Transthyretin is not necessary for thyroid hormone metabolism in conditions of increased hormone demand

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Abstract

Thyroid hormones circulate in blood mainly bound to plasma proteins. Transthyretin is the major thyroxine plasma carrier in mice. Studies in transthyretin-null mice revealed that the absence of transthyretin results in euthyroid hypothyroxinemia and normal thyroid hormone tissue distribution, with the exception of the choroid plexus in the brain. Therefore, transthyretin does not influence normal thyroid hormone homeostasis under standard laboratory conditions. To investigate if transthyretin has a buffer/storage role we challenged transthyretin-null and wild-type mice with conditions of increased hormone demand: (i) exposure to cold, which elicits thermogenesis, a process that requires thyroid hormones; and (ii) thyroidectomy, which abolishes thyroid hormone synthesis and secretion and induces severe hypothyroidism. Transthyretin-null mice responded as the wild-type both to changes induced by stressful events, namely in body weight, food intake and thyroid hormone tissue content, and in the mRNA levels of genes whose expression is altered in such conditions. These results clearly exclude a role for transthyretin in thyroid hormone homeostasis even under conditions of increased hormone demand.

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Introduction

Transthyretin (TTR) is synthesized by the liver and the choroid plexus (Harms *et al.* 1991) and acts as the major rodent plasma (Davis *et al.* 1970) and cerebrospinal fluid (Hagen & Solberg 1974) carrier for thyroxine (T_4) and the retinol-binding protein–retinol complex. TTR synthesis is a phylogenetically conserved event (Harms *et al.* 1991), and starts early during embryonic development. It has for a long time been suggested that TTR is involved in thyroid hormone homeostasis and hormone delivery to the brain (Dickson *et al.* 1987, Dratman *et al.* 1991, Chanoine *et al.* 1992). However, studies in a TTR-null mouse strain revealed that despite a 50% decrease in total T_4 plasma levels (Episkopou *et al.* 1993), no alterations are found in circulating free T_4 or total and free triiodothyronine (T_3) levels (Palha *et al.* 1994), and that TTR is not necessary for thyroid hormones to be normally distributed both to and within tissues (Palha *et al.* 1997, 2000, 2002). These studies, together with observations in other carrier protein deficiencies such as that of T_4-binding globulin (TBG) in humans (Refetoff 1989) and albumin deficiency in rats (Mendel *et al.* 1989) support the free hormone hypothesis for thyroid hormones. This hypothesis states that the biologically important pool of hormone in the circulation is in the free form (Mendel 1989). The role of carrier proteins in hormone delivery to tissues has been a matter of great debate (Schreiber *et al.* 1990, Palha 2002, Schreiber 2002). Individually, transport proteins might be redundant and part of a back-up mechanism for other carriers; they might not be essential but still contribute to hormone delivery to particular tissues, or they might represent important storage/buffer reservoirs required under conditions of increased or decreased hormone demand. Therefore, exposure to stressful conditions might provide further information on the physiological role of individual proteins.

Thyroid hormones are essential for the proper development of the central nervous system and regulate many different functions both during development and in the adult organism. Among these is the regulation of basal cellular metabolism (Silva 2003). The majority of thyroid hormone actions are mediated by the transcriptional activation or repression of target genes. T_3, the biologically

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active hormone, derived from circulating T_3 or from local deiodination of T_4 by deiodinases, interacts with nuclear receptors that bind to response elements in target genes (Ribeiro *et al.* 1995). Deiodinase enzymes are, therefore, important modulators of thyroid hormone action, and respond (both at the mRNA and protein and activity levels) in accordance with altered thyroid hormone availability (Bianco *et al.* 2002).

Exposure to cold induces a physiological adaptation of the basal cellular metabolism (Pecqueur *et al.* 2001), in which there is an increased rate of aerobic cellular metabolism and the production of heat by adaptive (or facultative) thermogenesis. Heat production is accomplished by the activation of uncoupling proteins that dissipate the proton gradient across the inner mitochondrial membrane (Nedergaard *et al.* 2001). In rodents, brown adipose tissue (BAT) is the major site of adaptive thermogenesis. BAT responsiveness to cold, initiated by norepinephrine (NE), elicits an increase in cAMP that results in the activation of several genes including those of uncoupling protein 1 (UCP1) (Bouillaud *et al.* 1984, Silva & Rabelo 1997) and deiodinase type 2 (DII) (Silva & Larsen 1983, Klingenspor 2003). Augmented T_3 resulting from increased DII activity also contributes to regulation of heat production by uncoupling proteins, enzymes of the basal cell metabolism and elements of the NE signaling pathway (Silva & Larsen 1983, Ribeiro *et al.* 2000, Silva 2001, Klingenspor 2003).

Extreme thyroid hormone deficiency is induced by ablation of thyroid tissue. Thyroid hormone plasma content decreases to less than 5% of normal values in 15 days after thyroidectomy in rats, but T_4 and T_3 are still detected in tissues after 4 months (Obregon *et al.* 1981). It appears reasonable to assume that the circulating pool of hormone bound to a carrier protein, namely to TTR, contributes to the amount of serum thyroid hormones after thyroidectomy.

In order to investigate whether TTR has a storage/buffer role in thyroid hormone homeostasis, we challenged TTR-null mice to cold exposure and removal of the thyroid gland, conditions of moderate and extremely increased hormone demand respectively.

Materials and Methods

Animals

All experiments were conducted using 1-month-old wild-type or TTR-null mice (Episkopou *et al.* 1993), in accordance with National and European Union guidelines for the care and handling of laboratory animals. Mice, under 12 h light cycles, were given chow and tap water freely.

Exposure to cold

Control animals kept at 23 ± 1 °C and cold-exposed animals kept at 4 ± 2 °C were individually housed and provided with minimal bedding material. Daily body weight and food intake were registered. Animals, fasted overnight, were killed by rapid decapitation 1 month after the onset of exposure to cold; serum and tissue samples of liver, kidney, interscapular BAT and brain (excluding cerebellum and brainstem) were rapidly frozen in dry ice and stored at -80 °C until analysis. Separate aliquots of liver and BAT samples were collected for RNA extraction.

