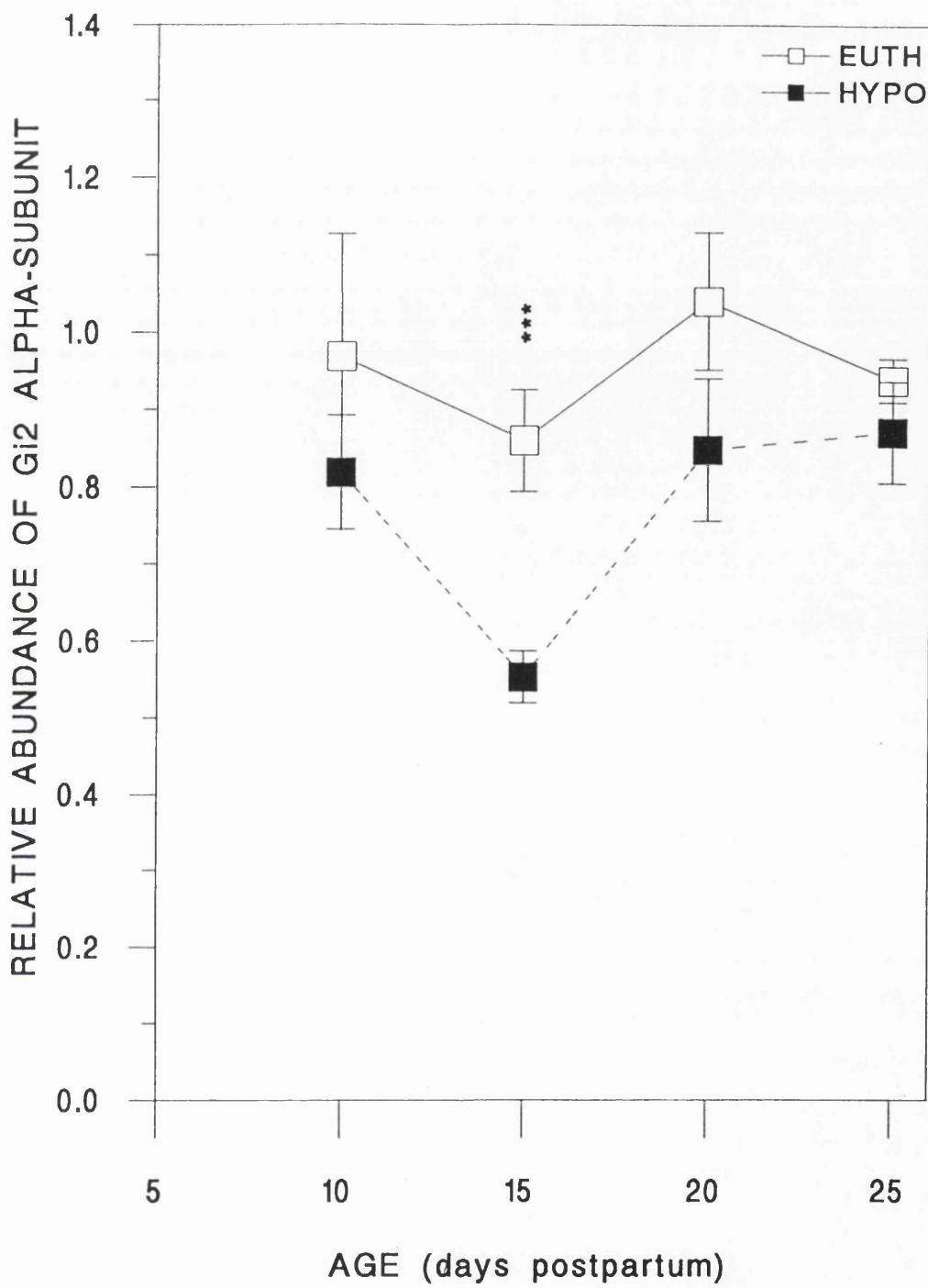
Figure 4.7 **EFFECT OF HYPOTHYROIDISM ON THE RELATIVE ABUNDANCE OF $G_i2\alpha$ IN SYNAPTOSOMAL MEMBRANES FROM RAT PUP FOREBRAIN.**

Synaptosomal membranes (100 μg) were resolved in 12.5% SDS gels. Following transfer to nitrocellulose sheets, $G_i2\alpha$ was probed with antiserum SG2 and detected with ^{125}I-labelled secondary antibody as described in section 2.9. The lower band of the doublet pertaining to $G_i2\alpha$ was consequently excised and quantitated via gamma counting.

Values are means ± S.E.M. (n=4-6) and expressed on a relative basis which is explained under the Materials and Methods section.

*** indicates P<0.01 for comparison of the hypothyroid (HYPO) and euthyroid (EUTH) states.

decrease was significant (33%, P<0.01). At the same time a 21% drop was also noted in the euthyroid state at this time but this change was not significant. In the cerebellum, $G_i2\alpha$ levels remained relatively constant in both states. Finally in contrast to the forebrain, hypothyroidism had no noteworthy effects on membranes isolated from the hindbrain at each developmental age studied.

The ratio of $G_i1\alpha$ to $G_i2\alpha$ (as detected by SG2) did not differ significantly in any of the brain regions studied between euthyroid and hypothyroid state, with the exception of day 10 forebrain and day 20 cerebellum. In the former, the ratio between the two α-subunits from hypothyroid animals was 1.5-fold higher than that from euthyroid animals (P<0.05). In the cerebellum region of day 20 animal, the reverse was observed with a 1.5-fold increase (P<0.01) detected in euthyroid animals.

Figure 4.8 <u>RELATIVE ABUNDANCE OF $G_i2\alpha$ IN SYNAPTOSOMAL MEMBRANES FROM RAT PUP WHOLE HINDBRAIN AND HINDBRAIN REGIONS.</u>

(a) Euthyroid ;   (b) Hypothyroid.

Synaptosomal membranes (100 μg) were resolved on 12.5% SDS-gels, electroeluted onto nitrocellulose sheets, and subjected to immunoblotting using SG2 antiserum. Bands were excised and counted for radioactivity (section 2.9). $G_i2\alpha$ was detected simultaneously with $G_i1\alpha$ as a doublet; the lower band pertained to $G_i2\alpha$.

Values are means ± S.E.M. (n=4-6) and expressed on a relative basis which is explained under the Materials and Methods section.

HIND, CE and MO indicate hindbrain, cerebellum and medulla oblongata respectively.

