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Transthyretin Regulates Thyroid Hormone Levels in the Choroid Plexus, But Not in the Brain Parenchyma: Study in a Transthyretin-Null Mouse Model*

JOANA ALMEIDA PALHA, RUI FERNANDES, GABRIELLA MORREALE DE ESCOBAR, VASSO EPISKOPOU, MAX GOTTESMAN, AND MARIA JOÃO SARAIVA

Unidade de Amilóide, Instituto de Biologia Molecular e Celular (J.A.P., R.F., M.J.S.), 4150–180 Porto, Portugal; Instituto Superior de Ciências da Saúde-Norte (J.A.P.), 4585-116 Gandra, Portugal; Unidad de Endocrinologia Molecular, Instituto de Investigaciones Biomédicas Alberto Sols, Consejo Superior de Investigaciones Científicas and Universidad Autónoma de Madrid (G.M.D.E.), 28029 Madrid, Spain; Medical Research Council Clinical Sciences Center, Mammalian Neurogenesis Laboratory, Hammersmith Hospital (V.E.), W12 ONN London, United Kingdom; Institute of Cancer Research, Columbia University (M.G.), New York, New York 10032; and Instituto de Ciências Biomédicas Abel Salazar (M.J.S.), 4099-003 Porto, Portugal

ABSTRACT

Transthyretin (TTR) is the major T_4-binding protein in rodents. Using a TTR-null mouse model we asked the following questions. 1) Do other T_4 binding moieties replace TTR in the cerebrospinal fluid (CSF)? 2) Are the low whole brain total T_4 levels found in this mouse model associated with hypothyroidism, *e.g.* increased 5′-deiodinase type 2 (D2) activity and RC3-neurogranin messenger RNA levels? 3) Which brain regions account for the decreased total whole brain T_4 levels? 4) Are there changes in T_3 levels in the brain? Our results show the following. 1) No other T_4-binding protein replaces TTR in the CSF of the TTR-null mice. 2) D2 activity is normal in the cortex, cerebellum, and hippocampus, and total brain RC3-neurogranin messenger RNA levels are not altered. 3) T_4 levels measured in the cortex, cerebellum, and hippocampus are normal. However T_4 and T_3 levels in the choroid plexus are only 14% and 48% of the normal values, respectively. 4) T_3 levels are normal in the brain parenchyma. The data presented here suggest that TTR influences thyroid hormone levels in the choroid plexus, but not in the brain. Interference with the blood-choroid-plexus-CSF-TTR-mediated route of T_4 entry into the brain caused by the absence of TTR does not produce measurable features of hypothyroidism. It thus appears that TTR is not required for T_4 entry or for maintenance of the euthyroid state in the mouse brain. (*Endocrinology* **141:** 3267–3272, 2000)

TRANSTHYRETIN (TTR) is a 55-kDa tetrameric protein (1) synthesized mainly by liver and the choroid plexus of the brain, a membrane specialized in the production of cerebrospinal fluid (CSF) (2). TTR expression, an early event in embryonic development, first occurs in the choroid plexus and later in the liver (3, 4). TTR, which represents about 20% of the total protein synthesized by the choroid plexus, is secreted into the CSF (5). As much as 25% of total CSF protein consists of TTR (6).

TTR is the major plasma carrier of thyroid hormones in rodents (7) and is the main thyroid hormone-binding protein in CSF of both rodent and humans (8). The high concentration of TTR in the CSF suggested a major role for TTR in mediating T_4 transfer from the blood into the brain across the blood-choroid-plexus-CSF barrier (9–13).

