Metabolic Brain Disease, Vol. 20, No. 2, June 2005 (© 2005)
DOI: 10.1007/s11011-005-4150-x

Changes in Antioxidant Status, Protein Concentration, Acetylcholinesterase, (Na^+,K^+)-, and Mg^{2+}-ATPase Activities in the Brain of Hyper- and Hypothyroid Adult Rats

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Received October 4, 2004; accepted November 18, 2004

It is a common knowledge that metabolic reactions increase in hyperthyroidism and decrease in hypothyroidism. The aim of this work was to investigate how the metabolic reactions could affect the total antioxidant status (TAS), protein concentration (PC) and the activities of acetylcholinesterase (AChE), (Na^+,K^+)-ATPase and Mg^{2+}-ATPase in the brain of hyper- and hypothyroid adult male rats. Hyperthyroidism was induced in rats by subcutaneous administration of thyroxine (25 μg/100 g body weight) once daily for 14 days, while hypothyroidism was induced by oral administration of propylthiouracil (0.05%) for 21 days. TAS, PC, and enzyme activities were evaluated spectrophotometrically in the homogenated brain of each animal. TAS, PC, and Mg^{2+}-ATPase activity were found unaffected in hyperthyroidism, while AChE and Na^+,K^+-ATPase activities were reduced by 25% ($p < 0.01$). In contrast, TAS, (Na^+,K^+)-ATPase and Mg^{2+}-ATPase activities were found to be increased (approx. 23–30%, $p < 0.001$) in the hypothyroid brain, while AChE activity and PC were shown to be inhibited (approx. 23–30%, $p < 0.001$). These changes on brain enzyme activities may reflect the different metabolic effects of hyper- and hypothyroidism. Such changes of the enzyme activities may differentially modulate the brain intracellular Mg^{2+}, neural excitability, as well as the uptake and release of biogenic amines.

Key words: Hyperthyroidism; hypothyroidism; rat brain; total antioxidant status; protein concentration; acetylcholinesterase; (Na^+,K^+)-ATPase; Mg^{2+}-ATPase.

INTRODUCTION

Thyroid hormones (THs) are recognized as key metabolic hormones stimulating the catabolism of glucose, fats, and proteins by increasing the levels of many enzymes that catalyze these metabolic reactions (e.g. liver exokinase) as well as mitochondrial enzymes involved in oxidative phosphorylation (Lodish *et al*., 1995; Smith *et al*., 2002). A characteristic response of the homeothermic animals with thyroid hormones is increased oxygen

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consumption, but several organs, including brain, are unresponsive to the calorigenic effects of the thyroid hormones (Goodman and Gilman, 2001; Silva, 1995). Since the demonstration and distribution patterns of THs receptors in the rat brain at both developmental and adult stages, many recent investigations have focused on THs activity within the central nervous system (CNS). Thyroid hormone plays a critical role in brain development and not only, mediating important effects within the CNS throughout life (Bernal and Nunez, 1995; Bradley *et al*., 1989; Cook *et al*., 1992; Gullo *et al*., 1987; Mellstrom *et al*., 1991; Oppenheimer and Schwartz, 1997; Rastogi *et al*., 1977). Metabolic effects such as those affecting mitochondrial respiratory enzyme activity, and acetate metabolism, have been shown to occur in the brain after THs administration (Chapa *et al*., 1995; Dembri *et al*., 1983). Oxygen free radicals (ROS: reactive oxygen species) can develop during several steps of normal metabolic events, as the mitochondrial electron transport chain, the microsomal electron transport chain, reactions of oxidant enzymes, and auto-oxidation reactions (Freeman and Crapo, 1982; Hauck and Bartke, 2000; Mates *et al*., 1999).

Variations of the THs levels can be one of the main physiological modulators of in vivo cellular oxidative stress due to their known effects on mitochondrial respiration (Guerrero *et al*., 1999). In particular, it has been suggested that the increase in ROS induced by THs can lead to an oxidative stress condition in several organs as liver, heart, skeletal muscles and in the brain with a consequent lipid peroxidative response (Asayama and Kato, 1990; Fernandez and Videla, 1989; Mano *et al*., 1995; Pasquini and Adamo, 1994). Superoxide and hydrogen peroxide, generated as by-products of oxidative metabolism in mitochondria, are removed by free radical-scavenging enzymes, such as superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), catalase, and glucose-6-phosphate dehydrogenase (G6PD) (Bilmen *et al*., 2001; Cheeseman and Slater, 1993; Lombardi *et al*., 2000; Sies, 1993).

