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Thyroid hormone exerts site-specific effects on SRC-1 and NCoR expression selectively in the neonatal rat brain

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Abstract

Thyroid hormone receptors (TRs) are ligand-gated transcription factors. Recently, many coregulator proteins have been identified that interact with steroid/TRs and are required for the activation or repression of hormone sensitive genes. We tested whether steroid receptor coactivator-1 (SRC-1) and nuclear corepressor (N-CoR) expression is altered by hypothyroidism in rat brains on gestational day 16 and postnatal day 15. We found that both SRC-1 and N-CoR mRNA levels were decreased in the cortex and dentate gyrus of 6-*n*-propyl-2 thiouracil treated rats only on P15, while mRNA levels for both genes were increased in the same CA3 region of the brains. These findings do not support the idea that cofactors are involved in the compensatory mechanisms for conserving TH action, but they do suggest that hypothyroidism affects the responsiveness of tissues to steroid hormones by altering the expression of necessary cofactors. © 2002 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Thyroid hormone; N-CoR; SRC-1; Brain development

1. Introduction

The importance of thyroid hormone (TH) in brain development is most often illustrated by the severe neurological deficits observed in humans and animals deprived of TH during development. For example, in geographic areas of iodine insufficiency where children are exposed to severe pre- and postnatal TH deficiency, the neurological and motor deficits, known as cretinism, are profound (Glinoer and Delange, 2000; Delange, 2000). However, measurable neurological deficits, including lower IQ and attention deficit, are also observed in less severe situations, such as in the case of low maternal TH in the absence of hypothyroidism (Haddow et al., 1999; Pop et al., 1999; Escobar et al., 2000). Likewise in experimental animals, TH is critical for brain development both before (Dowling et al., 2000, 2001) and after (Munoz and Bernal, 1997) birth.

A variety of compensatory mechanisms appear to function to ameliorate the deleterious effects of low TH. For example, TH uptake into cells increases during periods of hypothyroxinemia, presumably to increase the efficiency of TH delivery to cells (Evans, 1988; Everts et al., 1995; Kragie, 1996). In addition, tissue deiodinases are up-regulated during periods of hypothyroxinemia, presumably to increase the efficiency of converting thyroxine (T_4) to triiodothyronine (T_3) (Burmeister et al., 1997; Germain and Galton, 1997). These cellular responses are believed to be important for maintaining the level of intracellular TH during brain development despite fluctuations in the availability of TH.

Considering these effects, we postulated that important compensatory mechanisms may occur at the level of receptor function. Thyroid hormone receptors (TRs) are members of the steroid/thyroid superfamily of ligand-gated transcription factors that bind to hormone

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response elements in the promoters of responsive genes to affect their transcription (Evans, 1988; Lazar, 1994; Mangelsdorf and Evans, 1995). The ability of these receptors to affect gene transcription requires them to interact with nuclear cofactors, which are requisite mediators of ligand-dependent transcriptional activation or repression of hormone responsive genes (Glass and Rosenfeld, 2000). Cofactors are believed to remodel local chromatin structure enabling nuclear receptors to activate or repress gene regulation. Generally, the specific recruitment of a cofactor complex with histone acetyltransferase activity may play a regulatory role in activating gene transcription, whereas the recruitment of a cofactor complex with histone deacetylase activity may play a regulatory role in gene repression (Struhl, 1998). Therefore, it is possible that the sensitivity of tissue to TH may be modulated by the relative abundance of specific cofactors.

Two kinds of observations support the hypothesis that changes in cellular levels of specific cofactors can modulate cellular responsiveness to steroid/TH and may function as a compensatory mechanism to ameliorate the effects of hypothyroidism. First, ligand-dependent transcriptional activation by one nuclear receptor can be inhibited by ligand activation of another nuclear receptor in vitro, even though this second receptor does not directly regulate the affected gene (Barettino et al., 1994; Zhang et al., 1996). Thus, nuclear receptors compete for specific cofactors to transduce hormonal signals to transactivate gene expression. Second, overexpression of the cofactor steroid receptor coactivator-1(SRC-1) in MCF-7 cells results in an increase in the mitogenic response to estrogen (Tai et al., 2000). Thus, the sensitivity of a cell to a specific level of hormone may be determined, in part, by the availability of specific cofactors.

There are two categories of nuclear receptor cofactors in general: corepressors and coactivators (Glass and Rosenfeld, 2000; Leo and Chen, 2000). In the absence of TH, TRs are able to repress basal transcription via recruitment of the corepressors SMRT or NCoR (Koenig, 1998; Horlein et al., 1995). In the presence of TH, TRs release their corepressor and recruit a coactivator complex that includes SRC-1 (Koenig, 1998; Onate et al., 1995). The SRC family of coactivators is large and includes SRC-1, TIF2/GRIP1, and RAC3/pCIP (Leo and Chen, 2000). However, considering the background information concerning the interaction of TRs and cofactors (Koenig, 1998), we focused on SRC-1 and nuclear corepressor (N-CoR) specifically. In addition, we focused on two developmental periods during which TH is known to regulate gene expression: the gestational day 16 (G16) rat fetus (Dowling et al., 2000, 2001), and the postnatal day 15 (P15) rat pup (Oppenheimer and Schwartz, 1997; Schwartz, 1983). Moreover, we focused on specific regions of the brain in which TH is known to affect gene expression: the fetal rat cortex, and the postnatal rat hippocampus (Dowling et al., 2000; Iniguez et al., 1996).

