Hypothyroidism Decreases the Biogenesis in Free Mitochondria and Neuronal Oxygen Consumption in the Cerebral Cortex of Developing Rats

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Thyroid hormone plays a critical role in mitochondrial biogenesis in two areas of the developing brain, the cerebral cortex and the striatum. Here we analyzed, in the cerebral cortex of neonatal rats, the effect of hypothyroidism on the biogenesis in free and synaptosomal mitochondria by analyzing, in isolated mitochondria, the activity of respiratory complex I, oxidative phosphorylation, oxygen consumption, and the expression of mitochondrial genome. In addition, we studied the effect of thyroid hormone in oxygen consumption *in vivo* by determining metabolic flow through ^{13}C nuclear magnetic resonance spectroscopy. Our results clearly show that *in vivo*, hypothyroidism markedly reduces oxygen consumption in the neural population of the cerebral cortex. This effect correlates with decreased free mitochondria biogenesis. In contrast, no effect was observed in the biogenesis in synaptosomal mitochondria. The parameters analyzed were markedly improved after T_3 administration. These results suggest that a reduced biogenesis and the subsequent reduction of respiratory capacity in free mitochondria could be the underlying cause of decreased oxygen consumption in the neurons of the cerebral cortex of hypothyroid neonates. **(*Endocrinology* 150: 3953–3959, 2009)**

Thyroid hormones (T_4, T_3) play an important role in mammalian tissue development and metabolism. Most of the actions of these hormones are mediated by the binding of T_3 to specific nuclear receptors that are ligand-dependent transcription factors that bind to thyroid hormone response elements in target genes and regulate transcription initiation (1, 2). These receptors belong to the nuclear receptor superfamily that is one of the largest families of transcription factors, which include, among others, the receptors for steroid hormones, retinoids, vitamin D_3, peroxisomal proliferators, and many other transcription factors without known ligand (orphan receptors) (1). The activation of these receptors has been implicated in a broad range of cellular functions.

The brain is an important target for thyroid hormone action, particularly during late development, as shown by numerous clinical and experimental data (3, 4). In the rat brain, the expression of thyroid hormone receptors increases rapidly after birth reaching the highest value by d 6 of postnatal life (5, 6). In agreement with this, during the perinatal period, the consequences of hypothyroidism are more dramatic and results in numerous alterations, such as reduction in dendritic arborization of cerebellar Purkinje cells; impairment of nerve process development; poor connectivity among neurons; changes in microtubule content; and impaired myelin deposition, cell migration, and synaptogenesis (4).

The mitochondria are well-established targets of thyroid hormone action. The regulation by thyroid hormone of mitochondrial activity and biogenesis are extensively documented in numerous mammalian tissues such as liver, kidney, heart, and muscle (7). In these tissues the administration of T_3 increases oxygen consumption and oxidative phosphorylation, which underlies the calorigenic effect of this hormone. In the case of the brain, after initial contradictory reports (8–11), it is now well established that the mitochondria are targets of thyroid hormone

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Abbreviations: Ct, Cycle threshold; GABA, γ-aminobutyric acid; NADH, nicotinamide adenine dinucleotide hydroxide; NMR, nuclear magnetic resonance; TCA, tricarboxylic acid; TFA, trifluoroacetic acid.

action at least during development. In the brain of neonatal rats the expression of mitochondrial genes encoded both in nuclear and mitochondrial genomes is regulated by thyroid hormones (12). We have also demonstrated that congenital hypothyroidism decreases rhodamine 123 uptake, alters mitochondrial morphology (13), and decreases respiratory complex I activity and oxidative phosphorylation capacity in the presence of nicotinamide adenine dinucleotide (NADH)-generating substrates (14). These effects of thyroid hormone are dependent of the brain area analyzed, being the mitochondria in the cerebral cortex and the striatum sensitive to the action of this hormone (14). These results suggest that, in the developing rat, thyroid hormone regulates mitochondrial biogenesis through the control of the expression of mitochondrial genes and in consequence also controls the capacity of oxidative phosphorylation in specific regions of the brain such as the cerebral cortex and striatum. Two different mitochondrial fractions can be easily isolated from nervous tissues: free and synaptosomal. The synaptosomal fraction is unequivocally from neuronal origin, and consequently, any effect of thyroid hormone on this mitochondrial fraction could easily be ascribed to the neuronal population. In addition, the important role played by synaptosomal mitochondria in neurotransmitter release makes the analysis of the possible action of thyroid hormone more relevant.

