Abundance of the α-subunits of G_i1, G_i2 and G_o in synaptosomal membranes from several regions of the rat brain is increased in hypothyroidism

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1. Rats (4 weeks old) were made hypothyroid by treatment with propylthiouracil together with a low-iodine diet for a further period of 4 weeks. Synaptosomal membranes were obtained from six anatomical regions of the brain. 2. The abundances in these membranes of the G-protein α-subunits $G_i1\alpha$, $G_i2\alpha$ and $G_o\alpha$ were measured by quantitative immunoblotting. 3. Hypothyroidism significantly increased the abundances of all three G-protein subunits in membranes from the cerebral cortex and the striatum. In the medulla oblongata and the hippocampus the abundances of $G_i2\alpha$ and $G_o\alpha$ were increased significantly. By contrast, in the cerebellum only $G_o\alpha$ was increased, and in the hypothalamus only $G_i2\alpha$ was increased. 4. It is suggested that this up-regulation of G-protein abundances may modify signalling pathways and may contribute to the functional changes that are observed in the central nervous system in hypothyroidism.

INTRODUCTION

One arbitrary subdivision of the family of heterotrimeric G-proteins is based on whether or not their specific α-subunit is a substrate for ADP-ribosylation by pertussis toxin [1]. Outside of photoreceptor cells, five such α-subunits have so far been recognized showing considerable sequence similarity. The three products of the genes for $G_i1\alpha$, $G_i2\alpha$ and $G_i3\alpha$ subunits are widely expressed in mammalian cell types [2]. A fourth gene, which is particularly, but not exclusively, expressed in neuronal-type cells gives rise to two splice variants, $G_o1\alpha$ and $G_o2\alpha$ [3]. Intrinsic properties of these G-proteins are the subject of considerable investigation, but it is already apparent that they differ appreciably in their rates of binding and release of guanine nucleotides [4,5], thereby offering tissues options in terms of speed of response to cell signalling events. In neuronal tissues, occupancy of a wide range of receptors causes activation of K^+ channels and inhibition of voltage-sensitive Ca^{2+} channels. The pertussis-toxin-sensitive G-proteins (in particular 'G_o', $G_i1\alpha$ and $G_i3\alpha$) have been implicated in direct regulation of these effectors in response to receptor occupancy (for recent reviews see [6–10]). Other evidence [11,12] suggests that G_i2 may particularly mediate the other well-documented effect of 'G_i', the inhibition of adenylate cyclase. How specificity and discrimination is built into the way G-proteins can couple diverse receptors to effectors in different tissues is still not clear. One contribution to the solution of such problems may lie in the examination of pathophysiological states in which the abundance or function of particular G-protein subunits may alter. This may allow correlation with particular changes in receptor/effector coupling and in physiological responses of specific tissues. Recent examples of this approach have been the demonstration of down-regulation of the abundance of 'G_i' α-subunit(s) in hepatocyte membranes from diabetic rats [13], and in adipocyte membranes from *ob/ob* mice [14] together with a decrease in $G_i1\alpha$, $G_i2\alpha$ and $G_i3\alpha$ in adipocytes cultured with an A_1-adenosine-receptor agonist [15]. Hypothyroidism increases the responsiveness of adipocytes to agonists that inhibit adenylate cyclase and lipolysis [16–18] accompanied by a significant increase in the abundance of 'G_i' α-subunits in the plasma membrane [19–21]. More recently, Milligan & Saggerson [22] showed that broadly similar approx. 2-fold increases in abundance of $G_i1\alpha$, $G_i2\alpha$ and $G_i3\alpha$ contribute to this up-regulation of 'G_i' in fat tissue in hypothyroidism. Also, hypothyroidism has been found to increase the relative amounts of immunoreactive $G_i2\alpha$ and $G_i3\alpha$ by approx. 2-fold in rat heart ventricular membranes [23]. Saito *et al.* [24] demonstrated a small decrease in the abundance of an mRNA species corresponding to 'G_i' in rat cerebral cortex after corticosterone treatment. However, no significant changes in immunoreactivity corresponding to 'G_i' or 'G_o' α-subunits were noted in a brain crude membrane preparation after this treatment [24]. With the above exception [24], we are unaware of any studies reporting alteration of G-protein α-subunit abundance in the central nervous system as a result of alteration in endocrine state. The study of Mazurkiewicz & Saggerson [25] with rat brain synaptosomal membranes, however, suggested that such changes might be expected in hypothyroidism. This state enhanced both the inhibition of forskolin-stimulated adenylate cyclase by GTP and the effect of GTP to modify the affinity state of A_1 adenosine receptors in these membranes [25].

The purpose of the present study was therefore to test for altered abundance of α-subunits of pertussis-toxin-sensitive G-proteins in rat brain in the hypothyroid state. This was done by using purified synaptosomal membranes from six anatomical regions, which were probed for $G_i1\alpha$, $G_i2\alpha$ and '$G_o\alpha$'.

