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M A Iñiguez, … , G Morreale de Escobar, J Bernal

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Research Article

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Adult Rat Brain Is Sensitive to Thyroid Hormone

Regulation of RC3/Neurogranin mRNA

Miguel A. Iñiguez, Angeles Rodriguez-Peña, Nieves Ibarrola, G. Morreale de Escobar, and Juan Bernal
Instituto de Investigaciones Biomédicas, Consejo Superior de Investigaciones Cientificas, Facultad de Medicina, Universidad Autónoma de Madrid, 28029 Madrid, Spain

Abstract

The mammalian brain is considered to be poorly responsive to thyroid hormone after the so called "critical periods" of brain development, which occur in the rat before postnatal days 15–20. In a previous work (Muñoz, A., A. Rodriguez-Peña, A. Perez-Castillo, B. Ferreiro, J. G. Sutcliffe, and J. Bernal. 1991. *Mol. Endocrinol.* 5:273–280) we have identified one neuronal gene, RC3, whose expression is influenced by early neonatal hypothyroidism and thyroid hormone treatment. In the present work we show that adult-onset hypothyroidism leads to a reversible decrease of RC3 mRNA. Rats thyroidectomized on postnatal day 40 and killed three months later showed a decreased RC3 mRNA concentration in the cerebral cortex and striatum. The same effect was observed in animals made hypothyroid on postnatal day 32 and killed on postnatal day 52. RC3 expression was normal when hypothyroid animals were treated with T4 five days before being killed. In contrast, the mRNA encoding myelin proteolipid protein showed no changes in either experimental situation. RC3 mRNA levels were not affected by food restriction demonstrating that the effect of hypothyroidism was not related to the lack of weight gain. The control of RC3 mRNA is so far the only molecular event known to be regulated by thyroid hormone once the critical periods of brain development are over and could represent a molecular correlate for the age-independent, reversible alterations induced by hypothyroidism in the adult brain. (*J. Clin. Invest.* 1992. 90:554–558.) Key words: hypothyroidism • protein kinase C • dendrites • food restriction • striatum

Introduction

It is widely recognized that thyroid hormones are essential for normal mammalian brain development (1–3). In the human being, lack of adequate levels of thyroid hormones as occurs in endemic iodine deficiency or in untreated sporadic congenital hypothyroidism, may lead to severe and irreversible mental deficiency. The actions of thyroid hormones on brain development occur during narrow developmental windows, or "critical periods", and it is often stated that the brain is poorly responsive to thyroid hormone outside these periods. These concepts developed, among others, from the early work of Eayrs (4) who showed that when thyroidectomy in the rat was delayed beyond 24 days of age, many of the cortical function tests in the hypothyroid animals were not different from those of normal animals. Conversely, alterations induced by early hypothyroidism were reverted by thyroid hormone only if they were administered before 10–15 days of age. In the human being, the alterations induced by congenital hypothyroidism can only be prevented if treatment with thyroid hormone is started early after birth (1–3). However, and despite of the fact that the adult brain has traditionally been considered unresponsive to thyroid hormone, juvenile- or adult-onset hypothyroidism are frequently associated with mental disturbances such as slow ideation, impaired memory, somnolence, and other alterations (5). On the other hand, thyrotoxic patients have hyperactivity, insomnia, shortened reflex relaxation times, etc. In contrast to the effects during brain maturation, these alterations are reversible with adequate treatment.

A major problem in understanding thyroid hormone action on the brain is the lack of biochemical and molecular parameters influenced by thyroid hormones in this organ. Recently we have identified several brain genes whose expression at the mRNA level is influenced by neonatal hypothyroidism (6). Most of them were oligodendrocyte-specific genes encoding myelin proteins, and a neuronal specific gene, known as RC3 (7). This gene encodes an mRNA specifically expressed in the rat brain starting from the first few days after birth. Its expression is restricted to neuronal populations of the cerebral cortex, hippocampus, striatum, and olfactory bulb. The RC3 protein is identical to bovine neurogranin (8), a protein kinase C substrate probably involved in long-term potentiation mechanisms (Sutcliffe, J. G., personal communication). We demonstrated that neonatal hypothyroidism led to a decreased expression of this mRNA (6). Now we show that RC3 is also influenced by adult-onset hypothyroidism, being thus the only neuronal gene known to be modulated by thyroid hormone after the neonatal period, once the critical period of brain maturation has ended.

