Metab Brain Dis (2007) 22:31–38
DOI 10.1007/s11011-006-9034-1

SHORT COMMUNICATION

Effects of hyper- and hypothyroidism on acetylcholinesterase, (Na^+, K^+)- and Mg^{2+}-ATPase activities of adult rat hypothalamus and cerebellum

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Received: 15 February 2006 / Accepted: 8 May 2006 /
Published online: 14 December 2006

Abstract Thyroid hormones (THs) are recognized as key metabolic hormones, and the metabolic rate increases in hyperthyroidism, while it decreases in hypothyroidism. The aim of this work was to investigate how changes in metabolism induced by THs could affect the activities of acetylcholinesterase (AChE), (Na^+, K^+)- and Mg^{2+}-ATPase in the hypothalamus and the cerebellum of adult rats. Hyperthyroidism was induced by subcutaneous administration of thyroxine (25μg/100 g body weight) once daily for 14 days, while hypothyroidism was induced by oral administration of propylthiouracil (0.05%) for 21 days. All enzyme activities were evaluated spectrophotometrically in the homogenated brain regions of 10 three-animal pools. Neither hyper-, nor hypothyroidism had any effect on the examined hypothalamic enzyme activities. In the cerebellum, hyperthyroidism provoked a significant decrease in both the AChE (-23%, $p < 0.001$) and the Na^+, K^+-ATPase activities (-26%, $p < 0.001$). Moreover, hypothyroidism had a similar effect on the examined enzyme activities: AChE (-17%, $p < 0.001$) and Na^+, K^+-ATPase (-27%, $p < 0.001$). Mg^{2+}-ATPase activity was found unaltered in both the hyper- and the hypothyroid brain regions. In conclusion: neither hyper-, nor hypothyroidism had any effect on the examined hypothalamic enzyme activities. In the cerebellum, hyperthyroidism provoked a significant decrease in both the AChE and the Na^+, K^+-ATPase activities. The decreased (by the THs) Na^+, K^+-ATPase activities may increase the synaptic acetylcholine release, and thus, could

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result in a decrease in the cerebellar AChE activity. Moreover, the above TH-induced changes may affect the monoamine neurotransmitter systems.

Keywords Hyperthyroidism · Hypothyroidism · Rat brain · Acetylcholinesterase · (Na^+, K^+)-ATPase · Mg^{2+}-ATPase · Hypothalamus · Cerebellum

Introduction

Thyroid hormones (THs) are recognized as key metabolic hormones that play a critical role in brain development (and not only), mediating important effects within the central nervous system (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). Moreover, adult-onset thyroid dysfunction is associated with both neurological and behavioural abnormalities (DeGroot *et al*., 1984), emphasizing the importance of THs for normal brain function. Neurologically, hypothyroidism (Hypo) has been associated with cerebellar ataxia, confusion, delusions, memory impairment, hallucinations and psychotic behaviour. On the contrary, in the case of hyperthyroidism (Hyper), symptoms include irritability, nervousness, anxiety, sleep disturbances, tremulousness, delirium, stupor and even 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 was found that AChE is co-released 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), serotonin (Hernandez, 1989) and glutamate (Lees *et al*., 1990). 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).

In a previous study (Carageorgiou *et al*., 2005), Mg^{2+}-ATPase activity was found unaffected in the hyperthyroid rat whole brain, while AChE and Na^+, K^+-ATPase activities were found reduced. In contrast, Mg^{2+}- and (Na^+, K^+)-ATPase activities were found to be increased in the hypothyroid rat brain, while AChE activity was found decreased.

The aim of this work was to assess the activities of AChE, (Na^+, K^+)-ATPase and Mg^{2+}-ATPase in the hypothalamus and the cerebellum of adult rats with experimental hyper- and hypothyroidism.

Materials and methods

Animals

Albino Wistar adult male rats (six months 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 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, USA) 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 brain regions were 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 *et al*., 2000; Tsakiris, 2001). In the resulting supernatant, the protein content was determined according to the method of Lowry *et al*. (1951) and then the enzyme activities were measured.

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+}-ATPace activities

(Na^+, K^+)-ATPase activity 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 colour which developed was read at 390 mM.