Thyroidectomy

Total thyroid gland removal or sham surgeries were performed under ketamine (150 mg/kg) plus medetomidine (0·3 mg/kg) i.p. anesthesia. Since removal of thyroid follicles in adult rats by surgical thyroidectomy is not always totally successful, thyroidectomized animals received, 1 week later, 100 μCi ^{131}I to ensure complete removal of thyroid tissue. One week was usually left between the surgery and the radio-thyroidectomy, so that plasma thyroid-stimulating hormone (TSH) had time to increase and to ensure maximal uptake by remnants of the radioiodine. This, however, imposes some restrictions on the experimental procedures, because destruction of thyroid remnants and excretion of ^{131}I-labeled compounds from the body is not immediate and ^{131}I in serum and tissues may interfere with later determinations by RIA. This is the reason for waiting until a week before killing the animals. Moreover, previous work (Obregon *et al.* 1981) indicates that disappearance of T_4 and T_3 from plasma at 2 weeks after thyroidectomy occurs rapidly, as expected from the short biological half-lives, but both iodothyronines may still be found in many tissues. Because the parathyroids were removed along with the thyroid gland, thyroidectomized animals received 2% calcium lactate in their drinking water. Either 1 or 2 weeks after ^{131}I injection, animals were anesthetized as described above, and heparin 0·17% (20–30 μl) was administered through the vena cava prior to blood withdrawal, followed by perfusion with 20 ml 0·05 M pH 7·4 phosphate buffer containing 0·9% NaCl. Samples of plasma, liver, kidney, heart, cortex and cerebellum were collected, frozen and stored as described above. To assess complete thyroid removal, animals subjected to this procedure were considered only if the weight gain between the iodine injection and the last day of experiment was less than 15% of their initial body weight, and if plasma TSH values were markedly increased.

TSH determination

TSH plasma levels were measured using the specific RIAs developed for the rat provided by the Rat Pituitary Agency of the National Institute of Diabetes, Digestive and Kidney Diseases (NIDDK, NIH, Bethesda, MD, USA). Results were expressed in weight equivalents of the NIDDK r-TSH RP-3 preparation.

Thyroid hormone determination

T_4 and T_3 were measured in all samples after extraction and purification of the iodothyronines, as previously described (Morreale de Escobar *et al.* 1985, 1994). In brief, methanol was added to the frozen sample and it was homogenized. Tracer amounts of [^{131}I]T_4, and [^{125}I]T_3, were added to each homogenate, followed by extraction of endogenous and added tracers with chloroform–methanol (2:1). The iodothyronines were then back-extracted into an aqueous phase and purified using AG 1 × 2 resin columns (Bio-Rad, Hercules, CA, USA). After a pH gradient, the iodothyronines were eluted with 70% acetic acid and evaporated to dryness. Each extract was counted to determine the recovery of the [^{131}I]T_4 and [^{125}I]T_3 added to each sample during the initial homogenization process. The samples were then used to determine T_4 and T_3 content. Each sample was processed in duplicate at two dilutions. Concentrations were calculated from the respective RIAs, taking into account the individual recovery and tissue sample weight.

In the case of the cold-exposure experiment, in which the animals were not perfused, during the calculations we took into consideration the plasma content of each tissue, as previously determined (Palha *et al.* 1997).

RNA extraction and semiquantative RT-PCR

Total RNA was isolated from liver and BAT using Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. cDNA synthesis was performed using the SupersScript First-Strand Synthesis System for RT-PCR (Invitrogen) and semiquantitative multiplex PCR reactions were performed as previously described (Wong *et al.* 1994). Briefly, each PCR cycle was composed of the following steps: 94 °C for 30 s, 60 °C for 45s and 72 °C for 60s. A sequential series of PCR reactions using each primer pair was performed initially to determine the number of cycles in which the amplification would reside within the exponential phase of the amplification curve for both the gene under study and the housekeeping gene, hypoxanthine guanine phosphoribosyl transferase (HPRT). In accordance we chose 27, 26, 30 and 25 cycles for deiodinase type 1 (DI), DII, TBG or UCP1 respectively. Using an established 'primer-dropping' method (Wong *et al.* 1994), 21 cycles were used to amplify the HPRT gene in the same reaction in which the gene under study was amplified.

The oligonucleotide primers for DI, DII, TBG, UCP1 and HPRT were synthesized using the Primer3 software (Rozen & Skaletsky 2000) on the basis of the following GenBank sequences: AY575783 (TBG); NM007860 (DI); NM010050 (DII); U63419 (UCP1); XM356404 (HPRT). The sequences of oligonucleotide primers were: DI sense, CTGGAAAAGCTTTGCACTCC, DI anti-sense, AGGGTGACACTCTGGATTGG; DII sense, ATGGGACTCCTCAGCGTAGACTTGC, DII anti-sense, TGAACCAAAGTTGACCACCA; UCP1 sense, GTCCTAGGGACCATCACCA, UCP1 anti-sense, CCCGTGTAGCGGGGTTT; HPRT sense, GCTGG TGAAAAGGACCTCT, HPRT anti-sense CACAGG ACTAGAACACCTGC; TBG sense, CAACAGGGCT TCCAACATTT, TBG anti-sense, TGGTGCTCTTG TCCACTGAG.

Aliquots of the PCR products (10 μl) were separated by 2% agarose gel electrophoresis and stained with ethidium bromide. Gels were visualized with AlphaImager 2200 (AlphaInnotech, San Leandro, CA, USA) and analyzed densitometrically with the corresponding AlphaEase software. The expression level of the housekeeping gene HPRT was used as an internal standard, to which other PCR amplification products were normalized.

In another set of experiments, we confirmed the data obtained with the Quantum RNA 18S internal standards (Ambion, Austin, TX, USA) as housekeeping gene.

Statistics

Body weight and food intake comparisons were made using Student's *t*-test with $P<0{\cdot}05$ considered as statistically significant. T_4 and T_3 levels were compared by two-way (strain and exposure to cold) ANOVA, after testing for homogeneity of variance by Levene's test. Logarithmic transformation ensured homogeneity of variance when this was not found with the raw data. The F statistic was considered significant at $P<0{\cdot}05$.

Because removal of the thyroid results in a drastic reduction of thyroid hormone levels, it is not possible to apply a parametric statistical test that includes both the results from the sham-operated animals and those of the thyroidectomized ones. Therefore, we chose to compare, independently, the effect of thyroid removal in each experimental group (TTR-null vs wild-type) and the two strains within each experimental condition (sham or thyroidectomy), using Student's *t*-test.

All values presented are means ± S.D. from four to nine samples per group. Statistical analyses were performed with SPSS version 12·0 for Windows (SPSS, Inc., Chicago, IL, USA).