Methods

Male rats of a Wistar strain were used. They were housed, treated, and killed under recommended humane conditions. Hypothyroidism was induced by surgical thyroidectomy at the age specified for each experiment. In addition, 0.02% methyl-mercapto-imidazol was administered in the drinking water to the thyroidectomized rats until the end of the experiment. T4 was administered by one daily intraperitoneal dose of 2 μg/100 g body wt in saline containing 0.1% BSA, starting 15 d after thyroidectomy, with the last dose injected 24 h before death. The animals were killed under ether anesthesia, the brains quickly removed and placed on ice, and individual regions were dissected out and frozen on dry ice. Total RNA was isolated according to the method of Chomzynski and Sacchi (9) and was used to perform Northern blots on nylon filters (Nytran, Schleicher & Schnell, Dassel, Germany) following standard techniques (10). As a control for the amount of RNA present on the filters, ribosomal RNAs were stained in 0.02% methylene blue solution made in 0.3 M sodium acetate (11). In addition the

Address correspondence to Dr. Juan Bernal, Instituto de Investigaciones Biomédicas, C/Arturo Duperier 4, 28029 Madrid, Spain.

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filters were probed with a cDNA encoding cyclophilin (CF),[1] a ubiquitous mRNA present at adult levels from early fetal development (12), and that is not affected by thyroid status (6). To detect the RC3 mRNA we used a probe provided by Dr. J. G. Sutcliffe (Scripps Clinic, La Jolla, CA) containing the full length RC3 cDNA cloned in pHG327 (7). We also used full length cDNA probes encoding rat proteolipid protein (PLP [13]), the major myelin protein, cloned in pUC18, and neuron-specific enolase (NSE [14]) in pCD (also gifts of Dr. J. G. Sutcliffe). Labeling of the probes was by nick translation using the whole plasmids, with [^{32}P]dCTP (Amersham International, Amersham, Bucks., UK) to specific activities of $\sim 10^8$ cpm/μg. Hybridizations were performed at 42°C in the presence of 50% formamide, following standard procedures (10).

T4 and T3 were determined in plasma by highly sensitive and specific RIAs as adapted for the rat (15). The concentration of both iodothyronines in the cerebral cortex was determined using the same RIAs after extraction and extensive purification of the individual hemicortices, as previously described (16). In brief, homogenization in methanol is followed by extraction in chloroform-methanol, back-extraction into an aqueous phase, and purification of this phase through AG 1 × 2 resin columns (Bio-Rad Laboratories, Richmond, CA). The iodothyronines are eluted with 70% acetic acid, which is then evaporated to dryness. RIA buffer is added, and the samples are submitted to highly sensitive RIAs for the determination of T4 and T3, the limits of sensitivity being 2.5 pg T4 and 1.5 pg T3/tube. Cross-reactivities for the T4 RIA and T3 RIA were as recently reported (16). Each sample is processed in duplicate at two dilutions. Results are then calculated using individual recovery data obtained after addition of [^{131}I] T4 and [^{125}I] T3 during the initial homogenization process. The amounts of tracers added are such that the radioactivities carried over into the RIA tubes are too low to interfere with the determinations, representing 2–5% of the radioactivity added as labeled antigen.

Data from thyroid hormone concentrations in plasma and cortex were submitted to one-way analysis of variance, after testing for homogeneity of variance using Bartlett's procedure for groups of unequal size. Square root or logarithmic transformations usually ensured homogeneity of variance when this was not found with the raw data. Significance of differences between groups was assessed using the protected least significant difference test, and considered significant when $P < 0.05$. All these calculations were performed as described by Snedecor and Cochran (17).