2. Results

The goal of the present study was to determine whether changes in circulating levels of TH could affect the expression of SRC-1 and N-CoR in the developing brain. The working hypothesis was that the abundance of specific cofactors could modulate the sensitivity of cells to TH; thus, an important response to changes in TH availability could be compensatory changes in cofactor abundance. We focused on two developmental periods known to be sensitive to TH—G16 and P15. Therefore, we first had to determine that both SRC-1 and N-CoR are expressed in the brain at these periods. The developmental pattern of SRC-1 expression was reported by Misiti et al. (1999), which confirmed that SRC-1 expression is abundant in the developing brain at both G16 and P15. However, this had not been previously reported for N-CoR. Therefore, we first characterized the pattern of N-CoR expression during fetal and neonatal development (Fig. 1). N-CoR mRNA was broadly distributed throughout the fetus at all developmental times (G15 to adult). N-CoR was most intensely expressed in specific regions of the fetal nervous system including the ventricular zones of the forebrain and hindbrain on G15 and G16. As development proceeded on G18 and G21, N-CoR mRNA was pronounced in both the ventricular zone and superficial layers that contain the newest migrating neurons (Caviness et al., 1995). During early postnatal development, N-CoR expression was most prominent in the cortex, hippocampus, and cerebellum, with low expression in areas of white matter (e.g. corpus callosum), and 'patchy' expression in the caudate and dorsal thalamus (P3 to adult). Also, expression in the cortex changed noticeably from P11 to P14. On P11, expression was higher in the outer layers of the cortex and decreased in the inner layers. By P14, expression was uniform throughout the cortex and was also lower than that of P11. Expression in the cerebellum was pronounced throughout development and adulthood. These observations verified that N-CoR expression is abundant in the developing brain at G16 and P15.

To test whether the abundance of SRC-1 and/or N-CoR mRNAs change in relation to thyroid status, we manipulated circulating levels of TH in the fetus and in the neonate. The thyroid status of dams used to test the effect of TH on N-CoR and SRC-1 expression in the fetus has been published elsewhere (Dowling et al., 2001). Briefly, 6-*n*-propyl-2 thiouracil (PTU) treatment reduced circulating levels of total T_4 and free T_3

and elevated thyroid stimulating hormone (TSH). The injection of T_4 prior to sacrifice restored T_4 and T_3 and suppressed TSH. Because fetal thyroid function does not begin until approximately G17.5 (Fisher et al., 1977), we inferred that the fetus was exposed to changes in TH in proportion to that observed in the dam's circulation. This inference was confirmed by showing that the abundance of RC3/neurogranin in the fetal brain was affected by the experimental treatments. Likewise, the thyroid status of the P15 pups responded predictably to experimental treatment (Fig. 2). Specifically, circulating concentrations of total T_3 and total T_4 were significantly lower in pups derived from PTU-treated dams (T_4: $F_{1,18} = 22.06$, $P = 0.002$); (T_3: $F_{1,18} = 19.89$, $P = 0.003$) and restored by T_4 injections.

The body weight of the dams treated with PTU and/or T_4 until P15 was slightly, but significantly altered by treatments. Group weights (grams) were 380 ± 5 (control), 355 ± 6 (PTU), 317 ± 7 (T_4) and 337 ± 6 (PTU + T_4). The two-way analysis of variance (ANOVA) revealed a significant effect of T_4 ($F_{1,22} = 46$, $P < 0.0001$) and a significant interaction between PTU and T_4 ($F_{1,22} = 14$, $P < 0.001$). A pairwise comparison of means revealed that T_4-treated animals exhibited significantly lower body weights. These effects were not observed in dams sacrificed on G16.

Film analysis following the in situ hybridization revealed that manipulation of maternal thyroid status did not significantly affect the abundance of SRC-1 or N-CoR mRNAs in the fetal cortex (Fig. 3) [$F_{1,20} = 0.073$, $P = 0.7895$]. In contrast, the abundance of both N-CoR and SRC-1 mRNAs was affected in a site-specific manner by the manipulation of thyroid status in 15 day-old pups (Figs. 4 and 5). Specifically, PTU-treated