Results

Effects of cold exposure on thyroid hormone metabolism

Body weight and food intake We chose 1-month-old animals because they were expected to grow rapidly during the 1-month duration of the experiment, and an effect of the absence of TTR could be more easily disclosed. At the beginning of the experiment (day zero), wild-type and TTR-null mice had similar body weights both in the groups maintained at room temperature

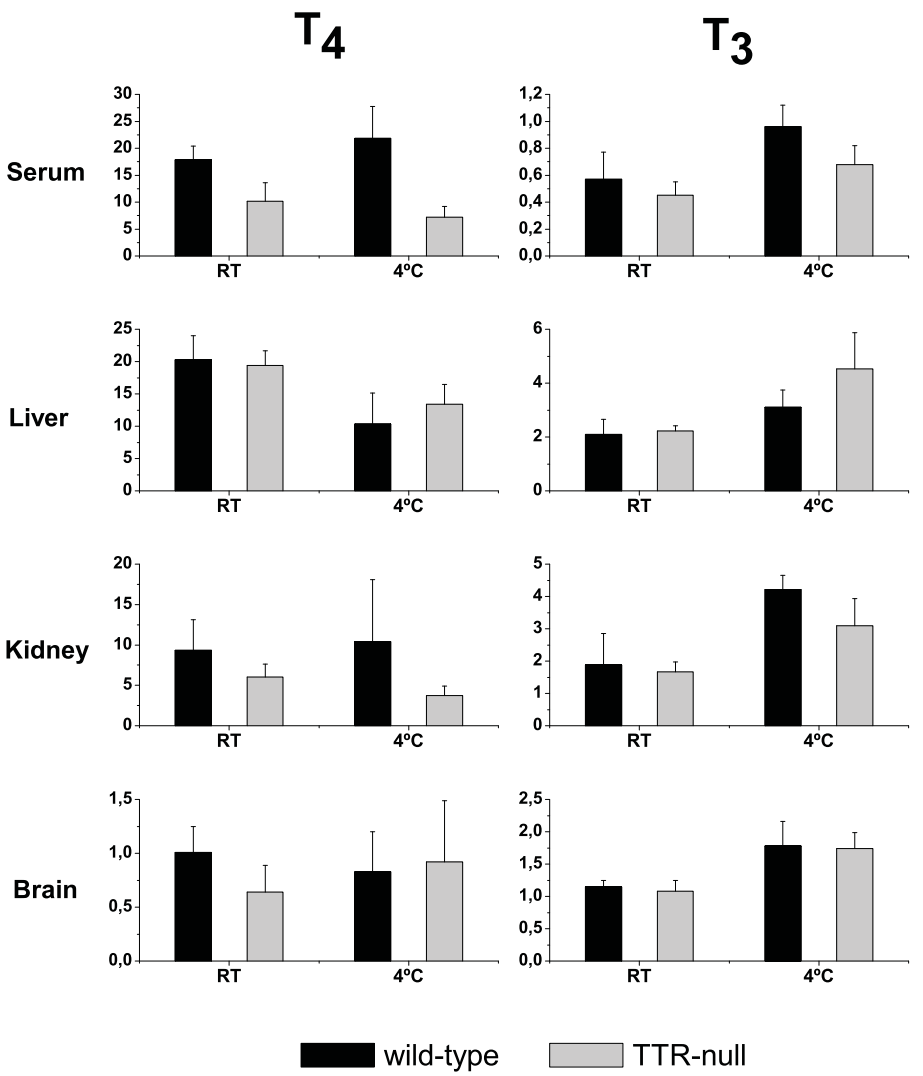

Figure 1 Effect of exposure to cold on T_4 and T_3 serum (ng/ml) and tissue (ng/g wet weight) content of wild-type and TTR-null mice. A one month exposure to cold induced similar changes in circulating and tissue T_4 and T_3 levels. The decreased T_4 serum levels previously described for the TTR-null mice were not further aggravated by exposure to cold. Statistical analysis is summarized in Table 1. RT, room temperature; 4 °C, cold-exposed.

(20·4 ± 2·9 and 18·7 ± 3·2 g respectively) and in the cold (20·7 ± 2·4 and 19·7 ± 1·6 g respectively). Increase in body weight at the end of the experiment (day 30) was similar in the animals kept at room temperature (wild-type: 22·4 ± 2·9 g and TTR-null: 24·3 ± 1·9 g) and in the cold (wild-type: 22·9 ± 1·7 g and TTR-null: 23·7 ± 1·3 g) and was not influenced by the absence of TTR.

As expected, exposure to cold led to statistically significant increases in food intake for both wild-type (8·2 ± 0·4 g/day) and TTR-null (9·0 ± 0·4 g/day) mice when compared with food consumption at room temperature (3·3 ± 0·6 and 3·7 ± 0·4 g/day respectively).

Thyroid hormone concentrations The effects of exposure to cold on thyroid hormone concentrations are presented in Fig. 1.

Except for the liver, exposure to cold did not induce changes in T_4 tissue content. In the liver, both wild-type and TTR-null mice presented a cold-induced reduction in T_4 levels ($F=27{\cdot}532$, $P<0{\cdot}001$). A slight decrease in T_4 kidney levels, which was not sufficient to result in a cold-induced statistically significant effect, was more pronounced in the TTR-null mice ($F=8{\cdot}196$, $P<0{\cdot}01$).

TTR total serum T_4 was strongly reduced in TTR-null mice both at room temperature and when mice were exposed to cold ($F=4{\cdot}562$, $P=0{\cdot}05$).

Table 1 Two-way ANOVA statistics for T_4 and T_3 concentrations in wild-type and TTR-null mice maintained at room temperature or after a 1 month exposure to cold

	Strain effect		Effect of exposure to cold		Strain vs exposure to cold interaction	
	F	*P*	F	*P*	F	*P*
T_4						
Serum	45·521	<0·001	0·344	NS	4·562	NS
Liver	0·452	NS	27·352	<0·001	0·513	NS
Kidney	8·196	<0·01	0·156	NS	0·791	NS
Brain	1·069	NS	0·150	NS	1·959	NS
T_3						
Serum	9·899	<0·001	23·390	<0·001	1·629	NS
Liver	3·973	NS	35·258	<0·001	2·176	NS
Kidney	4·996	<0·05	40·069	<0·001	2·564	NS
Brain	0·331	NS	43·709	<0·001	0·007	NS

NS, not-statistically significant.

The slight difference in total T_4 brain levels in animals kept at room temperature seemed to be attenuated by exposure to cold.

