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Splicing of juvenile and adult tau mRNA variants is regulated by thyroid hormone

(tau expression/RNase protection assay/brain development/hypothyroidism)

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ABSTRACT **The effect of thyroid hormone on the expression of tau transcripts was studied during postnatal brain development. The level of tau mRNA was only slightly changed postnatally in the cerebral hemispheres of hypothyroid rats, whereas the level of tau mRNA in the cerebellum was maintained at a higher level than in the euthyroid controls. As shown by *in situ* hybridization studies, such an alteration in tau mRNA expression can be ascribed to an effect of thyroid hormone on the rate of migration of the granule cells in the cerebellum; that tau mRNAs remain high in the cerebellum as long as the granule cells are migrating correlates with the observation that hypothyroidism slows the rate of migration of granule cells. RNase protection assays also showed that thyroid hormone deficiency delays the transition between the immature and mature tau transcripts in both brain regions. Thus, one of the effects of thyroid hormone is to regulate the splicing mechanism that allows replacement of the juvenile tau variants by the adult entities during neuronal differentiation.**

Thyroid hormone is essential for normal brain development, and dramatic abnormalities, including mental retardation, are associated with its deficiency (for review, see ref. 1). The most notable abnormalities are marked impairment of nerve process development and poor connectivity among neurons. In the cerebellum, for instance, the development of both dendrites and axons is impaired. Outgrowth of the dendritic tree of Purkinje cells is severely retarded during the first 3 postnatal weeks (2), whereas the axons of the granule cells, the parallel fibers, remain shorter during the same developmental period (3). Such a situation seems to be transient because normal cerebellar anatomy is partially restored at later stages. However, the cerebellar networks remain functionally defective in adulthood if proper thyroid hormone administration to hypothyroid animals is not performed soon after birth. This suggests that thyroid hormone is essential during a short developmental period to establish proper connectivity and to ensure in due time a normal program of neuronal development.

Because of the reduced nerve process outgrowth associated with hypothyroidism during the developmental stages both in the cerebral hemispheres and the cerebellum, this laboratory examined (4, 5) the polymerization of microtubules in the brain of rats deprived of thyroid hormone and found that the *in vitro* assembly of microtubules from hypothyroid rats was reduced and could be corrected by adding the microtubule-associated tau protein. Immature and mature variants of tau proteins have been identified (6, 7). In adults, four or five tau isoforms are present (8), whereas at immature stages two or three isoforms of lower apparent molecular weight are expressed (6). All of these juvenile and adult variants are produced from a single tau gene (9, 10) by a developmentally regulated alternative splicing mechanism (10). The sequence of these tau isoforms is highly homologous. The C-terminal domain of the juvenile and adult variants contains three (11) and four (12, 13) homologous repeats, respectively, that have been shown to represent the tubulin binding sites (11, 14). Exons present in the N-terminal region of the primary tau transcript are also differentially spliced during development (10, 14).

Both the mature (15) and the juvenile (16) variants of this protein are predominant components of axonal microtubules. The inhibition of axonal polarity by tau antisense oligonucleotides in primary cerebellar neurons (17) strongly supports previous findings (15, 16) suggesting that tau proteins are preferentially targeted to the axons and are required for axonal outgrowth. We show in this report that thyroid hormone deficiency alters the expression of tau mRNAs and delays the transition between the immature and mature tau transcripts. This latter observation suggests that thyroid hormone regulates, directly or indirectly, the timing of the splicing mechanism that during neuronal differentiation selects the exons present in the tau gene.

MATERIALS AND METHODS

Congenital hypothyroidism was induced by force-feeding pregnant rats with propylthiouracil (50 mg/day), a drug that blocks thyroid function, from 16 days of gestation until they were killed at postnatal day 3, 7, 14, or 21. The pups received the drug through the blood of the mother until birth and through the milk until weaning at postnatal day 21. As reported (18), the offsprings of propylthiouracil-treated rats are severely deficient in thyroid hormone.