Adult-onset thyroid dysfunction is associated with both neurological and behavioral abnormalities (DeGroot *et al*., 1984), emphasizing the importance of THs for normal brain function. Neurologically, hypothyroidism (Hypo) has been associated with loss of the alpha-rhythm during EEG examination, cerebellar ataxia, confusion, delusions, memory impairment, hallucinations, and psychotic behavior. On the contrary, in the case of hyperthyroidism (Hyper), symptoms include irritability, nervousness, anxiety, sleep disturbances, tremulousness, and increased frequency of the alpha-rhythm, even delirium, stupor, and coma (Adams and Rosman, 1971; Hall *et al*., 1986).

Acetylcholinesterase (AChE, EC 3.1.1.7) is a cholinergic enzyme, the role of which is very important in the acetylcholine (ACh) cycle, including the release of ACh (Kouniniotou-Krontiri and Tsakiris, 1989). In addition, it is found that AChE is coreleased from the dopaminergic neurons, implying an interaction between these two molecules which is important for the dopaminergic function (Klegeris *et al*., 1995). (Na^+,K^+)-ATPase (EC 3.6.1.3) is an enzyme implicated in neuronal excitability (Sastry and Phillis, 1977), metabolic energy production (Mata *et al*., 1980), as well as in the uptake and release of catecholamines (Bogdanski *et al*., 1968; Swann, 1984) and serotonin (Hernandez, 1987). Moreover, the role of Mg^{2+}-ATPase is to maintain high brain intracellular Mg^{2+}, changes of which can control rates of protein synthesis and cell growth (Sanui and Rubin, 1982).

The aim of this work was to assess the total antioxidant status (TAS), the protein concentration and the activities of AChE, (Na^+,K^+)-ATPase and Mg^{2+}-ATPase in the brain of adult rats with experimental hyper- and hypothyroidism.

MATERIALS AND METHODS

Animals

Albino Wistar adult male rats (6-month old) were used in all experiments. The rats were housed four in a cage, at a constant room temperature ($22 \pm 1^{\circ}$C) under a 12-h light: 12-h dark (light 08:00–20:00 h) cycle. Food and water were provided ad libitum. Animals were cared for in accordance with the principles of the "Guide to the Care and Use of Experimental Animals" (Committee on the Care and Use of Laboratory Animals, 1985).

Experimental Hyper- and Hypothyroidism

Hyperthyroidism was induced in rats by thyroxine administration. L-Thyroxine (T4) (Sigma, St. Louis, MO) was dissolved in 99% ethanol by adding a small volume (20 μL) of 25% NaOH and diluted 33 times by adding 0.9% NaCl to obtain a stock solution of 1 mg/mL. Before each injection, a fresh solution was made in 0.9% NaCl to obtain a concentration of T4 at 50 μg/mL. Thyroxine, 25 μg/100 g body weight was given subcutaneously once daily for 14 days. On the other hand, hypothyroidism was induced in rats by administration of 6-*n*-propyl-2-thiouracil in drinking water to a final concentration of 0.05% for 21 days. Each treatment results in a long-term moderate hyperthyroidism (Pantos *et al*., 1999) or hypothyroidism (Pantos *et al*., 2003). Two controls were used: (a) saline controls (SC) that were treated with subcutaneous injections of normal saline given once daily for 14 days (control of hyperthyroid rats), and (b) controls without any treatment (NTC) for 21 days (control for hypothyroid rats).

Tissue Preparation

The animals were sacrificed by decapitation and the whole brain was rapidly removed. The tissue was homogenized in 10 vol. ice-cold (0–4°) medium containing 50 mM Tris (hydroxymethyl) aminomethane-HCl (Tris-HCl), pH 7.4 and 300 mM sucrose, using an ice-chilled glass-homogenizing vessel at 900 rpm (4–5 strokes). Then, the homogenate was centrifuged at $1,000 \times g$ for 10 min. to remove nuclei and debris (Tsakiris, 2001; Tsakiris *et al*., 2000). In the resulting supernatant, the protein content was determined according to the method of Lowry *et al*. (1951) and then the enzyme activities and total antioxidant status (TAS) were measured.