In vivo and *in vitro* ^{13}C nuclear magnetic resonance (NMR) approaches have been found to provide comprehensive information on cerebral metabolism, mainly in the metabolic compartmentation of glutamate, glutamine, and γ-aminobutyric acid (GABA) (15–17). High-resolution *ex vivo* ^{13}C NMR spectroscopy after *in vivo* infusion of (1-^{13}C) glucose can be used to evaluate changes through metabolic pathways and also provide insights into the cellular localization of these changes thanks to the use of specific inhibitors such as trifluoroacetic acid (TFA), a well-known inhibitor of the glial tricarboxylic acid (TCA) cycle (18).

In this work we analyzed, in the cerebral cortex of neonatal rats, the effect of thyroidal state on the biogenesis in free and synaptosomal mitochondria and on oxygen consumption. To achieve these goals, we determined in isolated free and synaptosomal mitochondria the expression of mitochondrial genome, the activity of respiratory complex I, the capacity of oxidative phosphorylation and respiration, and *in vivo* the metabolic flow through the TCA cycle, as a measure of oxygen consumption, using ^{13}C NMR spectroscopy. Our results clearly show that the biogenesis of synaptosomal mitochondria is not dependent on thyroidal state. In contrast, the biogenesis of free mitochondria and also the oxygen consumption *in vivo* were dramatically decreased in the cerebral cortex of hypothyroid neonates.

Materials and Methods

Materials

Methyl mercaptoimidazole, T_3, glutamate, malate, succinate, pyruvate, ADP, ATP, D-luciferin, luciferase, and TFA were obtained from Sigma Chemical Co. (St Louis, MO); (1-^{13}C) glucose (99.9% ^{13}C) was obtained from Cambridge Isotope Laboratories (Andover, MA) and ^2H_2O (99.9% ^2H) from Apollo Scientific Ltd. (Stockport, Cheshire, UK). All other chemicals were reagent grade or molecular biology grade.

Animal treatment

All animal related procedures were approved by the Laboratory Animal Care and Use Committee of the Universidad Complutense de Madrid and were conducted in accordance with the guidelines of the European Communities Council, directive 86/609/EEC. All efforts were made to minimize animal suffering and reduce the number of animals used. Female Wistar rats were mated and the day of appearance of the vaginal plug was considered as d 0 of fetal age. To induce fetal and neonatal hypothyroidism, dams were given 0.02% methyl mercaptoimidazole in the drinking water at d 12 of gestation. Methyl mercaptoimidazole treatment was continued throughout the whole period of the experiment. This protocol ensures that the animals are hypothyroid throughout the neonatal age, as shown by the decrease growth rate and circulating levels of T_3 in 15-d-old neonates below the level of detectability. To determine reversibility of the effects caused by hypothyroidism treatment, half of the hypothyroid animals were daily injected with 20 μg T_3 per 100 g of body weight during 3 d (starting at postnatal d 12), a treatment shown to normalized mitochondrial activity (14). During this period the remaining animals received an ip injection of the same volume of saline. Except for the ^{13}C NMR spectroscopy studies, the animals were killed by decapitation at postnatal d 15, and the brain was quickly removed and the cerebral cortex dissected for free and synaptosomal mitochondria isolation. All the rats used in this study were housed in a humidity- and temperature-controlled room on a 12-h light, 12-h dark cycle, receiving water and food *ad libitum*.

Isolation of mitochondria

Cerebral cortex tissue was minced and homogenized manually in 0.32 M sucrose, 1 mM EDTA, 10 mM Tris-HCl (pH 7.4), and 0.1% BSA, centrifuged at 2000 × *g* for 4 min. The precipitate was discarded and the supernatant was centrifuged again at 12,500 × *g* for another 10 min. This precipitate was resuspended and free and synaptosomal mitochondria were isolated in Ficoll and Percoll gradients according to the procedures described by Lai *et al.* (19) and Dunkley *et al.* (20) respectively. The mitochondria were finally resuspended in the appropriate experimental solution without BSA and protein content was measured by Bradford's procedure using BSA as standard.

ATP production and respiratory complex I activity

Isolated mitochondria were incubated at 22 C in incubation media [45 mM sucrose, 100 mM KCl, 10 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 10 mM KH_2PO_4 and 0.5% BSA] with ADP (0.5 mM) and the following substrates: glutamate/malate (5 mM each) or succinate (5 mM). Samples were collected after 2, 4, 6, and 8 min of incubation and the amount of ATP determined by the luciferin/luciferase method (21). The rate of ATP synthesis was calculated with the straight line obtained by representing ATP concentration against time.

The activity of the rotenone-sensitive NADH-coenzyme Q1 reductase (complex I) was determined as previously described (22).