RNA Extraction and Hybridization Analysis. Euthyroid and hypothyroid rats were killed at various postnatal ages. The cerebral hemispheres and the cerebellum were dissected and quickly frozen in liquid nitrogen. Total RNA was extracted following the procedure of Chirgwin *et al.* (19). Northern blot analysis was carried out as described (20). A 1.6-kilobase tau cDNA, derived from the cDNA cloned from a 6-day-old mouse by Lee *et al.* (11), was labeled by nick-translation with [α-^{32}P]dCTP to a specific activity of 2×10^8 cpm/μg. This probe hybridizes with all the immature and mature tau mRNA variants (9).

Identical amounts of total RNA were loaded on the gels as measured by spectroscopy at 260 nm and by minigel analysis followed by densitometry. Relative amounts of tau mRNAs were measured by densitometric analysis of the radioautograms of the Northern blots. The reproductibility of these

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Abbreviations: EGL, external granular layer; ML, molecular layer; IGL, internal granular layer.

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measurements were controlled by quantitative dot-blot analysis and several experiments were performed for each developmental stage.

***In Situ* Hybridization.** *Probe preparation.* A tau cDNA insert (11) was subcloned into the *Pst* I and *Sac* I sites of the vector pGEM-4Z, using T4 DNA ligase and standard cloning procedures (21). Tau antisense and sense RNAs were generated by *in vitro* transcription as described (22) and labeled by ^{35}S-labeled UTP incorporation (Amersham; specific activity, 1250 Ci/mmol; 1 Ci = 37 GBq).

Hybridization conditions. Brains from euthyroid and hypothyroid rats were removed at postnatal day 3, 7, 14, or 21 and immediately fixed in 4% (wt/vol) paraformaldehyde. Sections (10 μm) were cut with a cryostat/microtome and collected on glass slides. Cryostat sections were fixed for 5 min in phosphate-buffered 4% paraformaldehyde and stored at 4°C in 70% ethanol until utilization. Just before hybridization, sections were rinsed with phosphate-buffered saline and then with 2× standard saline/citrate (SSC) and dehydrated in a graded series of ethanol solutions. They were then air-dried until used for hybridization. Hybridization was performed for 3 hr at 50°C in 50% (vol/vol) deionized formamide/10 mM dithiothreitol/10% (wt/vol) dextran sulfate/2× SSC/yeast tRNA (0.6 mg/ml)/sonicated salmon sperm DNA (1 mg/ml)/ ^{35}S-labeled tau antisense RNA (10^6 cpm/μl). In control sections, ^{35}S-labeled tau sense RNA of similar specific activity was used and did not hybridize significantly (data not shown). After hybridization, the slides were washed at 52°C for 15 min in 2× SSC/50% deionized formamide followed by a 15-min wash in 2× SSC at the same temperature. Thereafter, ribonuclease digestion was performed by incubating the slides for 30 min at 37°C with RNase A (100 μg/ml). Next, the slides were washed at 52°C for 10 min in 2× SSC/50% deionized formamide and dehydrated in a graded series of ethanol solutions. Finally, the slides were dipped in an aqueous solution of 50% (vol/vol) NTB3 photographic emulsion at 42°C and exposed at 4°C for 6 days. After development with D19 developer and fixing with Rapid Fix, the slides were washed with water, counterstained with Gomori stain, and examined with a Zeiss microscope (standard 16).

Ribonuclease Protection Analysis. The probe was prepared as follows. (*i*) A cDNA fragment was synthesized from total brain RNA isolated from a 21-day-old rat and amplified by the PCR with *Taq* DNA polymerase (Perkin–Elmer/Cetus) and the oligonucleotide primers 5′-TGTCTTGGCTTTGGCATT and 5′-ATAATTAATAAGAAGCTG. These primers allowed the PCR to cover the sequence between positions 801 and 1130 of the adult tau variants (i.e., 330 bases). Only part of this sequence, 243 bases, is present in the immature tau variants since they lack repeat 2 (see Fig. 3). (*ii*) The amplified cDNA fragment was cloned into pGEM-4Z and linearized with *Eco*RI or *Xba* I for the antisense and sense probe, respectively. (*iii*) Antisense RNA was transcribed with T7 RNA polymerase in the presence of [α-^{32}P]UTP; sense RNA was prepared under the same conditions with SP6 polymerase.