Thyroid hormones are essential for normal mammalian brain physiology and are particularly critical during development (14–16). Moreover, the adult brain is sensitive to thyroid hormones beyond the time of the so-called critical period of brain development. More than half of the T_3 (the biologically active thyroid hormone) in the cerebral cortex arises from local deiodination of T_4 by the enzymatic action of type 2 iodothyronine 5′-deiodinase (D2) (17, 18). In hypothyroidism, the brain compensates for the low levels of circulating hormones by increasing D2 activity (19). T_4 or T_3 can regulate brain D2 through both pre- and posttranslational mechanisms (20). Among the molecular events known to be controlled by thyroid hormones in the adult brain is the transcription of the RC3 gene, whose product is a brain-specific, calmodulin-binding protein kinase C substrate that has been implicated in postsynaptic events (21). A thyroid hormone-responsive element has been found in the human RC3 gene homolog (22). Previous analysis of thyroid hormone metabolism markers (23) revealed that TTR-null mice are hypothyroxinemic, but euthyroid (24). In addition, kinetic studies using [^{125}I]T_4 showed that TTR is not essential for T_4 uptake by the brain and other tissues (25). However, lower total T_4 levels were detected in the TTR-null whole brain. In the present work we use the TTR-null mouse strain to study further the involvement of TTR in the maintenance of thyroid hormone homeostasis in the brain.

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Address all correspondence and requests for reprints to: Dr. Joana Almeida Palha, Instituto de Biologia Molecular e Celular, Rua do Campo Alegre 823, 4150–180 Porto, Portugal. E-mail: japalha@ibmc.up.pt.

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Materials and Methods

Animals

Animals were handled in accordance with the NIH guidelines and the European Union rules for the care and handling of laboratory animals. Two- to 4-month-old 129 SV wild-type and TTR-null male mice (23) were kept under a 12-h light cycle and fed regular rodent chow and tap water *ad libitum*. Mice were anesthetized with ketamine/xylazine and killed by cardiac puncture, and brain and sera were immediately frozen in dry ice and kept at −70 C for further analysis. CSF was collected from the cisterna magna and pooled from several animals. An aliquot was kept for blood contamination analysis, and the remainder was immediately frozen at −70 C. No CSF samples were contaminated, in accordance with criteria previously described (26).

In vitro choroid plexus labeling

Pools of choroid plexuses from three wild-type or TTR-null mice were incubated in 300 μl leucine-free medium (DMEM, Life Technologies, Inc., Paisley, UK) containing 15 μCi [^{14}C]leucine (Amersham Pharmacia Biotech, Uppsala, Sweden; SA, 319 mCi/mmol). After 3-h incubation in 5% CO_2 at 37 C, cell pellets were resuspended in medium containing 0.1% Tween. Radioactive proteins present in both secretion media and cells were separated on SDS-PAGE gels and analyzed by autoradiography. As a standard we used a preparation containing ^{125}I-radiolabeled TTR and retinol-binding protein.

T_4 binding to CSF proteins

Native gel electrophoresis. Aliquots of 13 μl pooled CSF and 5 μl serum were incubated for 30 min at room temperature with 12 μl [^{125}I]T_4 (DuPont, Wilmington, DE; 5700 μCi/μg) diluted 4-fold in 200 mM glycine and 130 mM sodium acetate, pH 8.6 (7) and subjected to electrophoresis on 8% native polyacrylamide gels, using the same glycine-acetate as gel and running buffer, for 3–5 h at 40 mA at room temperature. The migration of albumin was determined in a serum sample incubated with bromophenol blue run on a different gel lane. Radioactive proteins were visualized by autoradiography 4 h after exposure.

In another experiment, 40 μl CSF were applied on a blue-Sepharose (Amersham Pharmacia Biotech) column (300-μl volume) equilibrated in 30 mM sodium phosphate, pH 7.0. The bound material was eluted in 0.5 M sodium thiocyanate in 30 mM sodium phosphate, pH 7.0. Both the bound- and unbound fractions were concentrated on Microcon 10 (Amicon, Beverly, MA) and then incubated with [^{125}I]T_4 and analyzed by electrophoresis as described above.

Chromatography on Biogel P-6 DG. Seventeen microliters of pooled CSF were incubated for 4 h at 4 C with 10^5 cpm [^{125}I]T_4 (DuPont; SA, 1250 μCi/μg) in TNE buffer (100 mM Tris, 100 mM NaCl, and 1 mM EDTA, pH 8.0; 200-μl final volume). Samples were applied to 1-ml Biogel P-6 DG columns (Bio-Rad Laboratories, Inc., Hercules, CA) (27) in TNE buffer immediately after loading 200 μl TNE/20% sucrose and were centrifuged for 1 min at 1000 rpm. The columns were then washed with 200 μl TNE, and the eluates, containing the protein-bound [^{125}I]T_4 were counted in a γ-counter. Free [^{125}I]T_4 remained bound to the gel, allowing calculation of the percentage of protein-bound [^{125}I]T_4 in CSF. A blank consisted of 10^6 cpm [^{125}I]T_4 in TNE buffer; the eluate of this preparation is considered background radioactivity.