Figure 4.8a   <u>EUTHYROID</u>
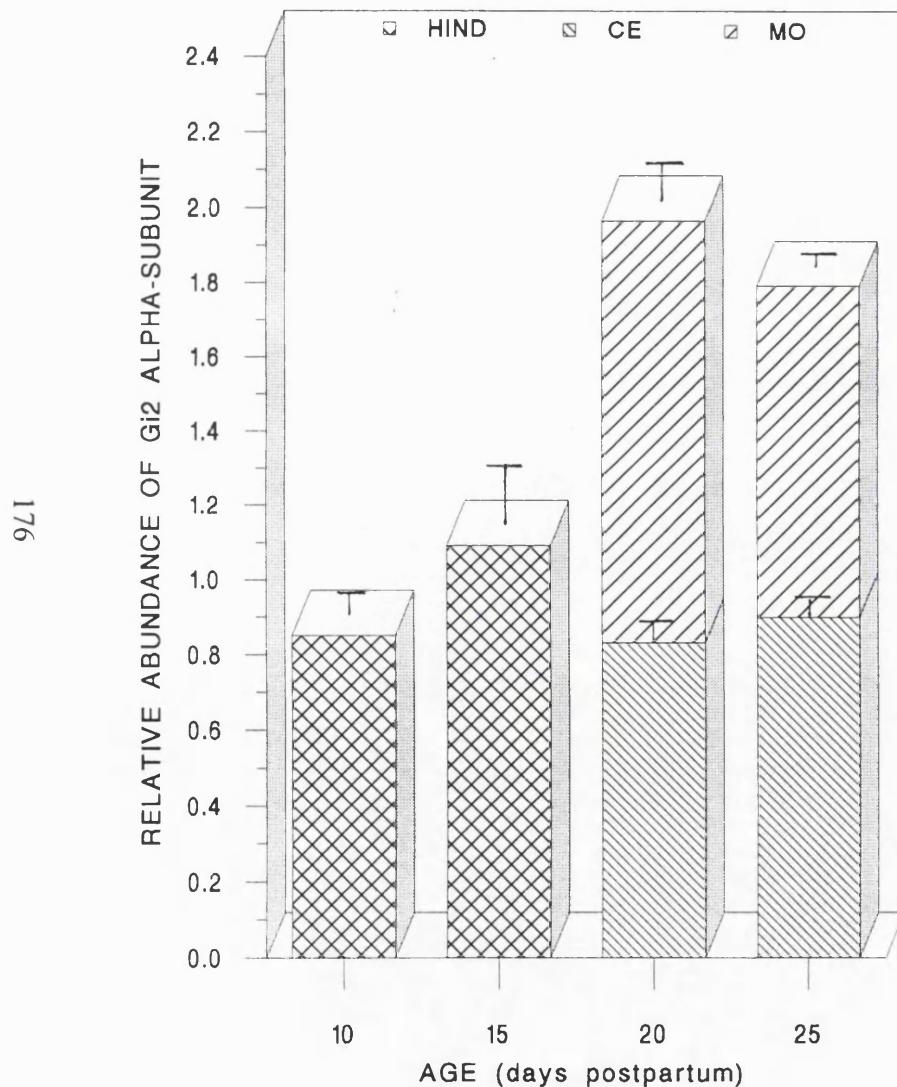
Figure 4.8b   <u>HYPOTHYROID</u>
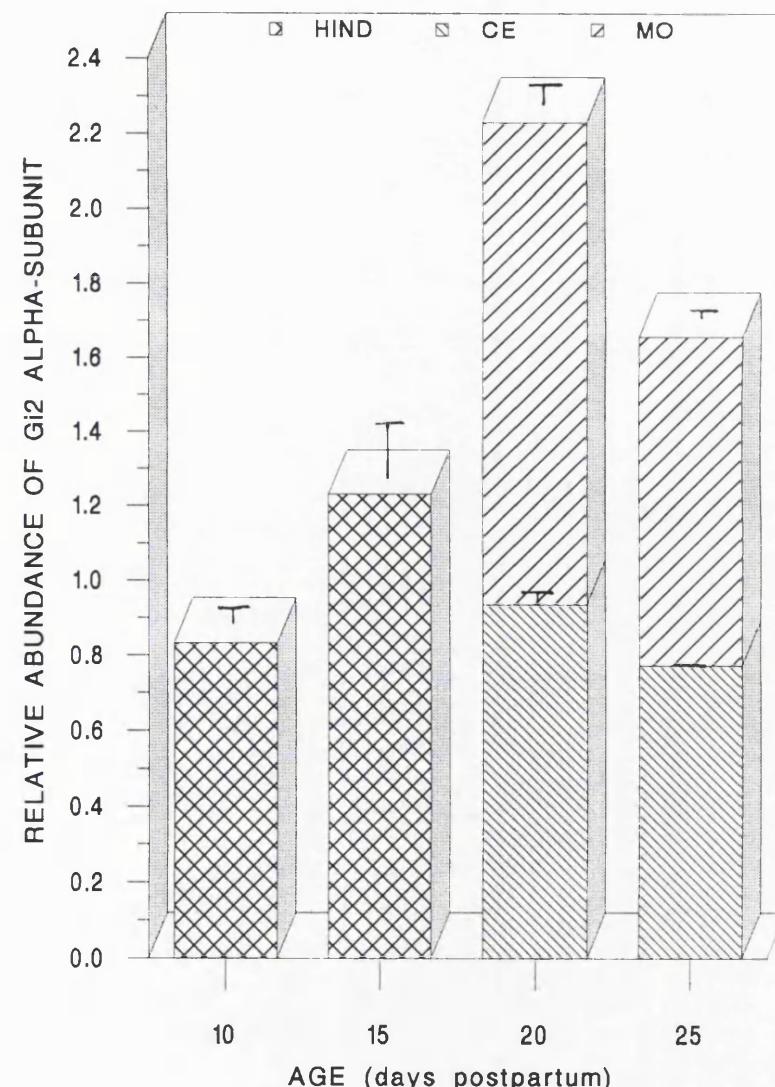
4.3.5 G-PROTEIN DISCUSSION.

Pre- and post-natal hypothyroidism resulting from maternal iodine deficiency, altered the ontogenic profile of $G_{q/11}\alpha$, $G_o\alpha$, $G_i1\alpha$ and $G_i2\alpha$ observed in forebrain synaptosomal membranes of euthyroid rat pups. Disruptions in the developmental appearance of the aforementioned proteins were also detected in hindbrain regions albeit to a lesser extent. Moreover with the exception of $G_i2\alpha$, all of the noteworthy changes observed in the hypothyroid forebrain at various developmental stages were that of α-subunit up-regulation. The majority of changes occurred around day 15 postpartum which in the hindbrain is a time of active cellular activity in terms of differentiation, maturation and synaptogenesis. Though these events are effectively over in the forebrain by this time, an abnormal thyroid state during the earlier periods of life leads to altered morphology and organisation of the neuronal network and therefore may affect changes via indirect rather than direct means. For example, impaired synaptogenesis as a consequence of lowered thyroid hormones, could have some bearing on synaptosomal membrane G-protein concentrations. This is the first demonstration of differential regulation in G-protein α-subunits of regional brain synaptosomal membranes by neonatal hypothyroidism.

Age dependent increases of $G_o\alpha$ and $G_i1\alpha$ in conjunction with δ-opiate receptor numbers which are responsible for the majority of inhibition of brain adenylyl cyclase, have been detected in crude membrane homogenates prepared from rat forebrain (Milligan *et al.*, 1987b; Asano *et al.*, 1988). Charpentier *et al.* (1993) showed that expression of $G_o\alpha$ in C6 glioma cells which do not normally express this G-protein, resulted in morphological changes and a decrease in cell growth rate. The appearance of $G_o\alpha$ and its closely associated protein, GAP 43, during specific times of embryogenesis in the CNS imply that

the α-subunit is not involved in neuroblast mitosis or migration but in the regulation of neurite extension, and/or outgrowth and recognition (Schmidt *et al.*, 1994). Alterations in the developmental profile of $G_o\alpha$ observed in this study may have severe consequences for the regulation of these cellular events in the forebrain and hindbrain in the hypothyroid neonate.

In contrast to the findings presented here, Wong *et al.* (1994) detected no significant differences in levels of $G_q\alpha$, $G_o\alpha$, $G_i1\alpha$, $G_i2\alpha$ and $G\beta$ at day 22 in hypothyroid animals. These discrepancies may be related to the use of a tissue homogenate versus a synaptosomal preparation and/or the use of methimazole to induce maternal hypothyroidism at day 14 gestation.

The substantial increase in the abundance of $G_{q/11}\alpha$ observed at 10 days postpartum in the hypothyroid forebrain implies the possibility of enhanced activation of PLC-β isoforms. These secondary effectors are present in the brain particularly in the hippocampus and the cerebral cortex (Gerfen *et al.*, 1988; Rhee *et al.*, 1991; Mailleux *et al.*, 1992). The functional significance of PLC in the brain is at present unknown but it would be reasonable to assume that the temporal appearance of this α-subunit may along with others, be vital in the regulation of various maturational and/or differential events during this important time.