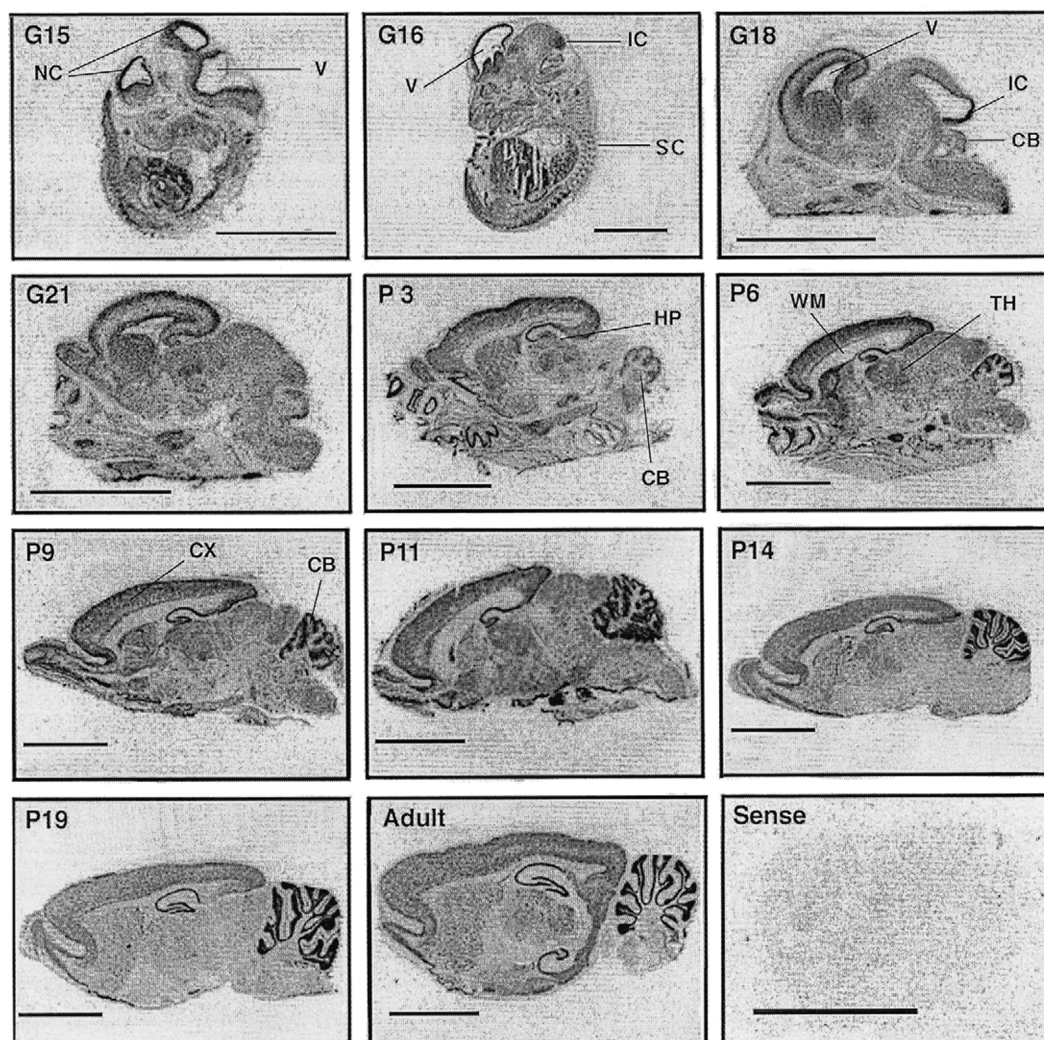

Fig. 1. Developmental profile of N-CoR expression in the rat. Images are derived from film autoradiograms produced by in situ hybridization. Age is noted in the top left-hand corner of each panel. Note the high relative expression in the developing cortex and cerebellum, as well as the change in cortical expression from P11 to P14. More detailed descriptions are provided in the text. Scale bar = 0.500 μm: Cx, cortex; IC, inferior colliculous; HP, hippocampus; NC, neocortex; V, ventricle; SC, spinal cord; TH, thalamus; CB, cerebellum; WM, white matter.

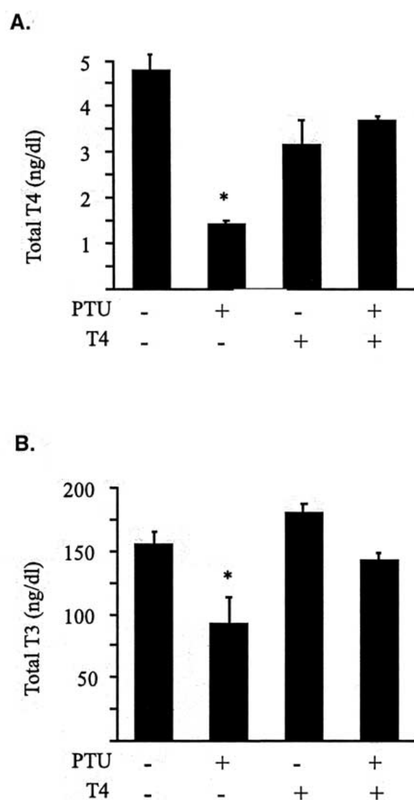

Fig. 2. Effect of PTU and T_4 treatment on the serum concentrations of total T_4 (A) and total T_3 (B) in postnatal day 15 pups. See Section 4 for details of thyroid hormone manipulation. Bars represent mean ± SEM. Treatment groups are indicated below the ordinate. *Significantly different from euthyroid pups ($P < 0.05$) using Bonferroni's t-test following two-way ANOVA.

pups exhibited significantly lower levels of N-CoR and SRC-1 mRNA in the cortex and dentate gyrus compared to controls, but elevated levels of these mRNAs in the CA3 subfield of Ammon's horn. The abundance of N-CoR or SRC-1 mRNA in CA1 was not significantly different among animals with different thyroid status. Moreover, in PTU-treated animals injected with T_4, N-CoR and SRC-1 mRNA levels were not different from those of the control animals in any of these hippocampal or cortical regions.

Because both N-CoR and SRC-1 expression was affected similarly by thyroid status in a site-specific manner, we tested the possibility that a systematic error of unknown origin may have been responsible for this finding. Therefore, we measured β-actin mRNA in the same regions of tissue sections taken from brains analyzed for cofactors. We have previously used β-actin as an internal control in studies focused on the regulation of the hypothalamic–pituitary–thyroid axis (Zoeller et al., 1990, 1993). Film analysis following the in situ hybridization for β-actin revealed no differences in expression among the treatment groups in any of the brain areas evaluated (Table 1). We also measured RC3/neurogranin mRNA in the striatum of these P15 brains to confirm that changes in thyroid status produced known effects in the P15 brain. Analysis of the film following in situ hybridization for RC3/neurogranin in the striatum of these animals revealed a significant effect of PTU [$F_{1,19} = 39.74$, $P < 0.001$], T_4 [$F_{1,19} = 28.12$, $P < 0.001$], and a significant interaction ($F_{1,19} = 25.0$; $P < 0.001$). Post-hoc analysis demonstrated that the abundance of RC3/neurogranin mRNA was significantly lower in the striatum of PTU-treated animals compared to that of the controls (Table 1; $t_{10} = 14.95$, $P < 0.001$), and was restored by T_4 treatment (Table 1).

