

Differential Effects of Triiodothyronine and the Thyroid Hormone Receptor β -Specific Agonist GC-1 on Thyroid Hormone Target Genes in the Brain

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The availability of synthetic thyroid hormone receptor agonists provides a valuable tool to analyze whether specific receptor isoforms mediate specific physiological responses to thyroid hormone. GC-1 is a thyroid hormone analog displaying selectivity for thyroid hormone receptor β . We have analyzed the effect of GC-1 on expression of thyroid hormone target genes in the cerebrum and cerebellum. Congenitally hypothyroid rats were treated with single daily doses of either T_3 or GC-1. Both compounds similarly induced Purkinje cell protein-2 (PCP-2) in the cerebellum. Expression of RC3 and Rhes in the caudate, and hairless, neurotrophin-3, Reelin, and Rev-ErbA α in the cerebellum, was analyzed by *in situ* hybrid-

ization on postnatal d 16. Hypothyroidism strongly decreased expression of RC3 and Rhes in the caudate, and hairless, Rev-ErbA α , and neurotrophin-3 in the cerebellum, and increased Reelin. T_3 treatment normalized the expression of all genes. However, GC-1 effectively normalized expression of Rhes and Reelin only. The lack of a GC-1 effect on most cerebellar genes can be explained by the known distribution of thyroid hormone receptor α and β isoforms. However, in the caudate, RC3 and Rhes are expressed in the same cells, and therefore, they may represent specific gene responses linked to specific thyroid hormone receptor isoforms. (*Endocrinology* 144: 5480–5487, 2003)

THE PHYSIOLOGICAL ACTIONS of thyroid hormone (T_3) are mediated through interaction with nuclear receptors, which are ligand-modulated transcription factors containing hormone and DNA-binding domains (1). There are several T_3 receptor proteins (TRs), encoded by two distinct genes, TR α and TR β . The TR α gene encodes three proteins, TR α 1, TR α 2, and TR α 3, that differ in their carboxyl terminus. TR α 1 binds T_3 and activates or represses target genes, whereas TR α 2 and TR α 3 do not bind T_3 and may antagonize T_3 action (2, 3). The TR α gene also produces two truncated proteins known as $\Delta\alpha$ 1 and $\Delta\alpha$ 2, which have a role in intestinal development (4). The TR β gene produces several amino-terminal protein variants, TR β 1, TR β 2, and TR β 3, and the truncated protein Δ TR β 3 (5), which lacks the DNA-binding domain and might therefore compete with T_3 receptors for available T_3 .

The physiological roles and specific functions of the T_3 receptor isoforms are being elucidated using two complementary approaches. One is the use of mutant mice that lack the expression of single or multiple products of the TR genes (6, 7). The phenotypes of TR-deficient mice, although not totally coincident with that of hypothyroid animals, have allowed relating some discrete specific functions to individual receptor isoforms. The TR β gene is involved in the regulation of TSH secretion, liver metabolism, and hearing (8, 9), and the specific product TR β 2 regulates the development of a specific subset of photoreceptors involved in color vision

(10); TR α 1 controls myocardial activity (11) and intestinal development (12, 13).

A different approach relies on the use of T_3 receptor isoform-specific agonists. GC-1 is a TR β -selective, T_3 analog that has an affinity for TR β equal to that of T_3 and one order of magnitude lower affinity for TR α 1 (14, 15). This compound, when administered to hypothyroid rats or mice, has similar effects as T_3 on plasma TSH, cholesterol, and triglycerides, liver malic enzyme, and brown adipose tissue uncoupling protein-1. These responses may therefore be linked to TR β . In contrast, GC-1 has no effect on heart function and heart gene expression, which are TR α related. In most cases, the differential effect of GC-1 on physiological endpoints agrees with the spatial distribution of thyroid hormone receptor isoforms, supporting the view that the receptor isoforms are equivalent in their gene targets, and their specific physiological functions depend on their tissue and cell distribution. However, during adaptive thermogenesis GC-1 was able to induce brown fat uncoupling protein-1 mRNA as T_3 did but did not potentiate adrenergic responsiveness. These results suggested that TR α and TR β are involved in two different pathways contributing to adaptive thermogenesis (16).

The brain is an important target of thyroid hormone, both in developing and in adult animals. All receptor isoforms are expressed in brain, although the TR α 1 protein accounts for about 70–80% of total receptor content (17, 18). In some regions, such as the cerebellum, expression of the TR α and TR β genes are segregated into specific cells, but in most regions both genes are coexpressed although in different proportions (19, 20). The relative importance of TR α vs. TR β may depend on its relative quantitative expression, but, given the extraordinary cell complexity of the brain, defini-

Abbreviations: BW, Body weight; CAT, chloramphenicol acetyl transferase; Hr, hairless; NT-3, neurotrophin-3; PCP-2, Purkinje cell-specific protein-2; TR, T_3 receptor protein; TRE, thyroid hormone-responsive element.

tion of specific functions of each isoform is a most difficult task. Recent indications of isoform specificity was provided recently by us showing that migration of granular cells in the cerebellum is a TR α -dependent response, whereas maturation of Purkinje cells depends on both TR α and TR β (21). Also, we found that hippocampal interneurons express differentially the TR α 1 isoform over TR β , and adult TR α 1-deficient animals have behavioral alterations that may be related to dysfunction of these interneurons (22). In this work we have analyzed the effects of GC-1 on expression of a panel of selected brain genes. We found that GC-1 was without effect on most genes expressed in the cerebellar granule cell layer in agreement with the predominant expression of TR α in these cells. However, in the caudate, GC-1 increased the expression of Rhes, but not RC3, suggesting that different receptor isoforms may regulate different sets of genes in the same cell.