MATERIALS AND METHODS

Reagents

Sources of these have been described previously [19,25]. In addition, $Na^{125}I$ was from Amersham International (Little Chalfont, Bucks., U.K.).

Antisera

All antisera used in this study have previously been described [19,26]. In summary, antisera for detection of G-protein

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Table 1. Effect of hypothyroidism on the abundance of $G_i1\alpha$ in synaptosomal membranes from six brain regions

Immunoblotting was performed with antiserum I1C and 96 μg samples of membranes in each case. Numbers of animals are indicated in parentheses.

Brain region	Rats...	$G_i1\alpha$ (d.p.m./100 μg of membrane protein)	
		Euthyroid	Hypothyroid
Striatum		241 ± 55 (5)	648 ± 96[C] (5)
Cerebral cortex		255 ± 20 (4)	876 ± 165[C] (5)
Cerebellum		262 ± 68 (5)	483 ± 89 (5)
Medulla oblongata		283 ± 34 (5)	276 ± 34 (5)
Hypothalamus		372 ± 89 (4)	186 ± 27 (4)
Hippocampus		379 ± 75 (5)	710 ± 144 (4)

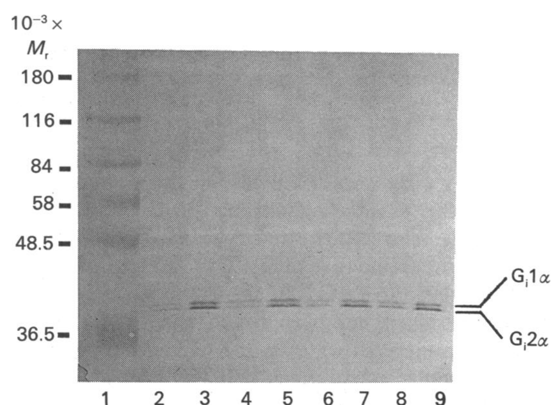

Fig. 1. Representative immunoblots showing the detection of $G_i1\alpha$ and $G_i2\alpha$ in synaptosomal membranes

Immunoblotting was performed with antiserum SG2 and 60 μg of membrane protein in each case. Lane 1, prestained M_r markers. Lanes 2 and 3, hippocampus. Lanes 4–9, cerebral cortex. Lanes 2, 4, 6, 8, euthyroid rats. Lanes 3, 5, 7, 9, hypothyroid rats.

α-subunits were raised in New Zealand White rabbits against glutaraldehyde conjugates of keyhole-limpet haemocyanin [from Novabiochem (U.K.) Ltd., Nottingham, U.K.] and synthetic peptides representing sections of the primary sequence of various pertussis-toxin-sensitive G-proteins. Antiserum SG2 was raised against the *C*-terminal decapeptide of the α-subunit of rod transducin (KENLKDCGLF) and identified both $G_i1\alpha$ and $G_i2\alpha$ [26]. Antiserum I1C was raised against a peptide (LDRIAQPNYI) which is equivalent to amino acids 159–168 of the α-subunit of G_i1. This antiserum is specific for $G_i1\alpha$ [26]. Antiserum OC1 was raised against a peptide (ANNLRGCGLY) which is equivalent to amino acids 345–354 of the α-subunit of G_o [1] and recognizes $G_o1\alpha$ and $G_o2\alpha$. Peroxidase-conjugated anti-rabbit IgG (raised in goat) was from ICN Immunobiologicals (Lisle, IL, U.S.A.). Anti-rabbit IgG was labelled with ^{125}I as described in [27].

Animals

These were male Sprague–Dawley rats bred at University College London. All animals had constant access to drinking water and to Rat & Mouse No. 3 Breeding Diet (Special Diet Services, Witham, Essex, U.K.), which contained (by wt.) 21 % digestible crude protein, 4 % digestible crude oil and 39 % starches and sugars. The light/dark cycle was 13 h/11 h, with light from 06:00 to 19:00 h. Rats to be made hypothyroid were selected at age 4 weeks (80–90 g body wt.) and then fed on an iodine-deficient version of the No. 3 Breeding Diet and drank water containing 0.01 % (w/v) 6-n-propylthiouracil [28,29]. The animals were killed 4 weeks after commencement of this treatment, when they weighed 140–170 g. Euthyroid age-matched controls fed on the normal diet weighed 260–280 g at the time of death.