Exposure to cold resulted in increased T_3 concentrations in all organs studied, and this was identical for both wild-type and TTR-null mice. However, in the serum this effect was not as pronounced in the TTR-null mice, since we observed a strain effect ($F=9·899$, $P<0·01$). T_3 derives mainly from local T_4 deiodination. Interestingly, and in accord with the lower T_4 levels we found in the kidney of cold-exposed TTR-null mice, the increase in T_3 induced by exposure to cold was less pronounced in the TTR-null mice ($F=4·996$, $P<0·05$).

A summary of the ANOVA is presented in Table 1.

Expression levels of DI, DII, TBG and UCP1 Animals left in the cold presented a 2·5-fold increase in the expression of liver DI ($P<0·05$) (Fig. 2a). This increase was similar in both wild-type and TTR-null mice.

The BAT mRNA levels of DII (Fig. 2b) were, as expected, increased by exposure to cold.

As seen on Fig. 2c, after 1 month at 4 °C, a statistically significant increase in the mRNA for UCP1 was similarly observed in wild-type and TTR-null mice.

In order to investigate whether TBG could compensate for the absence of TTR in the cold, we measured the levels of liver TBG mRNA. TBG mRNA was similarly down-regulated ($P<0·05$) by cold, in TTR-null and wild-type mice, five and four times respectively. No statistical differences were seen for the TBG expression levels between TTR-null and wild-type mice at room temperature or in the cold.

Effects of thyroidectomy on thyroid hormone metabolism in TTR-null mice

Thyroid hormone concentrations In the absence of thyroid, there is no synthesis or secretion of thyroid hormones, and in such animals we can assess the possible role of TTR in mobilizing existing hormone stores. The weight gain of the thyroidectomized mice between the radioiodine injection and the last day of experiment was less than 15% of their initial body weight; plasma TSH values were markedly increased (>16 ng/ml) after thyroidectomy and were clearly indicative of a hypothyroid state.

Two (Fig. 3) or 3 weeks (data not shown) after removal of the thyroid gland there was a drastic and similar reduction in T_4 and T_3 plasma and tissue levels, whether TTR was present or absent. While for the plasma T_4, the strain effect continued to be present when the thyroid was ablated, for the heart, a smaller T_3 decrease was observed in the TTR-null mice ($F=12·540$, $P<0·01$). A summary of the statistical analysis is presented on Table 2.

Expression levels of TBG Thyroidectomy resulted in the up-regulation ($P<0·05$) of liver TBG mRNA in both TTR-null and wild-type mice (1·7 and 1·5 times respectively); no differences were seen between TTR-null and wild-type mice either in the sham or in the thyroidectomized groups.

Discussion

Previous studies from our own and other laboratories have failed to establish a limiting role for TTR in ensuring adequate supplies of thyroid hormone to mouse tissues under conditions of normal laboratory rearing. The present work was carried out in an effort to address the question of whether, or not, there is a requirement for TTR when pathophysiological conditions impose increased demand for thyroid hormone availability to tissues. Surprisingly, the results did not show any major difference between the response of TTR-null and wild-type mice during exposure to cold or during hypothyroidism caused by thyroidectomy.

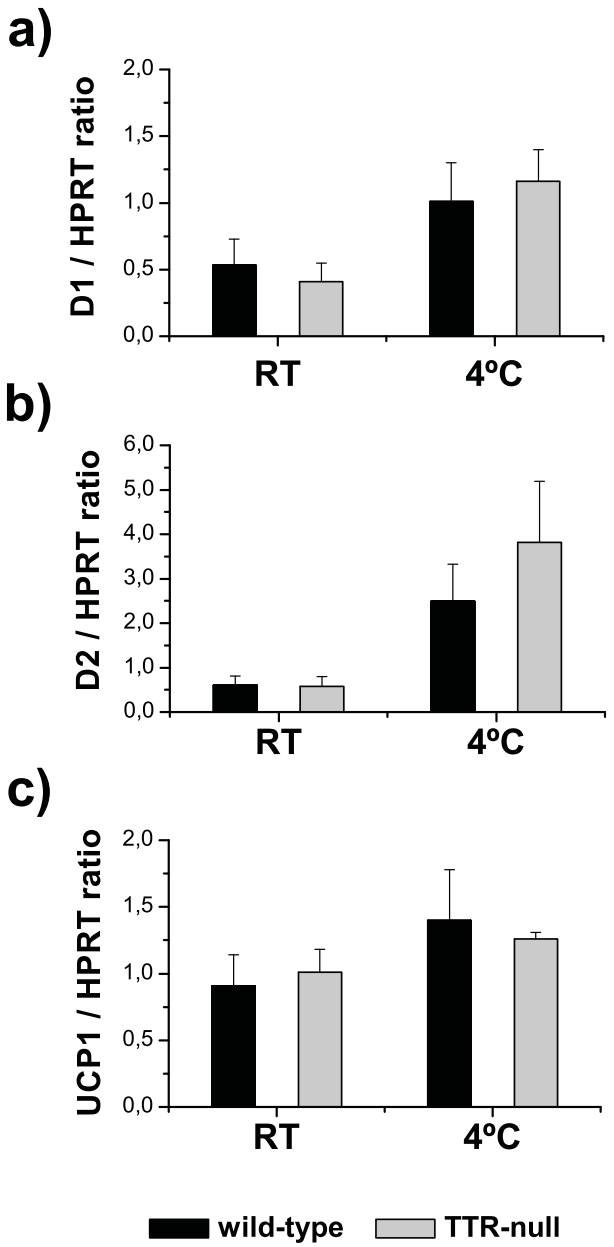

Figure 2 Cold induction of liver DI and BAT DII and UCP1 expression levels. TTR-null mice adapted to cold as the wild-type, as seen by the induction ($P<0{\cdot}05$) in the expression of genes coding for liver DI (a), and BAT DII (b) and BAT UCP1 (c). RT, room temperature; 4 °C, cold-exposed.

We have previously shown that TTR is not required for normal T_4 tissue uptake or distribution, and its absence does not impair hormone tissue contents, despite the low circulating levels of total T_4 (Palha *et al.* 1994, 1997). Even the absence of TTR from the choroid plexus, where it represents the major protein synthesized, does not influence the proper access and distribution of T_4 within the brain (Palha *et al.* 2000). Considering that the TTR-null mice have normal free thyroid hormone levels, the data obtained so far from studying these mice are in agreement with the free hormone hypothesis (Mendel 1989), as previously discussed (Palha *et al.* 1994).