Materials and Methods

Animals and treatments

Rats from the Wistar strain were bred in our animal facilities. Animal care procedures were conducted in accordance with the guidelines set by the European Community Council Directives (86/609/EEC). Animals were under temperature (22 ± 2 C) and light (12-h light, 12-h dark cycle; lights on at 0700 h) controlled conditions and had free access to food and water in the colony room. Hypothyroidism was induced by administering 0.02% 2-mercapto-1-methylimidazole (Sigma Chemical Co., St. Louis, MO) and 1% sodium perchlorate in the drinking water to pregnant dams from embryonic d 9 and throughout the experiment. Hormone treatments consisted of daily single ip injections of 15 ng T₃ (Sigma) per gram body weight (BW), which represents five times the total T₃ production rate of about 3 ng/g (23). The TR β -selective compound GC-1 was administered at a dose of 10 ng/g BW. We used the male neonates from 16 dams, *i.e.* four litters for each group of control, hypothyroid, and T₃- or GC-1-treated hypothyroid pups. Treatments were started on postnatal d 11 (P11), and the rats were killed 24 h after the last injection on P16, the time of maximum T₃ responsiveness of most brain target genes. For TSH, T₄, and T₃ determinations we used two pups from each litter, *i.e.* a total of eight pups per condition. For *in situ* hybridization, one rat of each litter, *i.e.* four rats per condition, was used after random selection. To check for effects of GC-1 on body growth, an additional group of animals was treated from P5 through P25. Plasma TSH levels were measured using immunoreactants kindly supplied by the Rat Pituitary Agency of the National Institutes of Arthritis, Diabetes, and Kidney Diseases of the National Institutes of Health (Bethesda, MD), as previously described (23). Thyroid hormone concentrations were measured as previously described (23). The TR α 1^{-/-} and wild-type mice were supplied by Björn Vennström (Karolinska Institute, Stockholm, Sweden). For *in situ* hybridization, four P20 wild-type or TR α 1^{-/-} mice from different litters were processed.

RNA analysis

Northern blotting was performed following standard methods (25) using 20 μ g of pituitary total RNA, 50 μ g of heart total RNA, and 0.5 μ g of cerebellum poly (A)⁺ RNA. The RNA was isolated from pooled tissues of 16 pituitaries, four hearts, and eight cerebella for each condition. A 1.6-kb fragment from the 5' end of the sarcoplasmic calcium adenosine triphosphatase (Serca-2) cDNA (a gift from Dr. W. Dillman, University of California, San Diego, CA), the full-length cDNA of the GH gene, and a 440-bp EcoR1-PstI fragment from the cDNA encoding the Purkinje cell-specific protein (PCP-2) (a gift from Dr. Cary N. Mariash, University of Minnesota, Minneapolis, MN) were used to prepare the radioactive probes by the random priming procedure using the Ready-To-Go DNA Labeling Beads (Amersham Pharmacia Biotech Inc., Piscataway, NJ). As a control for the amount of RNA present in the filters, blots were hybridized with a glyceraldehyde-3-phosphate dehydrogenase probe. The blots were quantitated after autoradiography using

the Scion Image program version 4.02 (Scion Corp., Frederick, MD; <http://www.scioncorp.com>).

In situ hybridizations

Animals were perfused transcardially with buffered 4% paraformaldehyde, the brains were cryoprotected, and 25- μ m sagittal sections were obtained in a cryostat. *In situ* hybridization with radioactive or digoxigenin-labeled riboprobes was performed on floating sections following procedures previously described in detail (26). Sense and antisense riboprobes were synthesized from the following cDNA templates as follows: Rhes, nucleotides 1431–1869; RC3, nucleotides 253–486; Reelin, nucleotides 1532–3071; neurotrophin-3 (NT-3), nucleotides 1–875; hairless (Hr), nucleotides 2703–5186; Rev-ErbA α , 600-bp PCR product corresponding to exon 1. For quantification, the autoradiographs were scanned and densitometry was measured with the Scion Image program.

Transfections and chloramphenicol acetyl transferase (CAT) assay

Cos-7 cells were grown and maintained in DMEM supplemented with 10% fetal calf serum. The cells were plated to a density of 250,000 cells/6-cm² plate the day before transfection. The cells were transfected by the calcium phosphate protocol (25) using 5 μ g of the CAT construct, 0.3 μ g of the expression vector containing the nuclear receptors, and 4 μ g of the plasmid PCH110, containing the gene for β -galactosidase as an internal control of transfection efficiency. At 16 h after DNA additions, the medium was changed to medium containing serum that had been depleted of thyroid hormones by treatment with Dowex resin. T₃ or GC-1 was added, and the cells were incubated for 24 h before harvesting for determination of β -galactosidase and CAT activities.

