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Triiodothyronine administration reverses vitamin A deficiency-related hypo-expression of retinoic acid and triiodothyronine nuclear receptors and of neurogranin in rat brain

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Recent studies have revealed that retinoids play an important role in the adult central nervous system and cognitive functions. Previous investigations in mice have shown that vitamin A deficiency (VAD) generates a hypo-expression of retinoic acid (RA, the active metabolite of vitamin A) receptors and of neurogranin (RC3, a neuronal protein involved in synaptic plasticity) and a concomitant selective behavioural impairment. Knowing that RC3 is both a triiodothyronine (T_3) and a RA target gene, and in consideration of the relationships between the signalling pathways of retinoids and thyroid hormones, the involvement of T_3 on RA signalling functionality in VAD was investigated. Thus, the effects of vitamin A depletion and subsequent administration with RA and/or T_3 on the expression of RA nuclear receptors (RAR, RXR), T_3 nuclear receptor (TR) and on RC3 in the brain were examined. Rats fed a vitamin A-deficient diet for 10 weeks exhibited a decreased expression of RAR, RXR and TR mRNA and of RC3 mRNA and proteins. RA administration to these vitamin A-deficient rats reversed only the RA hypo-signalling in the brain. Interestingly, T_3 is able to restore its own brain signalling simultaneously with that of vitamin A and the hypo-expression of RC3. These results obtained *in vivo* revealed that one of the consequences of VAD is a dysfunction in the thyroid signalling pathway in the brain. This seems of crucial importance since the down regulation of RC3 observed in the depleted rats was corrected only by T_3.

Vitamin A deficiency: Retinoic acid nuclear receptors: Triiodothyronine nuclear receptors: Brain: Neurogranin

Vitamin A and its derivatives (the retinoids) participate in many physiological processes including vision and reproduction (Sporn *et al.* 1994), and exert a wide variety of profound effects on vertebrate development, cellular differentiation and homeostasis (Chambon, 1996). In addition to the well-known and important role of retinoids and particularly retinoic acid (RA, the active metabolite of vitamin A) during the normal development of the central nervous system (Maden *et al.* 1998; Környei *et al.* 1999; Malik *et al.* 2000), various data suggest that retinoids play a significant role in the adult central nervous system. Initial investigations have shown that the adult brain: (i) is a retinoid-metabolizing tissue (McCaffery & Dräger, 1994; Connor & Sidell, 1997); (ii) contains cellular RA and retinol-binding protein as well as a high level of nuclear RA receptors (Krezel *et al.* 1999; Zetterström *et al.* 1999). The RA receptors, RAR (whose ligands are the all-*trans*-RA and 9-*cis*-RA isomers) and RXR (whose ligand is the 9-*cis*-RA isomer), are DNA-binding proteins which, upon activation by specific retinoid ligands, induce gene transcription by interacting with distinct promoter sequences in the target genes (Kastner *et al.* 1995). Therefore, changes at the retinoid level are capable of producing alterations in several neuronal target proteins and consequently may affect physiological process maintenance in the mature brain (Malik *et al.* 2000). Indeed, knockout mice for RARβ and RARβ–RXRγ display an alteration of long-term potentiation (the most widely studied form of synaptic plasticity, thought to underlie memory formation), as well as substantial performance deficits in a hippocampal-dependent spatial learning task (Chiang *et al.* 1998). A similar conclusion can also be drawn from recent studies using mice or rats receiving a postnatally induced vitamin A-deficient diet (Misner *et al.* 2001; Cocco *et al.* 2002). Moreover, our recent studies have shown that a moderate down regulation of retinoid-mediated transcription events naturally occurs with senescence (Enderlin *et al.* 1997). An administration of RA

Abbreviations: GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PCR, polymerase chain reaction; RA, retinoic acid; RAR, retinoic acid receptor; RC3, neurogranin; RXR, retinoid X receptor; T_3, triiodothyronine; TR, triiodothyronine nuclear receptor; tTG, tissue-type transglutaminase; VAD, vitamin A deficiency.