3. Discussion

The present results demonstrate that SRC-1 and N-CoR expression in the developing brain is regulated by TH. We observed three important features of this regulation. First, SRC-1 and N-CoR expression was affected by thyroid status in the brain of P15 pups, but not in the G16 fetus. Thus, TH effects on cofactor expression were temporally restricted to the postnatal period. Second, on P15, expression of SRC-1 and N-CoR mRNAs was selectively reduced by PTU treatment in the cortex and dentate gyrus, but was elevated by PTU treatment in the CA3 region of the hippocampus. Thus, TH effects on SRC-1 and N-CoR expression were site specific. Finally, T_4 administration ameliorated the ef-

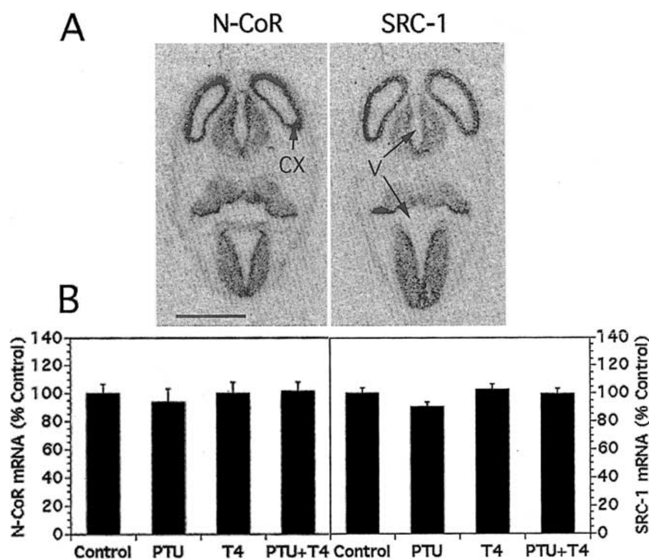

Fig. 3. Effect of maternal thyroid hormone on SRC-1 and N-CoR expression in the G16 fetal brain. (A) Film autoradiographic images of N-CoR and SRC-1 in the gestational day 16 rat-fetal cortex. Micrographs were taken from representative sections through fetal brains from control dams. Both genes were clearly expressed throughout the neocortex. Scale bar = 200 μm: CX, cortex; V, ventricle. (B) Film density measurements were taken from several brain regions of pups representing the four treatment groups. Bars represent mean ± SEM film density converted to percent control for the purpose of illustration. Neither gene was affected by thyroid hormone status.

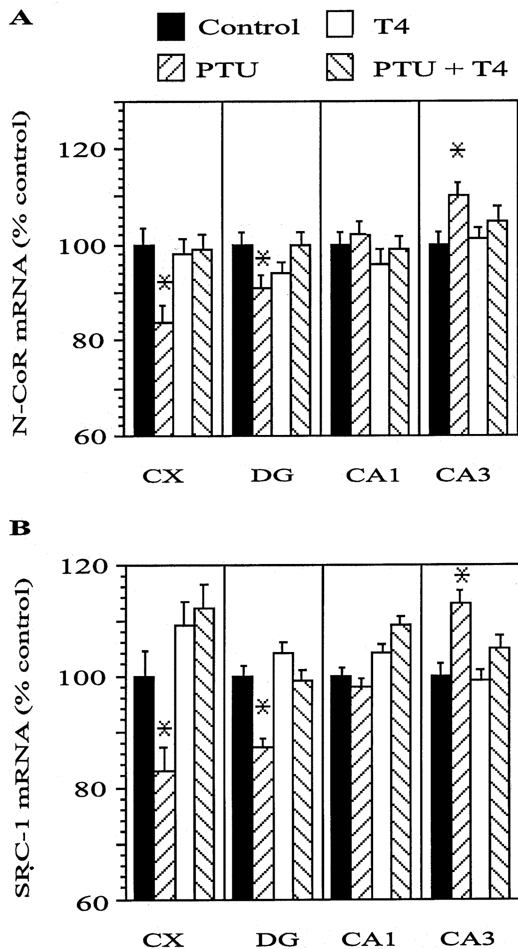

Fig. 4. Cofactor expression expressed as percent control in the P15 rat brain. (A) Effect of thyroid status on N-CoR mRNA levels in the P15 rat brain. Film density measurements were taken from several brain regions of pups representing the four treatment groups. Bars represent mean ± SEM film density converted to percent control for the purpose of illustration. Decreased levels of N-CoR mRNA, as reflected in film density, were observed in the dentate gyrus and cerebral cortex as well as increases in the CA3 subfeild of the hippocampus as compared to euthyroid controls. Average greyscale values of the pixels in the CX, DG, CA1, and CA3 of the control rats were 102.6, 152.5, 146.7 and 129.4, respectively. (B) Effect of thyroid status on SRC-1 mRNA levels in the developing brain. Film density measurements were taken from several brain regions of pups representing the four treatment groups. Bars represent mean ± SEM film density converted to percent control for the purpose of illustration. Decreased levels of SRC-1 mRNA, as reflected in film density, were observed in the dentate gyrus and cerebral cortex, as well as an increase in the CA3 subfield of the hippocampus as compared to euthyroid controls. Average grayscale values of the pixels in the CX, DG, CA1, and CA3 of the control rats were 48.2. 85.3, 84.7 and 78.5, respectively. *Significantly different from euthyroid pups ($P < 0.05$) using Bonferroni's t-test.

fects of PTU treatment on cofactor expression in all the three brain regions, indicating that the effects of PTU on SRC-1 and N-CoR expression were mediated by TH. These findings do not support the hypothesis that changes in cofactor expression compensate for changes in thyroid status. However, these findings indicate that TH alters cofactor expression in a developmentally and site-specific manner which may have important implications for the regulation of steroid hormone action on brain development.