T_4 and T_3 tissue content

Animals were anesthetized and perfused via the vena cava with 5 ml PBS containing 11 U heparin/ml, followed by 2 ml PBS; the portal vein was used as an exit for the perfusate. Cortex, cerebellum, hippocampus, and choroid plexus were dissected and frozen in dry ice. Samples were kept at −70 C until use. T_4 and T_3 contents were determined by RIA after extraction from tissue (28).

Northern blot analysis for RC3

RNA was isolated from brain using the Ultraspec RNA Isolation System (Biotecx Laboratories, Inc., Houston, TX), and 20-μg RNA samples were subjected to electrophoresis and blotted on nylon membranes using standard procedures. Probes were labeled using a random primer labeling kit (Stratagene, La Jolla, CA).

A rat RC3 complementary DNA (cDNA) probe was used to detect RC3 messenger RNA (mRNA) (29). Prehybridization was performed for 5 h at 42 C in 50% formamide, 3 × SSC (standard saline citrate), 5 × Denhardt's solution, 0.1% SDS, and 100 μg/ml salmon sperm DNA, and hybridization was performed under the same conditions for 20 h after the addition of 1×10^6 cpm probe/ml solution. Washes were performed at room temperature in 1 × SSC-0.1% SDS. A signal was observed after 6 h.

The membrane was normalized with cyclophilin message, a ubiquitous mRNA (30), using a rat cyclophilin cDNA probe. Prehybridization was for 5 h at 39 C in 53% formamide, 0.75 M sodium chloride, 25 mM PIPES (pH 6.8), 25 mM EDTA, 5 × Denhardt's solution, 0.1% SDS, and 100 μg/ml salmon sperm DNA, and hybridization was performed under the same conditions for 20 h after the addition of 1×10^6 cpm probe/ml solution. Washes were performed at room temperature in 2 × SSC-0.1% SDS and in 0.2 × SSC-0.2% SDS at 60 C. Dr. J. G. Sutcliffe (The Scripps Research Institute, La Jolla, CA) provided both the RC3 and cyclophilin cDNAs.

D2 activity measurements

Hippocampus and cortex samples, pooled from three animals, and individual cerebellum samples were homogenized in 470, 1000, and 350 μl 10 mM Tris-HCl (pH 7.2) and 0.25 M sucrose, respectively; and kept at −70 C until use. A previously described procedure (31) was used, with minor modifications. The 200-μl final mix consisted of 50 mM potassium phosphate (pH 7.2), 1 mM propylthiouracil, 1 mM EDTA, 10 mM dithiothreitol, 8 nM T_4, 2 μM T_3, 800 μg homogenate protein, and 10^5 cpm [^{125}I]T_4 (SA, 1250 μCi/μg; DuPont) previously purified on LH-20 Sephadex chromatography (32). Each sample, run in duplicate or triplicate, was incubated for 60 or 90 min at 37 C. Two types of blanks were used, some in which incubation was stopped at time zero by adding 100 μl BSA (70 mg/ml) and 500 μl 10% trichloroacetic acid, and others in which the homogenate was boiled before the addition of the labeled hormone and then incubated for 60 or 90 min at 37 C. The reaction was stopped by adding 100 μl BSA (70 mg/ml) and 500 μl 10% trichloroacetic acid. Samples were centrifuged for 5 min at 3500–4500 rpm. Iodine produced in the reaction was separated by Sephadex LH-20 chromatography (32). Radioactivity was measured by γ-spectrometry.

Protein concentrations were determined using a protein assay (Bio-Rad Laboratories, Inc.) with albumin as standard.

Statistics

Group values, expressed as the mean ± SD, were compared by Student's t test. $P < 0.05$ was considered statistically significant.