Data analysis

When data from only two groups were available, Student's *t* test was applied. For multiple comparisons we used one-way ANOVA and the protected least-significant differences test, after validation of the homogeneity of variances by the Levene test. For T₄ and T₃ concentrations in liver and neocortex, the analysis was carried out using only the three hypothyroid groups, treated and untreated. Including the control data in the set led to nonhomogeneity of variances, which was probably due to the large differences in values (*i.e.* one order of magnitude) between the control and the hypothyroid hormone concentrations. All calculations were performed with the SPSS statistical package (SPSS Inc., Chicago, IL).

Results

Thyroid hormone concentrations in tissues

Table 1 shows the T₄ and T₃ concentrations in the neocortex and in liver in control animals (C), untreated hypothyroid animals (H), and T₃- or GC-1-treated hypothyroid animals (H + T₃ and H + GC-1), respectively. The data were analyzed by ANOVA, and the result of the analysis is shown in the table footnote. Both T₄ and T₃ concentrations were strongly reduced in the hypothyroid animals. In liver, both T₃ and GC-1 treatment still decreased T₄ levels further, as a reflection of TSH suppression. The data show that GC-1 was as effective as T₃ or even more. In T₃-treated hypothyroid rats, T₃ was still increased 24 h after the last injection both in liver and in the neocortex, although residual T₃ was higher in the liver than in the neocortex. This probably reflects the fact that the entry of T₃ into the brain is restricted compared with the liver. The untreated hypothyroid and GC-1-treated animals showed the same concentrations of T₃, both in liver and in the neocortex, ensuring that the degree of hypothyroidism achieved in the two groups by the antithyroid treat-

TABLE 1. Thyroid hormone concentrations in neocortex and liver

	Control rats	Hypothyroid rats (H)		
		H	H + T ₃	H + GC-1
Neocortex				
T ₄	3.33 ± 0.45	0.31 ± 0.06	0.36 ± 0.08	0.26 ± 0.02
T ₃	2.35 ± 0.12	0.25 ± 0.12	0.67 ± 0.19 ^a	0.24 ± 0.05 ^b
Liver				
T ₄	31.56 ± 5.84	1.11 ± 0.18	0.53 ± 0.06 ^a	0.45 ± 0.08 ^{a,b}
T ₃	2.44 ± 0.28	0.23 ± 0.08	1.75 ± 0.18 ^a	0.20 ± 0.08 ^b

T₄ and T₃ concentrations were measured in the neocortex and liver of six rats per treatment killed on P16. Results are expressed as nanograms of T₄ or T₃ per gram of tissue wet weight (mean ± SD). Data from the three hypothyroid groups were subjected to one-way ANOVA, with the following results: neocortex T₄: F_{2,9} = 2.47; P = 0.14 neocortex T₃: F_{2,9} = 13.27; P = 0.021; liver T₄: F_{2,9} = 33.7; P = 0.0001; liver T₃: F_{2,9} = 190.6; P = 0.0000. The result of the *post hoc* analysis is indicated in the table as:

^a The differences were significant as compared with the untreated hypothyroid groups; and ^b the differences were significant comparing the T₃- and GC-1-treated groups.

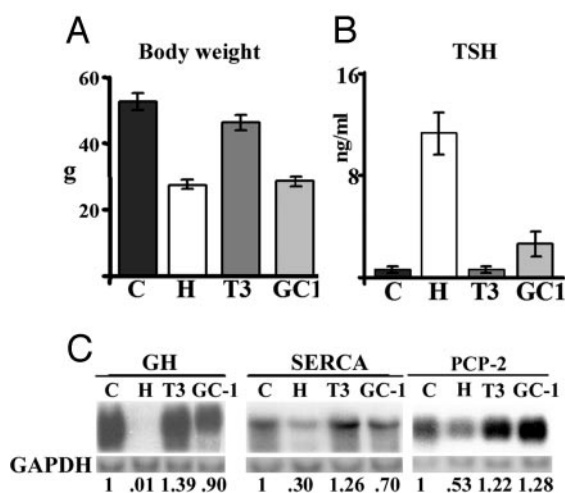


FIG. 1. Effects of T₃ or GC-1 on several endpoints of thyroid hormone action. **A**, Hypothyroid rats were treated with either T₃ (15 ng/g BW) or GC-1 (10 ng/g BW) starting on P5 until P25, and the weights were recorded. Shown are the weights of eight control rats (C), eight hypothyroid rats (H), and eight T₃- or GC-1-treated, hypothyroid rats. **B**, Serum TSH in P16 control (C) and hypothyroid (H) animals and in hypothyroid animals treated with either T₃ or GC-1 in single daily doses starting on P11. **C**, Effects of T₃ and GC-1 on pituitary GH, heart Serca-2, and cerebellar PCP-2 mRNAs. The numbers under the blots are the ratios of each target gene *vs.* the control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) after scanning the blots and densitometry measurements.

ment was similar. For the caudate and cerebellum, we measured only T₃ in untreated hypothyroid and control samples. The data showed that in the caudate T₃ decreased from 1.92 ± 0.37 ng/g in control animals to 0.17 ± 0.01 ng/g in hypothyroid animals (P < 0.001), and in the cerebellum from 2.04 ± 0.15 to 0.18 ± 0.03 ng/g (P < 0.001).