The observation that both SRC-1 and N-CoR expression was similarly affected by TH on P15 suggested to us the possibility that experimental variables of unknown origin may have produced a systematic error that led to these results. To preclude this possibility, we measured β-actin mRNA in the hippocampus, and RC3/neurogranin mRNA in the striatum of the same P15 brains used to measure relative levels of SRC-1 and N-CoR. We chose these genes for two reasons. First, we previously employed the use of a β-actin probe as a reference for in situ hybridization studies (Zoeller et al., 1990; Zoeller and Rudeen, 1992; Zhang et al., 1995) and found that its abundance was not altered under conditions in which TH are altered. Although others have found its abundance affected by thyroid status in the cerebral cortex (Poddar et al., 1996), this would clearly not preclude the possibility that its expression would be unaffected by thyroid status in the hippocampus. Therefore, this gene was used to control the possibility that the abundance of all mRNAs would appear

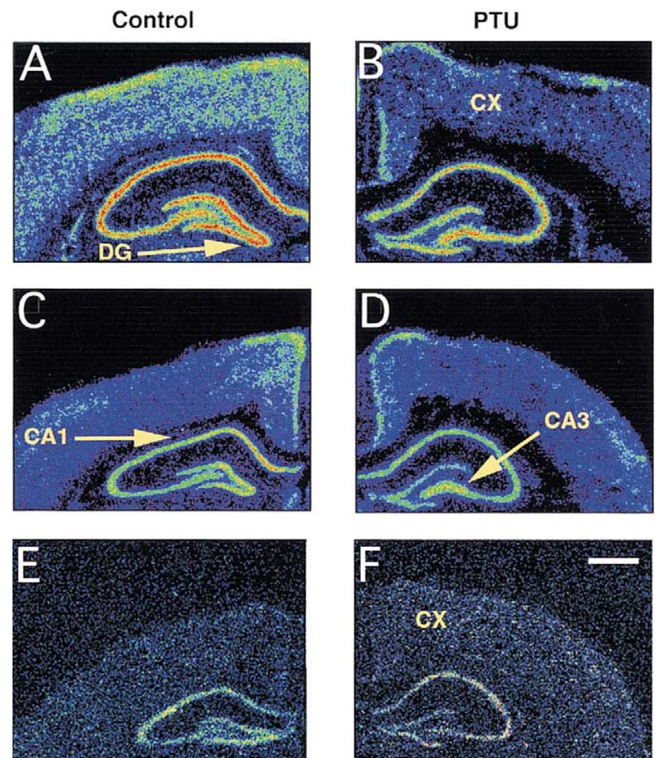

Fig. 5. Pseudocolor images of in situ hybridization for N-CoR (A, B), SRC-1 (C, D) and β-actin (E, F) in euthyroid (A, C, E) and PTU-treated (B, D, F) P15 rat brains. Relative levels of SRC-1 and N-CoR mRNAs were decreased in the CX and DG of PTU-treated rats (A, C vs. B, D). Relative levels of SRC-1 and N-CoR in the CA3 subfield were elevated in PTU-treated rats (A, C vs. B, D). The relative levels of β-actin mRNA were unaltered in the brains of PTU-treated rats (E vs. F). Scale bar = 200 μm: CX, cortex; CA3 and CA1 subfields of Hippocampus; DG: dentate gyrus.

Table 1
Effect of thyroid status on β-actin mRNA in cortex and hippocampus of P15 rat brain and RC3/neurogranin mRNA in striatum

	Control	PTU	T_4	PTU+T_4
β-Actin				
Cortex	100 ± 7.4	122.0 ± 14.2	106.3 ± 8.6	92.5 ± 1.5
CA1	100 ± 8.2	99.13 ± 5.91	124.5 ± 20.58	107.3 ± 10.06
CA3	100 ± 4.4	124.2 ± 18.3	103.3 ± 4.89	99.18 ± 9.15
Dentate	100 ± 4.9	138.5 ± 22.6	94.8 ± 5.64	93.8 ± 8.37
RC3/neurogranin				
Striatum	100 ± 1.08(6)	78 ± 10.5(6)*	100.6 ± 0.98(5)	98.1 ± 0.87(6)

Values represent mean ± SEM (*n*) of the film density following semi-quantitative in situ hybridization.

* Significantly different from control, $P<0.001$

to be altered in a pattern identical to that observed for SRC-1 and N-CoR in the hippocampus. We found that β-actin mRNA levels were not different among the treatment groups; thus, the differences we observed in the abundance of SRC-1 and N-CoR mRNAs in the P15 brain were not due to a systematic error. Second, previous reports have demonstrated that RC3/neurogranin expression is most sensitive to TH manipulation in the striatum (Iniguez et al., 1996). Therefore, we measured RC3/neurogranin mRNA in the striatum to verify that the observed differences in the circulating levels of TH in P15 pups produced measurable effects on known TH-responsive genes. As predicted, we found that RC3/neurogranin mRNA was reduced in PTU-treated animals and restored by the treatment with T_4 (Table 1). Therefore, the effects of manipulating thyroid status in the P15 pups on SRC-1 and N-CoR mRNAs were due to the effects of TH. Likewise, the lack of effect of TH on SRC-1 and N-CoR expression in the G16 brain was not due to the lack of effect of T_4 in these animals because we found that RC3/neurogranin expression was also regulated by T_4 in these tissues (Dowling and Zoeller, 2000).

The present findings that SRC-1 and N-CoR expression is sensitive to TH only during specific developmental times, and only in specific brain areas, are consistent with the known pleiotropic effects of TH on gene expression in the brain. For example, the expression of the transcription factor, Oct-1, is elevated by TH in the fetal cortex, but suppressed by TH in the adult cortex (Dowling et al., 2000). Moreover, RC3/neurogranin expression is sensitive to TH regulation only in some brain areas, despite the fact that RC3/neurogranin and TRs are coexpressed in brain areas in which TH has no effect on RC3/neurogranin (Guadano-Ferraz et al., 1997).