Results

In vitro choroid plexus synthesis of TTR

The absence of TTR in TTR-null choroid plexus was confirmed by metabolic labeling of choroid plexus explants. Figure 1 indicates that despite an overload of TTR-null secretion medium protein, no TTR was present in the choroid plexus secretion medium of the TTR-null mouse (lane 2 *vs.* lane 1). Identification of the TTR band in the wild-type choroid plexus secretion medium (lane 1) and cells (lane 3) was based on the migration of a TTR standard (lane 4). TTR was absent in choroid plexus cells and CSF of the TTR-null mice (data not shown). RT-PCR experiments further confirmed the absence of TTR transcription in the TTR-null choroid plexus (data not shown).

T_4 binding to CSF proteins

The electrophoretic pattern of T_4-binding proteins in rodent serum is known (33). T_4-binding globulin (TBG) mi-

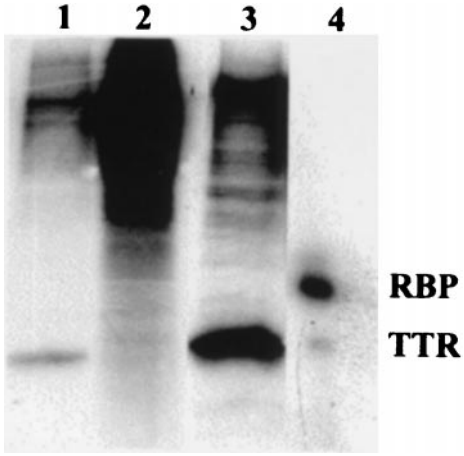

FIG. 1. SDS-PAGE analysis of *in vitro* labeled proteins of 1) wild-type choroid plexus secretion medium, 2) TTR-null choroid plexus secretion medium, and 3) wild-type choroid plexus cells. Lane 4 shows the migration of ^{125}I-radioladeled TTR and retinol-binding protein (RBP) standards. Note that no TTR is secreted from TTR-null choroid plexus.

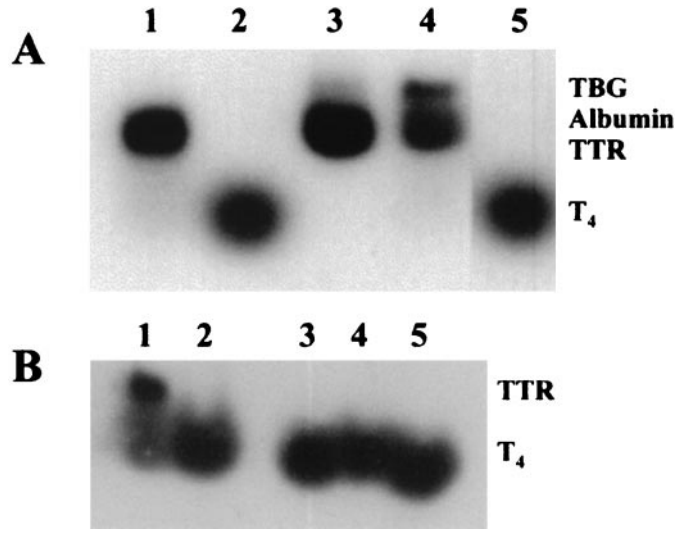

FIG. 2. A, Autoradiogram showing [^{125}I]T_4 migration patterns after incubation with 1) wild-type CSF, 2) TTR-null CSF, 3) wild-type serum, 4) TTR-null serum, and 5) buffer. In the wild-type CSF, [^{125}I]T_4 binding occurs in the region where TTR and albumin migrate (lane 1), whereas in the TTR-null CSF (lane 2), all radioactivity is recovered in the free form (compare with lane 5 where only buffer is present). Migration patterns of [^{125}I]T_4 in wild-type (lane 3) and TTR-null (lane 4) serum are shown for comparison (see *Results*). B, Autoradiogram showing [^{125}I]T_4 migration patterns after incubation with 1) wild-type CSF fraction unbound to blue-Sepharose, 2) wild-type CSF fraction bound to blue-Sepharose, 3) TTR-null CSF fraction unbound to blue-Sepharose, 4) TTR-null CSF fraction bound to blue-Sepharose, and 5) buffer. In the wild-type CSF, [^{125}I]T_4 binding is not seen in the blue-Sepharose-bound fraction (lane 2), indicating the absence of albumin, whereas it occurs to the blue-Sepharose-unbound fractions (lane 1) that contains TTR. The blue-Sepharose-bound (lane 3) and -unbound (lane 4) fractions from the TTR-null CSF do not bind [^{125}I]T_4, as all labels are recovered in the [^{125}I]T_4-free region (lane 5).