Effects of GC-1 administration *in vivo*

We compared the relative effects of GC-1 *vs.* T₃ treatment given in daily doses to hypothyroid rats on BW, circulating TSH, and GH and Serca-2 mRNAs in pituitary and heart, respectively. To assess the effects of T₃ and GC-1 on BW (Fig. 1A), these compounds were administered as single daily injections starting on P5, and the animals were killed at P25. At the end of the experiment, the BW of untreated hypothyroid rats was 56% of controls. In contrast to T₃, which

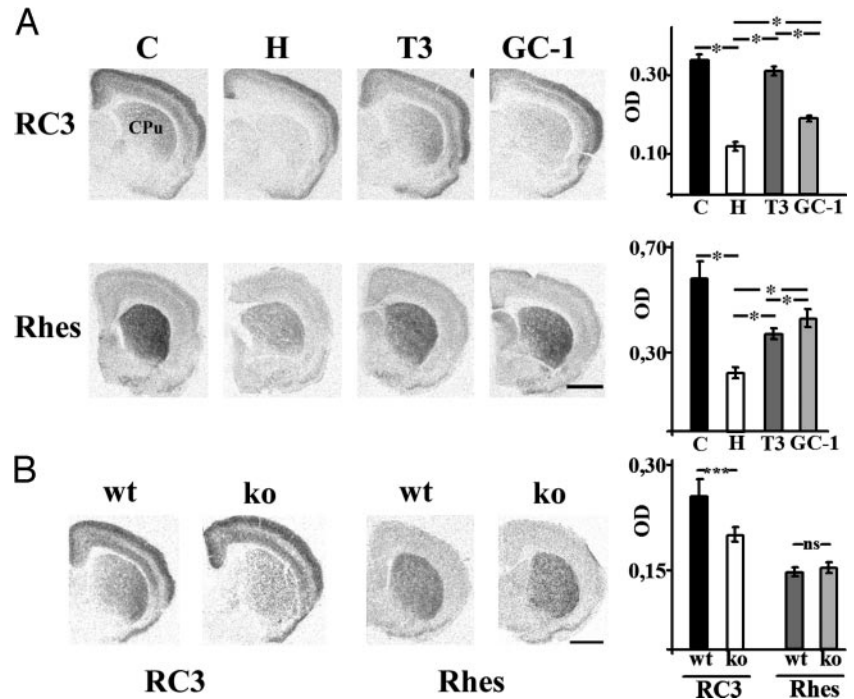
increased BW to near control levels, GC-1 had no effect. Circulating TSH and tissue mRNA levels were measured in P16 rats after treatment with either T₃ or GC-1 for 5 d. The elevated circulating TSH concentrations of hypothyroid animals were normalized by T₃ (Fig. 1B). TSH was also reduced by GC-1, but was less effective than T₃, the difference between the two treatments being statistically significant (P < 0.05).

The responses of selected mRNAs to T₃ or GC-1 administration is shown in Fig. 1C. Pituitary GH mRNA was undetectable in hypothyroid rats. Treatment with either T₃ or GC-1 increased mRNA levels, suggesting that thyroid hormone control of pituitary GH is exerted through TR β . We also analyzed the expression of a myocardial gene, the Serca-2, which is primarily regulated by TR α in the heart (27). The mRNA encoding Serca-2 was decreased in hypothyroid rats and was increased by T₃, whereas the effect of GC-1, if any, was weak. To ensure that GC-1 was reaching the brain and eliciting genomic effects, we analyzed the expression of PCP-2, a gene expressed in Purkinje cells and responsive to GC-1 administration (21). PCP-2 mRNA was decreased in hypothyroid rats and was increased by either T₃ or GC-1, with both compounds having similar effects.

Expression of thyroid hormone-dependent genes in the caudate

We then analyzed the effects of T₃ or GC-1 treatment on the expression of genes known to be under thyroid hormone regulation in different regions of the brain. For this task we selected two genes expressed in the caudate, RC3 and Rhes, and several genes expressed in the cerebellum, namely Hr, NT-3, Reelin, and Rev-ErbA α . The brains were collected after perfusion of the animals and processed for *in situ* hybridization. Figure 2A shows the patterns of expression of RC3 and Rhes in P16 normal, hypothyroid, and hypothyroid rats treated with either T₃ or GC-1. As shown previously, RC3 is expressed, among other regions, in the caudate nucleus (CPu) where it is under strong control on adequate supply of thyroid hormone (28). Rhes is another thyroid hormone-dependent gene abundantly expressed in the caudate (29). In agreement with previous data, we found that hypothyroidism resulted in a strongly decreased expression of both genes in the caudate. Densitometric analysis showed that RC3 decreased from 0.35 ± 0.01 (arbitrary units) in control to 0.11 ±

FIG. 2. Differential effects of T_3 and GC-1 on thyroid hormone target genes. Shown are representative *in situ* hybridization autoradiographs and to the right of the figures the quantification of the respective autoradiograph signal. A, RC3 and Rhes expression by *in situ* hybridization on coronal brain slices of P16 control (C), hypothyroid (H), and hypothyroid rats treated with T_3 or GC-1 starting on P11. RC3 was responsive to T_3 but not GC-1 in the caudate (CPu), whereas Rhes was responsive to both compounds. The data were analyzed by ANOVA, after confirming homogeneity of variances, with the following result: RC3, $F_{3,12} = 397.7$, $P = 0.0000$; Rhes, $F_{3,8} = 15.1$, $P = 0.001$. An asterisk between bars indicates statistically significant differences after the least-significant differences *post hoc* test. B, RC3 and Rhes expression in the caudate of wild-type (wt) and $TR\alpha 1^{-/-}$ (ko) mice. The data were analyzed by using the Student's *t* test for single comparisons. ***, $P < 0.001$; ns, nonsignificant. Scale bar, 1.4 mm.