Oxygen consumption

Oxygen consumption in freshly prepared mitochondria was measured polarographically with a Clark-type electrode (Oxigraph Hansatech, Norfolk, UK) at 30 C with constant stirring. Mitochondria (0.7 mg of protein) were suspended in 1 ml of respiratory medium [75 mM sucrose, 50 mM KCl, 5 mM KH_2PO_4, 0.5 mM EDTA, 5 mM $MgCl_2$, and 30 mM Tris-HCl (pH 7.4)] and the basal rate of respiration (state 4) determined in the presence of diverse substrates: glutamate/malate (5 mM each), pyruvate/malate (5 mM each), or succinate (5 mM). When succinate was the substrate, 5 μM rotenone was also added. The active state of respiration (state 3) was initiated by the addition of 0.5 mM ADP (22).

Mitochondrial gene expression

For total mitochondrial RNA and DNA extraction, 400 μg of mitochondrial protein were digested with proteinase K (6 μg/ml) at 37 C during 30 min in extraction media [LiCl 0.5M, urea 1 M, 1% sodium

dodecyl sulfate, 2.5 mM EDTA, and 20 mM sodium citrate (pH 8)]; organic extracted; and the nucleic acids precipitated, resuspended in diethylpyrocarbonate water, and its integrity analyzed and quantified.

cDNA was synthesized with the RETRoscript kit (Ambion, Austin, TX) using 2 μg of total nucleic acids and the downstream oligonucleotides used in PCR. In parallel a mock cDNA synthesis reaction (without reverse transcriptase) was performed for each sample. Real-time PCR was performed in an ABI Prism equipment using the SYBR Green PCR master mix (Applied Biosystems, Warrington, UK), 5 μl of mitochondrial nucleic acid, and 300 nM concentrations of specific primers in a final volume of 25 μl. Cycle threshold (Ct) values were calculated from the strait line created using different dilutions of sample. The primers used for the determination of the concentration of the transcripts of 12S rRNA and ND4 mRNA were: 12S, TCGATAAACCCCGTTCTACCTTAC and TGGTGATGATGGCGGTATATAGG, which amplified a fragment of 80 bp (from nucleotide 564 to 640 of the rat mitochondrial DNA) and ND4, AATAATCGCCCACGGCTTAAC and TTCGTTCGTAGTTGGTGTTTGC, which amplified a fragment of 65 bp (from nucleotide 11101 to 11166 of the rat mtDNA). The concentration of mtDNA was determined by measuring D loop, GCATGAAGGTCAGCACAAAGTC and TCGAGCTTTGTCTATGAGGATAAATG. The difference between Ct values of real and mock cDNA synthesis were used as a measure of gene expression in each sample. As expected, the same Ct values were obtained with mock and real cDNA synthesis when the D loop oligonucleotides were used for amplification. The final values are the average ± SE of the determination in at least five different samples and are normalized by the values found in the mitochondria of control animals.

^{13}C NMR spectroscopy studies

At least five animals per group were infused under deep anesthesia (1–2% of isoflurane in 1 liter/min O_2 through a nose cap). The infusion was performed through the jugular vein with (1-^{13}C) glucose (8 μmol min^{-1}/g^{-1}) during 60 min. Sodium TFA was coinfused (24 μmol/$min^{-1} \cdot g^{-1}$) with (1-^{13}C) glucose (23). The physiological state of the animal was constantly monitored, and blood samples were taken before and immediately after infusion for glucose levels determination. The metabolism of the rat brain was arrested using a microwave fixation system (Muromachi Kikai Co. Ltd., Tokyo, Japan), the brain rapidly removed, and the cerebral cortex dissected and frozen in liquid N_2. Perchloric acid extracts were prepared, neutralized with KOH, lyophilized, and resuspended in 99.9% ^2H_2O. High-resolution ^{13}C NMR spectroscopy and the quantification of absolute amount of ^{13}C incorporated in the different carbons was performed in a AVANCE 500WB NMR spectrometer (Bruker, Ettlingen, Germany) using a commercial (5 mm) triple resonance probe (^{1}H, ^{13}C, ^{2}H) optimized for direct ^{13}C NMR detection (24, 25). The unchanged myo-inositol was used as a robust reference from which all ^{13}C resonances can be normalized (25, 26). Additionally, *myo*-inositol is a good internal reference once its resonances do not change with thyroid status (25).

High-resolution proton-decoupled ^{13}C NMR spectra were obtained at 11.7 Tesla as described (16) and the absolute amount of ^{13}C incorporated in the different carbons and the fractional enrichment was determined as previously described (23, 26, 27).

Quantification of metabolite concentrations

The amino acid content of brain extracts was determined with an automatic amino acid analyzer Biochrom 20 (Pharmacia, Uppsala, Sweden) using a cationic exchange column and precolumn derivatization with ninhydrin as previously described (23).

