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Neuronal Mitochondrial Morphology and Transmembrane Potential Are Severely Altered by Hypothyroidism during Rat Brain Development*

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ABSTRACT

We recently demonstrated that thyroid hormone is an important regulator of mitochondrial gene expression during brain development. To gain further insights into the consequences of this regulation, we have performed functional and structural analysis of brain mitochondria from control and hypothyroid neonatal rats. Flow cytometric analysis showed a significant decrease in the mitochondrial transmembrane potential in hypothyroid animals compared with controls, which was reversed after 48 h, but not after 2 h, of thyroid hormone administration, suggesting that the functional alterations observed are the consequence of changes in mitochondrial gene expression. In addition, band shift studies showed a protein bound to the rat mitochondrial promoter differentially regulated by thyroid state. Electron microscopic analysis of cerebral cortex, striatum, and hippocampus revealed marked differences in the morphology of neuronal mitochondria from control and hypothyroid neonates. Hypothyroid mitochondria presented a decrease in the area of the inner membrane plus cristae in all areas studied, except for the hippocampal CA1 neurons and nonneuronal cell types. The observations reported here provide a basis for the known biochemical action of thyroid hormone on brain development. (*Endocrinology* **138:** 3771–3778, 1997)

THYROID HORMONE (T_3) is an important effector of development, cell growth, and metabolism of vertebrate organisms (1). Most of these effects are mediated by specific nuclear receptors that belong to a superfamily of nuclear receptors that includes receptors for steroids, vitamin D_3, retinoic acid, and peroxisome proliferators (2). These receptors act as transcription factors regulating gene transcription in response to the binding of their respective ligands (2). The hormonal regulation of gene expression is exerted by the receptors upon binding to hormone response elements, thus activating or repressing the transcription of specific target genes (3). Vertebrates produce different isoforms of thyroid hormone receptors from two related genes, α and β, which are differentially expressed in a tissue-specific manner (4, 5).

One of the most important effects of thyroid hormone concerns neonatal brain development. The association of congenital hypothyroidism with abnormal development and mental retardation has been extensively documented (6). In humans, thyroid hormone deficiency has been associated with irreversible mental retardation and profound neurological deficits, including deafness and movement disorders (7). Also, experimental hypothyroidism in the rat results in impaired brain development with a diminished interneuronal connectivity, decreased myelination, defective cell migration, and alterations in levels of neurotransmitters (6). There is a critical period during which appropriate thyroid hormone levels are essential for normal brain development. In humans, this period was considered to begin late in gestation and to extend through 1–2 yr of age. In rats, this critical period was thought to occur during the first 15 days after birth. This is an important period for neuronal differentiation, maturation, and development of neuronal processes. We have observed that during this time, both the number of T_3 receptors and the amount of receptor messenger RNA rise together with the level of T_3 in brain (5, 8). More recently, however, the idea has arisen that thyroid hormone is required earlier in development (9, 10). Understanding the mechanisms behind thyroid hormone effects on brain development has been a challenge for many years, and despite all the descriptions of functional and morphological defects in the developing brain in hypothyroidism, only recently have several reports shown specific genes that are targets of T_3 action in this tissue (10–14).

Thyroid hormone has been reported to play an important role in the regulation of mitochondrial function in several tissues, such as liver, kidney, and skeletal muscle. T_3 stimulates mitochondrial metabolism, and T_3 deficiency results in decreased respiratory activities (15, 16). In general, thyroid hormone causes an increase in the overall capacity of mito-

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chondrial electron transport chain proteins. This regulation of mitochondrial function takes place in the adult animal as well as during development (16–20). In accordance with its role in growth and development, thyroid hormone also plays an important role in hepatic mitochondrial maturation (18, 19). At least in part, thyroid hormone can affect mitochondrial respiratory function by controlling the expression of nuclear-encoded respiratory genes as well as mitochondrially encoded transcripts (20–22). However, each tissue responds in a characteristic fashion, and not all thyroid hormone effects are observed in all responsive tissues. Brain mitochondria have been generally considered to be nonresponsive to thyroidal state, as no changes in O_2 consumption in either adult or neonatal rats have been found (23). Nevertheless, some biochemical analyses have suggested that neonatal hypothyroidism causes an alteration of brain mitochondria (24, 25). Along this line, we have recently reported a significant decrease in mitochondrial gene expression during brain development as well as an induction after thyroid hormone treatment (10).

In view of these data, the present study was designed to determine whether alterations in brain mitochondrial gene expression in hypothyroid neonatal animals could result in alterations in mitochondrial structure and function and, therefore contribute to the well known dramatic consequences of thyroid hormone deficit during brain development. To this end, we performed a flow cytometric analysis together with electron microscopic studies of ultrathin sections of the cerebral cortex, striatum, and hippocampus of 15-day-old control and hypothyroid rats. Our results show a drop in mitochondrial transmembrane potential ($\Delta\Psi_m$) in hypothyroid brain, which is corrected after a long treatment with thyroid hormone. This is associated with alterations in the number of cristae membranes and large intracristal spaces in the neurons of all of the areas studied besides the pyramidal neurons of the CA1 field of the hippocampus and the nonneural population. In addition, to better define the role that T_3 plays in regulating mitochondrial transcription, we have analyzed the DNA-binding capacities of brain mitochondrial extracts from control, hypothyroid, and T_3-injected 15-day-old animals throughout an approximately 900-bp region containing well characterized control elements within the displacement loop region of the mitochondrial DNA (mtDNA). We have observed a T_3-dependent DNA-binding activity with a mtDNA sequence corresponding to a conserved region called conserved sequence block II (CSB-II).