Table 1 Effects of hyper- and hypothyroidism on hypothalamic acetylcholinesterase, (Na^+, K^+)-ATPase and Mg^{2+}-ATPase activities in adult rats

Treatment	Activities		
	AChE ($\Delta\overline{OD}$/min × mg protein)	Na^+, K^+-ATPase (μmol Pi/h × mg protein)	Mg^{2+}-ATPase (μmol Pi/h × mg protein)
Saline controls (SC), $N = 10$	0.614 ± 0.025	4.14 ± 0.33	7.42 ± 0.67
Hyper, $N = 10$	0.632 ± 0.024, *ns* (+3%)	4.35 ± 0.28, *ns* (+5%)	6.93 ± 0.62, *ns* (−7%)
Controls (NTC), $N = 10$	0.620 ± 0.029	4.40 ± 0.26	6.90 ± 0.71
Hypo, $N = 10$	0.621 ± 0.031, *ns* (0%)	4.30 ± 0.35, *ns* (−2%)	7.04 ± 0.42, *ns* (+2%)

Note. Each value indicates the mean ± SD of ten independent experiments (ten pools of three rat regions). The average value of each experiment was obtained from four evaluations in the homogenated brain region tissue of three animals. *ns* = non statistically significant as compared to the respective control values (for details see Materials and methods). There is no statistically significant difference between the two control groups.

Statistical analysis

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

Results

Table 1 presents the effects of hyper- and hypothyroidism on the hypothalamic AChE, (Na^+, K^+)- and Mg^{2+}-ATPase activities in adult rats. The examined rat hypothalamic enzymes do not seem to be affected by the experimental procedures followed. The effects of hyper- and hypothyroidism on the cerebellar AChE, (Na^+, K^+)- and Mg^{2+}-ATPase activities in adult rats, are shown in Table 2. In the cerebellum, hyperthyroidism provoked a significant decrease in both the AChE (−23%, $p < 0.001$) and the Na^+, K^+-ATPase activities (−26%, $p < 0.001$). Moreover, hypothyroidism had a similar effect on the examined enzyme

Table 2 Effects of hyper- and hypothyroidism on cerebellar acetylcholinesterase, (Na^+, K^+)-ATPase and Mg^{2+}-ATPase activities in adult rats

Treatment	Activities		
	AChE ($\Delta\overline{OD}$/min × mg protein)	Na^+, K^+-ATPase (μmol Pi/h × mg protein)	Mg^{2+}-ATPase (μmol Pi/h × mg protein)
Saline controls (SC), $N = 10$	0.474 ± 0.014	6.42 ± 0.57	8.47 ± 0.76
Hyper, $N = 10$	0.365 ± 0.017*** (−23%)	4.75 ± 0.29*** (−26%)	8.89 ± 0.53, *ns* (−5%)
Controls (NTC), $N = 10$	0.463 ± 0.018	6.00 ± 0.48	8.90 ± 0.85
Hypo, $N = 10$	0.384 ± 0.020*** (−17%)	4.38 ± 0.35*** (−27%)	8.81 ± 0.44, *ns* (−1%)

Note. Each value indicates the mean ± SD of ten independent experiments (ten pools of three rat regions). The average value of each experiment was obtained from four evaluations in the homogenated brain region tissue of three animals. *ns* = non statistically significant; ***$p < 0.001$; as compared to the respective control values (for details see Materials and methods). There is no statistically significant difference between the two control groups.

activities: AChE (-17%, $p < 0.001$) and Na^+, K^+-ATPase (-27%, $p < 0.001$). Mg^{2+}-ATPase activity was found unaltered in both the hyper- and the hypothyroid brain regions.

Discussion

The overall analysis of our data revealed that THs affected the examined parameters only in the cerebellum. None of the hypothalamic examined parameters was affected by any of the experimental procedures followed. This is in accordance with the fact that no findings have been reported so far, concerning the function of the hypothalamic AChE, (Na^+, K^+)- and Mg^{2+}-ATPase in hyper- or hypothyroidism. It may be suggested that the hypothalamus, because of its intimate relationship with both the endocrine and the autonomic system, appears to possess significant regulatory mechanisms, towards homeostatic functioning of its neuronal systems (Iversen *et al*., 2000).

The data concerning the effects induced by alterations of the thyroid state on adult rat brain AChE activity are limited. It appears that the present findings in the cerebellum (concerning AChE activity) are in accordance with previous findings (Carageorgiou *et al*., 2005) refereeing to the whole rat brain AChE activity (exhibiting a similar reduction in both hyper- and hypothyroid states).