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restores to pre-senescent levels the age-related decrease in brain expression of its own receptors and of neurogranin (RC3), a specific associated target gene (Iñiguez *et al.* 1994; Enderlin *et al.* 1997) involved in synaptic plasticity (Gerendasy & Sutcliffe, 1997; Pak *et al.* 2000), and concomitantly alleviates both the relational memory and hippocampal long-term potentiation seen in aged mice (Etchamendy *et al.* 2001). Together these data suggest that a fine regulation of retinoid-mediated gene expression is fundamentally important for optimal brain functioning.

A previous study, obtained in our laboratory using a murine model, has evidenced that vitamin A deficiency (VAD) leads to a reduced expression of brain retinoid nuclear receptors and that of RC3 as well as selective behavioural impairment (Etchamendy *et al.* 2000). Surprisingly, RA administration to these animals failed to fully normalize the expression of the genes studied and had no effect on the associated cognitive deficit. It is well known that the activity field and signalling pathway of retinoids and thyroid hormones, whose active metabolite is triiodothyronine (T_3), are in close relationship (Schräder & Carlberg, 1994; Chin & Yen, 1997). Moreover, alterations of thyroid hormone metabolism and functionality associated with VAD have been described (Ingenbleek & De Visscher, 1979; Okamura *et al.* 1981; Higueret & Garcin, 1984). Finally, RC3 is not only under the influence of retinoids (Iñiguez *et al.* 1994), but is regulated by thyroid hormone too (Guadaño-Ferraz *et al.* 1997; Morte *et al.* 1997; Martinez de Arrieta *et al.* 1999).

Thus, in the present work, the question arose regarding the possible implication of thyroid disorders in RA impairment in restoring neurological alterations to normal. Therefore, an examination was made first of the consequences of a vitamin A-deprived diet on T_3 and RA nuclear receptors expression (TR, RAR and RXR, respectively), and on two of their target genes, RC3, and tissue-type transglutaminase (tTG); the tTG is a protein whose expression is highly regulated by RAR (Chiocca *et al.* 1989) and is considered as a biological marker of early VAD (Savouré *et al.* 1996). Second, the effect of administration of RA and/or T_3 in vitamin A-deficient animals was studied.

Materials and methods

Experimental design

The study was conducted in accordance with the European Communities Council Directives (861609/EEC). All the experiments conformed to the Guidelines on the Handling and Training of Laboratory Animals. The experimental design of postnatal VAD was according to Audouin-Chevallier *et al.* (1993). Weaning male Wistar rats were purchased from Harlan (Gannat, France). They were maintained in a room with a constant airflow system, controlled temperature (21–23°C) and a 12 h light–dark cycle. The rats were allowed to have *ad libitum* access to food and tap water and were divided into two experimental groups: vitamin A-deficient (forty-eight animals); control (twelve animals). The vitamin A-deficient diet was composed as indicated in Table 1; the control diet was the same plus vitamin A (1515·15 RE/kg diet).

Table 1. Composition of the diet*

Ingredients	Amount (g/kg)
Vitamin-free casein†	180
Sucrose	305
Peanut oil	25
Rapeseed oil	25
Cellulose	20
Maize starch	400
Mineral mixture‡	35
Vitamin mixture§	10

* Vitamin A-sufficient diet according to Audouin-Chevallier *et al.* (1993). Chow was stored in sealed bags at 20°C and conserved after opening for a maximum of 1 week at 4°C.

† Vitamin-free casein from Touzard et Matignon, France.

‡ Mineral mixture no. 102 from INRA (Jouy en Josas, France) consisted of the following (g/kg chow): calcium phosphate dibasic, 500; sodium chloride, 74; potassium monohydrate citrate, 220; magnesium sulfate, 52; magnesium oxide, 24; manganous carbonate (430–480 g Mn/kg manganous carbonate), 3·5; iron citrate (160–170 g Fe/kg iron citrate), 6; zinc carbonate (700 g zinc oxide/kg zinc carbonate), 1·6; copper carbonate (530–550 g Cu/kg copper carbonate), 0·3; potassium iodate, 0·01; sodium selenite (456·5 g Se/kg sodium selenite), 0·022; potassium and chromium sulfate, 0·55; sucrose to make up to 1 kg.

§ Vitamin Diet Fortification Mixture without vitamin A no. 102 from INRA (Jouy en Josas, France) consisted of the following (g/kg chow): thiamine HCl, 0·6; riboflavin, 0·6; pyridoxine HCl, 3·0; D-calcium panthotenate, 1·6; folic acid, 0·2; D-biotin, 0·02; cyanocobalamin, 0·01; cholecalciferol, 0·00625; all-*rac*-α-tocopherol, 5; menadione, 0·05; ascorbic acid, 0·45; sucrose to make up to 1 kg.