Materials and Methods

Materials

Methyl mercaptoimidazole, T_3, rhodamine-123 (Rh-123), glutamate, malate, succinate, and ADP were obtained from Sigma Chemical Co. (St. Louis, MO). Radiolabeled [α-^{32}P]deoxy-CTP, [γ-^{32}P]ATP, and DNA labeling systems were purchased from Amersham Corp. (Aylesbury, UK) and Pharmacia LKB Biotechnology (Piscataway, NJ), respectively. All other chemicals were reagent or molecular biology grade.

Animal treatment

All animal experiments were conducted in accordance with the Spanish Guidelines for the Care and Use of Laboratory animals. Female Wistar rats were mated, and the day of appearance of the vaginal plug was considered day 0 of fetal age. To induce fetal and neonatal hypothyroidism, dams were given 0.02% methyl mercaptoimidazole in the drinking water on day 9 of gestation. To assure very low levels of T_3 in the brain, 5 days after birth the pups were injected with ^{131}I (150 μCi/100 g BW) to destroy all thyroidal tissue. Methyl mercaptoimidazole treatment was continued throughout the period of lactation. With this treatment, T_3 values were below the level of detectability. For T_3 treatment, hypothyroid animals were daily injected with 200 μg T_3/100 g BW and killed 48 h (flow cytometry analysis) or 72 h. (electron microscopy studies) later. After being weighed, the animals from all groups were killed by decapitation on postnatal day 15, and the brain was quickly removed and used for mitochondria isolation or electron microscopy studies.

Isolation of mitochondria

Purified brain mitochondria were prepared as described previously (26). The tissue was minced, washed, and homogenized manually in 0.32 M sucrose, 1 mM EDTA, 10 mM Tris-HCl (pH 7.4), and 0.1% BSA. The homogenate was centrifuged at 2,000 × *g* for 3 min. The precipitate was discarded, and the supernatant was centrifuged again at 12,500 × *g* for another 10 min to yield the crude mitochondrial pellet. This mitochondrial pellet was resuspended in 0.03 M sucrose, 0.12 M manitol, 0.025 mM EDTA, 10 mM Tris-HCl (pH 7.4), and 3% (wt/vol) Ficoll. This suspension (3 ml) was layered onto 7 ml of a discontinuous gradient of Ficoll (7.5–13%) and centrifuged at 99,000 × *g* for 30 min. The pellet was washed twice, centrifuged at 12,500 × *g*, and resuspended in homogenization medium.

Preparation of mitochondrial extracts

Mitochondrial extracts were prepared from brains of 15-day-old control, hypothyroid, and T_3-injected animals. Mitochondrial pellets, isolated as described above, were resuspended in lysis buffer [20 mM Tris HCl (pH 8.0), 0.2 mM EDTA, 10% glycerol, 0.35 M NaCl, 1% Triton X-100, and 1 mM dithiothreitol] containing 1 mM phenylmethylsulfonylfluoride. The mitochondrial suspension was vigorously vortexed and allowed to stand on ice for 15 min. The mitochondrial lysate was then spun at 45,000 rpm for 1 h. Protein was determined in the supernatant, and fractions were frozen and stored at −70 C.

Mobility shift assays

Three DNA probes were obtained by amplifying three different sequences of the rat mitochondrial promoter spanning nucleotides 15,401–15,750, 15,751–16,012, and 16,065–16,298 and labeled using Klenow DNA polymerase and [^{32}P]deoxy-CTP. Two double stranded oligonucleotides corresponding to two conserved sequence blocks in the mitochondrial promoter (CSB-II and CSB-III) and one oligonucleotide from nucleotides 16,167–16,192 were also obtained and labeled using [γ-^{32}P]ATP and T_4 polynucleotide kinase. The gel shift mixture (20 μl) contained 10 mM Tris-HCl (pH 8.5), 10 mM $MgCl_2$, 10 mM KCl, 1 mM dithiothreitol, 100 μg BSA, 2 μg poly(dI-dC), 10 μg mitochondrial protein, and 10^5 dpm of the indicated ^{32}P-labeled DNA probes or oligonucleotides. This mixture was incubated at room temperature for 30 min before electrophoresis on 4% nondenaturing polyacrylamide gels in 89 mM Tris, 89 mM borate, and 2 mM EDTA. Gels were run at 200 V for 2–3 h, dried, and exposed for autoradiography. The experiments were performed three times using different animals.

Flow cytometry analysis

Isolated mitochondria were analyzed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA), using CELLQUEST as the data acquiring and analysis software. Except in kinetic experiments, in which time was used as the limit of acquisition, 10,000 events were acquired by sample. The mitochondrial fractions were first treated with propidium iodide (5 μg/ml) to check the membrane integrity. All results presented here represent the propidium iodide-negative mitochondrial population. Rh-123 (5 nM) was added at the moment of running the mitochondria in the flow cytometer, and events were counted for 3 min. To study the effects of substrates on mitochondrial membrane potential,

mitochondria were incubated with Rh-123 for 10 min at room temperature. After this, we began time acquisition for 5 min, adding the substrates glutamate (2.5 mM) plus malate (2.5 mM), succinate (5 mM), and ADP (180 μM) every 90 sec. The mean fluorescence for each substrate was obtained by gating by time. Values obtained for each experimental group in the absence of substrates are called basal values. All experiments were carried out three times using different sets of animals.