Asano *et al.* (1989) detected constant levels of $G_i2\alpha$ at all ages studied which are consistent with the results shown here. $G_i2\alpha$ is present in high concentrations on the cell membranes of many cell types including the brain neuropile. $G_i2\alpha$ mRNA is widely expressed in a variety of tissues (Brann *et al.*, 1987; Kim *et al.*, 1988) prompting the suggestion that the $G_i2\alpha$ gene may be a housekeeping gene (Itoh *et al.*, 1988) and that the α-subunit is involved in regulating processes common in cells.

The increase in the glial/neuron ratio observed in the hypothyroid developing brain has been linked to cAMP concentrations. Segovia *et al.* (1994) observed that increased levels of cAMP induced differentiation of C6 cells towards a more astrocytic phenotype as reflected by increased expression of the astroglial marker GFAB mRNA (glial fibrillary acidic protein). The drop in $G_i2\alpha$ levels noted in the forebrain at day 15 may therefore have some bearing on the cellular phenotype of the brain region during neonatal hypothyroidism thereby altering the signalling ability of the brain.

The lack of noteworthy changes in the hindbrain, particularly during this vulnerable time was surprising but may be accounted for by the heterogeneity of the membrane preparation which possibly masks significant changes in either the cerebellum or medulla oblongata at this time. Disruptions in the normal expression of hindbrain G-protein α-subunits were however seen in the hypothyroid state. Given the fact that G-protein mediated signal transduction involves an amplification response, it is possible that small insignificant changes in G-protein abundance and/or function are sufficient to cause alterations in cell signalling processes of the brain. Relatively minor changes in G-protein subtypes therefore, may be associated with large changes in neuronal function (Innis and Aghajanian, 1987; Innis *et al.*, 1988). Innis *et al.* (1988) found that inhibition of $G_o\alpha/G_i1\alpha$ by only 10-15% leads to a 50% drop in the ability of neurotransmitters to exert their electrophysiological effects on specific neuronal cell types. Chronic administration of lithium results in a relatively small (20%) drop in $G_i1\alpha$ expression; but enough to exert a significant disruption upon $G_i1\alpha$ function (Colin *et al.*, 1991). In addition, as hypothyroidism affects a range of receptor systems and subsequently the cell's response to agonists/hormones (Bileziken and Loeb, 1983), impaired functioning of the brain during this time may be the result of the synergistic effects of hypothyroidism upon

G-proteins and receptors.

Ageing effects on G_i, G_o and G_s have also been studied in brain parietal cortex samples from humans. A gradual decline of G_i, G_s (52 kDa form) and G_o α-subunits were observed whereas the opposite was seen with the 45kDa form of G_s which rose (Young *et al.*, 1991). It is feasible to assume that age dependent changes in G-protein abundance play a crucial part in the various events that neurons and other brain cells go through during life.

The number of endogenous signalling agents and distinct receptors that mediate their action are large. Perturbations in the ontogenic appearance of G-protein subunits which couple receptors to intracellular effector enzymes, would result in impaired transmission of these signalling agents. Disruptions in the overall mechanics and agonist response of cells arising as a consequence of these changes, may underlie some of the symptoms observed in diseased states such as hypothyroidism.

4.4 EFFECTS OF HYPOTHYROIDISM ON ADENYLYL CYCLASE ACTIVITY IN BRAIN SYNAPTOSOMAL MEMBRANES OF 15 DAY-OLD RAT PUPS.

To further explore the consequences of hypothyroidism in signal transduction processes in the developing brain, the effect of various GTP concentrations upon adenylyl cyclase activity in synaptosomal membranes was investigated. Adenylyl cyclase activities under basal and forskolin (10 μM) stimulated conditions were measured in membranes from 15 day-old euthyroid and hypothyroid rat pups. This concentration of the diterpene causes near maximal stimulation of adult rat brain cyclase (Mazurkiewicz and Saggerson, 1989). The effects of GTP were investigated on the basis that effects of the nucleotide are mediated through G-proteins; therefore any hypothyroid induced changes in the activation or inhibition of cyclase by GTP should be indicative of alterations in the function or relative abundance of stimulatory and/or inhibitory G-proteins.

Adenylyl cyclase was assayed via a modified method of Cooper and Londos (1979) and Sharma *et al.* (1982), and activity of the enzyme assessed by measuring the amount of cAMP generated using an enzyme immunoassay procedure (see section 2.14). An attempt to duplicate the cAMP enzyme immunoassay (see section 2.15) met with little success due mainly to the failure in reproducing the same level of sensitivity obtained with the purchased assay kit.

Assays of forskolin-stimulated activities were performed in the absence and presence of 100 mM NaCl since this assay condition had previously revealed a difference in adult hypothyroidism in the extent to which higher concentrations of GTP inhibited forskolin-stimulated cyclase (Mazurkiewicz and Saggerson, 1989). In order to prevent degradation of cAMP, the phosphodiesterase inhibitor papaverine was present in the assay buffer. Metabolism of the substrate ATP by nucleotides present in the membrane preparation was

inhibited by the inclusion of creatine kinase and creatine phosphate which acts as a regeneration system. Alumina used prior to the cAMP assay did not remove cAMP from the solution but did remove residual ATP and other nucleotides which may have otherwise affected the sensitivity of the competition assay. Forskolin was included to assess the stimulatory activity of the secondary effector system and effects of GTP during development under euthyroid and hypothyroid conditions.

Hypothyroidism did not alter specific adenylyl cyclase activity in synaptosomal membranes from adult rats (Mazurkiewicz and Saggerson, 1989). Measurement of basal (i.e. in the absence of forskolin) and forskolin-stimulated cyclase activities were initiated by the addition of 2.5 μg and 1.0 μg of synaptosomal membrane protein respectively. Cyclase activity was linear with respect to time under both basal and forskolin-stimulated conditions using membrane from both forebrain and hindbrain regions (see Materials and Methods section).

4.4.1 EFFECTS OF GTP ON BASAL ADENYLYL CYCLASE ACTIVITIES IN FOREBRAIN AND HINDBRAIN SYNAPTOSOMAL MEMBRANES.

Basal adenylyl cyclase activities of forebrain synaptosomal membranes are shown in Figure 4.9a. Relative to activities measured in the euthyroid state, hypothyroidism had no effect upon basal activity in this brain region. In addition, with the exception of 10 μM GTP in hypothyroid membranes (P<0.02), basal rates were also unaffected by GTP. As in the forebrain, neither hypothyroidism nor GTP had any discernible effect on hindbrain cyclase activity (Figure 4.9b).

Figure 4.9 <u>EFFECTS OF GTP UPON BASAL ADENYLYL CYCLASE ACTIVITY IN BRAIN SYNAPTOSOMAL MEMBRANES FROM 15 DAY OLD PUPS.</u>

(a) Forebrain ;   (b) Hindbrain.

Synaptosomal membranes isolated from euthyroid and hypothyroid pups (section 2.4) were assessed for basal adenylyl cyclases activities at various concentrations of GTP (section 2.14).

Values represent means ± S.E.M. of 3-4 separate experiments.

* indicates $P<0.05$ for comparison of hypothyroid (HYPO) and euthyroid (EUTH) states.