RNase protection assays were performed as described by Goedert *et al.* (12) with some modifications. Total RNA (20 μg) was annealed to 10^6 cpm of ^{32}P-labeled probe for 5 min at 85°C in 80% formamide/40 mM Pipes, pH 6.7/0.4 M NaCl/1 mM EDTA. The hybridization was continued for at least 15 hr at 45°C. Single-stranded RNA was digested by a 60-min incubation at 30°C with RNase A (40 μg/ml) and RNase T1 (2 μg/ml). The RNases were then inactivated by treatment with SDS and proteinase K for 15 min at 37°C, and the protected RNA was extracted with phenol/chloroform. After ethanol precipitation, the protected RNA was dissolved in 1× TBE (89 mM Tris/89 mM boric acid/2 mM EDTA) and electrophoresed on a nondenaturing 8% polyacrylamide gel. The gel was dried and exposed at room temperature for 2–8 hr.

RESULTS

Expression of the tau mRNAs in the euthyroid and hypothyroid rat cerebral hemispheres and cerebellum was studied by Northern blot analysis with the 6-kilobase tau cDNA probe that recognizes all the mature and immature isoforms (9). Fig. 1 *A* and *B* shows that tau mRNA was markedly decreased (to

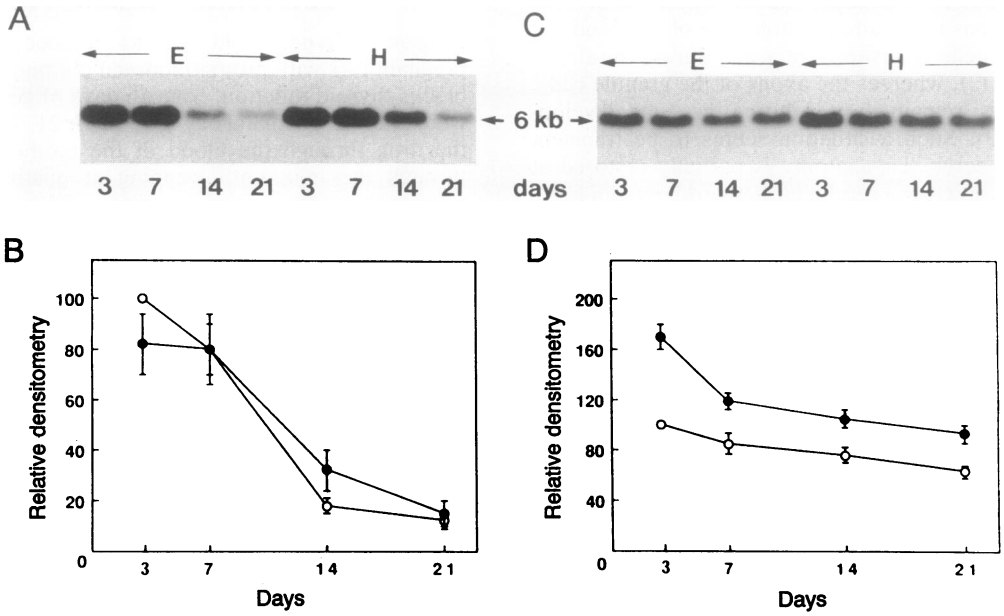

FIG. 1. Expression of tau mRNAs in the euthyroid and hypothyroid rat cerebral hemispheres (*A* and *B*) and cerebellum (*C* and *D*) at different stages of postnatal development. (*A* and *C*) Northern blot analysis of total RNA (20 μg per lane) extracted from euthyroid (lanes E) and hypothyroid (lanes H) rat cerebral hemispheres and cerebellum at postnatal days 3, 7, 14, and 21, as indicated. (*B* and *D*) Time course analysis of concentrations of tau mRNAs by relative densitometric measures of the Northern blots obtained from euthyroid (○) and hypothyroid (●) rats. The values are expressed as percentages of the maximal values of the control at day 3 and are the mean ± SEM of duplicate samples from three preparations.