We considered that cofactor levels might be altered by thyroid status to compensate for the changes in TH levels. Therefore, we predicted that PTU would increase SRC-1 mRNA levels and decrease N-CoR mRNA levels, thus increasing the cellular responsiveness to TH. We found that both SRC-1 and N-CoR expression was similarly regulated by the thyroid status in PTU-treated pups, which clearly is not consistent with our original hypothesis. However, these cofactors are shared by different receptor systems, suggesting that TH regulation of cofactor expression may be important for the ability of other receptor systems to function during development. For example, estrogen induces the expression of preproenkephalin (PPE) in neurons of the ventromedial hypothalamus (VMH), and this is linked causally to the expression of female sex behaviors (Dellovade et al., 1999, 1996). However, coadministration of TH inhibits the effect of estrogen on both PPE expression in the VMH and sex behavior, and the available evidence supports the view that this inhibition is caused by competition between liganded TR and ER for SRC-1 (Zhu et al., 1997, 2001; Kia et al., 2001). Thus, in vivo competition for cofactors may be physiologically important, and the observed regulation of N-CoR and SRC-1 may have important consequences for the ability of hormones to affect brain development.

The functions of SRC-1 in brain development are poorly understood at present. SRC-1 mRNA is expressed in many tissues throughout development (Misiti et al., 1999, 1998). Moreover, SRC-1 interacts with several members of the steroid/thyroid receptor superfamily, including retinoic acid, estrogen, glucocorticoid, progesterone and TRs (Onate et al., 1995; Robyr et al., 2000). However, SRC-1 deficient mice are viable, fertile animals that display no major neurological defects (Onate et al., 1995). This suggests that there are redundant mechanisms that may compensate for the lack of SRC-1. It is known that SRC-1 enhances transcriptional activation by TRs (Onate et al., 1995; Jeyakumar et al., 1997; Ikeda et al., 1999). Additionally, SRC-1 deficient mice exhibit pituitary resistance to TH in that TSH levels remain high despite elevated levels of TH in the blood (Weiss et al., 1999). Because excess TH causes neurological deficits not seen in SRC-1 deficient mice (Krude et al., 1977; Xu et al., 1998), this suggests that the SRC-1 knock-outs are resistant to TH on other tissues as well. Therefore, the observation that TH alters SRC-1 expression in the neonatal rat brain indicates that TH may be affecting the sensitivity of these brain structures to steroid hormone action.

The specific processes that N-CoR affect during development are also poorly understood. The significance of changes in N-CoR mRNA levels may not be as far reaching as changes in SRC-1 levels, because N-CoR appears to interact with fewer receptors (Robyr et al., 2000). In the absence of artificial receptor antagonists,

such as RU486, N-CoR has been found to mediate repression only with Reverb, retinoic acid receptors, and TRs (Horlein et al., 1995; Robyr et al., 2000). Therefore, changes in the levels of N-CoR may only affect signaling mediated by these receptors. Our results for the developmental profile of N-CoR mRNA show that it is expressed widely in the developing brain. Furthermore, it is expressed at high levels in thyroid sensitive tissues, such as the developing cerebral cortex, dentate gyrus and cerebellum. Our results suggest that TH control of N-CoR expression may also contribute to the hormonal responsiveness of tissues.

The present results do not permit us to differentiate between a direct or indirect effect of TH on the expression of SRC-1 and N-CoR. Moreover, it is important to note that SRC-1 and N-CoR represent only two of a very large family of cofactors involved in transducing steroid hormone signals within the nucleus (Glass and Rosenfeld, 2000; Leo and Chen, 2000; Fondell et al., 1999). In addition, the SRC-1 gene produces several splice variants, only one of which (SRC-1E) appears to interact significantly with the TR (Hayashi et al., 1997). We did not determine whether TH exerted selective effects on the SRC-1 splice variants because the cRNA probe we used in the present studies was cloned from a common region shared by all splice variants of SRC-1. Thus, it is possible that different cofactors or specific SRC-1 transcripts exhibit compensatory changes to ameliorate the effects of altered thyroid status. Finally, it is important to recognize that the film analysis we performed cannot provide information about the cellular levels of these mRNAs. However, considering the densely-packed distribution of cells expressing in the sentence these cofactors in the hippocampus, it is not likely that we could have performed single-cell analysis following in situ hybridization as we have performed on other genes (Zoeller et al., 1988, 1992, 2000). Thus, these data are similar to that of Northern analysis or solution protection assays in which cellular levels of target mRNAs are obtained only if the proportion of cells expressing the target mRNA does not differ among treatment groups—an assumption infrequently tested.

In conclusion, we show that N-CoR mRNA is widely distributed during development and that both SRC-1 and N-CoR mRNA levels are regulated by thyroid status in a time and tissue-specific fashion. This pattern is consistent with other patterns of direct regulation by TH. Considering that both SRC-1 and N-CoR interact with multiple members of the steroid/TH, changes in expression could affect tissue sensitivity to a number of hormonal signals in TH sensitive areas.

4. Experimental methods

4.1. Animals

All animal procedures were performed in accordance with the NIH Guidelines for animal research and were approved by the University of Massachusetts–Amherst Institutional Animal Care and Use Committee. To characterize the spatial and temporal changes in the expression of N-CoR and SRC-1 mRNA throughout normal development, nulliparous female Sprague–Dawley rats ($n = 5$; Zivic Miller) were maintained on rat chow and drinking water ad libitum. Dams were mated by pairing females with males overnight; the presence of sperm in a vaginal smear the following morning indicated mating and this day was defined as gestational day 1 (G1). Dams were decapitated following CO_2 inhalation at 12:00 hours on G15, G16, G18, and G21 and the fetuses were removed from the uterus and quick frozen on pulverized dry ice. The remaining dams carried the pregnancy to term. The resulting pups ($n = 1$ per postnatal day) were killed by decapitation at 12:00 hours on P3, P6, P9, P11, P14, and P19, and the intact head (P3–P9) or dissected brains (P14 to adult) were frozen on pulverized dry ice and stored at -80 °C until sectioned for in situ hybridization.