Figure 4.9a **FOREBRAIN**
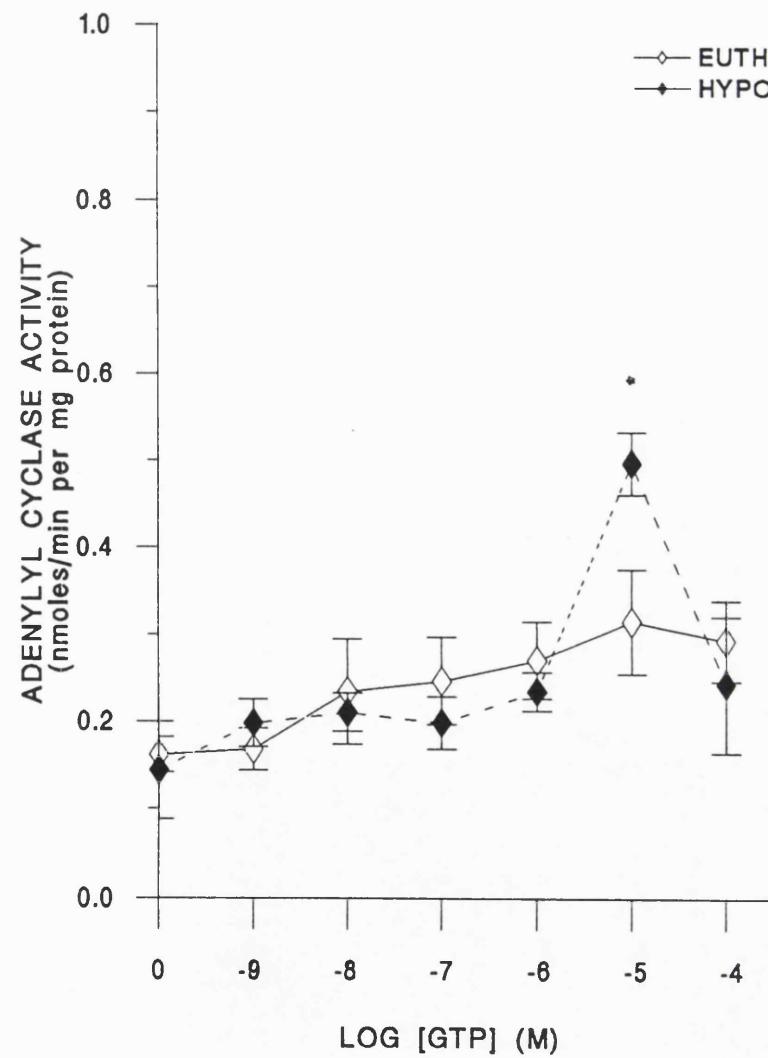
Figure 4.9b **HINDBRAIN**

184
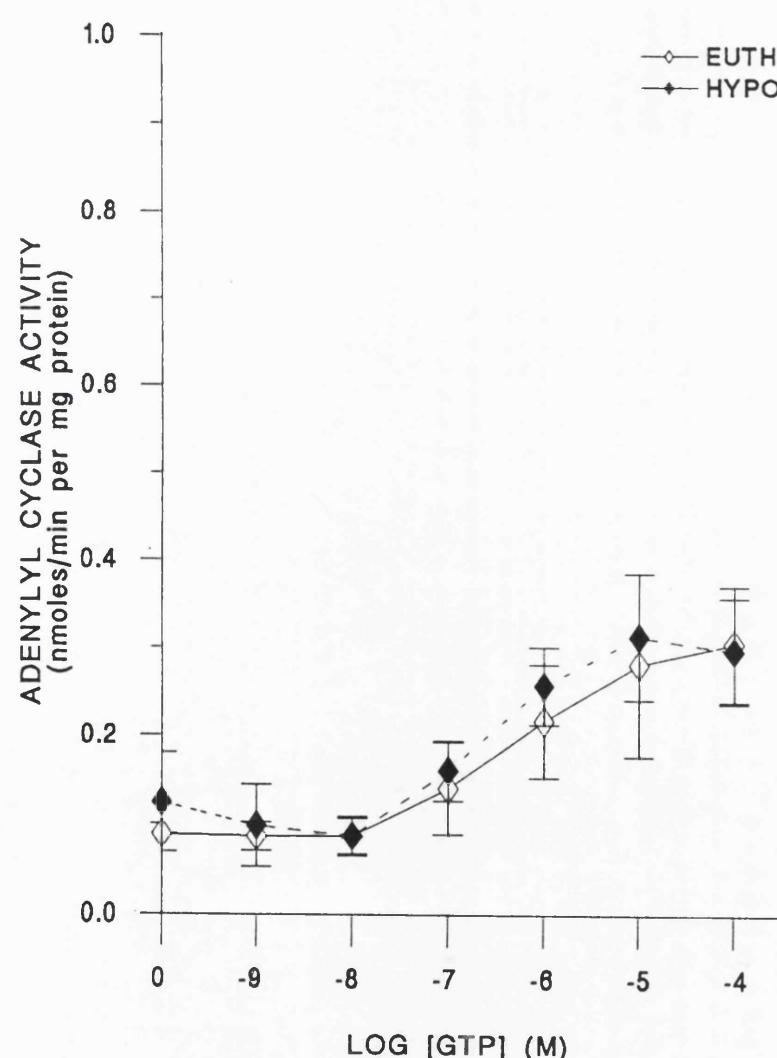
4.4.2 EFFECTS OF GTP ON FORSKOLIN-STIMULATED ADENYLYL CYCLASE ACTIVITIES IN FOREBRAIN SYNAPTOSOMAL MEMBRANES IN THE PRESENCE AND ABSENCE OF NaCl.

In the absence of NaCl, hypothyroidism was seen to have perceivable effects upon forskolin-stimulated cyclase activity in the forebrain as shown in Figure 4.10a. Firstly a significant decrease in the specific activity of cyclase compared with those seen in euthyroid animals was observed, suggesting a possible drop in abundance of cyclase protein in membranes isolated from hypothyroid animals. A drop in hypothyroid forskolin stimulated activity was significant at 0 M, and between 0.001 and 1 μM concentrations of GTP. Secondly whereas 0.1 μM GTP elevated forskolin-stimulated activity by 45% ($P<0.02$) in euthyroid membranes, the effect of the nucleotide was suppressed under conditions of low thyroid hormone and not significant. In the hypothyroid state, a less pronounced effect of the nucleotide was observed with maximal activity occurring at 10 μM GTP.

Figure 4.10b shows the effects of 100 mM NaCl upon forskolin-stimulated cyclase activities at various GTP concentrations during the euthyroid and hypothyroid state. In the presence of 100 mM NaCl similar changes to those seen in the absence of salt during hypothyroidism were seen. A significant decrease in the specific activity of cyclase as compared to euthyroid controls, was again observed. In this region of the brain 0.1 to 10 μM GTP significantly increased cyclase activity by 70-80% in euthyroid membranes; but as in the former case, during hypothyroidism this effect of GTP was muted and no longer statistically significant. Moreover an inhibitory effect of higher GTP concentrations which was previously seen in synaptosomal membranes from adult rats (Mazurkiewicz and Saggerson, 1989) was not observed here in membranes from euthyroid or hypothyroid neonates.

Figure 4.10 <u>EFFECTS OF HYPOTHYROIDISM UPON FORSKOLIN STIMULATED ADENYLYL CYCLASE ACTIVITY IN FOREBRAIN SYNAPTOSOMAL MEMBRANES FROM 15 DAY OLD PUPS IN THE PRESENCE AND ABSENCE OF NaCl.</u>

(a) without 100 mM NaCl ;   **(b) with 100 mM NaCl.**

Forebrain synaptosomal membranes were incubated with 10 μM forskolin at the indicated concentrations of GTP in the presence and absence of 100 mM NaCl as described in the Materials and Methods section.

Values represent means ± S.E.M. of 3-4 separate experiments.