Electron microscopy

Ultrastructural morphology of mitochondria was determined by transmission electron microscopy. The brains were cut in the coronal plane at 1-mm thickness, and sections of cerebral cortex, striatum, and hippocampus were rapidly fixed with 4% paraformaldehyde-4% glutaraldehyde in 0.1 M phosphate buffer. After fixation, the sections were osmicated in 1% osmium tetroxide for 2 h at 4 C and then dehydrated in an alcohol-acetone series. Tissues were embedded in araldite resin, and semithin (2.5 μm) sections were removed for optical microscopy. Ultrathin sections (50 nm) were mounted on copper mesh grids and stained with uranyl acetate and lead citrate before examination with a JEOL JEM 1010 electron microscope (JEOL, Peabody, MA). All tissue samples were first inspected on 2.5-μm sections by light microscopy so that the correct area could be determined. The most internal area of the cerebral cortex and the dentate gyrus and CA1 field of the hippocampus were selected. The studies were performed on three different groups of 15-day-old neonates [euthyroid, hypothyroid, and T_3 injected (200 μg/100 g BW)], each composed of three different animals. Three grids were used for each animal. Four different morphological groups of mitochondria were defined (see Fig. 3). They were further divided into two groups according to the degree of alteration observed: normal (N) and altered (A). After examining them we assigned each mitochondrium to the closest morphological group. This was performed without knowing the source of the tissue examined. Values presented in the histograms of Figs. 4, 5, and 6 were obtained after examining the morphology of about 200 mitochondria for each area of the brain (cerebral cortex, striatum, and hippocampus) and for each animal.

Results

Analysis by flow cytometry of mitochondrial modifications

We used the lipophilic dye Rh-123 to analyze mitochondrial function in the brains of 15-day-old neonatal rats during congenital hypothyroidism. Rh-123 is a cationic fluorochrome that has been reported to accumulate specifically in the mitochondria through a mechanism that depends on the mitochondrial membrane potential (27, 28). As shown in Fig. 1A, lower uptake of Rh-123 was observed in brain mitochondria from hypothyroid neonates. When average Rh-123-related fluorescence was calculated, a significant decrease was detected in the hypothyroid group, 61% of the values obtained in age-matched euthyroid animals (Fig. 1B). To evaluate whether the loss in the fluorescence associated with the uptake of Rh-123 would be reversible, hypothyroid neonates were injected 48 h before death with a dose of T_3 that saturates the nuclear receptor. As shown in Fig. 1, the drop in mitochondrial membrane potential was totally reversed by T_3 administration to hypothyroid animals, and mitochondria in this experimental group displayed a pattern of Rh-123 labeling similar to that observed for control animals.

As expected, the addition of mitochondrial substrates, succinate (5 mM) and glutamate plus malate (2.5 mM each), increased the uptake of Rh-123, and rotenone, an inhibitor of the respiratory chain, markedly diminished this uptake (Fig. 2A). When control and hypothyroid neonates were compared, we again found a significant decrease in Rh-123 fluorescence in the hypothyroid animals compared with that in

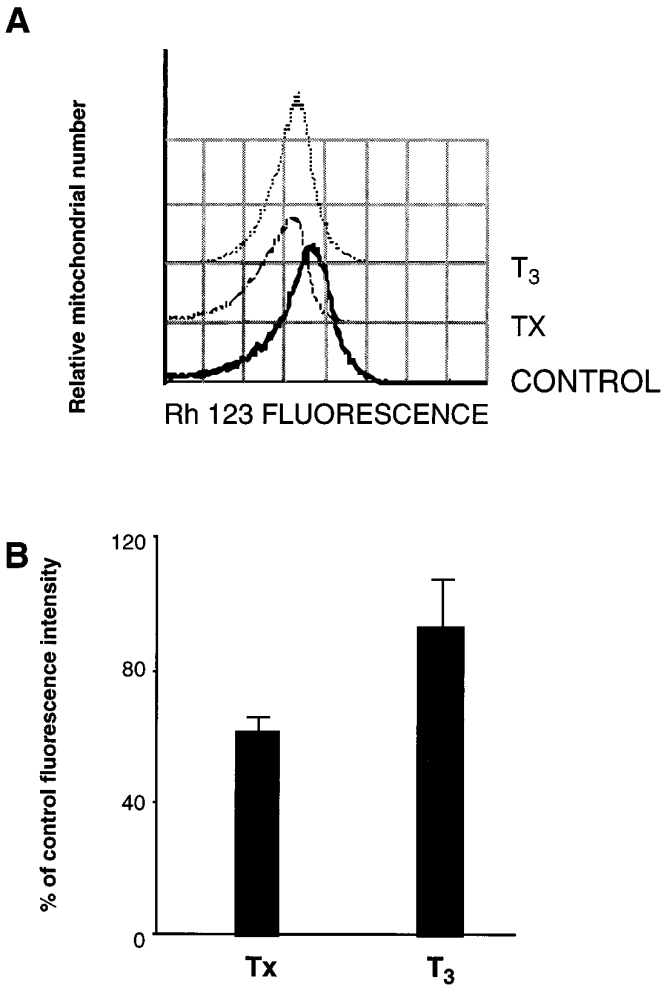

FIG. 1. Effect of thyroid hormone on basal Rh-123 uptake by isolated brain mitochondria. The fluorescence intensity of Rh-123-stained brain mitochondria from 15-day-old neonates was measured by flow cytometry. C, Control; Tx, hypothyroid; T_3, Tx injected with T_3. A, A representative experiment is shown. B, Quantitation of Rh-123 fluorescence. Values are the mean ± SD percentage from three different experiments. The value of mitochondrial fluorescence in control animals was considered 100% in each experiment.