Results

Two different kinds of experiments were performed. First, we studied whether long-term hypothyroidism starting after the first month of life had any effect on RC3 mRNA levels. Accordingly rats were thyroidectomized on postnatal day 40 (P40) and killed on P120. The brains were dissected out into individual regions and used to prepare total RNA. Northern blots were sequentially probed first with RC3 and then with PLP, and CF after stripping the previous probe (Fig. 1). The RC3 message consists of two mRNAs of 1.0 and 1.5 kb in size, which arise from the use of two alternative polyadenylation sites (7). As expected from the known regional distribution of RC3 mRNA, no signal was detected in cerebellum, mesencephalon, hypothalamus, and thalamus. RC3 was expressed in striatum and cortex, where its levels were lower in hypothyroid animals than in normals. These differences were not due to differences in RNA loaded on the gels as shown by the rRNA bands. After correction for CF mRNA content by densitometry, RC3 levels in cortex and striatum of normal animals were 1.5 and 1.7, respectively, whereas in hypothyroid animals the corresponding values were 1.1 and 0.9. Therefore, hypothyroidism led to a decrease of RC3 mRNA of $\sim$ 30% in the cortex and 50% in the striatum. In several experiments we have confirmed that the effect in the cortex, although measurable, is more modest than in the striatum. Using the PLP probe the two mRNAs of 1.6 and 3.2 kb, corresponding to the major spliced transcripts of the PLP gene (13), were detected in all brain regions, with stronger intensity in mesencephalon, thalamus, and hypothalamus. After correction for cyclophilin content, no differences were found in the abundance of PLP in any region after hypothyroidism.

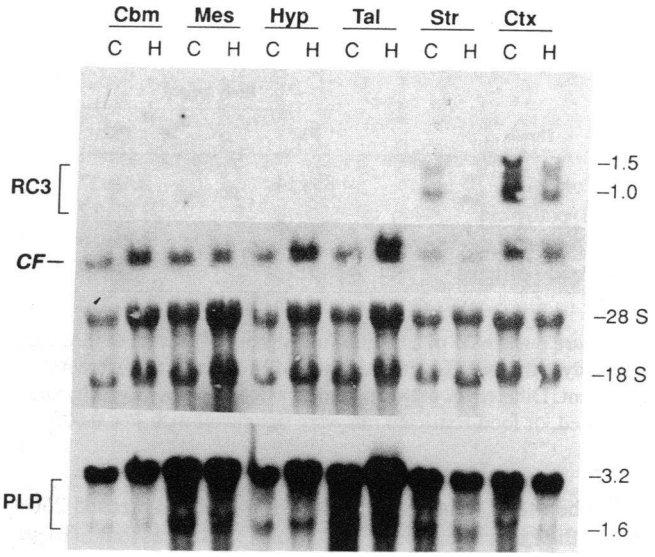

Figure 1. Effect of adult-onset hypothyroidism on RC3 mRNA expression. Northern blot hybridization analysis was performed using total RNA from pooled brain regions of five normal (*C*) and five hypothyroid animals (*H*) of 90 days of age. Animals were made hypothyroid by surgical thyroidectomy at 40 days of age. Brain regions analyzed were cerebellum (*Cbm*), midbrain (*Mes*), hypothalamus (*Hyp*), thalamus (*Tal*), striatum (*Str*), and cerebral cortex (*Ctx*). The blots were first stained with methylene blue to visualize 28S and 18S ribosomal RNAs, and then hybridized sequentially with labeled probes corresponding to the neuronal mRNA RC3 (mRNAs of 1.5 and 1.0 kbp), the myelin mRNA encoding proteolipid protein (PLP; mRNAs of 3.2 and 1.6 kbp), and cyclophylin (*CF;* mRNA of 1.1 kbp). Expression of RC3, but not PLP mRNAs, was lower in hypothyroid animals than in controls.

In a second type of experiments we assessed the effects of short-term hypothyroidism and of T4 treatment. Rats were thyroidectomized on postnatal day 32 (P32). After carefully controlling for body weights the group was split into two sets of rats of similar weights 15 d after thyroidectomy (P47), when the body growth had already slowed down and almost ceased. One of these groups was treated with one daily injection of T4 until 24 h before being killed. To rule out the possibility that the observed effects were due to nutritional alterations related to the poor weight increase of the hypothyroid animals, we also included a group of age-paired food-restricted rats that were given the same amount of food taken by the hypothyroid rats ($\sim$ 60% of normal intake). The growth curve (not shown) and final weights (Table I) of both groups of animals were identical. All the animals, including the control group, were sacrificed on

1. *Abbreviations used in this paper:* CF, cyclophilin; NSE, neuron-specific enolase; PLP, proteolipid protein.