0.01 in hypothyroid rats. T_3 treatment was able to increase RC3 expression to near normal levels (0.30 ± 0.02). However, the effect of GC-1 on RC3 was weak (0.17 ± 0.01). These results suggested that RC3 was regulated primarily by $TR\alpha 1$. Rhes decreased from 0.60 ± 0.16 in control rats to 0.22 ± 0.03 in hypothyroid animals. Both T_3 and GC-1 increased Rhes to similar levels of 0.40 ± 0.04 and 0.44 ± 0.08 , suggesting $TR\beta$ -mediated induction. Further indication for differential receptor isoform regulation was provided by examining the basal expression of RC3 and Rhes in $TR\alpha 1$ knockout mice (Fig. 2B). RC3 expression was decreased in the knockout with respect to the wild type (0.20 ± 0.01 vs. 0.28 ± 0.02), whereas Rhes did not change (0.13 ± 0.01 vs. 0.14 ± 0.01). In addition to a decreased RC3 expression, the normal dorsomedial to basolateral gradient of expression was also lost in the $TR\alpha 1^{-/-}$ mice.

The results reported above, suggesting differential regulation of RC3 and Rhes by TR isoforms, were somewhat unexpected. RC3 and Rhes were previously shown to be coexpressed in the same cells in the caudate. In this work we have also confirmed that the two mRNAs are colocalized in the same cells by performing a combined *in situ* hybridization technique. In agreement with previous data (29), we found that more than 90% of cells expressing RC3, which were digoxigenin labeled, were also radioactively labeled by the Rhes riboprobe. An example of a cell showing both labels is shown in Fig. 3A. The RC3 gene is regulated at the level of transcription and contains a thyroid hormone-responsive element (TRE) located in the first intron (30, 31). Therefore we checked the possibility that the RC3 TRE was not responsive to $TR\beta$. Cos-7 cells were cotransfected with $TR\alpha$ or $TR\beta$, together with RXR expression vectors and with a CAT reporter containing a single copy of the RC3 TRE upstream of the thymidine kinase promoter (Fig. 3B). CAT activity was measured after incubating the cells in the absence or presence

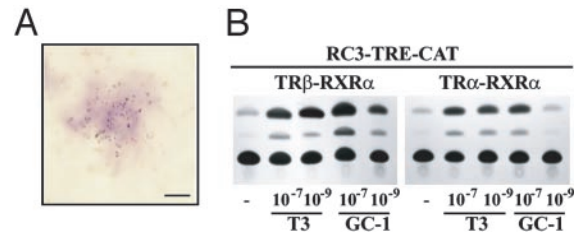


FIG. 3. A, Coexpression of RC3 and Rhes in the same cell. Shown is a cell of the caudate after a combined *in situ* hybridization using a digoxigenin-labeled riboprobe for RC3 and a radioactive probe for Rhes. Scale bar, 30 μ m. B, Transactivation assays in Cos-7 cells using the RC3 TRE upstream of the thymidine kinase promoter driving expression of the CAT gene. The cells were cotransfected with the reporter plasmid and expression vectors encoding $TR\alpha 1$ or $TR\beta 1$ and treated with either T_3 or GC-1 in the presence of thyroid hormone-deprived serum.

of T_3 or GC-1 at two different doses, 10^{-9} and 10^{-7} M, in the presence of 10% serum deprived of thyroid hormones. The activity of the CAT reporter, which was low in the absence of any additions, was stimulated by T_3 at the two concentrations used in the cells transfected with either $TR\alpha$ or $TR\beta$. The two concentrations of GC-1 were also effective in cells transfected with $TR\beta$. However, at the lowest concentration of 10^{-9} M, GC-1 had no effect on cells transfected with $TR\alpha$. Increasing the GC-1 concentration 100-fold elicited a clear reporter response. These results agree with the lower binding affinity of GC-1 to $TR\alpha 1$ vs. $TR\beta$. Therefore, the fact that RC3 in the caudate, in contrast to Rhes, was much less responsive to GC-1 than to T_3 was not due to a differential recognition of the RC3 TRE sequence by the GC-1- $TR\beta$.

Expression of cerebellar genes

Figure 4 shows representative autoradiographs after *in situ* hybridization analysis of selected genes in the cerebellum,