It is known that choline acetyltransferase (ChAT) activity (a—considered to be—specific marker for cholinergic neurons) decreases in the cerebellum of hyper- and increases in the cerebellum of hypothyroid suckling rats (Virgili *et al*., 1991). The level of ChAT activity correlates with Purkinje cell size, supporting the concept of a neurotrophic role of ACh (Clos *et al*., 1989). Moreover, in comparison with euthyroid suckling rats, the density of muscarinic receptors in the cerebellum was found to be 40% higher in hyperthyroidism and 30% lower in thyroid deficiency (Patel *et al*., 1980). ChAT and AChE are transiently expressed together in functionally non-cholinergic Purkinje cells. In contrast with most regions of the CNS, the high ratio of ChAT to AChE activity suggests an enhanced synthesis of ACh (Clos *et al*., 1989). It may be suggested that hyperthyroid cerebellum exhibits a diminished ACh synthesis, compared to the euthyroid one, followed by an increased muscarinic receptor density. Providing that AChE is the degradative enzyme of ACh, one could speculate that thyroxine plays a neurotrophic role and enhances the increase of ACh concentration in the synaptic cleft by reducing AChE activity and increasing the muscarinic receptor synthesis. The observed reduced AChE activity in the hyperthyroid cerebellum (Table 2) might be the result of a thyroxine-induced increase of intracellular calcium (Ernest, 1989), which could result in synaptosomal membrane changes and modulation of lipid(s)-protein(s) interaction(s).

In hypothyroidism, the observed reduced AChE activity in the cerebellum (Table 2) may be the result of a long-term diminution of the number of the active enzyme molecules in the membrane, mainly due to metabolic reasons. This is in accordance with the findings of Ahmed *et al*. (1993), where AChE activity was found reduced (by 45%) in the cerebellum of hypothyroid adult rats. Moreover, Li *et al*. (1996) suggested that iodine and THs deficiency can affect the maturation of ChAT and disturb the transformation and maturation of various molecular types of AChE. In addition to the reported low muscarinic receptor density, the reduced AChE activity might contribute to the clinically known hypothyroid cerebellar ataxia (Aminoff *et al*., 2005). The subject needs further investigation.

The observed inhibition of cerebellar Na^+, K^+-ATPase in hyperthyroidism (Table 2) is in accordance with previous findings in whole rat brain (Carageorgiou *et al*., 2005), and may be due to the increase of intracellular calcium by L-T4 (Ernest, 1989). However, the

hypothyroid cerebellar Na^+, K^+-ATPase activity was also found significantly decreased (Table 2), in contrast to the whole brain Na^+, K^+-ATPase activity that was previously reported to be enhanced (Carageorgiou *et al*., 2005). This region-specific finding seems to be in accordance with those of Atterwill *et al*. (1985), where hypothyroidism significantly reduced the Na^+, K^+-ATPase activity in the suckling rat cerebellum, but not in the forebrain. Moreover, Atterwill *et al*. (1985) observed a 20–45% reduction of the alpha (+) form of Na^+, K^+-ATPase (high ouabain affinity) against control cerebellum, compared to a 60–70% reduction in the activity of the alpha form of Na^+, K^+-ATPase (low ouabain affinity). The latter might be indicative of a retarded development of a selective cerebellar cell population, containing predominantly the alpha form of the enzyme.

It should be also noted that the decrease in Na^+, K^+-ATPase activity can enhance (at least in part) the ACh release (Meyer and Cooper, 1981), and thus, affect in a similar way the cerebellar AChE activity. Moreover, the observed alterations of Na^+, K^+-ATPase activity may modulate cerebellar catecholaminergic, serotoninergic and glutamatergic systems (Bogdanski *et al*., 1968; Hernandez, 1987; Lees *et al*., 1990, Swann, 1984), as well as neural excitability (Sastry and Phillis, 1977) and metabolic energy production (Mata *et al*., 1980). It must be also noted that in mature rat cerebellum, hypothyroidism produced a 61% increase of monoaminoxidase (MAO) activity (Ahmed *et al*., 1993).

The unaltered Mg^{2+}-ATPase activity in the hyperthyroid cerebellar tissue is in accordance with our earlier whole brain findings (Carageorgiou *et al*., 2005), while the hypothyroid cerebellar enzyme activity was not found increased as expected. The latter could be indicative of a different (state- and region-dependent) Mg^{2+}-ATPase behavior.

In conclusion: neither hyper-, nor hypothyroidism had any effect on the examined hypothalamic enzyme activities. In the cerebellum, hyperthyroidism provoked a significant decrease in both the AChE and the Na^+, K^+-ATPase activities. Moreover, hypothyroidism had a similar effect on the above enzyme activities. Mg^{2+}-ATPase activity was found unaltered in both the hyper- and the hypothyroid brain regions. These effects could be due to region- and state-specific differences in metabolism (provoking changes in the synthesis, the maturation and the function of at least some of the above enzymes), as well as to the THs-induced alterations of the intracellular calcium concentration and the monoaminergic systems' actions.

Acknowledgements This work was funded by the University of Athens and the University of Ioannina. Many thanks are expressed to the medical students Elena Gkrouzman, Zois Mellios and Christina Katsioni for their assistance.

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