the control group. The values presented in Fig. 2B show 117% of the basal signal in control animals, in contrast to 78% in hypothyroid neonates (~17% of increment over basal values in both cases). When hypothyroid animals were injected with a saturating dose of T_3 and killed 48 h later, not only was basal Rh-123 uptake normalized, but an increased response to the addition of substrates was observed (42% over the basal values obtained for the hyperthyroid group), suggesting a potentiation of mitochondrial activity in the hyperthyroid animals, as a saturating dose of T_3 was used. In contrast with the observed effect after 48 h of T_3 administration, no effect was noted at shorter (2 h) times, and only a partial recovery was observed 18 h later (data not shown). Taken together, these results suggest a deficiency of mitochondrial respiratory capacity in hypothyroid neonates that is corrected after thyroid hormone administration.

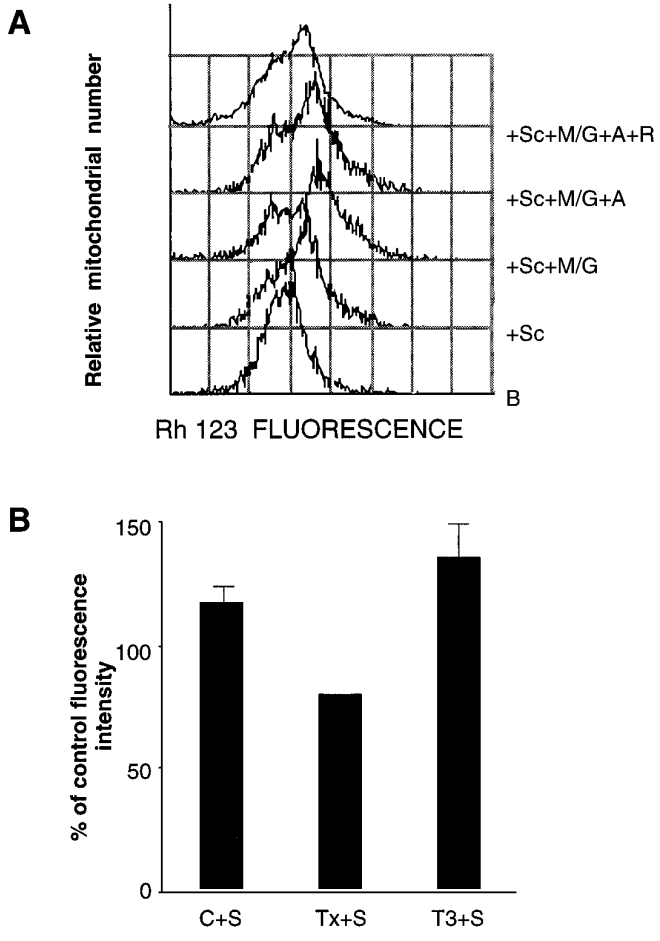

FIG. 2. Effects of different substrates on Rh-123 uptake by isolated brain mitochondria. The fluorescence intensity of Rh-123-stained brain mitochondria from 15-day-old neonates was measured by flow cytometry. C, Control; Tx, hypothyroid; T_3, hypothyroid injected with T_3. A, A representative experiment corresponding to a T_3-injected animal is shown (B, Basal; Sc, succinate; M/G, malate plus glutamate; A, ADP; R, rotenone). B, Quantitation of Rh-123 fluorescence in the presence of succinate, malate/glutamate, and ADP (S). Values are the mean ± SD percentage from three different experiments. The basal value of mitochondrial fluorescence of control animals was considered 100% in each experiment.

Electron microscopic analysis

To assess whether the decrease in mitochondrial membrane potential observed in hypothyroid animals could be related to an altered morphology of these organelles, we next performed a study of mitochondrial structure by electron microscopy in diverse areas of the brains of 15-day-old control, hypothyroid, and T_3-injected neonates. We observed that neuronal mitochondria of the cerebral cortex, striatum, and hippocampus were quite variable in size and shape; some of the mitochondria were enlarged, whereas others were globular organelles and distributed apparently at random throughout the cytoplasm. However, despite this variability in the morphology of mitochondria, these can be classified into two clearly distinguishable types (Fig. 3). Some of them, which we call normal, have the same basic architecture of typical neuronal mitochondria, with a highly folded internal membrane circumscribing a mitochondrial

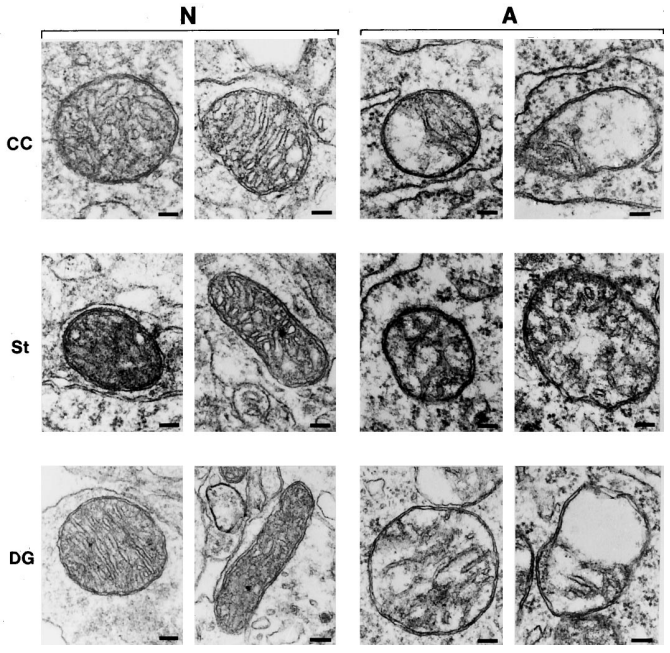

FIG. 3. Representative electron micrographs illustrating two different typical kinds of mitochondria. N, Normal morphology; A, altered morphology; CC, cerebral cortex; St, striatum; DG, dentate gyrus. *Bar scale* = 100 nm. Magnification, ×80,000.