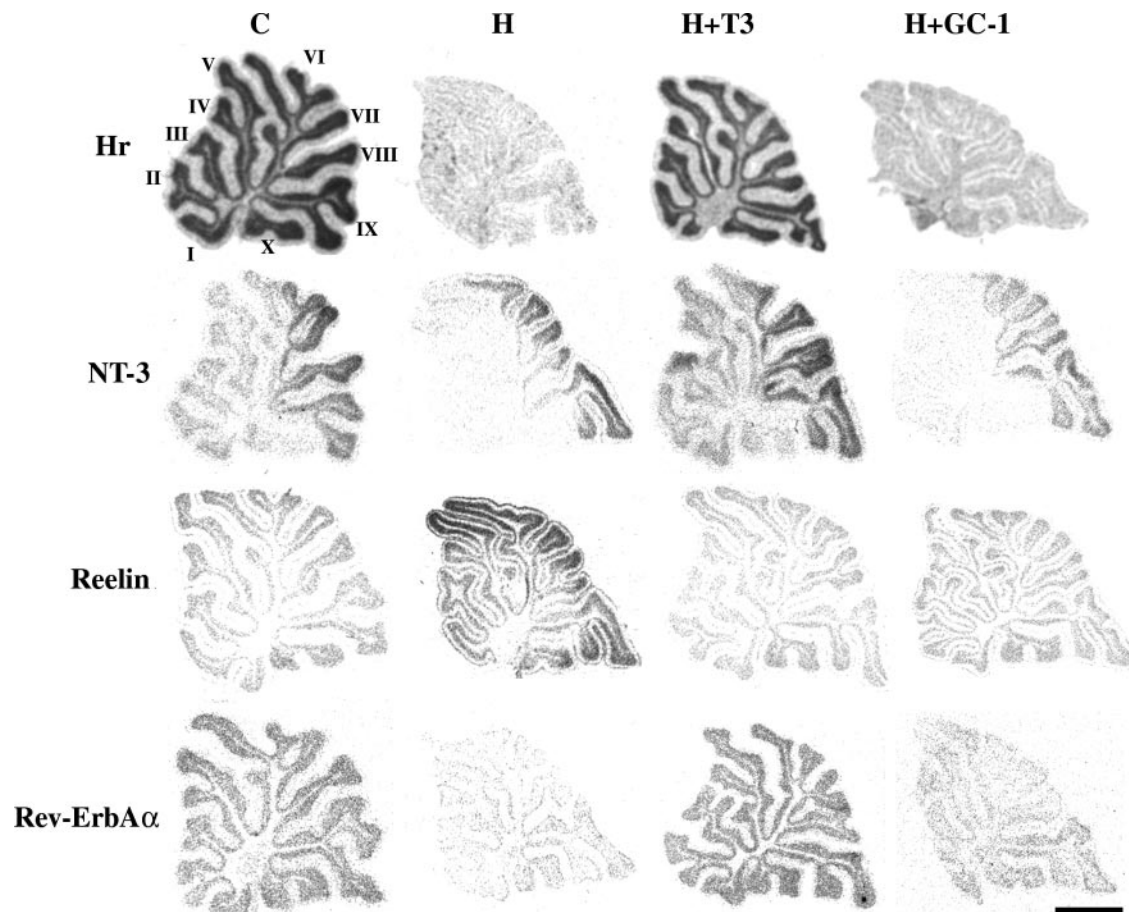


FIG. 4. Expression of cerebellar genes and the effects of T₃ and GC-1. Shown are sagittal slices of cerebellum (anterior lobes are to the left, posterior lobes to the right) after *in situ* hybridization and autoradiography of P16 control (C), hypothyroid (H), and hypothyroid rats treated with T₃ (H + T₃) or GC-1 (H + GC-1). Lobule numbering is indicated in the control section for Hr. Scale bar, 2.3 mm.

and the quantification of results is shown in Fig. 5. Hr was prominently expressed in the internal granular layer of normal animals and was strongly decreased in hypothyroid animals, confirming previous results by other investigators (32, 33). T₃ treatment, but not GC-1, was effective in increasing mRNA levels to normal. NT-3 is also regulated by thyroid hormone during brain development (34). NT-3 expression also occurred in the internal granular layer but was not homogeneous throughout the slice. Expression was stronger in posterior lobules VII, VIII, and IX. Hypothyroidism affected more strongly lobules I–VI and X. T₃ treatment normalized NT-3 expression, whereas GC-1 was without effect. Reelin was shown to be negatively regulated by thyroid hormone in the cerebellum (35). Accordingly, Reelin mRNA was increased in hypothyroidism, especially in central and posterior lobules, and decreased after T₃ treatment. At this stage of development, migration of granular cells was not complete, and Reelin mRNA was also present in the external germinal layer. GC-1 was as effective as T₃ in decreasing Reelin expression after administration to hypothyroid animals. Finally, we found that Rev-ErbA α is also thyroid hormone dependent in the cerebellum, a fact that was not known so far. It was strongly reduced by hypothyroidism in the granular cells, leaving a residual signal arranged in a row-like fashion possibly due to expression in either the Purkinje

or the basket cells. T₃ treatment increased Rev-ErbA α expression in the granular cells, but GC-1 was without effect.

Discussion

Previous studies showed that GC-1 is effective at reducing TSH and elevated cholesterol levels in hypercholesterolemic rats, and cholesterol levels in hypothyroid mice (27), but has no significant effect on heart rate in either case. TSH and cholesterol are primarily TR β -mediated responses, whereas heart rate is mediated by TR α 1 (11). In our hands, GC-1 administration to hypothyroid rats reduced plasma TSH, although, in agreement with other studies (15), was less effective than an equivalent dose of T₃. This might be due to the fact that pituitary TSH is under control of both TR β and TR α 1 (36). Also, as previously reported, GC-1 has no effect on body weight, despite the fact that it increased the expression of pituitary GH in a similar way as T₃. The increased pituitary GH after GC-1 treatment suggests that GH is under control of TR β . It has been proposed that GH is regulated specifically by TR β 2 (37) although TR β ^{-/-} or TR β 2^{-/-} mice have normal GH mRNA levels. In fact, in TR-deficient mice a decreased GH expression can only be achieved in TR α ^{-/-}TR β ^{-/-} mice, suggesting redundancy among TRs. In hypothyroid animals the situation is different from that of