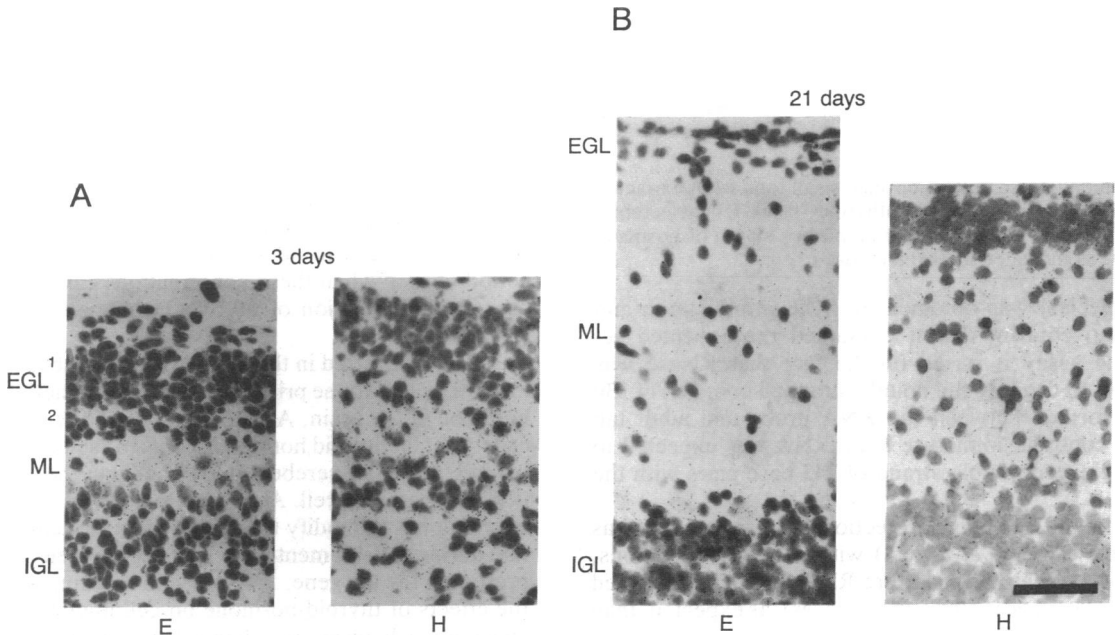

FIG. 2. *In situ* hybridization on cryostat sections of euthyroid (micrographs E) and hypothyroid (micrographs H) rat cerebellar hemispheres in the sagittal plane at postnatal days 3 (*A*) and 21 (*B*). (Bar = 50 μm.)

approximately one-eighth to one-tenth) between postnatal days 3 and 21 in the euthyroid cerebral hemispheres. A similar decrease has been shown to occur for tubulin and to depend on a post-transcriptional mechanism (23). The levels of tau mRNAs in the cerebrum were similar for hypothyroid samples and controls although they were slightly lower at day 3 and higher at days 14 and 21. In contrast, in the cerebellum (Fig. 1 *C* and *D*), significant quantitative changes in the expression of tau mRNAs were induced by thyroid hormone deficiency. The excess of tau mRNAs observed in hypothyroidism varied from 70 to 40% during development.