Statistical analysis

Statistical analysis was performed using the SPSS package (SAS Institute Inc., Cary, NC). When indicated, the statistical significance of the differences was assessed using the Student's t test. The threshold for statistical significance was set at $P < 0.05$.

Results

Congenital hypothyroidism does not alter oxidative phosphorylation and complex I activity in isolated synaptosomal mitochondria

Prior to the study of synaptic mitochondria function, we first analyzed the purity of synaptosomal fraction by electronic microscopy and flow cytometry (13). No differences were found between the synaptosomal fractions from the cerebral cortex of control and hypothyroid neonates. The percentage of particles microscopically identified as synaptosomes (presence of synaptic vesicles) and free mitochondria were approximately 60 and 10%, respectively, and cytometric analysis showed that 80% of the particles were positive for the anti syntaxin antibody in both groups of animals (data not shown). These results exclude any major interference of free mitochondria or other unwanted contaminants in later comparison of the activity of synaptosomal mitochondria between control and hypothyroid animals. In addition to this, the activity of citrate synthase was used as an index of purity in free mitochondrial fractions, and no differences were found between control and hypothyroid animals (data not shown). Oxidative phosphorylation studies show that in synaptosomal mitochondria, as previously shown in free mitochondria (14), ATP accumulates linearly in the experimental conditions used (Fig. 1A), and more importantly, no differences were found in the rate of ATP synthesis between control and hypothyroid mitochondria in the presence of glutamate/malate or succinate (complex I and complex II substrates) (Fig. 1B). The lack of differences in the presence of complex I substrates is clearly in contrast with the marked decrease (48%) in the rate of ATP synthesis previously observed by our group in the free mitochondria from the cerebral cortex of hypothyroid animals (14). This lack of difference suggests that we should not find any difference in the activity of complex I between synaptosomal mitochondria from control and hypothyroid neonates. On the contrary, and in

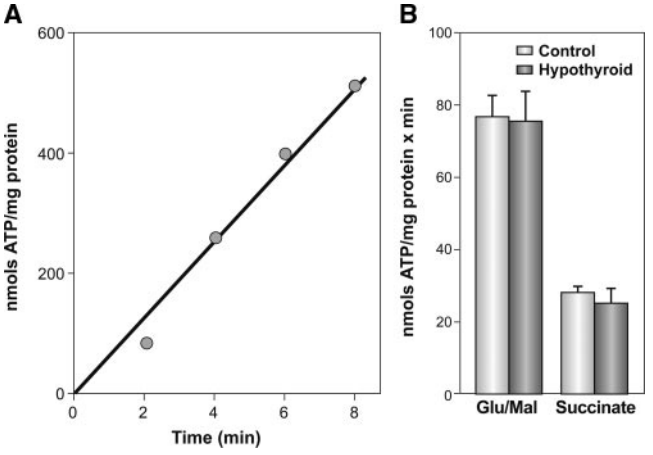

FIG. 1. Hypothyroidism does not alter oxidative phosphorylation in synaptosomal mitochondria. Synaptosomal mitochondria were obtained from the cerebral cortex of N15 (15 d old) control and hypothyroid rats and the oxidative phosphorylation capacity analyzed as indicated in *Materials and Methods*. A, A representative experiment of linear accumulation of ATP in a sample of control mitochondria in the presence of glutamate/malate (Glu/Mal) as substrates. B, Phosphorylation rate in the presence of different substrates. The values are the average of at least five different samples, and the *bars* represent the SEs.