Animals were fed these diets for 10 weeks. No difference between the different groups of rats was observed concerning the amount of food intake. At the time when the growth of the deficient animals slowed down, before weight reached a plateau and before the onset of apparent diseases was noted (these characteristics have previously been noted in the laboratory; Higueret & Garcin, 1982), some of depleted rats were injected daily (150 μg/kg body weight) for 4 d with RA (all-*trans*-RA, Sigma St Quentin Fallavier, France, no. R2625;) and/or T_3 (Sigma no. T2752) or vehicle only. Twelve rats were used for each treatment. Control rats were administrated with vehicle. The RA and T_3 were dissolved in a mixture (vehicle) containing polyethyleneglycol–NaCl–ethanol (70:20:10, by vol.). Rats were killed by decapitation. The brain and the liver were rapidly removed, weighed and stored at −80°C for subsequent analysis. Brains and livers of the different groups exhibited no weight differences.

Quantification of mRNA

Extraction of RNA was performed according to Chomczynski & Sacchi (1987).

Reverse transcription

The cDNA was synthesized with Superscript II reverse transcriptase (Invitrogen, Cergy Pontoise, France) according to the protocol recommended by the manufacturer with minor modifications. Briefly, 1 μg total RNA was incubated at 70°C for 10 min and then placed on ice before addition of reverse transcriptase reaction reagents with a specific reverse primer (120 ng) in a final volume of 20 μl. The reverse transcriptase reaction was performed

at 42°C for 60 min. Parallel reactions for each RNA sample were run in the absence of reverse transcriptase to assess the degree of any contaminating genomic DNA.

Analysis of gene expression using real-time polymerase chain reaction

The polymerase chain reaction (PCR) was carried out using a LightCycler system (Roche Diagnostics, Mannheim, Germany), which combines the processes of amplification and detection (by fluorescence) of a PCR product, thereby enabling on-line and real-time detection. To detect target-gene amplification products, LightCycler DNA Master SYBR Green I was used according to the manufacturer's instructions. The PCR reactions were performed in micro-capillary tubes in a final volume of 20 μl containing 1X LC-DNA Master SYBR Green I mix, 4 mM-$MgCl_2$, 0·5 μM of each primer and 2 μl cDNA. The amplification conditions were 95°C for 10 min to activate the polymerase, followed by forty cycles of denaturation at 95°C for 6 s, annealing at about 60°C (according to the gene studied) for 6 s, and elongation at 72°C for 10 s. After each elongation phase the fluorescence of SYBR Green I (a double-stranded DNA-binding dye) was measured and increasing amounts of PCR products were monitored from cycle to cycle. The forward and reverse primer sequences are shown in Table 2. For each primer pair used, melting curve analysis showed a single melting peak after amplification, indicating a specific product.

Quantification data were analysed using the LightCycler analysis software, version 3.5 (Roche Diagnostics, Mannheim, Germany). In this analysis, the background fluorescence was removed by setting a noise band. The log-linear portion of the standard amplification curve was identified and the crossing point was the intersection of the best-fit line through the log-linear region and the noise band. The standard curve was a plot of the crossing point *v.* the amount of initial cDNA used for amplification. Standard curves were generated from 4-fold serial dilutions of target and housekeeping (glyceraldehyde 3-phosphate dehydrogenase; GAPDH) cDNA preparation. The relationship between the crossing point and the initial amount of cDNA was found to be linear. The correlation coefficient (r) was 1 and PCR amplification efficiencies of the target and the housekeeping gene were similar and close to 100 %. These standard curves were used to estimate the concentration of both the target and the GAPDH gene in each sample. Then, the results were normalized by the ratio of the relative concentration of target to that of GAPDH in the same sample.