The differences between the cerebral hemispheres and the cerebellum might be explained by differences in the timing of neuronal differentiation in these two brain regions. In the cerebral hemispheres, all the neurons are present at birth whereas in the cerebellum the granule cells and the various types of interneurons are produced after birth and differentiate during the following 3 postnatal weeks (24). The granule cells and several types of interneurons proliferate postnatally in the upper part of the external granular layer (EGL1), then migrate from the lower part of this layer (EGL2) through the molecular layer (ML), and finally reach the internal granular layer (IGL) where they synapse with the dendrites of the Purkinje cells. Hypothyroidism slows the migration of the granule cells from the EGL1 through the ML (25). To investigate the distribution of tau transcripts in the various cerebellar layers, *in situ* hybridization experiments were performed with the ^{35}S-labeled tau antisense RNA probe; this probe detects all the various immature and mature tau mRNA variants. At day 3 (Fig. 2*A*) the tau RNA probe hybridized with the cells present in the EGL2, ML, and IGL in the control and hypothyroid cerebellum. Similar results were obtained at days 7 and 14 (data not shown). At day 21 (Fig. 2*B*), the EGL has almost disappeared in the controls, and there is almost no labeling in the ML. In contrast, the EGL is still present at this stage in the cerebellum of thyroid-hormone-deficient rats; the cells present in EGL2 as well as those that are still migrating in the ML are labeled. We have shown (26) that tau mRNAs remain at a high level and are untranslated in the granule cells as long as these cells have not reached their postsynaptic target, the Purkinje cells. It is not surprising, therefore, that thyroid hormone deficiency, which slows granule cell migration (25), also maintains higher levels of tau transcripts in the cerebellum. However, the mechanism of action of thyroid hormone on this developmental event remains unknown.

Northern blot and *in situ* hybridization analyses were unable to tell whether the transition between the immature and mature tau forms was regulated by thyroid hormone because the mRNAs encoding these variants were the same size, 6 kilobases (9), and had a highly homologous sequence (10–13). The C-terminal domain of the immature tau variants contains three homologous repeated sequences that represent tubulin binding sites (11). The adult tau variants contain four such repeats and also differ from the juvenile entities in some sequences of their N-terminal region (14). Such a difference in the number of repeats between the immature and mature tau forms allowed us to investigate whether the transition between the tau variants is regulated by thyroid hormone. To answer this question, ribonuclease protection assays were performed. An RNA probe (Fig. 3) containing the nucleotide sequence between positions 801 and 1130 of repeat 2 (which is expressed only in the adult tau variants) and of repeats 3 and 4 (which are common to the juvenile and

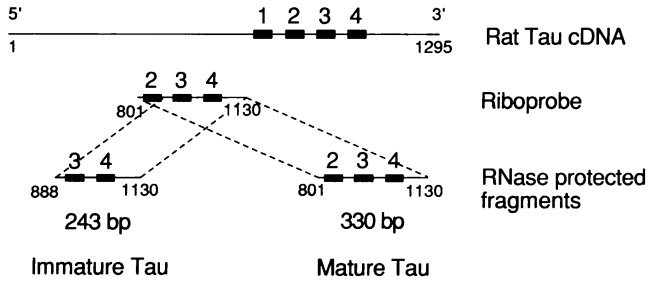

FIG. 3. Schematic representation of the predicted RNase protection fragments for the immature and mature tau variants. The boxes numbered 1 to 4 represent the four repeated homologous sequences present in the C-terminal end corresponding to adult rat tau mRNAs (21), which correspond to the microtubule binding sites. The RNA probe used for ribonuclease protection analysis is shown: it contains the nucleotide sequences present in the repeat 2 (which is only expressed in the adult tau variants) and in the repeats 3 and 4 (which are common to the juvenile and mature tau entities). Structures and sizes of predicted RNase protection fragments for the juvenile and mature tau variants are also schematically indicated.

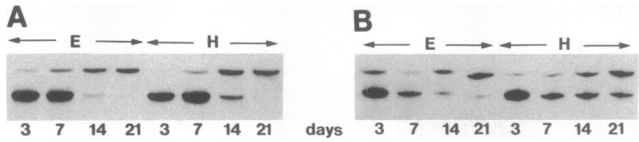

FIG. 4. Ribonuclease protection analysis of total RNA extracted from euthyroid (lanes E) and hypothyroid (lanes H) rat cerebral hemispheres (*A*) and cerebellum (*B*) at various stages of postnatal development, as indicated in postnatal days.

adult tau species) was obtained by PCR amplification and sequenced to confirm that it contained the expected sequence. The assay is based on the fact that RNase was expected not to degrade the double-stranded complex of 330 base pairs formed between the RNA probe and adult tau mRNA. In contrast, immature tau mRNA was expected to form an uncleaved double strand of 243 base pairs with the RNA probe.