grates more slowly than albumin, whereas TTR runs slightly faster than albumin, but cannot be resolved from albumin. As previously described (24), Fig. 2A shows that in the wild-type mouse, TTR and albumin were the major [^{125}I]T_4-binding proteins (lane 3). The TTR-null mouse serum showed less [^{125}I]T_4 binding at the albumin front, which corresponds to TTR mobility, and an additional band in the TBG region of the gel (lane 4). The appearance of the TBG band in the TTR-null serum did not correspond to increased TBG concentration, but, rather, to the shift of the binding toward TBG in the absence of TTR (24). In wild-type CSF, [^{125}I]T_4 labeling occurred in the region where TTR and albumin overlapped and appeared as a single band (lane 1). In TTR-null CSF (lane 2), [^{125}I]T_4 was recovered entirely in a band corresponding to free [^{125}I]T_4 (lane 5).

In an independent experiment, bromophenol blue, a dye that also binds to albumin, was included in the reaction mixture before electrophoresis. The lack of a blue band is in agreement with the absence of albumin in wild-type CSF (data not shown).

Because in the experimental conditions used, TTR and albumin cannot be resolved, we took advantage of the ability of blue-Sepharose to bind albumin to discriminate between TTR and albumin in wild-type CSF. The blue-Sepharose-bound and -unbound fractions of wild-type or TTR-null CSF were incubated with [^{125}I]T_4 and electrophoresed under native conditions. Figure 2B shows that no binding of [^{125}I]T_4 occurred to the blue-Sepharose-bound fraction in wild-type CSF (lane 2), indicating the absence of albumin. On the other hand, [^{125}I]T_4 bound to the blue-Sepharose-unbound wild-type CSF fraction (lane 1) that contained TTR. These results imply that TTR is the only T_4-binding protein in wild-type CSF. The blue-Sepharose-bound (lane 4) and -unbound (lane 3) fractions from the TTR-null CSF did not bind [^{125}I]T_4, as all labels were recovered in the free [^{125}I]T_4 region (lane 5). These observations further suggest the absence of T_4-binding protein in TTR-null CSF.

To confirm the absence of [^{125}I]T_4 binding in TTR-null CSF, we chromatographed CSF after incubation with [^{125}I]T_4 on Biogel P-6 DG. For wild-type mice, about 60% of the total [^{125}I]T_4 were recovered in the protein-bound fraction. In contrast, only approximately 4% of the total [^{125}I]T_4 were recovered in the protein-bound fraction of the TTR-null CSF, a value equivalent to the blank. Thus, both procedures indicated that TTR-null CSF contains no T_4-binding protein.

T_4 and T_3 tissue content

As shown in Table 1, no differences in T_4 and T_3 levels were found between wild-type and the TTR-null mutant cortex, cerebellum, and hippocampus. In TTR-null choroid plexus, T_4 and T_3 levels were significantly reduced, amounting to 14% and 48% of wild-type values, respectively.

RC3-neurogranin message levels in brain

Message levels of RC3 are normally decreased in adult rat brain in response to hypothyroidism (21). The expression of the two transcripts of this gene between the wild-type and TTR-null mice are compared in Fig. 3. No difference between these groups was observed, indicating that the brain was euthyroid with respect to this criterion.

TABLE 1. Thyroid hormone concentrations in brain regions and choroid plexus

	Cortex				Cerebellum			
	T_4 (pg/mg protein)	n	T_3 (pg/mg protein)	n	T_4 (pg/mg protein)	n	T_3 (pg/mg protein)	n
Wild-type	97 ± 34	9	114 ± 36	9	116 ± 30	9	55 ± 19	9
TTR-null	81 ± 24	9	106 ± 24	9	103 ± 17	9	57 ± 13	9

Values are the means ± SD.
[a] $P < 0.01$.