compartment filled with a dense matrix. On the other hand, we also observed some mitochondria with a morphology that we call altered, with a marked decrease in the area of the inner membrane, a reduction in the number of cristae, a destructurization of the matrix compartment, and clear vacuolization.

These two classes of mitochondria were visible in the three brain areas studied (Figs. 4-6A). Quantitation data of the different types of mitochondria are shown in Figs. 4–6B. The majority of neuronal mitochondria from the cerebral cortex of control animals presented a normal morphology, with a highly packed inner membrane and a regular disposition of the cristae; only a minor fraction (12%) belonged to the altered type. However, a great proportion of hypothyroid mitochondria harbor dramatic alterations in the mitochondrial structures, and in hypothyroid neonates, a significant increase in the number of altered mitochondria was observed compared with those found in age-matched euthyroid animals (57% and 12%, respectively; Fig. 4B). The changes observed consist mainly of a marked reduction on the surface of the inner membrane and vacuolization of the matrix compartment. Different stages of membrane disintegration, from minor disarrangements of the inner membrane to empty vesicular structures, can be seen. Similar results were obtained with the neurons of the striatum (Fig. 5) and the granular neurons of the dentate gyrus (Fig. 6). In contrast, we did not observe any difference in the ratio of normal to altered mitochondria between control and hypothyroid rats in the pyramidal neurons of the hippocampal field CA1 (data not shown). In these neurons, about a 50% of the mitochondria were normal, and the other 50% belonged to the altered type in both control and hypothyroid neonates. On the

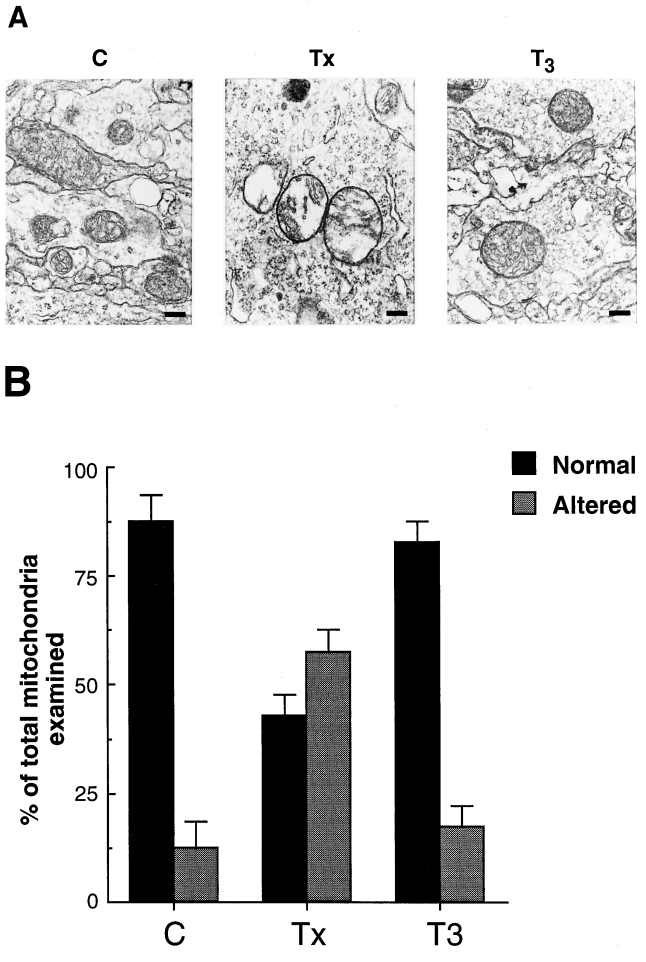

FIG. 4. Effect of thyroid hormone on neuronal mitochondrial morphology from cerebral cortex. C, Control; Tx, hypothyroid; T_3, hypothyroid injected with T_3. A, Representative electron micrographs. *Bar scale* = 80 nm. Magnification, ×30,000. B, Quantitation (percentage) of normal and altered mitochondria. Values are the mean ± SD from three different 15-day-old neonates.