Western blot analysis

Western blot analysis was performed according to the procedure of Watson *et al.* (1990) with minor modifications. Brain tissue from the control rats, the deficient rats and deficient rats treated with RA and/or T_3 was homogenized in 0·16 M-NaCl, 11 mM-sodium phosphate, pH 7·4. Just before homogenisation, 1·5 μm-PMSF was added. The homogenate was then centrifuged at 10 000 ***g***. A sample of the supernatant fraction was removed for protein assay. Then SDS and dithiothreitol were added to a final concentration of 1 % (w/v) and 50 mM, respectively. Proteins were separated electrophoretically by size in a 12 % (w/v) denaturing SDS-polyacrylamide gel and transferred to a nitrocellulose membrane by electroblotting. The membrane was pre-blocked with 5 % non-fat milk in PBS-Tween 20 buffer (145 mM-NaCl, 1·5 mM-monohydrate sodium phosphate, 8 mM-anhydrous sodium phosphate and 1 % Tween 20), incubated overnight with polyclonal rabbit anti-neurogranin antibodies (diluted 1:3000, Affinity Research Product, Le Perray en Yvelines, France, no. NA 1300) or monoclonal mouse anti-β-actin antibodies (1:8000; Sigma no. A-5441), and washed briefly with PBS-Tween 20 buffer. Immunoreactive polypeptide bands were visualized enzymically in a secondary antibody reaction using alkaline phosphatase-conjugated anti-rabbit (Sigma no. A-0545) or anti-mouse immunoglobulin G (Amersham, Orsay, France, no. Na 93;). The staining intensity of protein bands was determined using an image analyser (Bio 1D; Vilbert Lourmat, Marne La Vallée, France). The relative levels of RC3 and β-actin proteins were determined as a percentage of the RC3 and β-actin in control rats.

Table 2. Primers used for LightCycler™ real-time polymerase chain reaction (PCR) with sequences forward (F) and reverse (R) primers, and size of amplicon

PCR primer pair	Ref.	Sequence	Position	Product length (bp)
GAPDH	Sabath *et al.* (1990)*	F: 5-GAACATCATCCCTGCATCCA-3	1455–1474	
		R: 5-CCAGTGAGCTTCCCGTTCA-3	1532–1514	78
RARβ	Zelent *et al.* (1989)†	F: 5-CAGCTGGGTAAATACACCACGAA-3	786–808	
		R: 5-GGGGTATACCTGGTACAAATTCTGA-3	1012–988	227
RXRβ/γ	Mangelsdorf *et al.* (1992)‡	F: 5-AGGCAGGTTTGCCAAGCTTCTG-3	1361–1382	
		R: 5-GGAGTGTCTCCAATGAGCTTGA-3	1462–1441	102
TRα/β	Murray *et al.* (1988)§	F: 5-TCCTGATGAAGGTGACGGACCTGC-3	1247–1270	
		R: 5-TCAAAGACTTCCAAGAAGAGAGGC-3	1364–1341	118
RC3	Watson *et al.* (1990)‖	F: 5-GCTCCAAGCCAGACGACGATATTC-3	29–53	
		R: 5-CTTCTTCTATTTCTCGCCTCTCAC-3	152–128	127

GAPDH, glyceraldehyde 3-phosphate dehydrogenase; RAR, retinoic acid receptor; RXR, retinoid X receptor; TR, triiodothyronine receptor; RC, neurogranin.
* From murine GAPDH cDNA.
† From murine RARβ cDNA.
‡ From murine RXRβ cDNA.
§ From rat TRβ cDNA.
‖ From rodent RC3 cDNA.

Assay for tissue transglutaminase activity

Brain homogenates for the tTG assay were prepared as previously described by Alfos *et al.* (1996). Tissue transglutaminase-specific activity was measured by detecting the incorporation of [^{3}H]putrescine into N,N′-dimethylcasein.

Serum retinol assay

Serum retinol was assayed by HPLC according to the method of Leclercq & Bourgeay-Causse (1981).

Liver retinol and retinyl palmitate assay

Liver retinol and retinyl palmitate were assayed by HPLC according to the method of Savouré *et al.* (1996).

Proteins

Proteins were determined according to Bradford (1976) using a Bio-Rad protein assay (Bio-Rad Laboratories, Munich, Germany).

Statistical analysis

Values are given as means and standard errors of the mean. The statistical significance of differences between means was calculated by ANOVA followed by Student's *t* test ($P<0{\cdot}05$) using Minitab Statistical Software (State College, PA, USA).