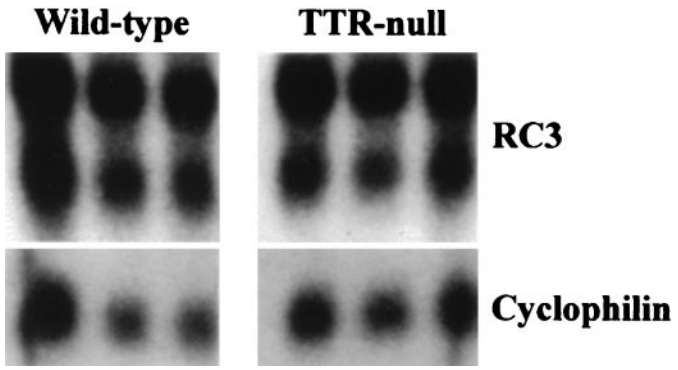

FIG. 3. Northern blot analysis of brain neurogranin message. Cyclophilin message normalizes the membrane RNA content on the same membrane and demonstrates that the contents of both RC3 transcripts are similar in TTR-null and wild-type mouse brains.

D2 activity measurements

D2 activities of brain regions were not statistically different in TTR-null and wild-type mice (see Table 2). Further measurements of deiodinase activity in synaptosomal fractions of both cortex and hippocampus likewise revealed no differences between TTR-null and wild-type mice (data not shown).

TABLE 2. Deiodinase type 2 activity in brain tissue homogenates

	% ^{125}I formed/ mg protein/time	n
Wild-type cortex (60 min)	8.35 ± 2.38	5
TTR-null cortex (60 min)	7.34 ± 1.66	6
Wild-type hippocampus (60 min)	3.93 ± 1.05	8
TTR-null hippocampus (60 min)	4.18 ± 0.57	9
Wild-type cerebellum (90 min)	3.92 ± 0.82	10
TTR-null cerebellum (90 min)	4.71 ± 1.16	9

Values are the means ± SD. No statistically significant differences found.

Discussion

We used a TTR-null mouse strain to study the role of TTR in brain thyroid hormone homeostasis. The data presented here indicate that in the absence of TTR, 1) the brain is euthyroid and 2) the concentration of thyroid hormones is significantly reduced in the choroid plexus, but not in the brain parenchyma.

T_4 binds only to TTR in the CSF of normal mice (8, 11). Using two independent methods, we show that T_4 is found exclusively unbound in TTR-null mice. Thus, no other CSF protein replaces TTR in the transport of T_4.

T_4 has been suggested to reach the brain through transport of the serum free T_4 fraction across the blood-brain barrier (34) as well as by association with TTR through the blood-choroid-plexus-CSF barrier (10, 12, 35). T_4 bound to CSF TTR could be delivered into different brain regions. Thus, after iv injection of [^{125}I]T_4, radioactivity first appeared in the choroid plexus, then in the CSF, and later in the cortex and striatum of the brain (9, 10).

We previously showed that TTR is not absolutely required for T_4 to reach the brain (24). However, we also found that whole brain total T_4 levels were decreased in the TTR-null mice (25). We report here that the T_4 levels in cortex, hippocampus, and cerebellum are normal. The decrease in T_4 levels previously measured in whole brain can be accounted for by the very marked reduction of T_4 levels in the choroid plexus of the TTR-null mice.

The TTR-bound fraction represents the major T_4 pool in the wild-type choroid plexus. Our results indicate that T_4 can enter the choroid plexus and, presumably, pass on into the CSF in the absence of TTR. Thus, T_4 entry into the choroid plexus may derive from the free T_4 pool. In fact, serum free T_4 is normal in the TTR-null sera (24).