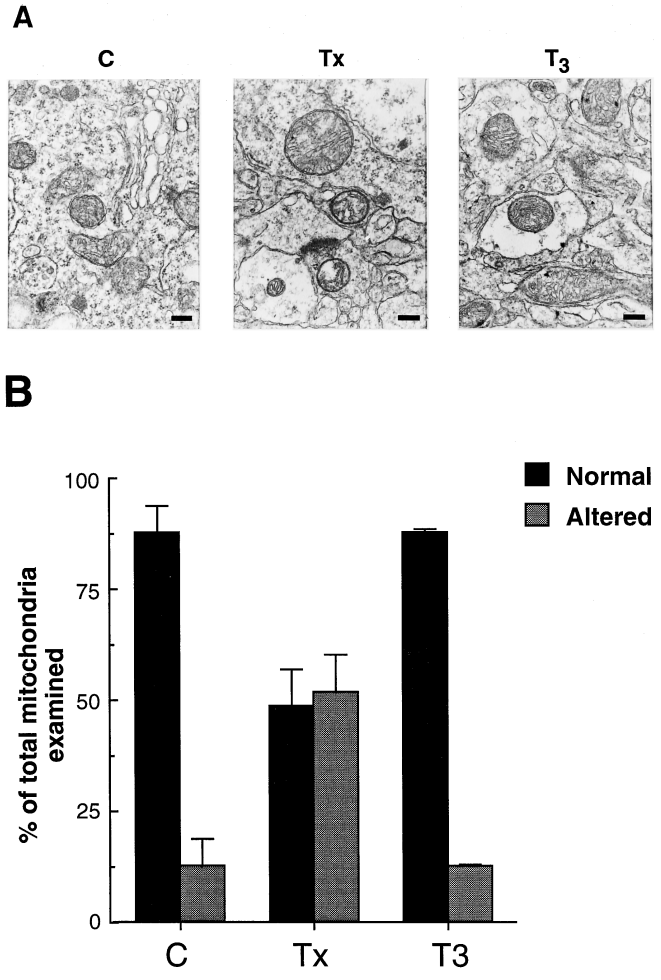

FIG. 5. Effect of thyroid hormone on neuronal mitochondrial morphology from striatum. C, Control; Tx, hypothyroid: T_3, hypothyroid injected with T_3. A, Representative electron micrographs. *Bar scale* = 80 nm. Magnification, ×30,000. B, Quantitation (percentage) of normal and altered mitochondria. Values are the mean ± SD from three different 15-day-old neonates.

other hand, nonneuronal cells, such as astrocytes and oligodendrocites, did not present a high proportion of altered mitochondria in the hypothyroid animals, and the ratio of normal to altered was the same in both control and hypothyroid groups, with a great percentage (80–90%) of mitochondria with no morphological alterations (data not shown). The fact that hypothyroidism did not affect the CA1 neurons and the nonneuronal cells suggests that the effect of thyroid hormone is specific and differential. Also, the aberrant mitochondrial structures were not preparation or fixation artifacts, as the integrity of other subcellular organelles (not shown in the pictures) affirms good subcellular preservation. Thyroid hormone administration to hypothyroid neonates resulted in a normalization of mitochondrial morphology. Quantitation of the number of altered mitochondria, compared with normal (Figs. 4–6B), resulted in a similar percentage of both populations between control and hyperthyroid animals. No effect of T_3 injection was observed in the neurons of the CA1 hippocampal field or in nonneuronal cells (data not shown).

These morphological alterations are in accordance with the flow cytometry data, showing a decrease in membrane potential, which is a reflection of a reduction in the respiratory activity of the mitochondria.

Gel retardation analysis of the mtDNA promoter region

Mammalian mitochondrial DNA is bidirectionally transcribed from a heavy strand and a light strand promoter. These promoters are both localized to the mayor regulatory region for mtDNA transcription and replication in the only extensive noncoding area, called the displacement loop (D-loop) (29). To gain further insight into how thyroid hormone could regulate mitochondrial gene expression, we next performed electrophoretic mobility shift assays to determine whether thyroid status could influence the binding of some mitochondrial protein to this regulatory region of the mtDNA. As indicated in *Materials and Methods*, three different DNA fragments, covering the region of the D-loop from nucleotides 15,401–16,298, were obtained. Brain mitochondrial extracts were prepared from euthyroid, hypothyroid, and hypothyroid T_3-injected animals and tested for DNA-

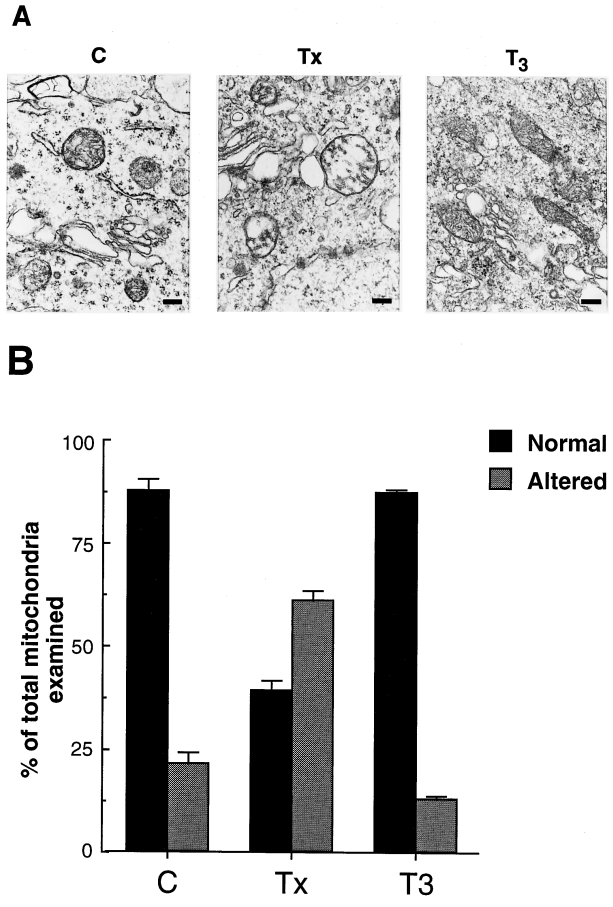

FIG. 6. Effect of thyroid hormone on neuronal mitochondrial morphology from dentate gyrus. C, Control; Tx, hypothyroid: T_3, hypothyroid injected with T_3. A, Representative electron micrographs. *Bar scale* = 80 nm. Magnification, ×30,000. B, Quantitation (percentage) of normal and altered mitochondria. Values are the mean ± SD from three different 15-day-old neonates.