However, carrier proteins are believed to have buffer/reservoir roles (Schreiber 2002), and this should become apparent if conditions of increased hormone demand are imposed. Exposure to cold represents a physiological situation of increased cellular metabolic activity for which thyroid hormones are required.

We chose 1-month-old animals because they were expected to grow rapidly during the 1-month duration of the experiment, and an effect of the absence of TTR could be more easily observed. In the cold, part of the reducing equivalents derived from fuel oxidation are lost to produce heat. Therefore, adaptation to cold includes an increase in food intake and, at least in the initial phases, increased mobilization of stored energy. If TTR-bound T_4 were important in the cold-induced increased demand for thyroid hormones, we would expect the TTR-null mice to adapt poorly or perhaps fail to survive at all in a cold environment. This was not the case. TTR-null mice adapted as well as the wild-type mice to a 1 month exposure to 4 °C: both genotypes responded with an increase in food intake and changes in serum and tissue thyroid hormone content. In addition, body weight gain was also similar in the two groups of animals.

The increased need for thyroid hormone induced by cold is reflected in the elevation of serum and tissue T_3, which we show here to be similar for TTR-null and wild-type mice. T_3, the biologically active thyroid hormone, derives mainly from the local deiodination of T_4 (Silva & Larsen 1985, Bianco *et al.* 2002). This explains the observed decrease in T_4 tissue content and increase in T_3 content associated with exposure to cold, especially in the liver, which produces a major fraction of circulating T_3 (Bianco *et al.* 2002). In accord, we observed an increase in the liver mRNA levels of DI after 1 month of cold exposure. The facts that kidney T_3 did not increase in the TTR-null as much as in the wild-type and that circulating T_3 mainly derives from thyroid and peripheral (liver and kidney) T_4 deiodination might explain that in the cold-exposed animals the increase in serum T_3 in the TTR-null was not as pronounced as in the wild-type mice.

Interestingly, exposure to cold did not induce changes in T_4 brain content within each genotype, despite the increase seen for T_3. There is controversy on the effect of cold on the activity of brain DII (Silva & Larsen 1983, Anguiano *et al.* 1995, Sullo *et al.* 2003). Whether the increase in T_3 observed by exposure to cold results from increased T_4 and/or T_3 delivery to the brain or altered DII and/or DIII activities remains to be elucidated. In any case, it is interesting to note that the slight, but statistically significant lower T_4 content found in the TTR-null brain at room temperature is not aggravated, but rather

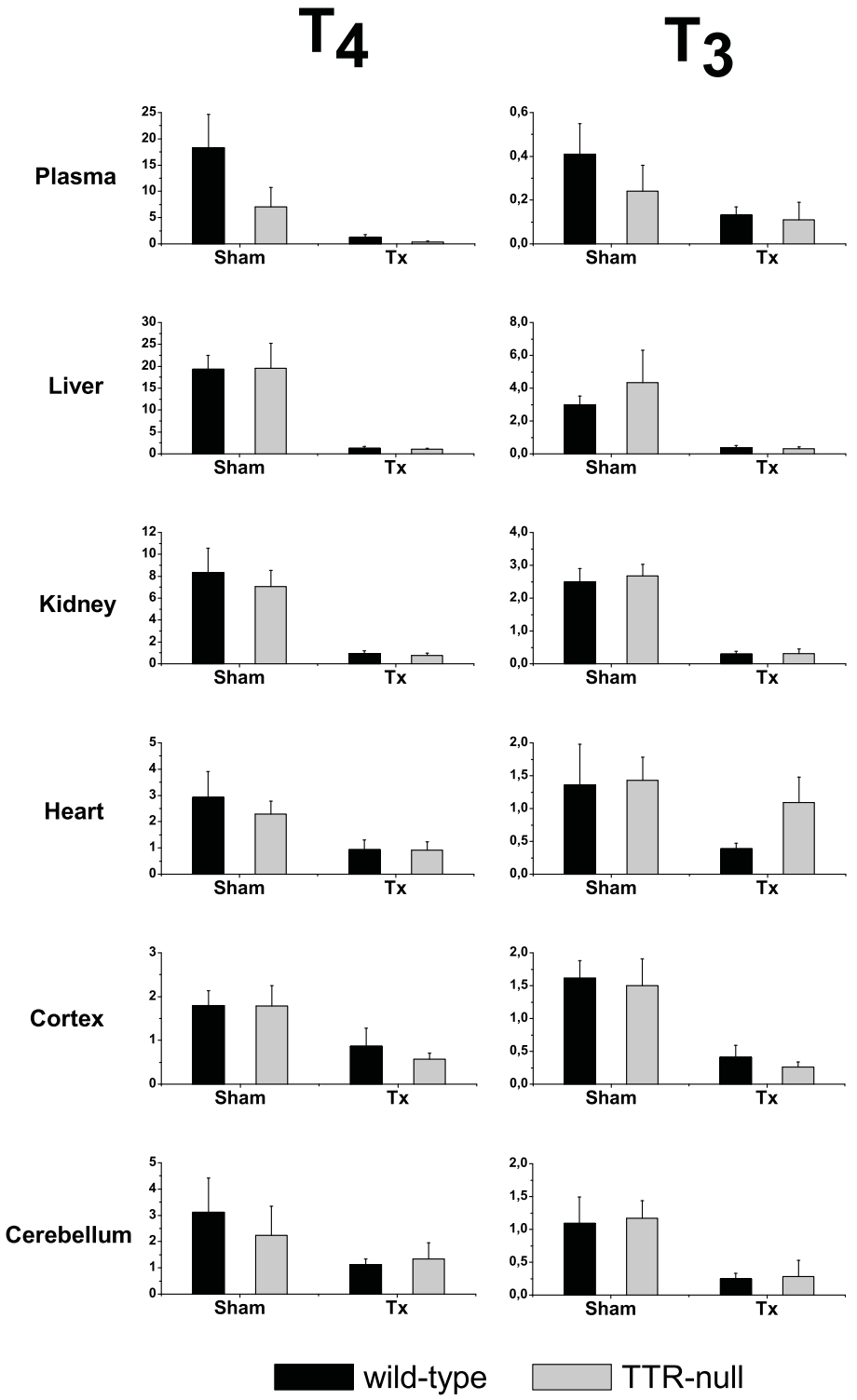

Figure 3 Effect of thyroidectomy on T_4 and T_3 serum (ng/ml) and tissue (ng/g wet weight) content of wild-type and TTR-null mice. Two weeks after complete ablation of the thyroid gland both TTR-null and wild-type mice presented drastic reductions in circulating and tissue T_4 and T_3 levels. Statistical analysis is summarized in Table 2. Sham, sham surgery; Tx, thyroidectomized.