Schreiber *et al.* (36) proposed that circulating T_4-binding proteins act as distributors of the hormone within the tissues. They also suggested that, given the redundancy of T_4-binding proteins (namely, T_4-binding globulin, albumin, and TTR), the absence of one protein would be compensated for by the others. This concept of a protein network may apply for serum (24). It cannot, however, apply for the blood-choroid-plexus-CSF barrier, as we found no T_4-binding protein in the CSF of TTR-null mice. Our results indicate that CSF TTR is not required for normal maintenance of thyroid hormone in brain. Whether the distribution of the hormone in the brain is equivalent in the TTR-null and wild-type mice is under investigation.

It has been suggested that one fifth of the T_4 present in the brain may originate from TTR-bound T_4 transported from the choroid plexus to the CSF (11). Our observations do not support the idea that TTR constitutes a major route of T_4 transfer into the brain parenchyma under normal physiological conditions (9, 10). A direct measure of T_4 levels in the CSF of wild-type and TTR-null mice is, unfortunately, not feasible, due to the large amounts of CSF required for the analysis.

To evaluate further the thyroid hormone status of the TTR-null mouse brain, we measured parameters known to be affected in altered thyroid states. Thyroid hormones regulate gene expression in the central nervous system, as in other tissues, through transcriptional activation (15). Therefore, we investigated the levels of steady state messenger RNA for RC3, a gene whose expression is decreased in adult hypothyroidism (21). Consistent with an euthyroid status, the

TABLE 1. *Continued.*

Hippocampus				Choroid plexus			
T_4 (pg/mg protein)	n	T_3 (pg/mg protein)	n	T_4 (pg/mg protein)	n	T_3 (pg/mg protein)	n
110 ± 27	7	40 ± 17	7	571 ± 138	5	23 ± 8	5
96 ± 19	11	51 ± 6	11	83 ± 41[a]	4	11 ± 2[a]	4

levels of RC3 messenger RNA were normal in the TTR-null brain.

The major portion of T_3 in the brain is locally produced by 5′-deiodination of T_4 by D2 (17, 18). To guarantee a sufficient supply of T_3, the activity of D2 increases in hypothyroidism, whereas D2 activity is decreased in hyperthyroidism (37, 38). In the adult brain, D2 mRNA is expressed in the cerebellar cortex, hippocampus, caudate-putamen, thalamus, and cerebellum (39). We reported previously that despite decreased whole brain total T_4 levels in the TTR-null mice, their normal whole brain T_3 levels were not accounted for by increased D2 activity (24). However, as regional differences may have been masked in the analysis of the whole brain, we performed regional D2 analyses. No differences were found in D2 activity in the cortex, cerebellum, and hippocampus of the TTR-null compared with wild-type mice. In addition, T_3 levels in the cortex, cerebellum, and hippocampus were equivalent in wild-type and TTR-null mice. The greatly reduced T_3 levels found in the TTR-null choroid plexus are probably a direct consequence of the low total T_4 levels found in this epithelium. The presence of deiodinases in the choroid plexus has not been reported. Taken together, our data provide strong evidence that the brain is euthyroid in the absence of TTR.

The normal free T_4 serum levels in the TTR-null mice (24) and the data presented here support the free hormone hypothesis for T_4 tissue uptake. In this hypothesis, the biological activity of hormones is a function of their unbound (free), rather than protein-bound, concentration (40).

We propose that under normal physiological conditions, TTR has a storage role for T_4 in blood, choroid plexus, and CSF. This function may be relevant under conditions of increased hormone demand. In the absence of TTR as a reservoir in the choroid plexus and CSF, T_4 exit from the CSF to the cerebral and, ultimately, the general circulation might be accelerated.

In summary, the data reported using TTR knockout mice bear on the role of TTR in thyroid economy in brains of normal mice. Because no other T_4-binding protein replaces TTR in the CSF and because a normal brain T_4 content (aside from the choroid plexus) is observed, we conclude that the blood-choroid-plexus-CSF barrier plays no essential role in entry or maintenance of T_4 in the brain. If the TTR-null mouse possesses compensatory mechanisms for brain CSF transport and distribution in the absence of TTR, our studies to date have not revealed them. To rule out the possibility that TTR might account for some quantitative or qualitative aspects of thyroid hormone transfer, we are investigating the distribution of radiolabeled T_4 and T_3 in the brains of TTR-null and wild-type mice.

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