binding activity. As shown in Fig. 7A, binding activity was highest in the extracts from control rat brain and was lowest in the hypothyroid extract when DNA fragment 16,065/16,298 was used. These differences were consistently observed. The binding activity was recovered 24 h after thyroid hormone administration to hypothyroid neonates. On the contrary, no differences were detected when the other mtDNA regulatory sequences, DNA fragments 15,401/15,750 and 15,751/16,012, were tested (data not shown). We next investigated more precisely the DNA sequence responsible for this DNA-binding activity. To this end, we designed three different oligonucleotides corresponding to two conserved sequence blocks in the D-loop region (CSB-II and CSB-III) as well as an oligonucleotide from nucleotides 16,167–16,192, all of them within the region that caused a retardation dependent on thyroid state. As shown in Fig. 7B, we observed that the T_3-dependent protein-DNA binding is localized to the CSB-II region. The other two oligonucleotides tested did not present any T_3-dependent DNA-binding activity. A 200-fold molar excess of unlabeled oligonucleotide abolished the formation of the shifted band, indicating specificity of binding. Unrelated oligonucleotides (CSB-III and oligonucleotide from nucleotides 16,167–16,192) did not prevent formation of the retarded band (data not shown).

Discussion

The present report clearly shows that brain mitochondria are targets of thyroid hormone action during development, as alterations in mitochondrial structure and function are observed in the brains of hypothyroid animals and are corrected after T_3 administration. These results are particularly important considering that until very recently (10) it was generally assumed that brain mitochondria were not responsive to thyroidal state, and because morphological alterations were detected in neuronal cells, but not in the glial population, suggesting that neurons are particularly sensitive to the lack of T_3, with the important consequences that this should have in brain development.

The general organization of the mitochondrial inner membrane cristae into the typical transverse alignment in control animals was largely absent in the mitochondria isolated from hypothyroid brain. These mitochondria were characterized by highly fractured and degenerated cristae and a clear vacuolation. The drop in mitochondrial membrane potential, observed by flow cytometry in total brain mitochondria of hypothyroid animals, is probably a reflection of the decreased surface area of the inner membrane observed in neuronal cells in the electron microscopic study because in this membrane all enzymes of the respiratory chain are present.

As we noted above, until recently it has been generally considered that brain mitochondria were insensible to the action of T_3. However, there were some reports indicating that mitochondrial alterations could be occurring in the brains of hypothyroid animals. An alteration in phospholipid composition and membrane fluidity of rat brain mitochondria has been reported (30) as well as some alterations of oxidative phosphorylation (31). We recently found that differences in thyroidal state during development result in profound alterations of mitochondrial gene expression (10). All of these data together with the results of the present study clearly indicate that mitochondrial function can be severely impaired in the hypothyroid brain, and that T_3 can counteract this event. These findings provide a biochemical basis for the known alterations of brain function as a consequence of the lack of thyroid hormone during brain development. This is particularly important in view of the growing evidence that primary defects in mitochondrial function are implicated in over 100 diseases, and that the clinical manifestations most often involve the central nervous system (Kearns-Sayre syndrome, MERFF, MELAS, *etc.*). In addition, mitochondrial dysfunction have now been found to occur in aging and in age-related degenerative diseases affecting brain function, such as Parkinson's and Alzheimer's diseases (32).

Alterations in mitochondrial morphology caused by hypothyroidism have been described in other tissues, such as liver and skeletal muscle (33, 34), and the modifications are essentially the same as those described here for the developing brain. In the liver, Jakovcic *et al.* (35) also reported hypothyroid mitochondria to be shorter and wider than those in controls, and some swelling after T_3 administration