*, ** and *** indicate $P<0.05$, <0.02 and <0.01 respectively for comparison of hypothyroid (HYPO) and euthyroid (EUTH) states.

A and C indicate $P<0.05$ and <0.01 respectively for significant effects of GTP.

Figure 4.10a   __without 100mM NaCl__
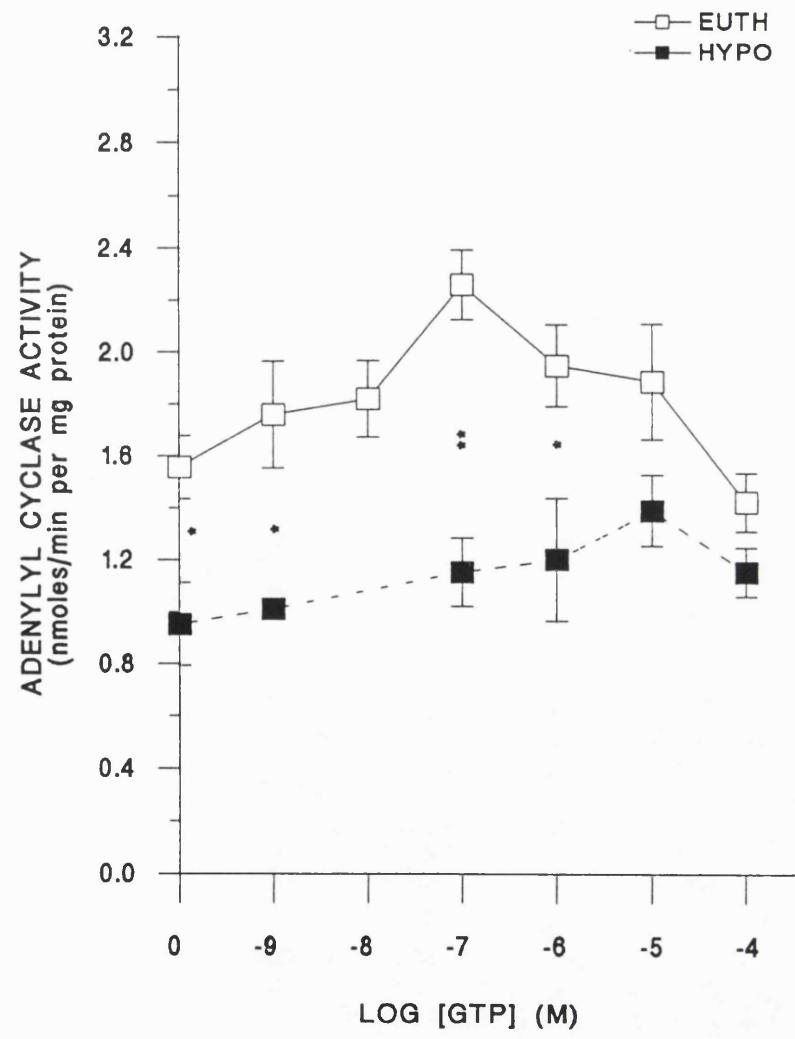
Figure 4.10b   __with 100mM NaCl__

187
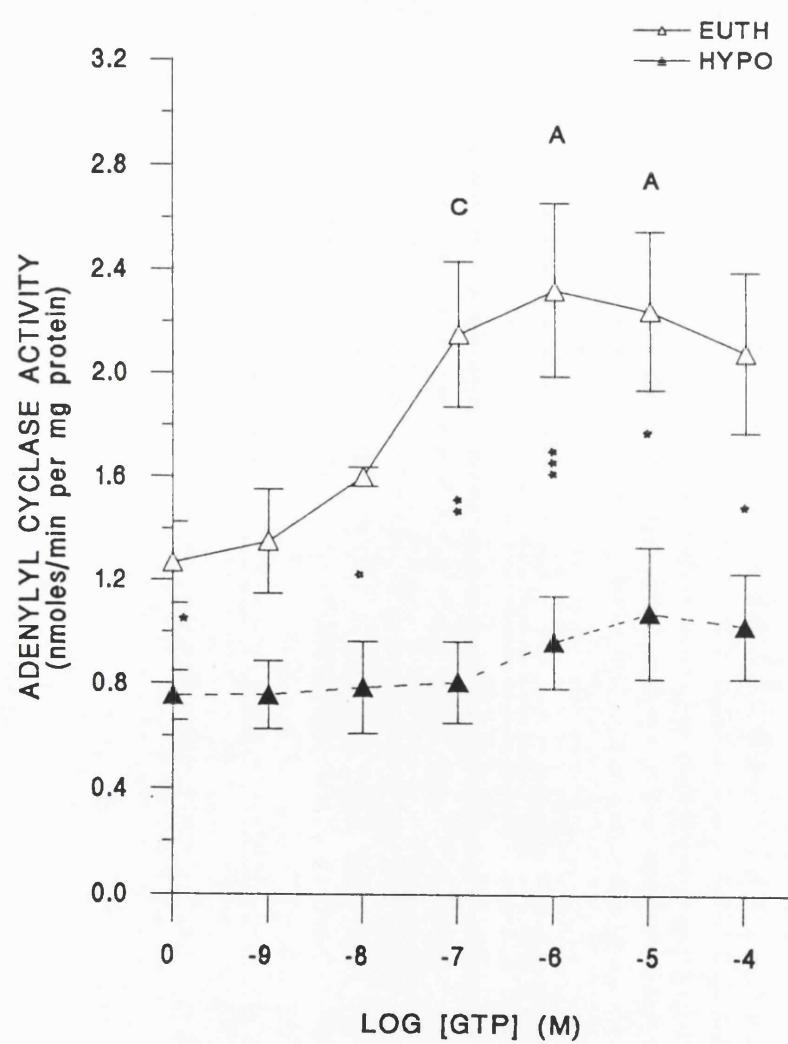
4.4.3 EFFECTS OF GTP ON FORSKOLIN-STIMULATED ADENYLYL CYCLASE ACTIVITIES IN HINDBRAIN SYNAPTOSOMAL MEMBRANES IN THE PRESENCE AND ABSENCE OF NaCl.

The effects of GTP on forskolin-stimulated adenylyl cyclase activity in the hindbrain differed vastly from those seen in the forebrain region. In the absence of Na^+, GTP had no effect upon cyclase activity in euthyroid synaptosomal membranes (Figure 4.11a). Hypothyroidism had no noteworthy effect on forskolin-stimulated activity of adenylyl cyclase in the hindbrain at 15 days postpartum and the only significant effect of the nucleotide was observed at 100 μM GTP ($P<0.05$).

When Na^+ was included in the assay buffer, a greater increase in the specific activity of cyclase was observed in the hypothyroid state as shown in Figure 4.11b. Another consequence of hypothyroidism was the enhanced stimulatory influence of GTP compared with the quite small effect observed in euthyroid membranes. Concentrations in excess of 0.1 μM had significant effects on hindbrain adenylyl cyclase activity of hypothyroid pups. In euthyroid membranes, the only significant effect by the nucleotide was seen at 10 μM GTP ($P<0.01$). Furthermore, an inhibitory effect of GTP at high concentrations (40% decrease in cyclase activity [$P<0.05$] between 10 μM and 100 μM GTP) which was not seen in hypothyroidism hindbrains or in forebrain, was also noted in euthyroid membranes.

Figure 4.11 <u>EFFECTS OF HYPOTHYROIDISM UPON FORSKOLIN STIMULATED ADENYLYL CYCLASE ACTIVITY IN HINDBRAIN SYNAPTOSOMAL MEMBRANES FROM 15 DAY OLD PUPS IN THE PRESENCE AND ABSENCE OF NaCl.</u>

(a) without 100 mM NaCl ;   **(b) with 100 mM NaCl.**

Hindbrain synaptosomal membranes were incubated with 10 μM forskolin at the indicated concentrations of GTP in the presence and absence of 100 mM NaCl as described in the Materials and Methods section.