To test whether the TH status alters the expression of N-CoR and/or SRC-1 in the fetus, timed-pregnant female Sprague–Dawley rats ($n = 24$; Zivic-Miller, Pottersville, PA) arrived at our facility on G2 and were maintained on drinking water containing either 0.04% 6-*n*-propyl-2 thiouracil (PTU) (Sigma Chemical Co., St. Louis, MO; $n = 12$) with 3% sucrose to reduce the bitterness associated with PTU, or 3% sucrose alone ($n = 12$). The goitrogen PTU blocks both TH synthesis and the conversion of T_4 to T_3 by type I 5′-deiodinase (Leonard and Koehrle, 1996; Chopra, 1996). The two solutions were provided fresh daily for 14 days. On G15, all animals received a subcutaneous injection of either T_4 (50 μg/kg body weight in 100 μl; Sigma; $n = 12$) or 100 μl saline ($n = 12$) at both 10:00 hours and 18:00 hours. This paradigm produced four groups of six animals each: no PTU + saline, no PTU + T_4, PTU + saline, and PTU + T_4. At 10:00 hours on G16, all dams were killed by decapitation and trunk blood was collected for the measurement of serum free T_3, total T_4, and thyrotropin (TSH). Fetuses were removed from the uterus, rapidly frozen on pulverized dry ice, and stored at -80 °C until they were sectioned for in situ hybridization.

To test whether TH status alters the expression of SRC-1 and/or N-CoR in the post-natal rat brain, timed-pregnant Sprague–Dawley rats ($n = 24$) were obtained from Charles River Breeding Laboratories Inc. (Wilmington, MA) and arrived at our facility on

G2. The animals were housed in individual Plexiglas cages and given food and water ad libitum. PTU and T_4 treatment began on GD8. Dams were weighed each morning to determine PTU doses. After weighing, the dams were given subcutaneous injections of thyroxine in sterile saline (20 μg/kg body weight) or saline alone. One hour before the end of the light cycle the dams were each given a Vanilla Wafer® (Keebler Company, Elmhurst, IL) dosed with PTU dissolved in ethanol (250 μg/100 kg body weight) or ethanol vehicle alone. Wafers were dosed in the morning and allowed to dry in a fume hood to prevent the consumption of alcohol by the dams. This paradigm produced four groups: control ($n = 8$), control + T_4 ($n = 6$), PTU + Saline ($n = 6$), and PTU + T_4 ($n = 6$). After birth, the dams continued to receive PTU which is transferred to the pups via the milk. On postnatal day 2, all litters were culled to two males and two females per dam and the T_4-supplemented pups began receiving daily injection of T_4 (20 μg/kg BW) or saline. On postnatal day 15 all pups were sacrificed by decapitation following CO_2 exposure. Trunk blood was collected for the measurement of total T_4 and T_3 levels, and the brain from one male pup was removed and frozen on dry ice and stored at −80 °C until sectioning for in situ hybridization.

4.2. Radioimmunoassay

The radioimmunoassay (RIA) for free T_3, total T_4 and TSH in the blood from dams on GD16 has been previously published. Total T_3 and T_4 in the P15 pups were measured with RIA kits (ICN, Costa Mesa, CA) and following the manufacturer's instructions. The limits of detection for free T_3 were 50–800 ng/dl. All samples were measured in duplicate in the same assay. TSH levels were not measured in the P15 pups.

4.3. Probes

To prepare templates for probe synthesis, fragments of rat N-CoR and SRC-1 were cloned by RT-PCR from total RNA isolated from 300 mg of adult cerebellar tissue. RNA was isolated using RNAzol B (Tel-Test Inc., Friendswood, TX) following the manufacturer's instructions. Reverse transcription to produce cDNA was performed with the RT-PCR kit from Stratagene (La Jolla, CA) and using the supplied random primers. Primers for PCR amplification of rat N-CoR and SRC-1 were synthesized by Gibco BRL (Gaithersburg, MD). Rat N-CoR forward and reverse primers corresponded to bases 3010–3030 and 3967–3943, respectively, of the mouse sequence (GenBank accession # U35312.1) (Horlein et al., 1995). Rat SRC-1 forward and reverse primers corresponded to bases 316–337 and 1179–1158, respectively, of the mouse sequence (GenBank accession # U64828) (Yao et al., 1996). Amplified fragments of N-CoR and SRC-1 were then cloned into the pCR II TOPO vector using the Topo TA cloning kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The authenticity of the DNA fragments was confirmed by sequence analysis. Specificity of probes was confirmed by Northern analysis (data not shown). Antisense probes were generated by linearizing the plasmid with EcoR V and transcribing with Sp6 RNA polymerase; sense probes were generated using BamH-1 and T7 RNA polymerase. The pRc/CMV cDNA containing RC3/neurogranin was kindly provided by Dr Juan Bernal (Madrid, Spain). The RC3/neurogranin cDNA was linearized with *Hin*dIII and transcribed in the presence of Sp6 RNA polymerase for complementary RNA synthesis; it was linearized with *Apa*I and transcribed in the presence of T7 RNA polymerase for sense-strand RNA synthesis. The sequence of the oligonucleotide probe for β-actin has been previously reported (Zoeller and Rudeen, 1992) and was synthesized by Gibco BRL (Gaithersburg, MD).