Results

Growth curve

Fig. 1 shows the effects of vitamin A-deprived diet consumption during 10 weeks in vitamin A-deficient and control rats. After 10 weeks the body weight of vitamin A-deficient rats was significantly lower than that of control. The average difference between body weights of the two groups after 10 weeks was 36·35 g.

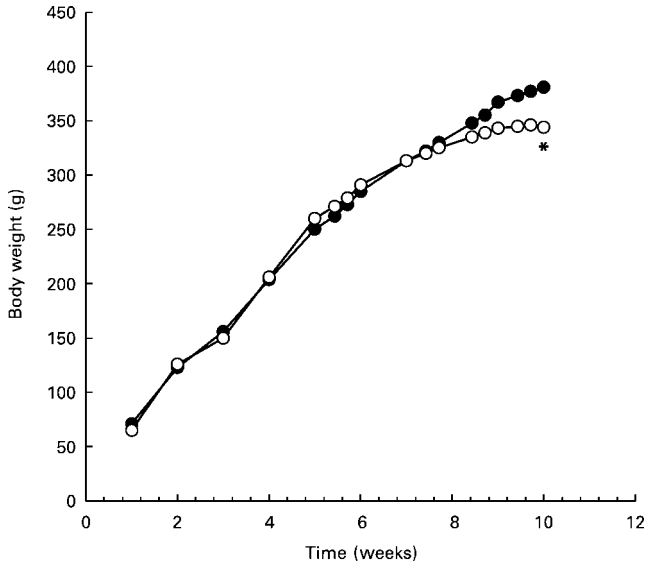

Fig. 1. Effect of 10 weeks consumption of vitamin A-depleted diet on body weight. Each point is the mean for twelve rats. (–●–), control rats; (–○–), depeleted rats. *Mean value was significantly different from that of the control animals.

Effect of vitamin A deficiency on liver retinol and retinyl palmitate

The liver retinyl palmitate and retinol concentrations were measured in control and depleted rats. Retinyl palmitate appeared nearly undetectable in vitamin A-deficient rat liver (<1 *v.* 355 (SEM 29) nmoles/g liver in control rats) after 10 weeks of depleted diet consumption. On the other hand, liver retinol concentrations were 67 % lower than in control animals (5·9 (SEM 1·8) *v.* 18·0 (SEM 2·3) nmoles/g liver).

Effect of vitamin A deficiency on serum retinol and triiodothyronine

Serum retinol concentration was significantly diminished by VAD (0·31 (SEM 0·03) *v.* 1·31 (SEM 0·10) μmol/l in depleted and control rats, respectively). In contrast, T_3 serum levels were unchanged in deficient animals compared with controls (1·06 (SEM 0·11) *v.* 1·01 (SEM 0·07) nmol/l in depleted and control rats, respectively). Data are for the measures performed on six animals.

Effect of vitamin A deficiency on nuclear receptors and target genes

The results are summarized in Tables 3 and 4.

*Effect on all-*trans*-retinoic acid nuclear receptor, retinoid X receptor and tissue transglutaminase*

In accordance with our previous results obtained in the brain of vitamin A-depleted mice (Enderlin *et al.* 2000), the expression of RARβ, RXR β/γ and the activity of tTG decreased in the rat brain after 10 weeks of the depleted diet. Indeed, in these rats, the amounts of RARβ and RXRβ/γ mRNA were lower (−36 and −24 %, respectively) than in the brain of control rats (Table 3). Simultaneously, a significantly reduced tTG activity (−35 %) was observed (234 (SEM 20) *v.* 362

Table 3. Influence of vitamin A-deficient diet on the abundance of retinoic acid (RARβ) and triiodothyronine (TRα/β) nuclear receptor (RXRβ/γ) mRNA in rat brain†

(Mean values and standard errors of the mean for six animals)

	mRNA (% GAPDH)					
	RARβ		RXRβ/γ		TRα/β	
	Mean	SEM	Mean	SEM	Mean	SEM
Controls	0·150	0·010	0·920	0·046	7·96	0·48
Depleted	0·096*	0·009	0·700*	0·035	5·55*	0·36

GAPDH, glyceraldehyde 3-phosphate dehydrogenase; RAR, retinoic acid receptor; RXR, retinoid X receptor; TR, triiodothyronine nuclear receptor.
* Mean values were significantly different from those of the control animals ($P<0{\cdot}05$). (ANOVA followed by Student's *t* test).
† For details of diet and procedures, see Table 1 and pp. 192–193.