Isolation of synaptosomal membranes

Six brain regions were dissected [30,31] and pooled from 4–6 rats. These were homogenized and fractionated on a Ficoll gradient to obtain synaptosomes as described in [32] with some minor modifications [31]. Synaptosomes were resuspended in 5 mM-Tris/HCl buffer (pH 8.0). After sonication for 30 s, the suspensions were left on ice for 30–45 min to achieve lysis of the synaptosomes, followed by centrifugation for 45 min at 105000 $g_{av.}$. The resulting synaptosomal membrane fractions were resuspended in 2–3 ml of 50 mM-Tris/HCl buffer (pH 7.4) and stored at −70 °C. Protein contents of the preparations were measured by the Lowry method [33], with BSA as standard. Enrichments of these fractions in the marker acetylcholinesterase and relative freedom from the myelin marker 2′,3′-cyclic nucleotide phosphodiesterase were very similar to that found in [31].

Immunoblotting

SDS/PAGE was performed with synaptosomal membranes from euthyroid and hypothyroid rats. When immunoblotting to detect $G_i1\alpha$ or $G_o\alpha$ alone (antisera I1C and OC1 respectively), electrophoresis was performed in gels containing 10 % (w/v) acrylamide and 0.27 % (w/v) bisacrylamide. When resolution and simultaneous detection of $G_i1\alpha$ and $G_i2\alpha$ was required, the gels contained 12.5 % acrylamide and 0.063 % bisacrylamide [19,26]. Proteins were subsequently transferred to nitrocellulose sheets and blocked for 3 h at 37 °C in 20 mM-Tris/HCl buffer (pH 7.5) containing 500 mM-NaCl and gelatin (3 %, w/v). The primary antisera were then added [1:100 (v/v) dilution in the Tris/NaCl/gelatin medium] and incubated overnight at 25 °C. The primary antisera were then removed and the blots washed [twice with water and then twice with 20 mM-Tris/50 mM-NaCl medium containing Tween 20 (0.5 ml/l)]. A mixture of anti-rabbit IgG conjugated with peroxidase and ^{125}I-labelled anti-rabbit IgG was then added, left for 2–3 h, and the blots were then extensively washed first with Tris/NaCl/Tween 20 medium and finally with Tris/NaCl medium. The blots were developed with 2.8 mM-4-chloro-1-naphthol + H_2O_2 (0.5 %, v/v) as the substrates for the peroxidase to make cross-reacting bands visible. After extensive washing with Tris/NaCl/Tween 20 medium and then with Tris/NaCl medium, the blots were air-dried and radioactivity in excised cross-reacting bands was quantified by γ-radiation counting. Equivalent-sized unstained regions of the nitrocellulose sheets were counted for radioactivity to determine background, which was subtracted from all values. Individual blots were normalized relative to each other by comparison against a standard sample of cerebral cortex from normal rats which was run every time. Preliminary experiments established that quantification by using each antiserum was linear with amount of electrophoresed protein in the range that was tested.

Statistical methods

All values are means ± S.E.M. Significance of differences was assessed by Student's *t* test for unpaired samples: throughout, $^{A}P < 0.05$, $^{B}P < 0.02$, $^{C}P < 0.01$, $^{D}P < 0.001$ respectively.

Table 2. Effect of hypothyroidism on the abundance of $G_i2\alpha$ in synaptosomal membranes from six brain regions

Immunoblotting was performed with antiserum SG2 and 96 μg samples of membranes in each case. Numbers of animals are indicated in parentheses.

Brain region	Rats...	$G_i2\alpha$ (d.p.m./100 μg of membrane protein) Euthyroid	Hypothyroid
Medulla oblongata		138±37 (6)	550±78^D (3)
Striatum		186±18 (6)	558±134^A (6)
Cerebellum		186±66 (6)	413±122 (6)
Hippocampus		208±52 (6)	814±178^C (5)
Hypothalamus		227±22 (6)	599±85^C (5)
Cerebral cortex		294±52 (6)	796±111^C (5)

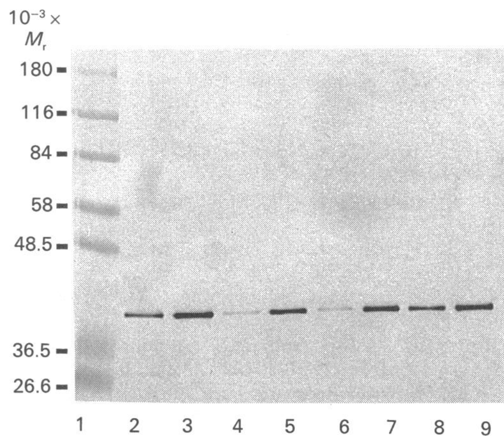

Fig. 2. Representative immunoblots showing the detection of $G_o\alpha$ in synaptosomal membranes

Immunoblotting was performed with antiserum OC1 and 10 μg of membrane protein in each case. Lane 1, prestained M_r markers. Lanes 2 and 3, cerebral cortex. Lanes 4 and 5, cerebellum. Lanes 6 and 7, hippocampus. Lanes 8 and 9, striatum. Lanes 2, 4, 6, 8, euthyroid rats. Lanes 3, 5, 7, 9, hypothyroid rats.