Values represent means ± S.E.M. of 3-4 separate experiments.

** and **** indicate P<0.02 and <0.001 respectively for comparison of hypothyroid (HYPO) and euthyroid (EUTH) states.

A, B and C indicate P<0.05, <0.02 and <0.01 respectively for significant effects of GTP.

Figure 4.11a   without 100mM NaCl
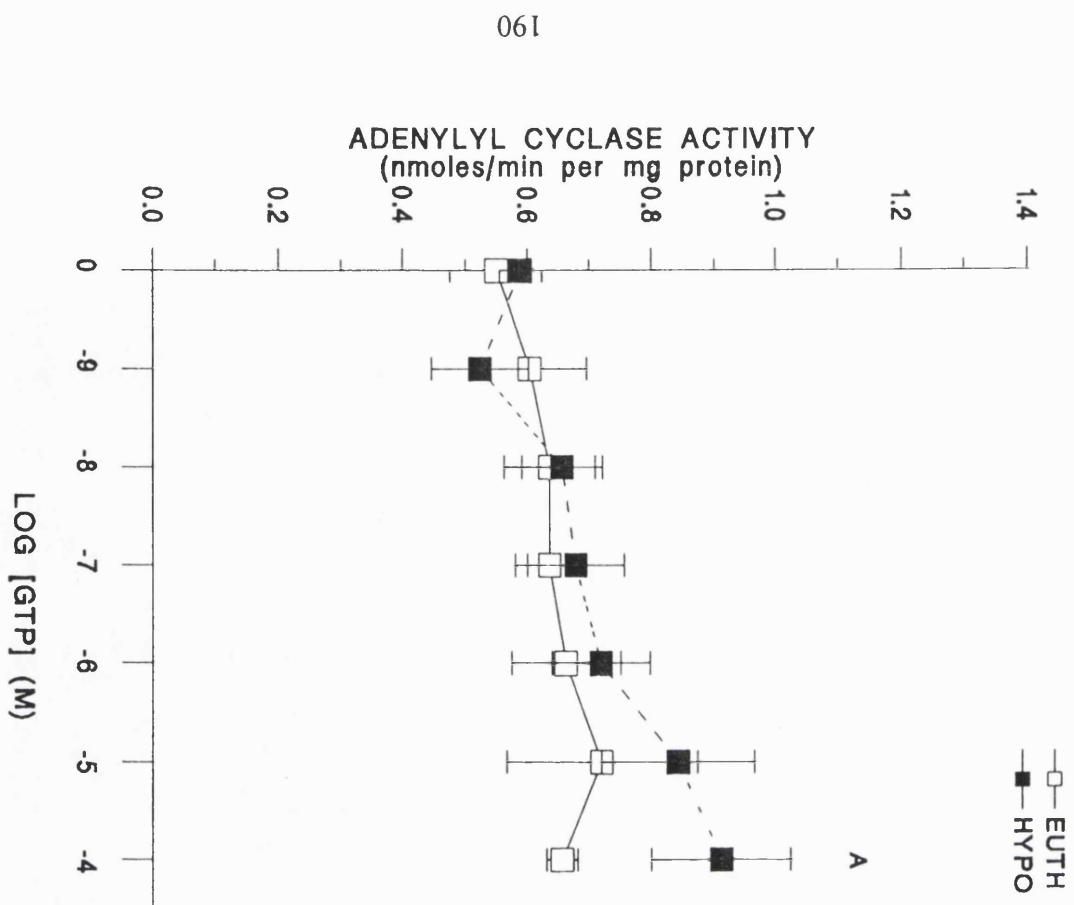
Figure 4.11b   with 100mM NaCl
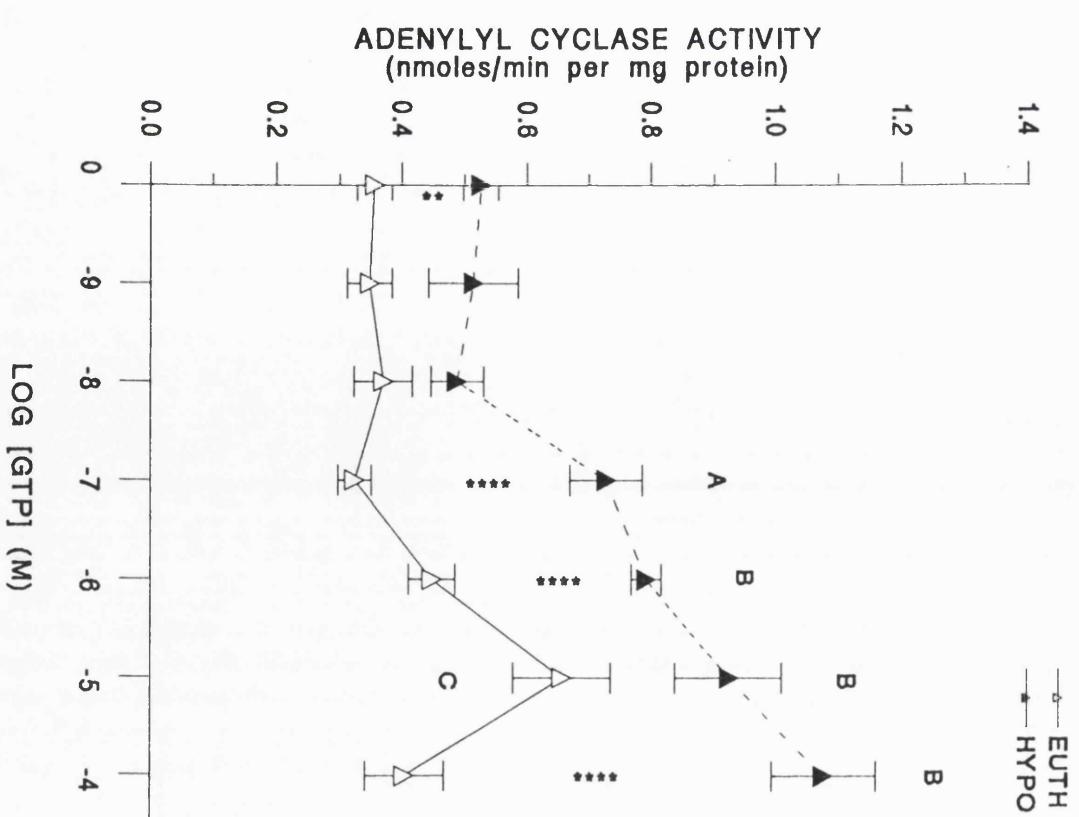
4.4.4 ADENYLYL CYCLASE DISCUSSION.