Table 4. Influence of vitamin A deficiency on the abundance of neurogranin mRNA and protein in rat brain†

(Mean values and standard errors of the mean for six animals)

	Neurogranin			
	mRNA (% GAPDH)		Protein (% controls)	
	Mean	SEM	Mean	SEM
Controls	102	6	100	5
Depleted	74*	4	63*	3

GAPDH, glyceraldehyde 3-phosphate dehydrogenase.
*Mean values were significantly different from those of the control animals ($P<0{\cdot}05$) (ANOVA followed by Student's t test).
† For details of diet and procedures, see, Table 1 and pp. 192–193.

(SEM 20) fmol/h per mg protein in depleted and control rats, respectively).

Effect on triiodothyronine nuclear receptor

Together with a reduced expression of RARβ and RXRβ/γ, VAD led to a decrease in TRα/β mRNA of about 30 % (Table 3). This decrease had previously been shown only in the liver of depleted rats (Pailler-Rodde *et al.* 1991).

Effect on neurogranin

The VAD was accompanied by an alteration in the expression of RC3, a T_3 and RA target gene. Indeed, in depleted rats, a reduced expression of mRNA and protein (−27 and −37 %, respectively) was observed (Table 4). In contrast, immunoblots of depleted and control homogenized brains with β-actin antibody (detected as a single band migrating at 42 kDa) revealed no differences of intensity between these two groups. The results concerning RC3 were in agreement with previous results showing a similar decrease in depleted mice (Etchamendy *et al.* 2000).

Effect of retinoic acid and/or triiodothyronine administration on nuclear receptors and target genes

The results are summarized in Figs. 2, 3 and 4.

Effect on retinoic acid receptor, retinoid X receptor and tissue-type transglutaminase

Following RA and/or T_3 administration in vitamin A-depleted rats, increased amounts of RARβ (+34 % with RA, + 35 % with T_3 and +57 % with RA and T_3) and RXRβ/γ (+ 31 % with RA, +24 % with T_3 and +30 % with RA and T_3) mRNA were observed (Fig. 2). This also led to an increase in tTG activity of about 85 % with RA, 50 % with T_3 and 90 % with RA and T_3 (Fig. 3).

Effect on triiodothyronine nuclear receptor

In vitamin A-deficient rats, the abundance of TRα/β mRNA was unchanged after administration of RA. On the other hand, an increase in mRNA level after administration of T_3 with or without RA of about 27 and 23 %, respectively, was observed (Fig. 2).

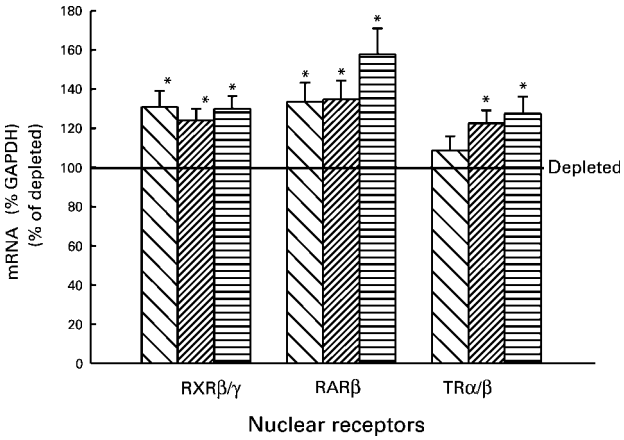

Fig. 2. Effect of retinoic acid (RA) and/or triiodothyronine (T_3) administration (150 μg/kg body weight per d for 4 d) on retinoid X receptor (RXRβ/γ), retinoic acid receptor (RARβ) and triiodothyronine receptor (TRαβ) mRNA levels in the brain of vitamin A-deficient rats. Data represent the mean values of the measures performed on six animals with the standard errors of the mean represented by vertical bars. *Mean value was significantly different from that of depleted animals ($P<0{\cdot}05$). Data were analysed using ANOVA followed by Student's t test. (▨), depleted +RA; (▨), depleted + T_3; (▤) depleted + RA + T_3; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Effect on neurogranin

Concerning RC3, the administration of T_3 with or without RA increased RC3 mRNA (+ 35 and + 32 %, respectively) and protein (+ 131 and + 38 %, respectively) abundance, whereas no change was observed after administration of RA (Fig. 4). Moreover, immunoblots revealed no treatment-related differences between groups concerning the intensity of β-actin.