Total Antioxidant Status

Total antioxidant status was evaluated in each fresh homogenized rat brain. The total antioxidant capacity was measured spectrophotometrically by a commercial kit (Randox Laboratories Ltd., Cat No NX2332) as previously reported (Carageorgiou *et al*., 2004; Tsakiris *et al*., 2000). ABTS (2,2′-azino-di-[3-ethylbenzthiazoline sulphonate]) was incubated with a peroxidase (metmyoglobin) and H_2O_2 in order to produce the radical cation

$ABTS^{\bullet+}$. The latter had a relatively stable blue–green color, which was measured at 600 nm. This study took place in order to investigate the possible free-radical production (low total antioxidant status) or the possible protective role of free-radical-scavenging enzymes in the brain (high total antioxidant status value).

Determination of Brain Acetylcholinesterase Activity

AChE activity was determined by following the hydrolysis of acetylthiocholine according to the method of Ellman *et al*. (1961), as described by Tsakiris (2001). The incubation mixture (1 mL) contained 50 mM Tris-HCl, pH 8, 240 mM sucrose, and 120 mM NaCl. The protein concentration of the incubation mixture was 80–100 μg/mL. The reaction was initiated after addition of 0.03 mL of 5,5′-dithionitrobenzoic acid (DTNB), and 0.05 mL of acetylthiocholine iodide, which was used as substrate. The final concentration of DTNB and substrate were 0.125 and 0.5 mM, respectively. The reaction followed spectrophotometrically by the increase of absorbance ($\Delta\overline{OD}$) at 412 nm.

Determination of Na^+,K^+-ATPase and Mg^{2+}-ATPase Activities

(Na^+,K^+)-ATPase was calculated from the difference between total ATPase activity (Na^+,K^+,Mg^{2+}-dependent ATPase) and Mg^{2+}-dependent ATPase activity. Total ATPase activity was assayed in an incubation medium consisting of 50 mM Tris-HCl, pH 7.4, 120 mM NaCl, 20 mM KCl, 4 mM $MgCl_2$, 240 mM sucrose, 1 mM ethylenediamine tetraacetic acid K_2-salt (K^+-EDTA), 3 mM disodium ATP, and 80–100 μg protein of the homogenate in a final volume of 1 mL. Ouabain (1 mM) was added in order to determine the activity of Mg^{2+}-ATPase. The reaction was started by adding ATP and stopped after an incubation period of 20 min by addition of 2 mL mixture of 1% lubrol and 1% ammonium molybdate in 0.9 M H_2SO_4 (Bowler and Tirri, 1974; Tsakiris, 2001). The yellow color which developed was read at 390 mM.

Statistical Analysis

The data were analyzed by a two-tailed Student's *t*-test. *P* values of <0.05 were considered statistically significant.

RESULTS

Figure 1 presents TAS values in the brain of hyper-, hypothyroid, and control rats. There was no change in brain TAS in hyperthyroid animals, while a 20% increase ($p < 0.001$) was observed in hypothyroid animals (compared to controls). Figure 2 illustrates brain total protein concentration values in the above treatments. Protein concentration was constant ($p > 0.05$) in hyperthyroid animals, while it was found inhibited by 35% ($p < 0.001$) in hypothyroid rats. Body and brain weights are illustrated in Table 1. We