Table 2 Student's *t*-test for T_4 and T_3 concentrations in thyroidectomized (Tx) wild-type and TTR-null mice. Comparison between strains was made for each experimental condition and between experimental conditions for each strain; *P* values are presented

	Strain comparison		**Thyroid removal effect**	
	Sham	Tx	Wild-type	TTR-null
T_4				
Plasma	<0·01	<0·01	<0·001	<0·001
Liver	NS	NS	<0·001	<0·001
Kidney	NS	NS	<0·001	<0·001
Heart	NS	NS	<0·001	<0·001
Cortex	NS	NS	<0·001	<0·001
Cerebellum	NS	NS	<0·001	NS
T_3				
Plasma	NS	NS	<0·001	NS
Liver	NS	NS	<0·001	<0·001
Kidney	NS	NS	<0·001	<0·001
Heart	NS	<0·01	<0·001	NS
Cortex	NS	NS	<0·001	<0·001
Cerebellum	NS	NS	<0·001	<0·001

NS, not-statistically significant.

disappears, upon exposure to cold. It is possible that in the absence of a carrier/buffer protein such as TTR in the choroid plexus, and in conditions of increased hormone demand, T_4 has more ready access to the brain via the blood–choroid plexus–CSF route.

Thyroid hormones are important for adaptive thermogenesis in BAT. Adaptation to cold is mediated by production of heat, a process that is accomplished by the dissipation of the protein gradient by the BAT-specific mitochondrial protein UCP1. By inducing the expression of UCP1, both T_3 and NE (Bianco *et al.* 1988) contribute to the maintenance of a normal thermal condition. UCP1-induced activation by T_3 is, in itself, indirectly dependent on sympathetic stimulation of DII (Klingenspor 2003). The absence of TTR did not influence the expected increase in BAT DII mRNA upon exposure to cold. With respect to UCP1, acute (Bianco *et al.* 2002) and chronic (Zaninovich *et al.* 2002, Liebig *et al.* 2004) exposure to cold is described as inducing mRNA levels and protein activity. In accord, in our experiment, the levels of UCP1 BAT mRNA were increased in both wild-type and TTR-null mice 1 month (chronic) after exposure to cold, although the increase is relatively mild compared with the changes described after acute exposure.

We expected that in such conditions of increased cellular metabolism as those required in adaptive thermogenesis, the absence of the major thyroid hormone carrier would trigger hypothyroidism and cold intolerance. In fact, hypothyroid rodents (Bianco *et al.* 2002, Zaninovich *et al.* 2002) and mice depleted of all thyroid hormone receptors (Golozoubova *et al.* 2004) are cold intolerant. With the present data we clearly show that TTR does not seem to play a buffer or storage role useful for conditions of increased hormone demand. Interestingly, exposure to cold in guinea pigs has been described as decreasing plasma T_4-binding capacity, which was attributed to a decrease in circulating albumin (Yamada *et al.* 1969). Here we show that, in the cold, TBG mRNA levels are also down-regulated, similarly in TTR-null and wild-type mice. The lower plasma T_4-binding capacity induced by cold would further aggravate impaired response to cold of TTR-null mice, if TTR were important for hormone delivery to tissues, which the present study excludes.

Total thyroidectomy induces marked hypothyroidism and may ultimately result in death. In such extreme situations, it is expected that viability might be prolonged by an increased availability of stored hormone. We chose thyroidectomy to evaluate the importance of TTR-bound T_4 in an acute and extreme condition of thyroid hormone deprivation. Removal of the thyroid gland induces growth arrest (Evans *et al.* 1964) and both the TTR-null and the wild-type mice presented a marked decrease in body weight gain in comparison with their sham-operated counterparts. Two weeks after ablation of the thyroid, TTR-null and wild-type mice showed similar drastic reductions in T_4 and T_3 plasma and tissue levels, which were not further aggravated 3 weeks after thyroid ablation (data not shown). These observations are in accord with those previously reported in rats. In rats, thyroid hormones tissue levels are strongly reduced upon thyroidectomy and this decrease is not significantly aggravated up to 4 months after thyroidectomy (Obregon *et al.* 1981). In the present case, it is clear that the TTR-bound T_4 does not delay and/or attenuate the decrease in tissue thyroid hormones, as measured at 2 and 3 weeks after removal of the thyroid gland. TBG mRNA levels, known to be up-regulated in hypothyroidism and to correlate with TBG serum levels (Vranckx *et al.* 1990), were similarly induced by thyroidectomy in TTR-null and wild-type mice. Therefore, as we have previously shown for TTR-null mice kept in normal housing conditions (Palha *et al.* 1994), TBG does not compensate for the absence of TTR.

Taken together, adaptation to a moderately or extremely increased need for thyroid hormone does not appear to be influenced by the presence of a TTR-bound T_4 pool. Therefore, TTR does not seem to be important as a reservoir for thyroid hormones in conditions of increased hormone demand. This conclusion raises two major issues, namely, the redundancy of TTR as a plasma hormone carrier, and the importance of further investigating the function of TTR. Redundancy might be expected when other proteins fulfill identical carrier roles, as is the case for albumin and TBG for thyroid hormones. Studies in Nagase analbuminemic rats have excluded a major role for albumin in thyroid hormone homeostasis in normal conditions (Mendel *et al.* 1989). A single report indicates that analbuminemic rats when fasted and exposed to

cold survive less than controls (Kawaguchi *et al.* 1986). However, since albumin is a carrier for many other ligands, including free fatty acids, known to influence survival in stressful situations such as exposure to cold, this is not the appropriate model to study the role of albumin as a thyroid hormone reservoir in conditions such as this. TBG is the major thyroid hormone carrier in man. There are cases of humans with partial or total TBG deficiency, which results in euthyroid hypothyroxinemia (Refetoff 1989, Bartalena 1993). Even though exposure to cold is known to induce alteration in thyroid hormone metabolism in humans (Solter *et al.* 1989, Sawhney *et al.* 1995, Do *et al.* 2004), to the best of our knowledge there are no reports on the ability of these individuals to adapt to cold. Because TTR-null mice are also euthyroid hypothyroxinemic, it is reasonable to believe that humans with TBG deficiency would normally adapt to cold.