Discussion

Our data showed that rats fed a vitamin A-deprived diet exhibited a hypo-activity of the retinoid signalling pathway, characterized by a decreased amount of RARβ and RXRβ/γ mRNA and tTG activity in the brain with respect to control rats. Comparable results have already been obtained in the rat brain (Verma *et al.* 1992; Yagamata *et al.* 1993), and recently in vitamin A-deficient mouse brain (Enderlin *et al.* 2000). The present study also revealed that VAD impaired the cellular action of T_3 with consequences in the brain. Indeed, it provides the first evidence for a decreased expression of the TR mRNA in vitamin A-depleted rat brain, and, as previously observed in mice (Etchamendy *et al.* 2000), a hypo-expression of the amount of mRNA as well as of proteins of RC3 which is a T_3 target gene. These results were coherent with data obtained in vitamin A-deficient rat liver, which have revealed a decreased transport of T_3 into target cells (Higueret & Garcin, 1984; Pailler-Rodde *et al.* 1991). Besides, in the present work, the cellular activity

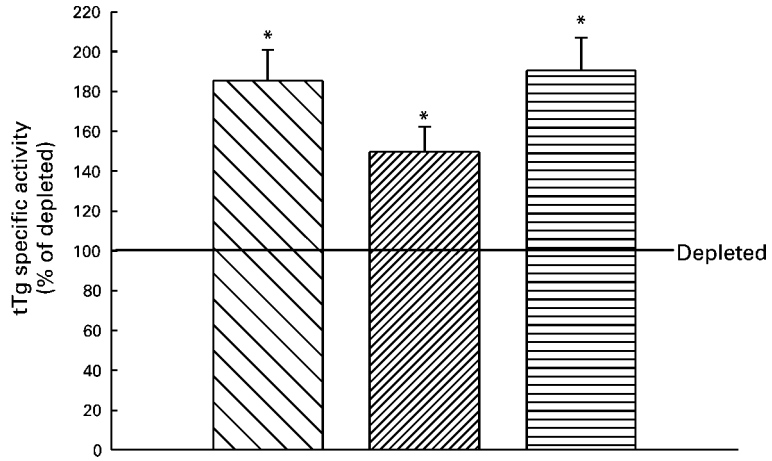

Fig. 3. Effect of retinoic acid (RA) and/or triiodothyronine (T_3) administration (150 μg/kg body weight per d for 4 d) on tissue-type transglutaminase activity in the brain of vitamin A-deficient rats. Data represent the mean values of the measures performed on six animals, with the standard errors of the mean represented by vertical bars. *Mean value was significantly different from that of depleted animals ($P<0{\cdot}05$). Data were analysed using ANOVA followed by Student's *t* test. (▧), depleted +RA; (▨), depleted + T_3; (▤) depleted + RA + T_3.

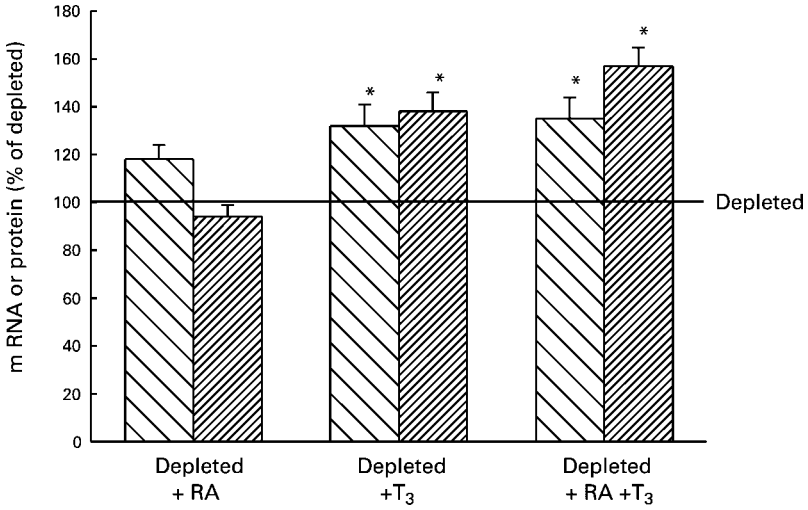

Fig. 4. Effect of retinoic acid (RA) and/or triiodothyronine (T_3) administration (150 μg/kg body weight per d for 4 d) on neurogranin mRNA (▧) and protein (▨) levels in the brain of vitamin A-deficient rats. Data represent the mean values of the measures performed on six animals, with the standard errors of the mean represented by vertical bars. *Mean value was significantly different from that of depleted animals ($P<0{\cdot}05$). Data were analysed using ANOVA followed by Student's *t* test.