cAMP triggers key processes in cell differentiation in virtually all organisms and cell types. During critical periods of development, a rise in cAMP initiates the change-over from cell replication to differentiation (Claycombe, 1976) thereby simultaneously providing a negative signal for one developmental process and a positive signal for another. The onset of specific stimulatory G-proteins and cyclases in the brain during development appear to be closely linked (Rius et $al.$, 1994). Five isoforms of adenylyl cyclase are expressed in the mammalian brain (Tang and Gilman, 1992), all of which can receive stimulatory input through $G_s\alpha$ (Taussig et $al.$, 1994). Adenylyl cyclase isoforms I, V and VI can receive inhibitory input via $G_i\alpha$ (types 1,2 and 3) whereas isoforms II and IV are unaffected by G-protein α-subunits (Taussig et $al.$, 1994). In principle a change in the GTP activation/inhibition profile of adenylyl cyclase in a membrane preparation could result from either an alteration in the ratio of abundances of stimulatory/inhibitory G-proteins (G_s forms versus G_is and G_os) or from an alteration of the proportions of cyclase isoforms that are expressed. Perinatal hypothyroidism decreases the level of the short form of $G_s\alpha$ by 70% and 83% at 17 and 22 days postpartum respectively in rat cerebral cortex membranes (Wong et $al.$, 1994) but has no effect on the abundance of the long form of $G_s\alpha$. This might be expected to decrease the extent to which GTP can stimulate forskolin-activated cyclase in forebrain membranes as seen in Figure 4.10. It is also possible that an increase in the abundance of inhibitory G-protein(s) may contribute to the failure of GTP to significantly activate cyclase in the hypothyroid state. The level of $G_i1\alpha$ however was unchanged at this time (Figure 4.5) and $G_i2\alpha$ was decreased by 36% (Figure 4.7). By contrast, $G_o\alpha$ which can also inhibit Type I cyclase (Taussig et $al.$, 1994), was 90% greater in membranes isolated from hypothyroid forebrain at 15 days

(Figure 4.3). Furthermore, the G-protein is much more abundant in the brain than $G_i1\alpha$ and $G_i2\alpha$. If Type I adenylyl cyclase was the dominant form present in these membranes then the larger increase in $G_o\alpha$ might outweigh the smaller decrease in $G_i2\alpha$ observed in terms of the overall change in the GTP activation/inhibition profile of the cyclase.

At day 15 in whole hindbrain membranes, no significant changes in the abundance levels of $G_o\alpha$, $G_i1\alpha$ or $G_i2\alpha$ were seen. It is possible that this heterogeneous preparation masks significant changes in either the cerebellum or medulla oblongata at this time. However at the following 20 day time point there were still no signs of hypothyroid-induced changes in abundances of $G_o\alpha$ or $G_i\alpha$ in either of these more discrete regions (Figures 4.4 and 4.6). The implication is that the enhanced stimulatory response to GTP of adenylyl cyclase in the hindbrain (as seen in Figure 4.11) is unlikely to be the consequence of decreased levels of the inhibitory G-proteins. Rather, further studies should address the possibilities of whether $G_s\alpha$ is increased in hindbrain regions by hypothyroidism or whether the spectrum of adenylyl cyclase isoforms is altered in this state. Type II and IV cyclases can be activated directly by $G_s\alpha$ and indirectly by G_o, G_i and G_q (Taussig *et al.*, 1994). An increase in Types II or IV cyclase relative to Types I, V or VI (which are inhibited by G_o and G_i) could therefore lead to a cyclase profile which is more susceptible to stimulation by GTP. Adenylyl cyclase can be regulated indirectly by $G_q\alpha$ (Taussig *et al.*, 1994). Normally associated with PLC-β activation, the α-subunit of G_q can, in collaboration with G_s or G_i, exert stimulatory and inhibitory effects upon the secondary effector. Consequently, changes in the level of this G-protein may also affect the response of cyclase to GTP.

Lastly, many cloning techniques have resulted in the recent discoveries of the various isoforms of adenylyl cyclase to date (Tang and Gilman, 1992) and the possibility exists

for the present number of cyclase isoforms to increase in the near future. Perhaps one or

more of these as yet unidentified cyclases possesses the biochemical properties which can

account for the effects observed in the present study.

CHAPTER 5
GENERAL DISCUSSION

5.   <u>GENERAL DISCUSSION.</u>

Hypothyroidism both pre- and postnatal, has severe repercussions on the development of the central nervous system (Ford and Cramer, 1977; Dussault and Ruel, 1987). These effects, due to the vulnerability of the brain during this time, may last all through life giving rise to differences in mental and/or behavioural capacity (Man et al., 1971). Dysfunction varies from abnormalities in mental and motor function through to overt cretinism characterised by mental retardation, deaf mutism and spastic diplegia (see Introduction). Three main situations have been recognised in which alterations of the central nervous system (CNS) have been associated with impaired thyroid function: iodine deficiency, congenital hypothyroidism of the foetus/child and maternal hypothyroidism (low circulating T_4 during pregnancy) (Morreale de Escobar et al., 1993; Porterfield and Hendrich, 1993).

In order to study the consequences of pre- and postnatal thyroid hormone deficiency on the brain and other organs, the hypothyroid state has to be induced during the developmental period. This has been accomplished in a variety of ways ranging from thyroidectomy (of dams or pups) to administration of antithyroid drugs, e.g. propylthiouracil and methimazole. The time of insult also differs among groups with some initiating treatment before, and others after, parturition. Given the carefully controlled timing of developmental events in the brain, variations in the induction of insult will lead to a wide range of subclinical defects of varying severity. In the present study, pups born of dams maintained on a propylthiouracil/low iodine diet regime throughout the course of gestation and weaning, were used to investigate the effects of developmental hypothyroidism. Problems of still birth and infant deaths were overcome by using dams that were a minimum of 280 g in body weight and had previously delivered at least one

normal litter. The rate of development was retarded in hypothyroid pups such that they were considerably smaller than their control counterparts. Abnormal physical and behavioural characteristics, similar to those observed previously, were also noted (Hamburgh *et al.*, 1964; Eayrs, 1971; Legrand, 1984). The thyroid status of the aforementioned animals was also confirmed following measurements of plasma free T_3 and T_4 levels (Chapter 3). From day 15 onwards, weights of both forebrain and hindbrain regions were significantly lower in pups born of hypothyroid dams and probably reflect the summation of several factors including altered brain cell size and number, packing density, migration and proliferation (Balazs, 1973; Clos and Legrand, 1973; Himwich, 1973; Lauder, 1979). Additional plasma measurements of the main metabolic fuels indicated that the impaired development of the brain and body noted in hypothyroid animals was unlikely to be the result of serious under-nutrition (Chapter 3).

Given the fact that an extensive number of cellular events are involved in the development and maturation of the brain, it is reasonable to expect that many of the features associated with abnormal thyroid states arise as a consequence of altered cell signalling processes. In the adult brain, substantial region specific increases in the abundance of $G_i1\alpha$, $G_i2\alpha$ and $G_o\alpha$ have been observed in synaptosomal membranes from hypothyroid rats (Orford *et al.*, 1991), whereas some opposite trends were detected following short term administration of T_3 (Orford *et al.*, 1992). Several recent studies of various treatments or disease states have drawn conclusions from measurements of the abundance of G-protein components in homogenates or crude membrane fractions from the brain. This study has centred its observations on synaptosomal membranes, the rationale being that any changes observed there are more likely to reflect potential changes in neuron/neuron signalling events. The animal model of developmental hypothyroidism revealed a disruption in the normal

expression and functioning of G-protein α-subunits during early postnatal development of the brain and provided information on the normal profile of the development of these signalling components. The majority of these alterations were apparent in hypothyroid pups between 10 and 20 days postpartum, a period which in euthyroid pups coincides with a peaking of microneurogenesis, gliogenesis (particularly of oligodendrocytes) and myelination together with continuing growth and differentiation of neurons (Morgane *et al.*, 1992). Furthermore, plasma thyroid hormone levels peak at this stage of life (Figure 3.7) with hormone levels significantly lower in hypothyroid animals (Figure 3.7).