It is also possible that in the absence of TTR a compensatory mechanism was developed to substitute TTR in hormone binding. It has been suggested that prostaglandin D2 synthase (L-PGDS) could be such a protein (Beuckmann *et al.* 1999); however, we have no evidence that L-PGDS is able to bind thyroid hormone *in vivo* (data not shown). Using electrophoretic and chromatographic approaches we see no other protein replacing TTR in T_4 binding (Palha *et al.* 2000). It is interesting to note that TTR, also a carrier for retinol, does not seem to impair retinoid metabolism under basal conditions, despite the low retinol found in the serum of TTR-null mice (Wei *et al.* 1995).

Despite these considerations, it is difficult to accept that TTR, the major protein synthesized by the choroid plexus and whose expression is highly conserved throughout evolution and starts early during embryonic development, plays no more than a biologically redundant role. We therefore consider it important to further investigate the role of TTR. We rather believe that the biological relevance of TTR is more than that initially described in the literature, as a carrier for thyroid hormones. Recently it has been shown that TTR is involved in depression-like behavior and exploratory activity, possibly by modulating noradrenergic transmission in the limbic forebrain (Sousa *et al.* 2004), and that it acts as a cryptic protease (Liz *et al.* 2004).

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References

Anguiano B, Quintanar A, Luna M, Navarro L, Ramirez del Angel A, Pacheco P & Valverde C 1995 Neuroendocrine regulation of adrenal gland and hypothalamus 5′deiodinase activity. II. Effects of splanchnicotomy and hypophysectomy. *Endocrinology* **136** 3346–3352.

Bartalena L 1993 Studies on thyroxine-binding globulin. *Journal of Endocrinological Investigation* **16** 353–371.

Beuckmann CT, Aoyagi M, Okazaki I, Hiroike T, Toh H, Hayaishi O & Urade Y 1999 Binding of biliverdin, bilirubin, and thyroid hormones to lipocalin-type prostaglandin D synthase. *Biochemistry* **38** 8006–8013.

Bianco AC, Sheng XY & Silva JE 1988 Triiodothyronine amplifies norepinephrine stimulation of uncoupling protein gene transcription by a mechanism not requiring protein synthesis. *Journal of Biological Chemistry* **263** 18168–18175.

Bianco AC, Salvatore D, Gereben B, Berry MJ & Larsen PR 2002 Biochemistry, cellular and molecular biology, and physiological roles of the iodothyronine selenodeiodinases. *Endocrine Reviews* **23** 38–89.

Bouillaud F, Ricquier D, Mory G & Thibault J 1984 Increased level of mRNA for the uncoupling protein in brown adipose tissue of rats during thermogenesis induced by cold exposure or norepinephrine infusion. *Journal of Biological Chemistry* **259** 11583–11586.

Chanoine JP, Alex S, Fang SL, Stone S, Leonard JL, Korhle J & Braverman LE 1992 Role of transthyretin in the transport of thyroxine from the blood to the choroid-plexus, the cerebrospinal-fluid, and the brain. *Endocrinology* **130** 933–938.

Davis PJ, Spaulding SW & Gregerman RI 1970 The three thyroxine-binding proteins in rat serum: binding capacities and effects of binding inhibitors. *Endocrinology* **87** 978–986.

Dickson PW, Aldred AR, Menting JG, Marley PD, Sawyer WH & Schreiber G 1987 Thyroxine transport in choroid plexus. *Journal of Biological Chemistry* **262** 13907–13915.

Do NV, Mino L, Merriam GR, LeMar H, Case HS, Palinkas LA, Reedy K & Reed HL 2004 Elevation in serum thyroglobulin during prolonged Antarctic residence: effect of thyroxine supplement in the polar 3,5,3′-triiodothyronine syndrome. *Journal of Clinical Endocrinology and Metabolism* **89** 1529–1533.

Dratman MB, Crutchfield FL & Schoenhoff MB 1991 Transport of iodothyronines from bloodstream to brain: contributions by blood:brain and choroid plexus:cerebrospinal fluid barriers. *Brain Research* **554** 229–236.

Episkopou V, Maeda S, Nishiguchi S, Shimada K, Gaitanaris GA, Gottesman ME & Robertson EJ 1993 Disruption of the transthyretin gene results in mice with depressed levels of plasma retinol and thyroid-hormone. *PNAS* **90** 2375–2379.

Evans ES, Rosenberg LL, Evans AB & Koneff AA 1964 Relative sensitivity of different biological responses to small quantities of thyroxine and triiodothyronine. *Endocrinology* **74** 770–779.

Golozoubova V, Gullberg H, Matthias A, Cannon B, Vennstrom B & Nedergaard J 2004 Depressed thermogenesis but competent brown adipose tissue recruitment in mice devoid of all hormone-binding thyroid hormone receptors. *Molecular Endocrinology* **18** 384–401.

Hagen GA & Solberg LA Jr 1974 Brain and cerebrospinal fluid permeability to intravenous thyroid hormones. *Endocrinology* **95** 1398–1410.

Harms PJ, Tu GF, Richardson SJ, Aldred AR, Jaworowski A & Schreiber G 1991 Transthyretin (prealbumin) gene-expression in choroid-plexus is strongly conserved during evolution of vertebrates. *Comparative Biochemistry and Physiology. B, Comparative Biochemistry* **99** 239–249.

Kawaguchi T, Shimode M, Matsushita H & Nagase S 1986 Frequent administration of uric acid extends survival of fasting analbuminemic rats under cold environment. *Japanese Journal of Physiology* **36** 295–303.

Klingenspor M 2003 Cold-induced recruitment of brown adipose tissue thermogenesis. *Experimental Physiology* **88** 141–148.

Liebig M, von Praun C, Heldmaier G & Klingenspor M 2004 Absence of UCP3 in brown adipose tissue does not impair nonshivering thermogenesis. *Physiological and Biochemical Zoology* **77** 116–126.

Liz MA, Faro CJ, Saraiva MJ & Sousa MM 2004 Transthyretin, a new cryptic protease. *Journal of Biological Chemistry* **279** 21431–21438.

Mendel CM 1989 The free hormone hypothesis: a physiologically based mathematical model. *Endocrine Reviews* **10** 232–274.

Mendel CM, Cavalieri RR, Gavin LA, Pettersson T & Inoue M 1989 Thyroxine transport and distribution in Nagase analbuminemic rats. *Journal of Clinical Investigation* **83** 143–148.

Morreale de Escobar G, Pastor R, Obregon MJ & Escobar del Rey F 1985 Effects of maternal hypothyroidism on the weight and thyroid hormone content of rat embryonic tissues, before and after onset of fetal thyroid function. *Endocrinology* **117** 1890–1900.