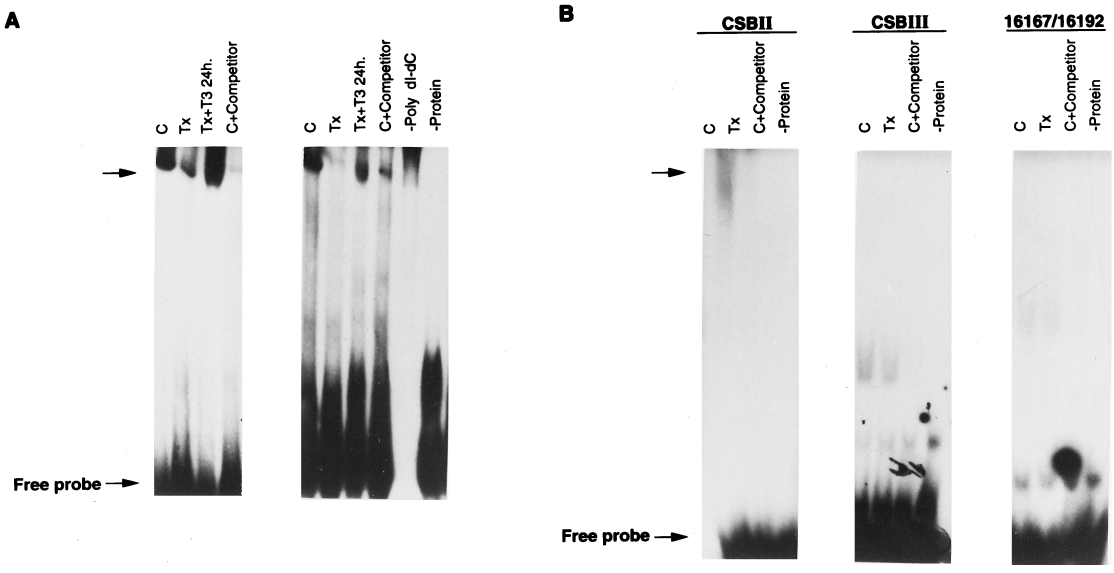

FIG. 7. DNA-binding activity of the mitochondrial protein extracts. Binding and electrophoresis conditions are described in *Materials and Methods*. A, Binding to DNA fragment 16,065/16,298. The *left and right panels* correspond to two different animals. B, Binding to oligonucleotides CSB-II, CSB-III, and 16,167/16,192.

has also been observed (33). In skeletal muscle, the administration of T_3 to hypothyroid animals results in an increase in the number of cristae, accompanied by some hypertrophy (33). The results of the flow cytometric analysis and electron microscopic studies shown here also revealed small differences in size between the brain mitochondria of control and hypothyroid neonates. Although the alterations found in mitochondrial morphology in the hypothyroid animals are essentially the same between liver and brain, our data, showing a drop in brain mitochondrial transmembrane potential in these animals, are different from the situation in the liver. There are several reports showing a lowering of the mitochondrial membrane potential after T_3 administration to euthyroid rats (36, 37). It has been proposed that the decrease in $\Delta\Psi_m$ in hyperthyroid liver mitochondria is the result of a nonspecific thyroid hormone-induced increase in proton conductance of the mitochondrial inner membrane (proton leak). Thus, there is a clear difference in the response of brain and liver mitochondria to thyroid hormone status, suggesting a tissue-specific effect of thyroid hormone on brain mitochondria.

An interesting point is the observation of a clear differential responsiveness to T_3 in the mitochondria of the different neurons. Whereas granular neurons mitochondria of the dentate gyrus respond to T_3 in the same fashion as those of the cerebral cortex or striatum, mitochondria of the pyramidal neurons from the CA1 field of the hippocampus are not sensitive to thyroidal state. These results together with recent reports showing a differential effect of thyroid hormone on brain gene expression depending on the area studied and the age of the animal (14, 38) point to a very complex effect of hypothyroidism on brain development, where different kinds of neurons respond to this hormone in very different fashions. The variability in the response to thyroid hormone suggests a modulation of T_3 action on gene expression for other cellular factors differentially expressed in different brain cell types.

The next step toward a better understanding of the role of T_3 in mitochondrial function must include studies of the molecular mechanisms underlying T_3 action. There are some reports showing very rapid effects of thyroid hormone on hepatic mitochondrial activity *in vivo* (39), which have been suggested to be mediated by a direct effect of the T_3 metabolites 3,3′-diiodothyronine and 3,5′-diiodothyronine on the mitochondria (40). This is in contrast to the effect of thyroid hormone that we have observed on brain mitochondrial activity, as this one requires a much longer period to be observed, suggesting that it is related to changes in mitochondrial gene expression (10). Although the presence of thyroid hormone receptors has been recently described in rat liver mitochondria (41, 42), a better explanation of the T_3 effects on these organelles could be the existence of a T_3-regulated mediator between T_3 nuclear receptors and the mitochondria, as the effects of thyroid hormone on the expression of nuclear and mitochondrially encoded genes are coordinate (10, 43). Good candidates for mediators are the nuclear respiratory factor-I protein (44), which is known to regulate the mitochondrial transcription factor A (mtTFA) (45), the mtTFA itself, or another protein necessary for mitochondrial transcription and not yet characterized.

In an attempt to know the possible existence of one of these mediators, in this work we have analyzed the protein-DNA binding activity on the regulatory region of the mtDNA and its response to thyroidal status. The mammalian mitochondrial DNA possesses a region called the D-loop, which is the main noncoding region and the most variable part of vertebrate mitochondrial genomes. This region contains the origin of heavy strand DNA replication and the promoters for heavy and light strand transcription (29). Within this region there are three conserved sequence blocks, called CSB-I, CSB-II, and CSB-III, that are thought to be involved in mtDNA replication and transcription. Our findings show that rat brain mitochondrial extracts contain a protein(s) that binds to a specific

sequence (CSB-II) in the mitochondrial regulatory region. The binding activity of the protein(s) was influenced by the thyroid status of the animal, suggesting that T_3 regulates mitochondrial gene expression through the control of proteins that bind to regulatory sequences responsible for controlling mitochondrial transcription. However, additional studies will be necessary to identify these proteins and establish their function and whether they could act as a link between the nuclear receptor for T_3 and its action in the mitochondria.

In summary, this work provides evidence that the changes in mitochondrial gene expression during development in the hypothyroid neonatal brain, previously reported by our laboratory (10), result in an impaired mitochondrial function and in the appearance of a population of neuronal mitochondria with severely altered structure. This work thus shows for the first time that the lack of thyroid hormone in the developing brain can severely alter mitochondrial structure and function and, in consequence, provides a cellular basis for the observed dependency on T_3 of the developing brain. Finally, our findings supply initial information about the mechanism by which thyroid hormone can influence brain mitochondrial gene expression.

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