Labeled probes for use in in situ hybridization were generated by in vitro transcription. The transcription was performed in a final volume of 10 μl. RNA was synthesized in the presence of 1 μg DNA linearized plasmid, 500 μM each of GTP, ATP and CTP, and 12 μM UTP (^{33}P-UTP + UTP at a molar ratio of 9:3). Antisense and sense probes were transcribed and hybridized with each set of tissue. After transcription, the DNA template was digested using DNAse I (Boerhinger Manheim, Indianapolis, IN), and the probe was purified by phenol–chloroform extraction followed by two ethanol precipitations. The oligonucleotide probe for β-actin was 3′ end labeled using terminal deoxynucleotidylexotransferase as previously described (Zoeller and Rudeen, 1992).

4.4. In situ hybridization

Frozen tissues were sectioned at 12 μm in a cryostat (Reichert-Jung Frigocut 2800N; Leica, Deerfield, IL). Frontal sections were collected from the cortex of one G16 fetus per dam, and sagittal sections were collected from the brains of animals in the developmental study. Coronal sections were taken from the level of the rostral hippocampus of the P15 pups. Sections were thaw-mounted onto gelatin-coated microscope slides and stored at −80 °C until hybridization. Prior to hybridization, slides were warmed to room temperature and fixed in 4% formaldehyde in phosphate buffered saline (PBS) for 30 min. Slides were washed twice in PBS for 2 min followed by 10 min in 0.25% acetic anhydride dissolved in 0.1 M triethanolamine hydrochloride–0.9% NaCl (pH 8.0). Slides were washed in two times concentrated standard saline citrate (SSC; 1 × SSC = 0.15 M NaCl/0.015 M sodium citrate), dehydrated through a graded series of ethanol, delipidated

in a 5-min chloroform bath, rehydrated to 95% ethanol and air dried.

Fifty microliters of hybridization buffer was applied to each slide, covered with a 22 × 30 mm glass coverslip and incubated at 52 °C for 16–18 h in covered plastic chambers with free standing water to maintain humidity. The hybridization buffer contained 50% formamide, 2 × SSC, transfer RNA (250 μg/ml), 1% sodium pyrophosphate, 10% dextran sulfate, Denhardt's solution (0.02% each of bovine serum albumin, Ficoll, and polyvinylpyrrolidone), 200 mM dithiothreitol, and 3–4 × 10^6 cpm probe per slide. After hybridization, coverslips were removed in 1 × SSC and washed four times in 1 × SSC for 15 min. Slides were then washed twice in 2 × SSC/50% formamide for 20 min at 52 °C, rinsed twice in 2 × SSC for 10 min followed by a 10 min rinse in RNAase buffer (0.5 M NaCl, 0.01 M Tris, 1 mM EDTA) at 37 °C then treated with 100 μg/ml RNAase A in RNAase buffer at 37 °C. After RNAase treatment, slides were washed twice in 2 × SSC for 10 min, followed by a stringent wash in 2 × SSC/50% formamide at 52 °C. The slides were finally rinsed twice in 1 × SSC at room temperature, then in 70% ethanol to remove salts but preserve the hybrids, and allowed to air dry. Hybridization for β-actin was performed as previously reported with the exception that the hybridization was performed at 52 °C. All tissues to be hybridized with a specific probe were included in a single run; thus, they were pretreated, hybridized and washed in the same solutions. Moreover, all slides to be included in a single statistical analysis were placed on a single film for analysis. These methods allow a valid comparison of mRNA levels among treatment groups.

Following in situ hybridization, all slides were arranged in large X-ray cassettes and apposed to BioMax film (35 × 45 cm; Kodak, Rochester, NY). The developmental tissue was apposed to film for 7 days to visualize SRC-1 mRNA and 4 days to visualize N-CoR probe. To visualize SRC-1 and N-CoR mRNA in fetal tissue, slides were apposed to film for 4 days. The postnatal tissue was apposed to film for 48 h to visualize N-CoR and SRC-1 mRNA. To visualize β-actin, postnatal tissue was exposed to film for 7 days. Radioactive ^{14}C-standards (American Radiolabeled Chemicals Inc., St. Louis, MO) were simultaneously apposed to all films to verify that the film was not overexposed. The hybridization signal was analyzed as described previously (Zoeller et al., 2000) using a Macintosh 7600 computer and the public domain NIH Image program (W. Rasband, NIMH). This system was interfaced with a Dage-MTI 72 series video camera equipped with a Nikon macro lens mounted onto a bellows system over a light box. This system has been used in our lab extensively and has produced consistent and repeatable results. Changes in mRNA levels found using our in situ hybridizations and image analysis protocols have been confirmed using northern blots (Dowling et al., 2000). Relative levels of mRNA in the fetal tissue, represented by film density, for both N-CoR and SRC-1 were evaluated in the neocortex. In the postnatal tissue, relative mRNA levels were evaluated in the dentate gyrus, CA1 and CA3 regions of Ammon's horn and in the cortex. The resulting values were averaged over four sections for each brain. Two operators who were unaware of the identity of the signal took measurements, and concordance between operators was always observed.

4.5. Statistical analysis

All tissues to be evaluated in a single statistical test were included in the same in situ hybridization assay and film. Pre- and posthybridization washes were performed together in a 100-place stainless steel slide rack, and all slides were placed against a single 35 × 45 cm film. A two-way ANOVA was performed on hormone levels and imaging data using the STATVIEW and SUPERANOVA statistical packages (Abacus Concepts, Berkeley, CA), with main effects of PTU and T_4 supplementation. The ANOVA was followed by Bonferroni's *t*-tests to compare individual means.

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