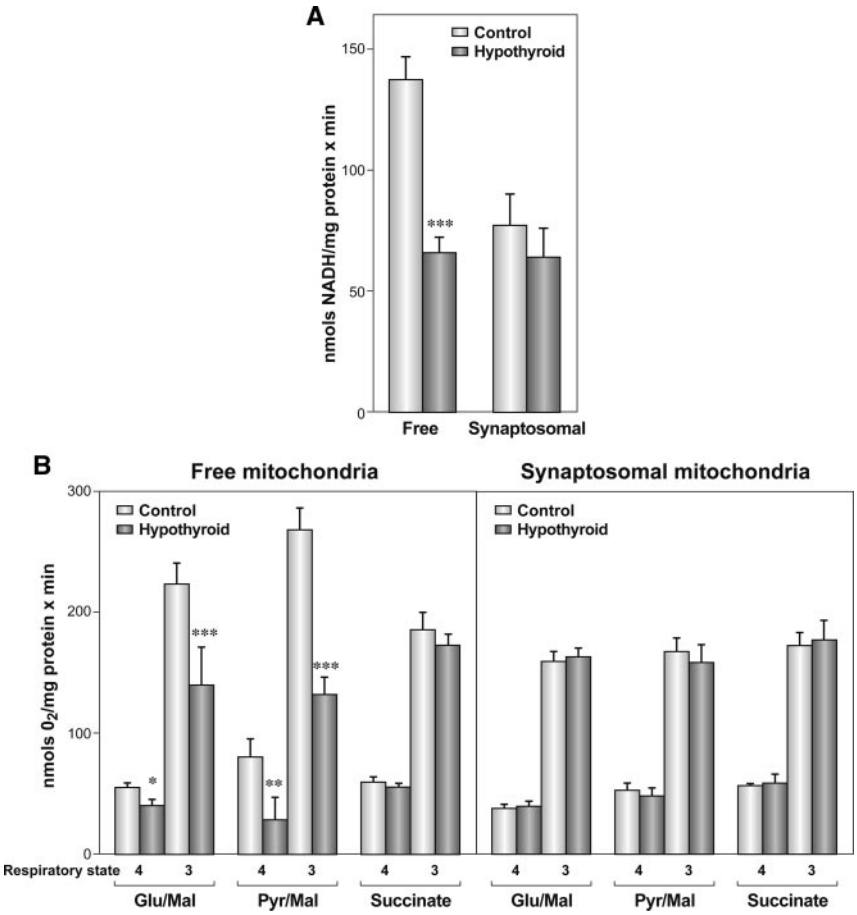

FIG. 2. Differential effect of hypothyroidism on complex I activity and oxygen consumption in free and synaptosomal mitochondria. Free and synaptosomal mitochondria were obtained as described from the cerebral cortex of N15 control and hypothyroid rats, and the activity of complex I and oxygen consumption were analyzed as indicated in *Materials and Methods*. A, Rate of complex I activity (NADH oxidized). B, Oxygen consumption in the presence of different substrates. The values are the average of at least five different samples, and the *bars* represent the SEs. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ *vs.* the corresponding control. Glu/Mal, Glutamate/malate.

accordance with previous results (14), we should find a clear difference in free mitochondria. Then, to clarify this point, we compared complex I activity in isolated free and synaptosomal mitochondria from the cerebral cortex of control and hypothyroid animals. As shown in Fig. 2A, no differences were observed in complex I activity in the synaptosomal fraction between control and hypothyroid animals. In contrast, a marked decrease (50–60%) was observed in the free mitochondria fraction from the same group of hypothyroid animals (Fig. 2A).

Congenital hypothyroidism decreases respiration only in isolated free mitochondria

These results suggest that a decreased capacity in oxygen consumption should be found only in the free mitochondrial fraction of hypothyroid animals and only in the presence of complex I but not in the presence of complex II substrates. Then we next tested this hypothesis and, as shown in Fig. 2B, a decrease in oxygen consumption was observed only in the free mitochondrial fraction of the cerebral cortex of hypothyroid animals in the presence of complex I substrates, glutamate/malate (36%), and pyruvate/malate (50%), whereas no differences were detected in the presence of the complex II substrate succinate. Regarding synaptosomal mitochondria, we did not find any significant difference with any of the substrates analyzed (Fig. 2B).

Congenital hypothyroidism alters mitochondrial gene expression only in free mitochondria

As suggested in a previous work from our laboratory, alteration of mitochondrial gene expression could be the leading cause of mitochondrial dysfunction observed in the hypothyroid animals (14). Consequently, alterations in mitochondrial gene expression in the cerebral cortex should be specific of free mitochondria. We tested this hypothesis by analyzing the content of ND4 and 12S transcripts in both fractions of mitochondria from control and hypothyroid neonates. As can be observed in Fig. 3 a decrease in 12S rRNA and ND4 mRNA (~60% in both cases) was observed in the free mitochondria of hypothyroid animals and no differences were detected in the synaptosomal fraction.

Congenital hypothyroidism reduces, in intact neonatal rats, the metabolic flow through TCA cycle in the neuronal population of the cerebral cortex