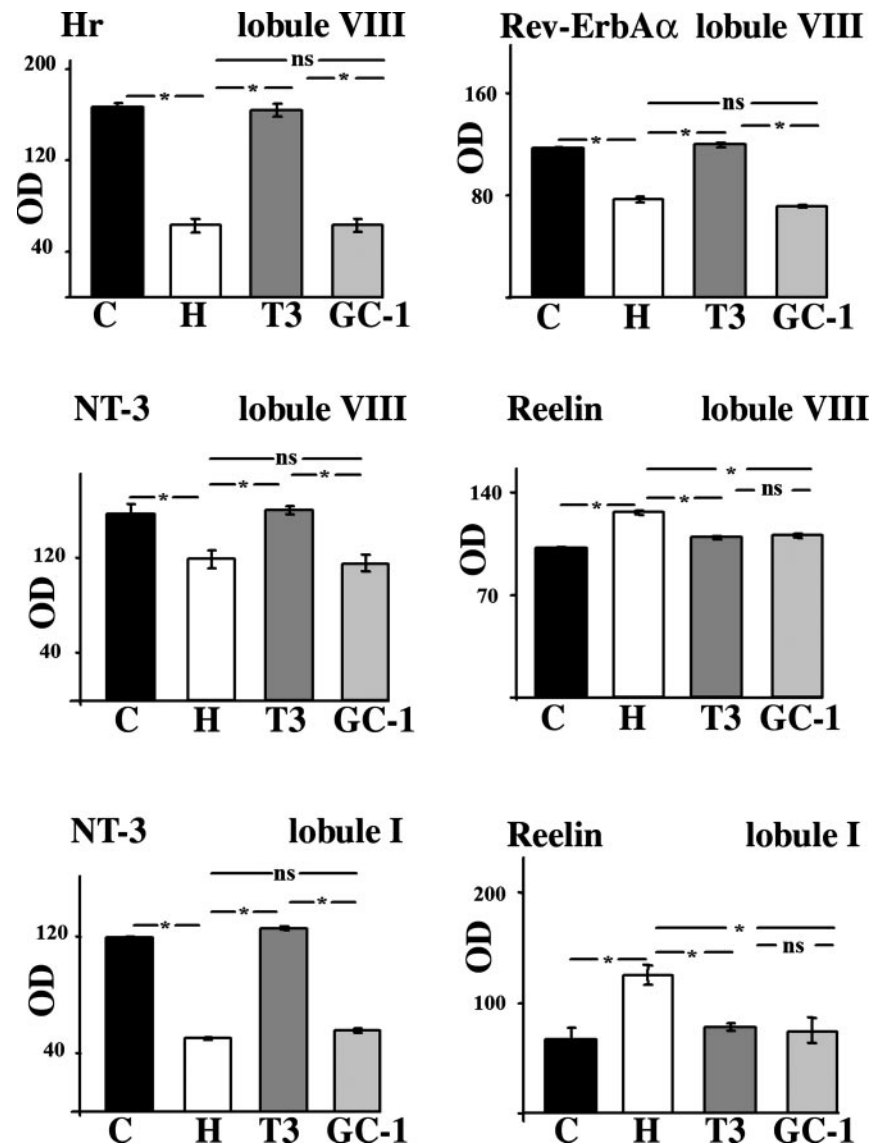


FIG. 5. Quantification of the autoradiograph data of Fig. 4. The figure shows the mean \pm SD of densitometric analysis performed on the autoradiographs after *in situ* hybridization of cerebellar genes. Four slices for each gene from different pups were used for measurements. The data for Hr and Rev-ErbA α were taken from lobule VIII and for NT-3 and Reelin from lobules I and VIII. The data were analyzed by ANOVA with the following results: Hr, $F_{3,12} = 387.5$, $P = 0.0000$; Rev-ErbA α , $F_{3,12} = 899.2$, $P = 0.0000$; NT-3, lobule I, $F_{3,12} = 4338.3$, $P = 0.0000$; NT-3, lobule VIII, $F_{3,12} = 40.0$, $P = 0.0000$; Reelin, lobule I, $F_{3,12} = 30.2$, $P = 0.0000$; Reelin, lobule VIII, $F_{3,12} = 422.2$, $P = 0.0000$. Statistically significant differences between groups are shown by *asterisks*.

receptor-deficient animals, because the unliganded receptors may repress the target genes. Therefore the suppressed GH gene expression in hypothyroid animals might be due to the repressor activity of unliganded receptors. From our results it appears that liganded TR β is sufficient to normalize GH expression. In the myocardium, Serca-2 mRNA, primarily a TR α 1 response (27), was decreased in hypothyroid rats and increased by T₃ but not by GC-1. Therefore our control experiments indicated that at the doses used, GC-1 was acting primarily, if not exclusively, through TR β 1.