of malic enzyme, which is controlled by T_3 in rat liver, kidney and heart (Jeannin *et al.* 1998) was decreased by about 50 % in vitamin A-deficient rat liver (data not shown) indicating that the cellular action of T_3 is decreased in vitamin A-deficient rats. Thus, the decreased amount of TR mRNA observed in depleted rats would be, indeed, the result of a decreased T_3 cellular bioavailability.

Present data are coherent with several findings which revive the concept of permanent interactions between thyroid hormone and vitamin A metabolisms. For example: (i) epidemiological data suggest that low serum retinol levels favour the appearance of goitrous disease in a manner comparable to I deprivation (Ingenbleek & De Visscher, 1979); (ii) the enhancement of retinoid pathways seems to depend on the secretory rate of transthyretin (which conveys thyroid hormone) (Ingenbleek & Bernstein, 1999). Moreover, previous study has revealed that the modulation of the binding properties of RAR as well as of TR by RA was dependent on thyroid status (Pallet *et al.* 1994).

As observed in vitamin A-depleted mice, the reactivation by RA treatment of its own signalling (auto-regulation) was demonstrated in depleted rats by a normalization of the expression of brain receptor (RARβ and RXRβ/γ) and tTG activity. Nevertheless, whereas in rats that are not vitamin A-deficient, RA is able to up regulate RC3 (Enderlin *et al.* 1997; Etchamendy *et al.* 2001), in vitamin A-deficient rats the administration of RA failed to normalize the expression of RC3 as well as of TR. Thus, to evaluate the involvement of T_3 on RA signalling in depleted rats, RA administration was compared with T_3 administration or T_3 and RA co-administration. Interestingly, the results showed that the administration of T_3 alone is able to reverse its own brain hypo-signalling

(auto-regulation) simultaneously with that of RA (hetero-regulation) and the hypo-expression of RC3 mRNA and proteins. Moreover, our experiments revealed a synergetic effect of RA and T_3, first on the mRNA expression of RAR which increased by about 35 % and 57 % after either T_3 or T_3 + RA administration, respectively, and second, on the protein expression of RC3. Therefore, the regulation of RC3 by RA in VAD is dependent on T_3 levels in spite of a RA-responsive element in the RC3 gene promoter.

Our results indicate that one of the consequences of VAD is a dysfunction in the thyroid signalling pathway in the brain. This seems of crucial importance since the down regulation of RC3 observed in the depleted rats was corrected only by T_3. It seems that in vitamin A-deficient rats, hypo-activity of T_3 signalling becomes a limiting factor, which impairs RA from exerting its modulator effect. In comparison with previous works in depleted mice, showing that a sufficient level of vitamin A was required for the maintenance of mature brain function, the novel finding here is that vitamin A seems effective through the preservation of the integrity of the T_3 signalling.

Vitamin A deficiency seems to be associated with integrative and probably adaptive processes, suggesting that many physiological functions, at least vitamin A and T_3 signalling, are mobilized and become stabilized at new levels far from homeostatic equilibrium. In our opinion, this situation corresponds to the allostatic state described by Sterling & Eyer (1988) where the organism resets the parameters of its physiological systems at a new set point, and matches them appropriately to the chronic lack of vitamin A. If the lack continued, the allostatic maladaptation would lead to breakdown (neurobiological disorders) and illness.

More generally our results suggest that vitamin A-depleted animals develop signs of cellular hypothyroidism, since rats exhibit thyroid disorders characterized by alterations of the brain T_3 signalling and related target-gene hypo-expression which is reversed only by T_3 administration. Given the VAD-related neurological alterations, further investigation would provide insights into VAD management, a public health problem in many areas of the world, according to its severity.

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