Table I. Effects of Hypothyroidism, Food Restriction, and T4 Treatment on Body Weight

Group	Body weight P32	Body weight P52
Control	80±14	200±37
Hypothyroid	79±10	130±12
T4-treated		139±15
Food-restricted	80±13	132±10

Results are mean±SD of six animals per group. Differences in weight among hypothyroid, T4-treated, and food-restricted rats were not significant. Differences between control rats with either hypothyroid, T4-treated, or food-restricted rats were significant with $P < 0.005$.

P52. The mean weight of the normal animals increased 250% during the experimental period (Table I), whereas that of hypothyroid as well as food-restricted rats increased by 160%. T4 treatment led to a slight increase in mean body weights but the differences with either control or hypothyroid rats were not significant.

Hypothyroid rats had sharply reduced circulating levels of T4 (4% of normal), and undetectable levels of T3 in their plasma (Fig. 2). Hormonal reductions were also apparent in the cerebral cortex, were the concentrations of T4 and T3 were ~ 20% of normal. Food restriction did not alter plasma or cortex T4 but lowered plasma T3 by 25% and cortex T3 by almost 40%. After T4 treatment plasma T4 and T3 were still very low, and cortex T4 and T3 increased roughly to ~ 30 and 50%, respectively, of normal control values.

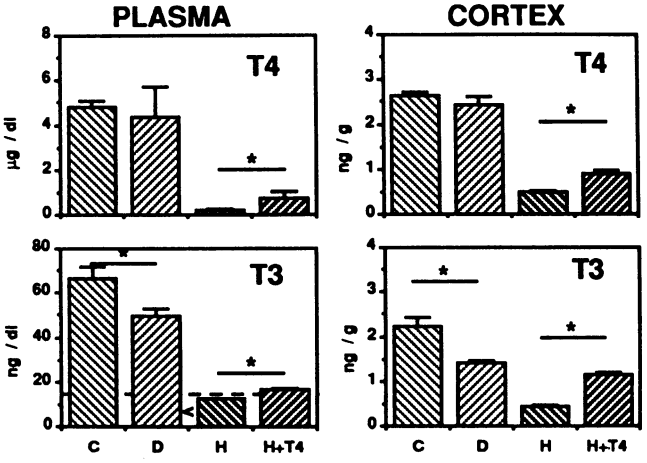

Figure 2. Effect of hypothyroidism, T4 administration, and food restriction on thyroid hormone concentrations. Rats were thyroidectomized (*H*) at 32 days of age (P32) and killed at P52, together with normal animals of the same age (*C*) and thyroidectomized animals that received a single daily dose of 2 μg of T4/100 g body wt (*H + T4*). Food-restricted rats (*D*) were normal, age-paired animals that were given the same amount of food taken daily by the hypothyroid rats so that their growth curve was identical. Results shown are mean±SEM of six to eight animals per group. Asterisks indicate a statistical significant difference at $P < 0.05$ or less, between C and D, or between H and H + T4. Plasma and cortex T4 and T3 for H and H + T4 animals were lower than for either C or D animals, except for T3 in the cortex which was the same for H + T4 and D groups.