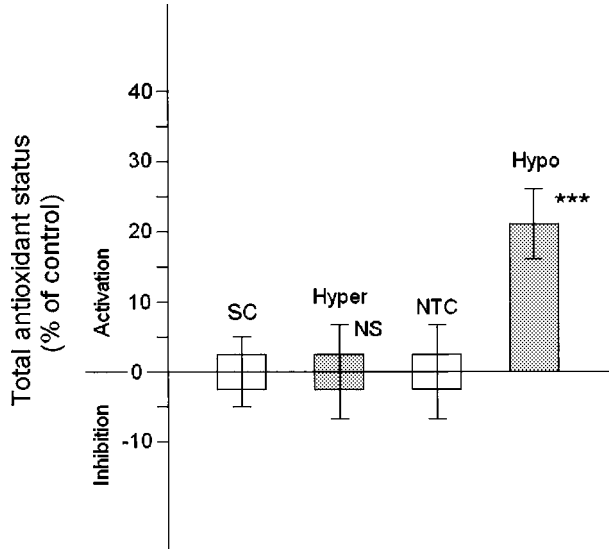

Figure 1. Total antioxidant status (TAS) values in the rat brain of hyperthyroid (Hyper), hypothyroid (Hypo) and controls. Saline controls (SC): NaCl of 0.9% subcutaneously (s.c.) for 14 days; Hyperthyroidism: thyroxine (25 μg/100 g body weight) s.c. once daily for 14 days; Hypothyroidism: propylthiouracil (0.05%) orally for 21 days; Controls without treatment (NTC): for 21 days. TAS values were determined in each fresh homogenized adult rat whole brain. Its value of brain homogenate in the saline control was 80 $\pm$ 6 mmol/L and that of the nontreated control was 85 $\pm$ 8 mmol/L. Each value indicates the mean $\pm$ SD of eight independent experiments (eight rats). The average of each experiment arose from three evaluations in the homogenated brain of each animal. NS: nonstatistically significant; $^{***}p < 0.001$; as compared to their respective control values (for details see Materials and Methods section). There is no statistically significant difference between the two controls.

observe that brain weight was not affected ($p > 0.05$) by the experimental procedure, while body weight was decreased by 38% ($p < 0.001$) in hypothyroid rats and remained rather constant ($p > 0.05$) in hyperthyroid animals. Similar results have been reported by Asayama *et al.* (1987) and Pantos *et al.* (2003). Table 2 shows the influence of experimental hyper- and hypothyroidism on brain AChE, (Na^+,K^+)-ATPase and Mg^{2+}-ATPase activities. We observed a decreased brain AChE activity (-25%; $p < 0.001$) in both hyper- and hypothyroid animals. In addition, (Na^+,K^+)-ATPase activity was found decreased by 27% ($p < 0.01$) in hyper-, and increased by 30% ($p < 0.001$) in hypothyroid rat brain. Mg^{2+}-ATPase activity was found unaffected in hyper- ($p > 0.05$), but activated by 26% ($p < 0.001$) in hypothyroidism.

DISCUSSION

The overall analysis of our data reveals that THs affected more or less the examined parameters of adult rat brain. A strong, dependent relationship seems to exist between the CNS and THs. Certain metabolic reactions increase in parallel with hyperthyroidism, such as those affecting mitochondrial respiratory enzyme activity (Dembri *et al.*, 1983), and

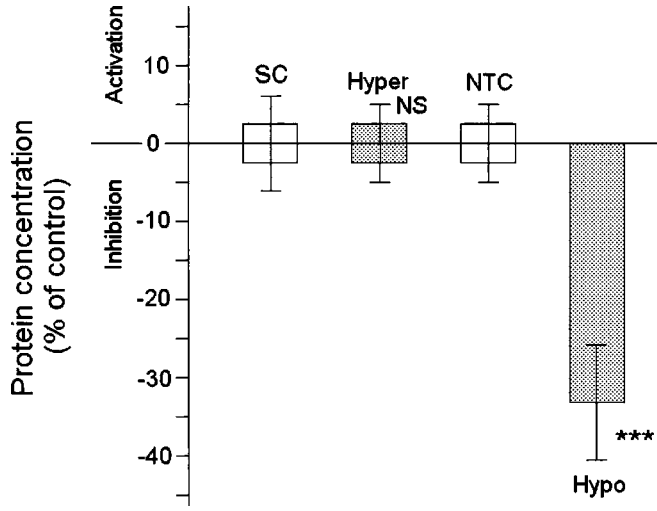

Figure 2. Protein concentration values in the brain of saline control (SC), hyperthyroid (Hyper), nontreatment control (NTC) and hypothyroid (Hypo) rats. The SC value of brain homogenate was 5.5 ± 0.3 mg/mL, while that of the NTC brain homogenate was 5.1 ± 0.2 mg/mL. Each value indicates the mean $\pm$ SD of eight independent experiments (eight rats). The average of each experiment arose from three evaluations in the homogenated brain of each animal. NS: nonstatistically significant; $^{***}p < 0.001$; as compared to the respective control values. There is no statistically significant difference between the two controls.

acetate metabolism (Chapa *et al*., 1995). Hyperthyroid animals appear to have a shorter lifespan and, at advanced age, present a myelin deficit. This may be due to the damage produced by the oxidative stress generated by an excess of THs (Pasquini and Adamo, 1994). However, this brain oxidative stress (inhibited TAS) was not found in the group of hyperthyroid animals in our study (0.25 mg/kg T4 daily for 14 days) (Fig. 1). It is likely that free-radical production is counteracted by the increased activities of brain radical-scavenging enzymes, such as catalase, Mn-superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) (Mano *et al*., 1995), as an adaptive long-term mechanism, or may be due to the fact that the brain does not respond to the calorigenic effect of THs (Silva, 1995; Goodman and Gilman, 2001).