The lack of effect of hypothyroidism specifically on the biogenesis and activity in synaptic mitochondria could be interpreted in different ways. It could be possible that only the biogenesis in glial, but no neuronal mitochondria is under the control of thyroid hormone. However, we think that this explanation is improbable because the glial mitochondrial volume is too small (28) to account for the described effect of hypothyroidism (14), and consequently, the neuronal mitochondria is most likely implicated. Therefore, to better address the question of cell types affected, we analyzed the respiratory activity in the neuronal population of the cerebral cortex *in vivo*. To do so, we measured the incorporation of ^{13}C from (1-^{13}C)-glucose into glutamate C4, glutamine C4, and GABA C2 in the presence of TFA, a potent glial TCA cycle inhibitor (18). Figure 4A shows representative high-resolution ^{13}C NMR spectra obtained from perchloric acid extracts of brains from control, hypothyroid and T_3-treated hypothyroid rats. A comparison of all the spectra indicates that the ^{13}C from the (1-^{13}C) glucose has been selectively incorporated into specific cerebral metabolites, like glutamate, glutamine, GABA, alanine, and aspartate. In contrast, myoinositol carbons were not noticeably *de novo* labeled because *myo*-inositol had a low turnover and did not become enriched after 60 min (1-^{13}C) glucose infusion. Figure 4B summarizes the amounts of ^{13}C incorporated in the ^{13}C NMR observable carbons of glutamate, glutamine, and GABA in the three groups of animals studied. Congenital hypothyroidism reduced 60–70% ^{13}C incorporation from (1-^{13}C)

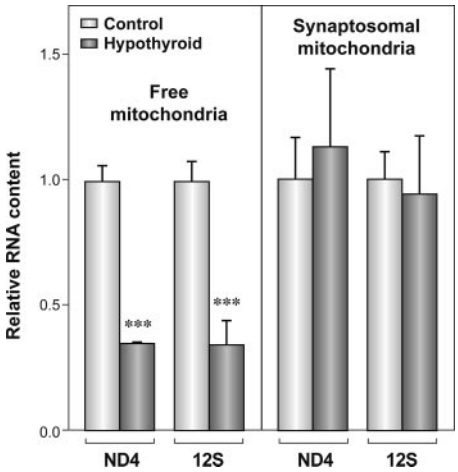

FIG. 3. Differential effect of hypothyroidism on mitochondrial gene expression in free and synaptosomal mitochondria. Total mitochondrial nucleic acids were isolated from free and synaptosomal mitochondria from the cerebral cortex of control and hypothyroid N15 rats and the relative amount of ND4 and 12S rRNA determined. Values are normalized to the corresponding controls and represent the average of determinations in at least five different samples. *Bars* represent the SE. ***, $P < 0.001$.

glucose in the carbons investigated: glutamate C4, glutamate C4, and GABA C2, reflecting a decrease in the *de novo* synthesis of these amino acids from (1-^{13}C) glucose. After the T_3 treatment, the incorporation of ^{13}C returned to values close to those obtained in the control animals in virtually all carbons investigated, indicating that thyroid hormone clearly improved neuronal TCA cycle flow. Figure 4C shows the total cerebral amounts of these metabolites under the different experimental conditions used. T_3-treated animals present slight increases in the total cerebral glutamate, glutamine, alanine, and aspartate concentrations. GABA was not significantly affected by T_3 treatment. Values of combined fractional ^{13}C enrichment are depicted in Fig. 4D, showing a very similar behavior to that shown in the ^{13}C incorporation results. Changes in the fractional ^{13}C enrichment are represented because they reflect the *de novo* synthesis of amino acids, whereas increased total amount of ^{13}C in amino acids might also be due to reduced amino acid turnover. No differences were observed in the plasma levels of glucose neither before nor after glucose infusion (3.2 ± 0.9 and 3.8 ± 0.7 mM before and 30 ± 3.6 and 36.6 ± 2.4 mM after glucose infusion in control and hypothyroid animals, respectively), therefore indicating that the (1-^{13}C) glucose was not differently diluted by endogenous glucose to explain the decrease in apparent flow in TCA cycle.

These results clearly show that the activity of neuronal mitochondrial is under the control of thyroid hormone. Similar absolute values and differences among the three experimental groups were observed when TCA cycle flow was measured in the absence of TFA (glial plus neuronal TCA cycle flow) (data not shown).

Discussion

In this work we have shown that the biogenesis and respiratory capacity in free mitochondria are decreased in the cerebral cortex of hypothyroid neonates, whereas no differences were observed