The effects of hypothyroidism, T4 treatment, and food restriction on RC3 mRNA abundance are shown in Figs. 3 and 4. Fig. 3 shows the result of Northern blot hybridization to total RNA from striata obtained from normal, hypothyroid, and T4-treated hypothyroid rats. The samples loaded on each lane of the gel were pooled samples from two animals. Visual inspection of the blots revealed that hypothyroid animals contained less RC3 mRNA than either normal controls or hypothyroid animals treated with T4. In contrast no effect of hypothyroidism was evident when the blot was probed with a cDNA encoding neuron-specific enolase. We have previously shown that NSE mRNA is not altered by neonatal hypothyroidism (6), and the same can be said of adult-onset hypothyroidism. The same blot, hybridized with the PLP probe showed that, in agreement with the result of Fig. 1, late-onset hypothyroidism had no effect on the levels of PLP mRNA. Fig. 3 also shows that differences in the amounts of RC3 mRNAs between hypothyroid animals and controls or T4 treated animals, were not the result of differences in the amount of RNA loaded. It can be seen that within each group of animals individual differences in the intensity of the RC3 or PLP mRNA bands correlated with the intensity of rRNA staining. The bands were also quantified by densitometry and corrected for CF content as above. The results are shown in Fig. 4 (*left* and *middle panels*). RC3 mRNA content in hypothyroid animals was 35% of controls and was increased by T4 treatment approaching normal levels (*middle panel*). In contrast, no parallel changes were observed for PLP (*left panel*), although its mean levels were slightly

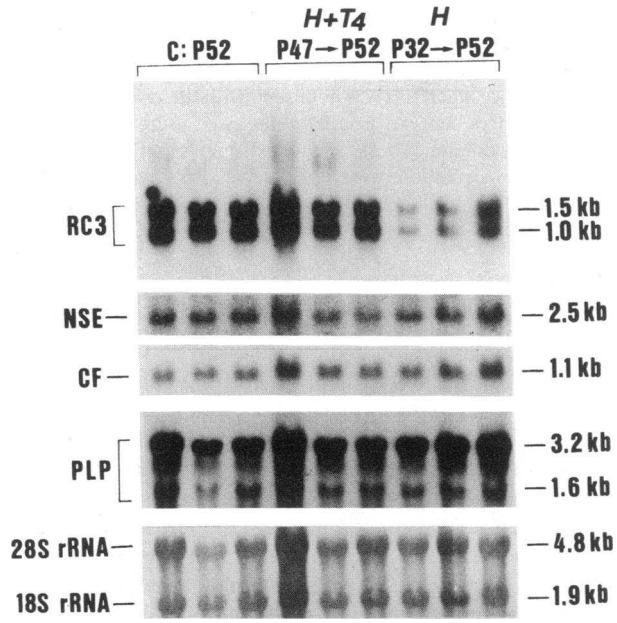

Figure 3. Effect of hypothyroidism and of T4 treatment on RC3 mRNA expression. Rats were thyroidectomized (*H*) at 32 days of age (*P32*) and sacrificed at P52, together with normal animals of the same age (*C*) and thyroidectomized animals that received a single daily dose of 2 μg of T4/100 g body wt (*H + T4*). Individual striata were collected and pooled in duplicates for total RNA isolation. Northern blots were performed as described in the legend to Fig. 1 and were hybridized sequentially with labeled probes corresponding to RC3, proteolipid protein (*PLP*), cyclophylin (*CF*), and neuron-specific enolase (*NSE*). Hypothyroidism decreased expression of RC3, an effect that was prevented by T4 treatment.

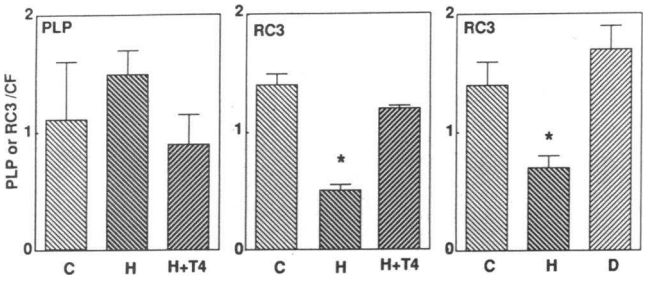

Figure 4. Effect of hypothyroidism, T4 administration, and food restriction on mRNA abundance. The data reported were obtained after quantification by densitometry of the PLP and RC3 bands after Northern blot hybridization, and are expressed as the ratio to the signal obtained for the control mRNA cyclophylin. *C,* normal animals. *H,* hypothyroid animals. *H + T4,* hypothyroid animals treated with T4. *D,* food-restricted normal animals. Data are mean±SD. Asterisks indicate a significant difference with the control group at $P < 0.005$.

higher in hypothyroid animals. Fig. 4 (*right panel*) shows that food restriction, in contrast to hypothyroidism, did not lower RC3 levels.