There are very few data on GC-1 effects on brain. In this work we have analyzed the effect of GC-1 on gene expression of selected genes in the cerebrum and in the cerebellum. In agreement with previous work (21), GC-1 was effective in inducing PCP-2 mRNA in the cerebellum. GC-1 is therefore able to enter the brain and effect genomic actions. Cerebellar PCP-2 is specifically transcribed in the Purkinje cells, which express primarily TR β 1, so that the PCP-2 response to thyroid hormone is presumably mediated by TR β 1. To gain more insight into the roles of TR α and TR β in the brain, we

selected RC3 and Rhes because they are coexpressed in the caudate, where they show a strong dependence on thyroid hormone. RC3 is a protein kinase C substrate involved in Ca²⁺ and glutamate receptor signaling at the postsynaptic terminal (38). It is regulated by thyroid hormone at the level of transcription, presumably through a TRE located in the first intron. Rhes is a novel Ras-related protein greatly enriched in the striatum (39), also induced by thyroid hormone by a mechanism that remains unknown. Surprisingly, Rhes was responsive to T₃ and GC-1, whereas RC3 was induced by T₃, with GC-1 having little effect. The reasons for the weak induction of RC3 by GC-1 are at present unknown. RC3 and Rhes are coexpressed in the main cells of the caudate, the medium-sized, spiny, γ -aminobutyric acid (GABA)ergic cells. These cells express both TR α and TR β , as shown by colocalization analysis with RC3 (40). Therefore, GC-1 should have elicited the same effect on RC3 as on Rhes. The possibility that the sequence of the RC3 TRE was not recognized by the GC-1-activated TR β was discarded by transactivation analysis that showed that TR β was able to induce

the activity of a reporter gene after GC-1 addition to the cells. A preliminary conclusion of these experiments is that RC3 and Rhes represent thyroid hormone responses elicited through specific receptor isoforms in the same cell. One can speculate that in the environment of the RC3 intronic TRE, TR α 1 might interact more productively than TR β with the transcriptional machinery. The role of TR α 1 in regulation of RC3, but not Rhes, agreed with data from TR α 1^{-/-} mice showing that RC3, but not Rhes, was significantly decreased in the absence of TR α 1.

The effects of T₃ and GC-1 on genes expressed in the granular cells of the cerebellum suggest that regulation of these genes is exerted primarily through TR α 1. This is in agreement with the fact that granular cells express predominantly TR α 1 (19, 20). In a previous work we have shown that GC-1 did not induce granular cell migration, despite partially correcting the arrested Purkinje cell differentiation caused by early postnatal hypothyroidism (21). An exception to this pattern of regulation was Reelin, which was down-regulated by GC-1 in a similar manner as by T₃, despite granule cell expression. Thyroid hormone positively regulates Reelin in the cerebral cortex, whereas in the cerebellum, the pattern of regulation is complex, switching from a positive to a negative regulation as development proceeds (35). This complex pattern of regulation suggests that other influences, for example Purkinje cell-derived brain-derived neurotrophic factor, and possibly other factors, are involved in Reelin regulation. Therefore, the effects of GC-1 on Reelin expression by granule cells may be an indirect consequence of an effect on the Purkinje cells.

Among the cerebellar genes studied we show that Rev-ErbA α is under thyroid hormone regulation, a fact that was previously unknown. Rev-ErbA α plays an important role during cerebellar development. During the first week after birth it is expressed in the Purkinje cells, and as development proceeds, its expression increases in the internal granular layer and basket cells and decreases in the Purkinje cells (41). Accordingly, we found that at P16 most Rev-ErbA α signal was present in the internal granular layer where it is under strong regulation by thyroid hormone. We believe that our findings are important because deletion of this gene in mice induces a cerebellar phenotype similar to that of hypothyroidism, *i.e.* stunted growth of Purkinje cells and delayed migration of granule cells (41). Therefore regulation of Rev-ErbA α expression might be at least partly responsible for the effects of thyroid hormones on cerebellar development.

A point of concern has been the availability of GC-1 to the brain after *in vivo* administration. A full pharmacokinetic analysis of GC-1 has not been carried out, but a preliminary study to measure plasma levels and tissue distribution from a single dose has been reported (27). These results indicate that GC-1 has a substantially shorter serum half-life than T₃, but the efficiency of absorption into most tissues is not dramatically different from that of T₃. In brain, which is the relevant tissue for this paper, T₃ has a tissue/plasma ratio of 0.56, whereas the ratio for GC-1 is 0.13, indicating that GC-1 absorbs into the brain from plasma four times less efficiently than T₃. For comparison, the same analysis on the liver, in which GC-1 displays potent lipid-lowering activity, shows that GC-1 is absorbed about three times less efficiently than

T₃. The results described in this paper demonstrate that GC-1 administration *in vivo*, at the doses used, is as effective as T₃ in cells containing primarily TR β 1 as Purkinje cells, whereas it has no effect on cells containing primarily TR α 1 as cerebellar granule cells.

In conclusion, the TR β -specific T₃ analog GC-1 is able to induce typical gene responses to thyroid hormone in the brain, and the patterns of regulation agree with the known distribution of thyroid hormone receptor isoforms in the cerebellum. In the caudate, however, where TR α and TR β are expressed in the same cells, the lack of effect of GC-1 on RC3 suggests that this gene is specifically regulated by TR α . The data further suggest that regulation by thyroid hormone receptor isoforms follows two types of specificity: one, which is due to specific cell type expression of receptor isoforms, and an additional one, which is due to specific gene recognition by individual isoforms.

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