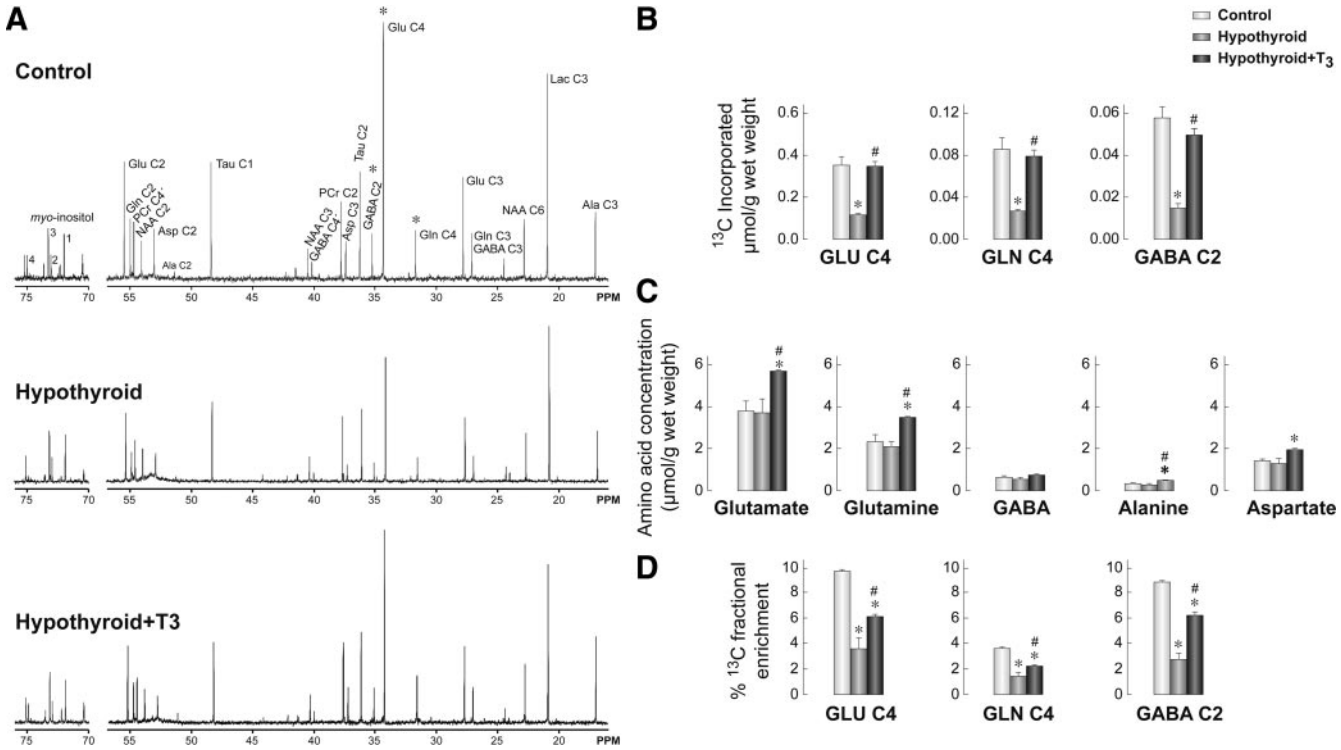

FIG. 4. Congenital hypothyroidism decreases metabolic flow through neuronal TCA cycle. A, Expansions of representative proton-decoupled ^{13}C NMR spectra of extracts from the cerebral cortex of control, hypothyroid and T_3-treated hypothyroid rats infused with (1-^{13}C) glucose. Only the 16–57- and 71–76-ppm regions are shown. Ac, Acetate; Ala, alanine; Asp, aspartate; Glu, glutamate; Gln, glutamine; NAA, *N*-acetylaspartic acid; PCr, phosphocreatine; tau, taurine; PPM, parts per million. *Asterisks* indicate peaks that were significantly different among groups. B, ^{13}C-label incorporation in some individual carbon positions after infusion of (1-^{13}C) glucose. C, Cortical tissues content of diverse amino acids in the same extracts used for ^{13}C NMR. D, Combined fractional ^{13}C enrichment of glutamate, glutamine, and GABA, determined as indicated. Values are the average of at least the determination in five different samples, and the *bars* represent the SE. *, $P < 0.05$ *vs.* control; #, $P < 0.05$ *vs.* hypothyroid.

in the behavior of synaptosomal mitochondria between control and hypothyroid animals. In parallel we observed that the metabolic flow through neuronal TCA cycle is markedly reduced, being all these changes almost completely reversed by the administration of T_3.

The metabolic flow through the TCA cycle is tightly coupled to the activity of the mitochondrial respiratory chain, and therefore, the rate of oxygen consumption can be inferred from the rate of TCA cycle using known stoichiometry (17). Our results showing a 60–70% decreased ^{13}C incorporation from (1-^{13}C) glucose suggest that a 60–70% decrease in oxygen consumption takes place in the neuronal population of the cerebral cortex of hypothyroid neonatal rats. These results shed light on the apparently contradictory results previously published concerning the effect of thyroid hormone on brain oxygen consumption in neonatal rats. Initial reports showed that the administration of thyroid hormone to developing animals increases oxygen consumption in brain slices (8, 10). However, later data failed to confirm these results in isolated mitochondria and brain slices (9, 11). The results obtained in the present study represent a significant advance over the above-mentioned studies because they have been performed *in vivo*.