The results of the RNase protection assays, performed as described by Goedert *et al.* (12) with some modifications, showed (Fig. 4) that the immature RNA fragment protected from RNase cleavage is smaller (243 bases), as expected, than that produced from the adult tau transcript (330 bases). Control experiments were also performed with a sense RNA probe; as expected, RNase treatment resulted in complete degradation of both the immature and mature mRNA samples. The assays performed with euthyroid cerebral hemispheres (Fig. 4*A*) show the following two results. (*i*) Adult transcripts increase in concentration after postnatal day 7 but, as reported (12), are already present in low amounts at early stages. It is not clear whether this means that both immature and mature tau forms are coexpressed at early stages in the differentiating neurons or whether, more probably, adult tau transcripts are present in a small population of neurons that is already differentiated in the immature brain. (*ii*) High levels of immature transcripts are present at postnatal days 3 and 7 and markedly decrease at days 14 and 21. In the euthyroid cerebellum (Fig. 4*B*), the situation is similar, but the immature transcripts have already decreased at the end of the first postnatal week. The transition between the immature and the mature tau transcripts also occurs in the hypothyroid cerebral hemispheres and cerebellum after the first postnatal week (Fig. 4). However, high levels of immature transcripts are maintained at later stages in both regions. For instance at days 14 and 21, immature tau transcripts are still very high in the hypothyroid cerebellum whereas they are present in trace amounts in the controls at the same stages. The effects in the cerebrum are apparent only at postnatal day 14 [i.e., at a period that has been shown to be critical for thyroid hormone action (18)]. Thus, one of the effects of thyroid hormone on neuronal differentiation is to modify the timing of the transition between the immature and mature tau variants.

DISCUSSION

The observation (9, 10, 12, 13) that the adult tau variants contain one additional repeat compared to the immature forms (i.e., an additional tubulin binding site) is consistent with previous experiments that showed (27) that the microtubule polymerization activity of the immature species is lower than that of the adult. One may assume that the expression of adult tau variants in adulthood allows the polymerization of microtubules that are more stable than those assembled in the axons at earlier developmental stages. Thus, microtubule and, therefore, neurite stabilizations (28) occur later in the hypothyroid brain because the transition between immature and mature tau variants is delayed. In other words, the hypothyroid brain would remain immature for a longer period of time during the critical period of neuronal differentiation (i.e., when cerebral connectivity is established). Although thyroid hormone is required during a short postnatal period, a change in timing of neuronal differentiation might contribute to the defective development of nerve processes seen in hypothyroidism. Such an impairment in neurite outgrowth is transient but, by desynchronizing the program of differentiation, might explain the permanent poor connectivity seen in adulthood. Actually, there is a short period of time when these abnormalities can be reversed by proper administration of thyroid hormone (for review, see ref. 1).

The data reported in this work raise a number of questions that are related to the primary mechanism of action of thyroid hormone in the brain. As shown by the *in situ* hybridization experiments, thyroid hormone seems to regulate the levels of tau mRNA in the cerebellum by changing the rate of migration of the granule cell. Another effect of this hormone on tau expression is to modify the timing of the splicing mechanism that during development allows differential selection of exons present in the tau gene. It remains to be determined whether the effects of thyroid hormone on cell migration and on the splicing mechanism are related. It is also not clear whether the post-transcriptional effect of thyroid hormone on the splicing mechanism is direct or, more probably, mediated by gene expression. It is widely accepted that the cellular actions of thyroid hormone are mediated by nuclear receptors that bind to thyroid hormone response elements associated with target genes and stimulate or inhibit expression of these genes (for reviews, see refs. 29 and 30). One may assume, therefore, that a putative gene responsive to thyroid hormone would regulate the splicing mechanism during a critical period of brain development. Variants of a large number of proteins are produced by alternative splicing (for review, see ref. 31) and yet we do not know how the splicing mechanism is regulated during development. The data reported herein suggest that, at least for the production of various tau protein isoforms during brain development, the splicing mechanism is regulated by thyroid hormone. Even if this effect of thyroid hormone is indirect it may explain the impairment in neurite outgrowth induced by thyroid hormone deficiency in the newborn.

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