Table 1. Experimental Hyper- and Hypothyroidism on Body and Brain Weight in Adult Rats

Treatment	Body weight (g)	Brain weight (g)
Saline controls (SC), $N = 8$	353 ± 35	1.761 ± 0.098
Hyper, $N = 8$	320 ± 26 (NS)	1.819 ± 0.082 (NS)
Controls (NTC), $N = 8$	340 ± 30	1.819 ± 0.082
Hypo, $N = 8$	$210 \pm 11^{***}$ (-38%)	1.816 ± 0.100 (NS)

Note. Each value indicates the mean $\pm$ SD of eight independent experiments (eight rats). NS: nonstatistically significant, $^{***}p < 0.001$; as compared to control values (for details see Materials and Methods section). There is no statistically significant difference between the two controls.

Table 2. Experimental Hyper- and Hypothyroidism on Brain Acetylcholinesterase, (Na^+,K^+)-ATPase and Mg^{2+}-ATPase Activities in Adult Rats

Treatment	Activities		
	AChE (Δ $\overline{OD}$/ min $\times$ mg protein)	Na^+,K^+-ATPase (μmol Pi/h $\times$ mg protein)	Mg^{2+}-ATPase (μmol Pi/h $\times$ mg protein)
Saline controls, $N = 8$	1.108 ± 0.036	6.32 ± 0.38	8.31 ± 0.66
Hyper, $N = 8$	$0.831 \pm 0.042^{***}$ (-25%)	$4.61 \pm 0.41^{***}$ (-27%)	7.98 ± 0.79 (NS) (-4%)
Controls (NTC), $N = 8$	1.126 ± 0.030	6.17 ± 0.30	8.65 ± 0.50
Hypo, $N = 8$	$0.853 \pm 0.051^{***}$ (-23%)	$8.22 \pm 0.66^{***}$ ($+30\%$)	$10.47 \pm 0.84^{***}$ ($+26\%$)

Note. In saline controls, NaCl of 0.9% (placebo) was administered subcutaneously for 14 days, while controls (NTC) were without any treatment for 21 days. Hyperthyroidism (Hyper) was induced in rats by thyroxine (25 μg/100 g body weight) administration given subcutaneously once daily for 14 days. Hypothyroidism (Hypo) was induced in animals by oral administration of propylthiouracil (0.05%) for 21 days. The animals were sacrificed by decapitation. Each value indicates the mean $\pm$ SD of eight independent experiments (eight rats). The average value of each experiment came from four evaluations in the homogenated whole brain of each animal. NS: nonstatistically significant; $^{***}p < 0.001$; as compared to the respective control values (for details see Materials and Methods section). There is no statistically significant difference between the two controls.

In hypothyroidism, brain TAS was found to be activated (Fig. 1). This may be due to decreased metabolic reactions and the low generation of free radicals (Swaroop and Ramasarma, 1985), as well as to the stimulated activity of total SOD, Cu,Zn-SOD, GSH-Px (Mano *et al*., 1995) and the high activity of G-6-PD (Yilmaz *et al*., 2003). In addition, it has been proposed that hypothyroidism provides in vivo protection against free radical induced damage and this cellular defense mechanism may be acting differently from antioxidant defense systems (Venditti *et al*., 1997). However, Yilmaz *et al*. (2003) have concluded that hypothyroidism, produced via an i.p. administration of propylthiouracil (in a different dose: 10 mg/kg) for 15 days, may cause peroxidation in the tissues of 1- and 2-month old female rats. In our experiments, adult male rats were used, which received 0.05% propylthiouracil for 3 weeks. In the study of Yilmaz *et al*. (2003), G-6-PD did not change in the brain of 30-day-old rats, but increased in the 60-day-old animals. It seems likely that antioxidant enzyme activities in experimental hypothyroidism depend on several factors such as sex, age and tissue, as well as on the severity of the induced hypothyroidism. The subject needs further investigation.

Brain protein concentration remained unaltered in our conditions of hyperthyroidism (Fig. 2), possibly due to the unaffected TAS. In contrast, the observed diminished protein concentration in hypothyroidism may be due to decreased protein synthesis during the lower metabolic rate in this condition.