Previous reports implicated thyroid hormone in mitochondrial biogenesis in the developing brain (12–14, 29, 30). Specifically, we have shown that hypothyroidism decreases mitochondrial gene expression (12), alters the morphology of mitochondria and decreases mitochondrial membrane potential (13) and oxidative phosphorylation capacity (14) in the brain of neonatal rats. We have also demonstrated that these effects depend on the area of the brain analyzed, being the cerebral cortex a very sensitive area (14). These effects of thyroid hormone on mitochondrial biogenesis could obviously underlay, or at least play an important role, in the observed decrease in *in vivo* respiration shown in this work. In addition to this, other factors could also contribute to the observed decrease in oxygen consumption. In this sense it is interesting to point out that the synaptic density is decreased in the cerebral cortex of hypothyroid rats (4), and consequently, a reduction in synaptic activity and the subsequence reduction in energy demands could be one of those other factors.

In the present study, in addition to assessing the metabolic flow through the TCA cycle, usually considered a measurement of respiratory activity, in neurons of the cerebral cortex of neonatal rats in different thyroidal states *in vivo*, we have analyzed in detail the biogenesis and respiratory activity in the synaptosomal and free mitochondrial fractions. Our results clearly show that hypothyroidism decreases gene expression, complex I activity, oxidative phosphorylation, and respiratory capacity only in free mitochondria, being the synaptosomal fraction unaffected by the thyroidal state. In agreement with previous data (14), the biogenesis of respiratory complex I seems to be critical for the action of thyroid hormone and only a decrease in oxidative phosphorylation and respiration was observed in free mitochondrial in the presence of complex I substrates, such as glutamate/malate and pyruvate/malate, but not in the presence of succinate, suggesting that no other respiratory complex was critically reduced. In this sense is interesting to note that a decrease in complex I activity is the most frequent dysfunction of the respiratory chain observed in mitochondrial pathologies (31, 32).

The administration of T_3 to hypothyroid animals almost abrogated the decrease in *in vivo* respiration and the other mitochondrial deficits observed in these animals (14). Interestingly, although the dose of T_3 used was higher than the physiological one, all mitochondrial parameters analyzed returned to control values 72 h after T_3 administration, as previously shown for oxidative phosphorylation, complex I activity, and mitochondrial gene expression (14). This situation is different from the one observed in other tissues in which mitochondrial function is under thyroid control, such as liver, in which administration of pharmacological levels of T_3 increases oxygen consumption well above control values (11). This is probably the consequence of the high receptor occupancy found in the brain of control animals (33).

The differential regulation by thyroid hormone of mitochondrial biogenesis in synaptosomal and free mitochondria shown in this paper further supports the already well-established differences between these two mitochondrial fractions in TCA cycle enzymes, respiratory activity, and lipid composition (34–37). Free mitochondria are the sum of glial plus neuronal (those in dendrites and body) mitochondria; therefore, the observed differences in this fraction between control and hypothyroid neonates could be due to cell type differences, glia *vs.* neurons. We do not think that our findings necessarily reflect a difference between neuronal and glial cells. We discount this possibility on the grounds that the glial mitochondrial volume is too small (28) to account for the extensive differences observed. Instead we suggest that the differential regulation noted here is a consequence of the mitochondrial heterogeneity that can be found within the neuronal population (38, 39). However, the precise mechanism underlying this differential response to thyroid hormone between free and synaptosomal mitochondrial remains to be ascertained. In this regard it is interesting that in cultured dorsal root ganglia neurons, Miller and Sheetz (40) have shown that high potential mitochondria are preferentially transported toward the growth cone. Taking into account our previous observation that the average transmembrane potential is reduced in free mitochondria of the brain of N15 hypothyroid rats and that the mitochondrial population is heterogeneous with both low- and high-potential mitochondria (13), it is tempting to speculate that the mechanism proposed by Miller and Sheetz (40), if operative in the cerebral cortex of the rat, could prevent or reduce the location of mitochondria with lower potential, and probably lower complex I activity, in presynaptic terminals. In addition to this, several transcription factors have also been described to be present in the mitochondrial matrix (41), which could mediate the regulation of mitochondrial gene expression by the local production of different mediators (42).

In summary, our results clearly show that in hypothyroid neonates the respiratory activity is decreased in the neuronal population of the cerebral cortex. This effect is accompanied by a decrease in mitochondrial biogenesis specifically in free mitochondria, as shown by reduced mitochondrial gene expression, complex I activity, and respiration and oxidative phosphorylation in hypothyroid animals. We hypothesize that the effect of thyroid hormones on free mitochondrial biogenesis could underlay their effects on res-

piration. Finally, the results presented in this paper are particularly important in the light of growing evidence showing an implication of mitochondrial alteration in diverse neurological pathologies, particularly in neurodegenerative diseases (43).

Acknowledgments

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