Discussion

In a recent survey of brain genes that might be influenced by neonatal hypothyroidism and thyroid hormone administration we found that the expression of the neuronal gene RC3 is altered by neonatal hypothyroidism (6). RC3 was recently cloned by Watson et al. (7) by subtracting cerebellar RNA sequences from cerebral cortex RNA, and it is identical to the protein kinase C substrate p17 or neurogranin purified by Baudier and colleagues (8). Protein kinase C is abundant in the brain and it has been implicated in important processes such as neuronal differentiation, transmitter release, and neuroplasticity (18). RC3/neurogranin has sequence similarity with another kinase C substrate, the growth cone–associated protein, GAP-43, or neuromodulin (8, 19), which in contrast to RC3 is not regulated by thyroid hormone (Iñiguez, M. A., A. Rodriguez-Peña, N. Ibarrola, A. Muñoz, and J. Bernal, manuscript submitted for publication). RC3/neurogranin is one of the few neuronal genes that has been shown to be regulated by thyroid hormone at the mRNA level. Other mRNAs that we found to be affected by neonatal hypothyroidism were nonneuronal and encoded myelin proteins (6). In the present work we show that RC3 is sensitive to thyroid hormone in rats after the first month of life. This is the first indication that thyroid hormone action in the adult brain also involves regulation of specific mRNA species, a concept that has been demonstrated in other tissues (20), but has faced numerous difficulties in the case of the brain. In clear contrast with RC3, the myelin mRNA encoding PLP, which is affected by neonatal hypothyroidism (6) is apparently unaffected by juvenile hypothyroidism. The effect on RC3 is not a general neuronal effect, since another neuronal gene, NSE, was not affected.

The results shown in this work rule out the possibility that the observed effects of hypothyroidism on RC3 mRNA abundance are the consequence of a nutritional deficit due to the lower food intake of hypothyroid animals compared with normals. Food restriction resulted in a weight gain identical to that of hypothyroid rats but did not alter RC3 mRNA abundance. On the other hand, food restriction lowered T3, but not T4, concentrations in plasma and brain. Other authors have also shown a lowering effect of food restriction on plasma T3 concentration (21, 22). There are few data on the effect of food restriction on brain thyroid hormone concentration. Van Doorn et al. (21) found a decreased concentration of T3 in the cerebral cortex, which was partially due to decreased local generation of T3, whereas Kaplan and Yaskoski (23) found lower activity of cortex T4 5′ deiodinase in neonatal, but not in adult, rats as a result of food deprivation. In a recent study Sanchez and Jolin (22) reported no effect of food deprivation on cortex nuclear T3 receptor occupancy. Therefore it is likely that despite the decreased T3 concentration in cortex tissue the actual receptor occupancy is normal, which would agree with the lack of effect of food restriction on the RC3 message.

Although the mRNA encoding RC3 is so far the only mRNA shown to be regulated by thyroid hormone in the brain of young adult rats, some end points of thyroid hormone action during brain development retain their capacity to respond to thyroid hormone in juvenile and adult rats (24–27). Alterations of dendritic spines in neonatal- and adult-onset hypothyroidism have been described previously by Morreale de Escobar and co-workers (24–26). Early hypothyroidism, i.e., thyroidectomy performed on P10, induces changes in the number and distribution of dendritic spines along the apical shaft of pyramidal cells of the visual cortex. This effect could be prevented completely only if T4 treatment was started early after thyroidectomy. However, hypothyroidism induced by thyroidectomy on P40 or P120 also induced rapid changes, i.e., within five days (26), in dendritic spines and late T4 treatment had an ameliorating effect, suggesting that these morphological effects were reversible. More recently Gould et al. have reached similar conclusions regarding the effects of adult hypothyroidism and hyperthyroidism of dendritic spine density of pyramidal cells of the hippocampus (27). Since the RC3 protein has been shown by immunohistochemistry to be localized in the dendrites the control of the RC3 gene might be related to these morphological changes.

In conclusion, the control of the RC3 gene expression at the mRNA level might represent the first disclosed molecular correlate of age-independent and reversible, clinical, and morphological effects of thyroid hormone action within the brain.

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