Table 3. Effect of hypothyroidism on the abundance of $G_o\alpha$ in synaptosomal membranes from six brain regions

Immunoblotting was performed with antiserum OC1 and 10 μg samples of membranes in each case; $n = 5$ in every case.

Brain region	Rats...	$G_o\alpha$ (d.p.m./100 μg of membrane protein) Euthyroid	Hypothyroid
Medulla oblongata		1320±211	2746±422^B
Hypothalamus		2006±422	2429±844
Cerebellum		3115±844	8712±317^D
Hippocampus		3538±264	8078±1320^B
Striatum		3696±422	9979±1795^C
Cerebral cortex		5808±633	10613±1478^B

RESULTS

Hypothyroidism caused widespread increases in the abundance of the α-subunits $G_i1\alpha$, $G_i2\alpha$ and $G_o\alpha$ through the regions of the brain, although there were some region- and α-subunit-specific deviations from this trend.

Considering individual α-subunits: when antiserum I1C was used, $G_i1\alpha$ (Table 1) was the least variable, only showing significant increases in the striatum and cerebral cortex (some increase, though not significant, was also seen in the cerebellum and the hippocampus). By contrast, there was no increase whatever in the abundance of $G_i1\alpha$ in the medulla oblongata, and in the hypothalamus there was a non-significant ($P < 0.1$) decrease. Attempts to quantify $G_i2\alpha$ specifically by using an antiserum directed against the sequence LERIAQSDYI [26] (corresponding to amino acids 160–169 of $G_i2\alpha$) were successful with the synaptosomal membranes, since the cross-reactive bands were barely visible on staining with the peroxidase-coupled second antibody and ^{125}I in these bands was barely above background. We therefore resorted to quantification of the 40 kDa band on immunoblots probed with antiserum SG2, which cross-reacts with both $G_i1\alpha$ and $G_i2\alpha$ (Fig. 1 and Table 2). $G_i2\alpha$ showed the largest increases in abundance. These were significant in all regions except the cerebellum, and were approx. 3–4-fold. The detection of $G_o\alpha$ (Fig. 2 and Table 3) was achieved with substantially smaller amounts of synaptosomal membrane than was needed from $G_i1\alpha$ or $G_i2\alpha$, presumably reflecting its high abundance in brain [34,35]. $G_o\alpha$ abundance was increased by 2–3-fold in all regions except hypothalamus, where little change was seen.

Considering individual regions: hypothalamus and cerebellum showed the least marked changes, exhibiting significant increases only in $G_i2\alpha$ or $G_o\alpha$ respectively. Striatum and cerebral cortex showed the greatest degree of variation, with the abundance of all three α-subunits being significantly increased in the hypothyroid state.

DISCUSSION

Thyroid hormone plays an essential role in pre- and peri-natal growth and development of the mammalian brain [36,37]. However, it must be stressed that induction of hypothyroidism in the present study was not commenced until processes such as dendritic arborization, myelinization and expression of enzymes of neurotransmitter synthesis that occur in the early 'critical period' were essentially complete. The changes reported here should therefore be indicative of adult hypothyroidism, i.e. myxoedema. It has frequently been inferred that adult brain is not sensitive to thyroid hormone, perhaps because it has not been possible to demonstrate metabolic changes characteristic of thyroid hormones in other tissues, e.g. increased O_2 consumption or the induction of NADP–malate dehydrogenase and glycerolphosphate oxidase [38–41]. Nevertheless, adult brain contains significant contents of intracellular thyroid hormone [42,43] and high-affinity nuclear receptors for the same [44–46]. In addition, thyroid status has been shown to affect cathecholamine and 5-hydroxytryptamine synthesis [47], Type II 5′-deiodinase activity [48–50], 5′-nucleotidase ectoenzyme activity [31] and actin polymerization [51]. There is also a large clinical literature to show that adult hypothyroid patients have generally decreased brain excitability and many symptoms attributable to functional impairment of the central nervous system, e.g. loss of alpha rhythm, cerebellar ataxia, increased threshold to light and sound stimuli, increased sensitivity to depressant actions of morphine and phenothiazines, tiredness, memory impairment, anxiety, depression and, occasionally, dementia or coma [52–56].

The up-regulation of $G_i1\alpha$, $G_i2\alpha$ and $G_o\alpha$ seen in the present study may be expected to strengthen signal inputs leading to inhibition of neurotransmitter release through inhibition of adenylate cyclase, inhibition of voltage-sensitive Ca^{2+} channels or increased K^+ conductance [57–62].

Further studies should be directed towards refining understanding of the localization of these changes in G-protein abundance, elucidating the molecular mechanisms through which thyroid-hormone deficiency causes these changes and relating these alterations at the molecular level to clinical situations.

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