The effects induced by alterations of the thyroid state on rat brain AChE remain largely unknown. Virgili *et al*. (1991) have reported a decreased AChE activity in the prefrontal cortex and in the striatum in hypothyroid rats. We also found decreased AChE activity, but in the whole brain of adult rats. In hyperthyroidism, we observed a decrease in the whole brain AChE activity, while Almeida and Santos (1993) found no change in AChE activity in T3 treated rats. In addition, Smith *et al*. (2002), with a different treatment protocol of L-T4 administration (2.5 mg/kg for 4 days and 5 or 10 mg/kg every third day for 28 days i.p.), found increased AChE activity in both frontal cortex and hippocampus in the first dosage regiment. However, this enzyme activity was shown to be increased only in the hippocampus in the second dosage regimen (28 days). In our experiments, we used a different dose and way of administration of L-T4 (0.25 mg/kg per day for 14 days s.c.) and produced a moderate hyperthyroidism, while AChE activity was measured in the whole rat brain. Providing that AChE is the degradative enzyme of ACh, one could speculate that thyroxine improves learning and memory because of the inhibition of AChE, by the increase of ACh in the synaptic cleft. However, this cannot explain the decreased AChE activity in the hypothyroid brain, in which diminished learning and memory functions are reported (Gerges *et al*., 2004). On the other hand, Smith *et al*. (2002) suggest that the increased cholinergic activity by L-T4 administration improves cognitive performance. In this case, the degree of hyperthyroidism may be related to the kind of changes occurring in the examined enzyme activities. The observed inactivated AChE in hypothyroid brain might be the result of the decreased protein concentration, possibly inducing a long-term diminution of the number of the active enzyme molecules in the membrane. In contrast, the observed inhibited AChE activity in the hyperthyroid brain (Table 2) might be the result of a thyroxine-induced increase of intracellular calcium (Ernest, 1989), which results in synaptosomal membrane changes and modulation of the lipid(s)-protein interaction(s).

The observed inhibition of brain Na^+,K^+-ATPase in hyperthyroidism (Table 2) may be due to the increase of intracellular calcium by L-T4 (Ernest, 1989). In contrast, the increased

activity of Na^+,K^+-ATPase in hypothyroidism can be related to the observed increase in TAS (Tsakiris *et al*., 2000). Changes in brain Na^+,K^+-ATPase activity have been associated with modulations of brain electrical activity (EEG) (Abdel-Latif *et al*., 1967). Therefore, the opposite changes of Na^+,K^+-ATPase activity in the above treatments might be related to the reported increased frequency of the alpha-rhythm of EEG in hyperthyroidism and the loss of the alpha-rhythm in hypothyroidism (Adams and Rosman, 1971; Hall *et al*., 1986). Additionally, the opposite changes of Na^+,K^+-ATPase activity may differentially modulate the brain neural excitability (Sastry and Phillis, 1977), metabolic energy production (Mata *et al*., 1980), as well as the uptake and release of catecholamines (Bogdanski *et al*., 1968; Swann, 1984) and serotonin (Hernandez, 1987).

Changes in brain Mg^{2+}-ATPase activity (Table 2) may be related to the changes observed in TAS (Fig. 1). The increased activity of Mg^{2+}-ATPase in hypothyroidism could be an adaptive mechanism, maintaining the brain intracellular Mg^{2+} at high levels, changes of which may modulate rates of protein synthesis and cell growth (Sanui and Rubin, 1982).

In conclusion: TAS, protein concentration and Mg^{2+}-ATPase activity were found to be unaffected in the brain of hyperthyroid adult male rats, while AChE and Na^+,K^+-ATPase activities were found to be reduced. In contrast, TAS, (Na^+,K^+)-ATPase and Mg^{2+}-ATPase activities were found to be increased in the hypothyroid brains, while AChE activity and protein concentration were shown to be inhibited. These effects could be due to differences in metabolism and TAS values, as well as to the intracellular calcium and magnesium concentration. Such changes in the enzyme activities may differentially modulate the brain neural excitability, uptake and release of biogenic amines.

ACKNOWLEDGMENTS

This work was funded by the University of Athens. Many thanks are expressed to Ioannis Paizis, Constantinos Bakogiannis, Nick Mavromatis, and Maria Stoupaki for their assistance.

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