To recap, significant up-regulation of $G_q\alpha$ (day 10 postpartum), $G_o\alpha$ (days 15 and 20 postpartum) and $G_i1\alpha$ (day 20 postpartum) was detected in forebrain membranes of hypothyroid animals. By contrast, down-regulation of $G_i2\alpha$ was noted at 15 days postpartum. Synaptosomal membranes from hindbrain regions revealed very few hypothyroid-induced changes. The only significant effects were seen in $G_i1\alpha$ at day 10 (total hindbrain) and day 25 (medulla oblongata). The lack of significant change observed in the hypothyroid hindbrain was surprising considering the prolific level of cellular activity which occurs during this time. However as previously mentioned, a small insignificant change may be all that is required to bring about a response downstream. Furthermore, in both brain regions, hypothyroidism perturbed the normal developmental profiles of all four $G\alpha$-subunits (Chapter 4). At 15 days postpartum compared to euthyroid values, specific forskolin-stimulated cyclase activity was decreased in forebrain membranes and elevated in hindbrain membranes from hypothyroid pups. GTP effects on adenylyl cyclase were also altered in membranes from both brain regions during the hypothyroid state (Chapter 4).

Changes in brain G-protein abundance coupled with regional-specific alterations of the

activation/inhibition pattern of adenylyl cyclase by GTP observed at day 15 indicate that hypothyroidism causes a distinct perturbation of the normal developmental and functional activities of these signalling components.

G-proteins are vastly outnumbered by receptors and while an α-subunit may be activated by 30 or more receptors, conversely individual receptors can be activated by more than one G-protein thereby initiating more than one signalling pathway (Abou-Samra et al., 1992; Gudermann et al., 1992; Allgeier et al., 1994; Chabre et al., 1994). By contrast, effectors discriminate better among G-protein α-subunits; for example adenylyl cyclase is only activated by $G_s\alpha$ and phospholipase C-β (PLC-β) by $G_{q/11}\alpha$. In the mammalian CNS, adenylyl cyclase can be activated via adrenergic (β), dopaminergic (D_1 and D_5), histaminergic (H_2) and serotonergic (5-HT_4) receptors whereas inhibition occurs through adrenergic (α_2), dopaminergic (D_2 and D_5) and $GABA_B$ (reviewed by Watson and Arkinstall, 1994). The findings presented here point to possible implications in terms of altered input, in particular adenylyl cyclase types I, V and VI. Adenylyl cyclase however is not the sole effector regulated by G-proteins in the brain. Altered expression of α-subunits in the hypothyroid state would also have consequences on the regulation of PLC-β and ion channels. These effectors also respond to selective stimulatory and inhibitory inputs from specific receptor types (Nicoletti et al., 1986; Fisher and Agranoff, 1987; Dolphin, 1990; Fisher et al., 1992; Hille, 1992). Activation of PLC-β isoforms result in the formation of two secondary messengers, IP_3 and DAG. The latter stimulates the protein kinase C system which is thought to play a part in the processing of neuronal signals and modulation of synaptic transmission (Taneka and Saito, 1992; Saito et al., 1993). Hyperpolarization of neurons and inhibition of transmitter release have been attributed to pertussis-toxin sensitive G-protein α-subunits ($G_i\alpha$ and $G_o\alpha$) interacting with

ion channel effectors (see Introduction). The majority of cellular responses are positively and negatively controlled by distinct G-protein-coupled pathways. This mode of regulation means that constitutive activation or loss of function of one of these pathways would lead to an abnormality in end organ response.

G-protein mediated signal transduction is not limited to the α-subunit component. Originally consigned to the role of negative regulator, (i.e. deactivating the α-subunit by forming the inactive heterotrimer [Gilman, 1987]) this opinion has changed following the discovery that $\beta\gamma$ complexes could also regulate effectors (reviews in Clapham and Neer, 1993; Iñiguez et al., 1993; Buckley et al., 1995). In the case of adenylyl cyclase, the pattern of regulation by $\beta\gamma$ is specific to effector subtype (Tang and Gilman, 1991) whereas PLC-β (soluble) activation is independent of α (Smrcka and Sternweis, 1993a). Activation of a particular G-protein may therefore result in the modulation of two separate effectors courtesy of its α and $\beta\gamma$ subunits respectively. Alternatively an individual receptor type may initiate a signalling pathway(s) via the synergistic activation of more than one G-protein. It is through a complex web of interactions that receptors, G-proteins and effectors are linked with signals that converge to shared targets and diverge from shared detectors (reviewed by Taylor, 1990a). Evidently there is still much to be learned about the selectivity of G-protein α and $\beta\gamma$ subunit interactions with their molecular partners, the receptor and effector.

As mentioned in the Introduction, the numerous functions of the brain are shared among the various regions of the organ, with each assigned specific roles and duties. Future studies should be directed to extending the measurements performed in the present study at a more detailed anatomical level. Measurements carried out in this study focused upon forebrain and hindbrain regions which represent gross anatomical structures. Although the

hindbrain was further dissected at days 20 and 25, this was not performed on the forebrain. Changes noted in synaptosomal membranes from this region are therefore the summation of several brain regions (e.g. cortex, hippocampus). Moreover, the lack of change in crude membrane Gα levels (Table 4.2) may reflect the heterogeneity of the preparation. Miniaturisation of the current membrane procedure would overcome the present problem of isolating adequate amounts of material from detailed dissected regions of the brain.

Only four α-subunits were measured via quantitative immunoblotting in this study. The wide availability of antisera directed against other Gα-subunits and G$\beta\gamma$, would enable the application of this study to other G-proteins, particularly in the case of $G_o\alpha$ splice variants and $G_{q/11}\alpha$. The two splice variants of $G_o\alpha$ are regulated independently of each other during neural development with a transient increase in $G_oA\alpha$ observed in differentiating neurons (Asano $et\ al.$, 1992). In addition antisera are available which can discriminate between $G_q\alpha$ and $G_{11}\alpha$ (Smrcka and Sternweis, 1993b; James $et\ al.$, 1994) and this would be useful in light of their regional-specific distributions within the brain (Milligan, 1993). An immunohistochemical approach using the aforementioned antisera on brain slices would enable insight into changes without the need for detailed dissection. Alternatively, detection of G-protein could be carried out using photo-affinity labels specific for cell signalling components (Zor $et\ al.$, 1995). The present findings also indicate it may be productive to conduct these current measurements on synaptosomal membranes earlier on in development. Recent studies on embryonic tissues have indicated signs of abnormal cell signalling in perturbed physiological states (Menco $et\ al.$, 1994; Rius $et\ al.$, 1994).

Investigations on adenylyl cyclase activity could be extended to include other times during

development as well as to other effectors (e.g. PLC-β isoforms). Studies may include assaying enzyme activities and assessing the regulatory effects of guanine nucleotides and receptor agonists. It would also be of interest to define the ontogenetic profile of these effectors in the brain and whether they are disrupted during times of hypothyroidism. Molecular biology studies (e.g. Northern blotting, hybridisation) would enable insight into whether changes in α-subunit levels can be attributed to altered mRNA abundance or to altered transcription activity. Recent findings by Greenwood and Jope (1994) have demonstrated G-Protein proteolysis by calpain (calcium-dependant neutral protease) prompting the idea that this may be one method by which G-protein concentrations are regulated. Measurements of mRNA abundances of the various cyclase isoforms would further aid in the elucidation of any changes in the adenylyl cyclase profile during development in the euthyroid and hypothyroid state.

In summary, G-proteins play a major role in receptor coupled signal transduction pathways. Modifications in their function and concentration have been shown to have important ramifications on the cellular response to external stimuli. Investigations have only recently begun to link these changes to abnormalities in physiological systems. It is imperative that investigations should concentrate on elucidating the manner by which components involved in cellular communication are ordered and regulated, and how they are modified during critical times of development and differentiation. Only by understanding the "rules" that dictate communication can we then begin to correct the inappropriate response to external signals that contribute to abnormal cell function seen in diseased states such as neonatal hypothyroidism.

CHAPTER 6
REFERENCES

6. REFERENCES

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