Morreale de Escobar G, Calvo R, Escobar del Rey F & Obregon MJ 1994 Thyroid hormones in tissues from fetal and adult rats. *Endocrinology* **134** 2410–2415.

Nedergaard J, Golozoubova V, Matthias A, Asadi A, Jacobsson A & Cannon B 2001 UCP1: the only protein able to mediate adaptive non-shivering thermogenesis and metabolic inefficiency. *Biochimica et Biophysica Acta* **1504** 82–106.

Obregon MJ, Mallol J, Escobar del Rey F & Morreale de Escobar G 1981 Presence of L-thyroxine and 3,5,3′-triiodo-L-thyronine in tissues from thyroidectomized rats. *Endocrinology* **109** 908–913.

Palha JA 2002 Transthyretin as a thyroid hormone carrier: function revisited. *Clinical Chemistry and Laboratory Medicine* **40** 1292–1300.

Palha JA, Episkopou V, Maeda S, Shimada K, Gottesman ME & Saraiva MJM 1994 Thyroid-hormone metabolism in a transthyretin-null mouse strain. *Journal of Biological Chemistry* **269** 33135–33139.

Palha JA, Hays MT, Morreale de Escobar G, Episkopou V, Gottesman ME & Saraiva MJM 1997 Transthyretin is not essential for thyroxine to reach the brain and other tissues in transthyretin-null mice. *American Journal of Physiology* **35** E485–E493.

Palha JA, Fernandes R, Morreale de Escobar G, Episkopou V, Gottesman M & Saraiva MJ 2000 Transthyretin regulates thyroid hormone levels in the choroid plexus, but not in the brain parenchyma: study in a transthyretin-null mouse model. *Endocrinology* **141** 3267–3272.

Palha JA, Nissanov J, Fernandes R, Sousa JC, Bertrand L, Dratman MB, Morreale de Escobar G, Gottesman M & Saraiva MJ 2002 Thyroid hormone distribution in the mouse brain: the role of transthyretin. *Neuroscience* **113** 837–847.

Pecqueur C, Couplan E, Bouillaud F & Ricquier D 2001 Genetic and physiological analysis of the role of uncoupling proteins in human energy homeostasis. *Journal of Molecular Medicine* **79** 48–56.

Resetoss S 1989 Inherited thyroxine-binding globulin abnormalities in man. *Endocrine Reviews* **10** 215–293.

Ribeiro RC, Apriletti JW, West BL, Wagner RL, Fletterick RJ, Schaufele F & Baxter JD 1995 The molecular biology of thyroid hormone action. *Annals of the New York Academy of Sciences* **758** 366–389.

Ribeiro MO, Lebrun FL, Christoffolete MA, Branco M, Crescenzi A, Carvalho SD, Negrao N & Bianco AC 2000 Evidence of UCP1-independent regulation of norepinephrine-induced thermogenesis in brown fat. *American Journal of Physiology. Endocrinology and Metabolism* **279** E314–E322.

Rozen S & Skaletsky H 2000 Primer3 on the WWW for general users and for biologist programmers. *Methods in Molecular Biology* **132** 365–386.

Sawhney RC, Malhotra AS, Nair CS, Bajaj AC, Rajan KC, Pal K, Prasad R & Basu M 1995 Thyroid function during a prolonged stay in Antarctica. *European Journal of Applied Physiology and Occupational Physiology* **72** 127–133.

Schreiber G 2002 The evolutionary and integrative roles of transthyretin in thyroid hormone homeostasis. *Journal of Endocrinology* **175** 61–73.

Schreiber G, Aldred AR, Jaworowski A, Nilsson C, Achen MG & Segal MB 1990 Thyroxine transport from blood to brain via transthyretin synthesis in choroid plexus. *American Journal of Physiology* **258** R338–R345.

Silva JE 2001 The multiple contributions of thyroid hormone to heat production. *Journal of Clinical Investigation* **108** 35–37.

Silva JE 2003 The thermogenic effect of thyroid hormone and its clinical implications. *Annals of Internal Medicine* **139** 205–213.

Silva JE & Larsen PR 1983 Adrenergic activation of triiodothyronine production in brown adipose-tissue. *Nature* **305** 712–713.

Silva JE & Larsen PR 1985 Potential of brown adipose tissue type II thyroxine 5′-deiodinase as a local and systemic source of triiodothyronine in rats. *Journal of Clinical Investigation* **76** 2296–2305.

Silva JE & Rabelo R 1997 Regulation of the uncoupling protein gene expression. *European Journal of Endocrinology* **136** 251–264.

Solter M, Brkic K, Petek M, Posavec L & Sekso M 1989 Thyroid hormone economy in response to extreme cold exposure in healthy factory workers. *Journal of Clinical Endocrinology and Metabolism* **68** 168–172.

Sousa JC, Grandela C, Fernandez-Ruiz J, de Miguel R, de Sousa L, Magalhaes AI, Saraiva MJ, Sousa N & Palha JA 2004 Transthyretin is involved in depression-like behaviour and exploratory activity. *Journal of Neurochemistry* **88** 1052–1058.

Sullo A, Brizzi G & Maffulli N 2003 Serotonin effect on deiodinating activity in the rat. *Canadian Journal of Physiology and Pharmacology* **81** 747–751.

Vranckx R, Rouaze M, Savu L, Nunez EA, Beaumont C & Flink IL 1990 The hepatic biosynthesis of rat thyroxine binding globulin (TBG): demonstration, ontogenesis, and up-regulation in experimental hypothyroidism. *Biochemical and Biophysical Research Communications* **167** 317–322.

Wei S, Episkopou V, Piantedosi R, Maeda S, Shimada K, Gottesman ME & Blaner WS 1995 Studies on the metabolism of retinol and retinol-binding protein in transthyretin-deficient mice produced by homologous recombination. *Journal of Biological Chemistry* **270** 866–870.

Wong H, Anderson WD, Cheng T & Riabowol KT 1994 Monitoring mRNA expression by polymerase chain reaction: the 'primer-dropping' method. *Analytical Biochemistry* **223** 251–258.

Yamada T, Fukuda H, Takemura Y & Shichijo K 1969 Effect of cold on plasma protein-thyroxine interaction in the guinea pig. *Metabolism* **18** 339–347.

Zaninovich AA, Raices M, Rebagliati I, Ricci C & Hagmuller K 2002 Brown fat thermogenesis in cold-acclimated rats is not abolished by the suppression of thyroid function. *American Journal of Physiology. Endocrinology and Metabolism* **283** E496–E502.

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